

A TRYPSIN-SOLUBILIZED LACCASE FROM PHARATE PUPAL INTEGUMENT OF THE TOBACCO HORNWORM, *MANDUCA SEXTA**

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Abstract—A laccase-like enzyme was solubilized from *Manduca sexta* pharate pupal integument by trypsin treatment and partially purified by ammonium sulfate precipitation, gel filtration, ultracentrifugation and anion exchange high performance liquid chromatography (HPLC). The molecular weight of the laccase as determined by gel filtration HPLC was 250 ± 30 kDa. The most purified preparation contained a single oxidase with typical laccase activity as determined by substrate and inhibitor specificities, enzyme kinetics, and a single band of enzyme activity on nondenaturing polyacrylamide gels with syringaldazine (SYR) and 3,4-dihydroxyphenylalanine (DOPA) substrates. The specific activity of the most purified enzyme preparation was $18.0 \mu\text{mol}$ of quinone and $1.1 \mu\text{mol}$ of *p*-quinoneimine formed $\text{min}^{-1} \text{mg}^{-1}$ from 2-methylhydroquinone (MHQ) and DOPA, respectively, at pH 6. The pH optimum of the laccase was 6 for MHQ and about 7 for *N*-acetyldopamine. Incubation of the *M. sexta* laccase with selective laccase and tyrosinase inhibitors demonstrated that the enzyme is generally inhibited like fungal laccase with *p*-diphenolic substrates and like mushroom tyrosinase with *o*-diphenolic substrates. *Manduca* laccase affinity for MHQ was 400-fold greater than that for DOPA and the catalytic efficiency (V_{max}/K_m) for oxidation of the *p*-diphenol was 2000-fold higher than that for the *o*-diphenol. Topical application of SYR to pharate pupal forewing cuticle revealed laccase activity *in situ*. The natural tanning precursor, *N*- β -alanyldopamine (NBAD), was the best *o*-diphenolic substrate tested. *N*- β -Alanyl-norepinephrine was also formed at a high rate when NBAD was incubated with *M. sexta* laccase, but at a low rate with fungal laccase, suggesting specificity for side chain oxidation through a *p*-quinone methide intermediate. The results indicate that *M. sexta* laccase is important for oxidizing cuticular *o*-diphenols to *o*-quinone and *p*-quinone methide tanning agents for sclerotization of the *M. sexta* pupal exoskeleton.

Key Word Index: *Manduca sexta*, laccase, tyrosinase, phenoloxidase, cuticle, sclerotization, catecholamines, *o*-diphenol, quinone, DOPA, *p*-diphenol, *N*- β -alanyldopamine, β -hydroxylation, insect, tobacco hornworm

INTRODUCTION

The insect exoskeleton is stiffened by the process of sclerotization, in which cuticular proteins become crosslinked by diphenolic bridges with increasing insolubility and dehydration (Brunet, 1980; Lipke *et al.*, 1983; Andersen, 1985; Kramer and Hopkins, 1987). Several kinds of phenoloxidases that catalyze the oxidation of phenolic compounds have been implicated in the sclerotization of insect cuticle

(Andersen, 1976, 1979; Brunet, 1980). Although the role of laccases (EC 1.10.3.2) and other oxidases, such as tyrosinases (EC 1.10.3.1) and peroxidases (EC 1.11.1.1), in the tanning process is still uncertain, the possibility exists that laccases are the primary enzymes involved in sclerotization, whereas tyrosinases catalyze wound healing reactions (Andersen, 1985).

Laccases and tyrosinases are differentiated principally by their substrate specificities (Robb, 1984; Reinhammar, 1984). Tyrosinase will catalyze the hydroxylation of monophenols (EC 1.14.18.1) and the oxidation of *o*-diphenols but not *p*-diphenols, whereas laccase will catalyze the oxidation of both types of diphenols, but not the hydroxylation of monophenols (Webb, 1984). Reports of *p*-diphenols, such as hydroquinone, in insect cuticle have been rare (Dennell, 1958), and verification of the presence of those compounds by more critical analytical methods has not been done. Thus, *o*-diphenols appear to be the major precursors for quinonoid tanning agents of

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insect cuticle, and they include two *N*-acylcatecholamines, *N*-acetyldopamine (NADA)* (Karlson and Sekeris, 1962) and *N*- β -alanyldopamine (NBAD) (Hopkins *et al.*, 1982). The cuticular β -hydroxylated metabolites of NADA and NBAD, *N*-acetylnorepinephrine (NANE) and *N*- β -alanyl-norepinephrine (NBANE), may also be tanning agent precursors (Morgan *et al.*, 1987).

In previous work on the tanning chemistry of *M. sexta* pupal cuticle, we studied the catecholamine substrates involved in cuticular sclerotization and melanization (Hopkins *et al.*, 1982, 1984; Morgan *et al.*, 1987), as well as a tyrosinase that may be involved in the tanning process (Aso *et al.*, 1984, 1985). The fact that soluble and insoluble laccases commonly occur in tanning cuticle suggests that these relatively nonspecific phenoloxidases play an important role in sclerotization (Andersen, 1985). In this study, we describe the partial purification and characterization of a trypsin-solubilized laccase, which occurs in *M. sexta* pharate pupal integument, and compare its properties with those of a fungal laccase and mushroom tyrosinase, as well as with other insect laccases that have been reported in the literature.

MATERIALS AND METHODS

Insects

M. sexta was reared as described by Bell and Joachim (1976) at $27 \pm 1^\circ\text{C}$ and using a nondiapausing photoperiod of 16 h light–8 h dark.

Chemicals

Substrates and inhibitors were obtained from Sigma Chemical Co., St Louis, Mo. or Aldrich Chemical Co., Inc. Milwaukee, Wis. Mushroom tyrosinase and *Pyricularia oryzae* laccase were obtained from Sigma Chemical Co. *N*- β -Alanyldopamine-3-*O*-glucopyranoside (NBADG) was purified from *M. sexta* pharate pupal hemolymph by reversed phase HPLC (Morgan *et al.*, unpublished data).

Enzyme purification

Integuments (15 g) of bar stage pharate pupae were homogenized in 0.1 M Tris–HCl buffer containing 1 M sodium chloride and 50 mM sodium ascorbate pH 7.8. The precipitate from this extract was treated

with 50 mg trypsin in 0.1 M phosphate buffer pH 7 containing 50 mM sodium ascorbate at 37°C for 1 h, after which 1 mM phenylmethylsulfonyl fluoride was added to inhibit further proteolysis. After centrifugation, the supernatant from the trypsin treatment was dialyzed against 0.1 M sodium phosphate buffer containing 12 mM thiourea pH 7. Enzyme was precipitated from the dialysate by the addition of ammonium sulfate to 40% saturation. The precipitate was redissolved by dialysis against 50 mM MOPS pH 7 containing 12 mM thiourea and then gel filtered on Sephacryl S-400 (Pharmacia) using 50 mM MOPS pH 7 at a flow rate of 0.9 ml min^{-1} . To further purify the enzyme and also determine the sedimentation coefficient, the most active fraction from gel filtration that oxidized DOPA was subjected to ultracentrifugation in a 10–40% sucrose gradient using a Beckman VTi 50 rotor at 50,000 rpm for 5 h. After storage at -20°C , fractions from the Sephacryl S-400 chromatography were further purified by anion-exchange HPLC using a Synchrom AX-300 column (Alltech, 4.6 mm \times 25 cm), 25 mM Tris–HCl pH 7.5, and a linear gradient from 0 to 0.5 M NaCl at a flow rate of 1 ml min^{-1} . The native molecular weight of the enzyme was determined by HPLC using a TSK 4000 gel filtration column (Toyo Soda, Tokyo) and 25 mM Tris–HCl buffer pH 7.5 containing 0.1 M NaCl at a flow rate of 1 ml min^{-1} . Protein standards were aprotinin, 6.5 kDa, as the inclusion volume marker; bovine serum albumin, 66 kDa; alcohol dehydrogenase, 150 kDa; β -amylase, 200 kDa; apoferritin, 443 kDa; thyroglobulin, 669 kDa; and DNA, >1000 kDa, as the void volume marker. Protein was determined by the Coomassie blue dye binding method (Bradford, 1976) and activity by DOPA oxidation (see Enzyme Assays section).

Electrophoresis

Denaturing electrophoresis in the presence of SDS was performed by the Laemmli method (1970), and nondenaturing gels were run according to the procedure of Davis (1964). A Bio-Rad Protein II slab gel apparatus was employed for all electrophoresis. Gels were stained for protein with Coomassie blue R-250 or the Bio-Rad silver stain and for activity using DOPA or SYR as substrate. Substrate (5 mM DOPA or $20 \mu\text{M}$ SYR) was dissolved in 0.1 M sodium phosphate buffer pH 6 and 100 ml was used to incubate gels at room temperature.

Enzyme assays

Reaction mixtures, in a 1 ml volume (except for carbon monoxide inhibition experiments), contained 0.1 M sodium phosphate buffer pH 6, 0.3 mM substrate (except SYR, which was used at $10 \mu\text{M}$) and an aliquot (usually 50 μl) of an enzyme preparation. For the determination of pH optima, 0.1 M sodium citrate-phosphate buffers were utilized. Spectrophotometric determinations of reaction rates were made with a Cary model 118 UV/VIS spectrophotometer at 25°C . Product formation was calculated using the following molar extinction coefficients: NBAD, NADA and NBANE quinones, $\epsilon_{390 \text{ nm}} = 1100 \text{ M}^{-1} \text{ cm}^{-1}$; SYR quinone methide, $\epsilon_{525 \text{ nm}} = 65,000$; dopachrome, $\epsilon_{475 \text{ nm}} = 3600$; and 2-methyl

*Abbreviations used: kDa, kilodalton; NBAD, *N*- β -alanyldopamine; NADA, *N*-acetyldopamine; NBANE, *N*- β -alanylnorepinephrine; SYR, syringaldazine; HPLC, high performance liquid chromatography; DOPA, 3,4-dihydroxyphenylalanine; MHQ, 2-methylhydroquinone; PTU, 1-phenyl-2-thiourea; NPD, naphthalene diol; CTAB, cetyl trimethylammonium bromide; CO, carbon monoxide; PHZ, phenylhydrazine; NBADG, NBAD-3-*O*-glucoside; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; K_m , Michaelis constant; V_{max} , maximum reaction velocity; K_i , inhibition constant; ND, not determined, NR, no reaction; HQ, hydroquinone; DOPAC, 3,4-dihydroxyphenylacetic acid; DA, dopamine; CAT, catechol; MC, 4-methylcatechol; MOPS, morpholinopropane sulfonic acid; Tris, Tris-(hydroxymethyl) aminomethane.

p-benzoquinone, $\epsilon_{250\text{ nm}} = 21,000$. When the laccase concentration in the reaction mixture was relatively low, a lag period, often lasting 5 min or longer, occurred during assays that utilized SYR as the substrate. The lag period was shortened or eliminated when higher enzyme concentrations were used. Values of K_m and V_{max} were calculated according to the Lineweaver-Burk method (1934).

All inhibitors were added to the reaction mixtures immediately before adding enzyme except phenylhydrazine (PHZ, a noncompetitive inhibitor), which was incubated with enzyme solution for 1 h prior to assay. Carbon monoxide (CO) was dissolved into reaction mixtures of 5 ml with stirring for 1 h before adding enzyme in a glass 20 ml vial sealed with a septum and containing 15 cm³ of CO. In controls, CO was replaced by nitrogen. Inhibition constants were determined using the Hunter-Downs method (Hunter and Downs, 1945; Webb, 1963).

NBADG reaction with enzymes was determined by reverse phase HPLC using an octadecyl bonded phase (Alltech, 5 micron particle size) column heated to 30°C, a mobile phase consisting of 135 ml methanol, 890 ml water, 75 mg sodium octyl sulfate, 100 mg EDTA, 8 ml phosphoric acid and concentrated sodium hydroxide to bring the pH to 2.7, and a Bioanalytical Systems model LC-4 amperometric detector set at 0.72 V. Reaction mixtures (0.1 ml) contained 0.13 mM NBADG and 0.1 M sodium phosphate buffer pH 6.

HPLC analysis was used to quantify hydroxylation products. For assay of *o*-hydroxylation of tyrosine, 0.3 mM substrate was incubated with the enzyme and either 0 or 60 μ M dopamine, a cofactor that decreases the lag time for hydroxylation (Pomerantz, 1966). At the end of the 10 min incubation, a 0.1 ml aliquot was added to 50 μ l of 0.9 M perchloric acid containing α -methyl DOPA as an internal standard. The 10,000 *g* supernatant was analyzed by HPLC with electrochemical detection using a 26% acetonitrile mobile phase with SDS as the ion pair (Morgan *et al.*, 1987). For analysis of the β -hydroxylation of NBAD to form NBANE, 10 μ l aliquots of the incubation mixture were added to 90 μ l of methanol containing a strong nucleophile (0.8 M sodium benzene sulfinate) or methanol containing a reducing agent (50 mM ascorbic acid). The samples were left at 5°C for several hours, recovered from alumina essentially as described by Murdock and Omar (1981), and analyzed by HPLC with electrochemical detection. The amount of β -hydroxylated products from NBAD (NBANE plus NBANE quinone) that was present after 10 min of incubation was estimated by analysis of samples treated with methanol and ascorbic acid, whereas the amount of substrate remaining at 10 min was estimated by analysis of samples treated with methanol and benzene sulfinate. The reaction of benzene sulfinate with NBAD quinone prevented any spontaneous reduction of quinone to diphenol.

Pupal forewing cuticles were stained for laccase activity *in vivo* by scraping epidermis from the underside of the outer integument of unsclerotized pupal forewings at 1, 2.5 and 5 h after pupal ecdysis and then topically applying 10 mM SYR dissolved in methanol.

RESULTS

Enzyme purification

From *M. sexta* bar stage pupal integument, a phenoloxidase was solubilized by treatment with trypsin and the enzyme preparation was subjected to thiourea treatment, ammonium sulfate precipitation, ultracentrifugation [Fig. 1(A)], gel filtration [Fig. 1(B)] and anion exchange HPLC. The resulting enzyme exhibited a native molecular weight of 250 ± 30 kDa determined by gel filtration HPLC and a sedimentation coefficient of approx. 11 Svedberg units. Coomassie blue or silver staining of this preparation subjected to denaturing electrophoresis (SDS-PAGE) revealed about four bands with apparent molecular weights of 70–90 kDa. These bands were due either to trypsin treatment, which may have resulted in microheterogeneity, or to other proteins (impurities) present in the preparation. Nondenaturing PAGE yielded a single broad band when stained for activity with the substrates DOPA (Fig. 2) and SYR (not shown). SYR or *N,N'*-bis-(3,5-dimethoxy-4-hydroxybenzylidene) hydrazine is a compound that has been employed as a specific substrate for laccases and peroxidases in plants (Harkin and Obst, 1973 a,b; Leonowicz and Grzywnowicz, 1981) which we have found useful as a stain for the *Manduca* laccase in electrophoresis gels. The reaction proceeded quickly in the gel with a pinkish color forming after 10–15 min. However, the color was temporary and faded after a few hours. Since only one band of protein oxidized both DOPA and SYR, there was only a single laccase-like enzyme present in the preparation. The fraction from Sephacryl S-400 gel filtration was stable for several months when frozen at -20°C and was subsequently used for most of the substrate specificity and inhibitor studies. The final specific activity at pH 6 and 25°C of the most highly purified laccase preparation from anion-exchange HPLC was 18 μ mol 2-methyl *p*-benzoquinone formed $\text{min}^{-1}\text{mg}^{-1}$ at pH 6 from 2-methylhydroquinone (MHQ) and 1.1 μ mol dopachrome formed $\text{min}^{-1}\text{mg}^{-1}$ from DOPA at pH 6.

pH dependence

The pH optimum of the *Manduca* laccase with MHQ was pH 6, whereas the pH profile for NADA was broader with > 85% of maximal activity from pH 5.5–7.0 and the optimum at about pH 7 (Fig. 3). A pH 6, 0.1 M sodium phosphate buffer was routinely used for all subsequent studies.

Substrate specificity

A spectrophotometric comparison of the relative activities of the *Manduca* oxidase preparation with those of a fungal laccase and mushroom tyrosinase on a variety of *o*-diphenolic and *p*-diphenolic substrates confirmed that the substrate specificity of the *Manduca* enzyme fits the criteria for a laccase (Table 1). Neither the *Manduca* enzyme nor the fungal laccase hydroxylated tyrosine (< 0.1 nmol DOPA produced $\text{min}^{-1}\text{mg}^{-1}$), even in the presence of a diphenolic activator (60 μ M dopamine). Under identical assay conditions, mushroom tyrosinase catalyzed DOPA formation at a rate of 743–885 nmol

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A B

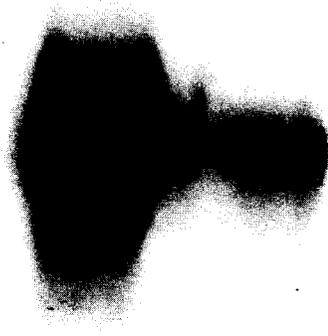


Fig. 2. Nondenaturing 7.5% polyacrylamide electrophoretic gel of *M. sexta* laccase with DOPA staining. Lane A, Sephacryl S-400 active fraction and lane B, anion exchange HPLC active fraction.

Table 1. Specific activities (nmol min⁻¹ mg⁻¹) of *Manduca sexta* laccase, fungal laccase and mushroom tyrosinase toward *o*- and *p*-diphenolic substrates

Substrate	<i>M. sexta</i> laccase	Fungal laccase	Mushroom tyrosinase
DOPA	230 ± 10	378 ± 15	3600 ± 15
NBAD	4350 ± 140	2100 ± 100	10200 ± 300
NBANE	465 ± 38	764 ± 38	1669 ± 201
NBADG	NR	NR	NR
SYR	155 ± 5	1150 ± 50	NR
MHQ	9220 ± 460	4140 ± 230	43 ± 2
TYR	< 0.1	< 0.1	886

*Substrate concentration was 0.3 mM except for SYR which was 10 μM. Mean values ± SEM (n = 3) except TYR where n = 2. See Introduction section for list of abbreviations.

fungal oxidases. This result demonstrated that the diphenol oxidizing activity of the laccases does not extend to glycoside conjugated *o*-diphenols. The high specific activity towards the *p*-diphenolic substrate MHQ and the lack of hydroxylating activity towards tyrosine showed that the *M. sexta* trypsin-solubilized diphenoloxidase is a true laccase (EC 1.10.3.2; Webb, 1984).

β-Hydroxylation of the catecholamine side chain

When the *Manduca* laccase was incubated with 0.3 mM NBAD at pH 6, *β*-hydroxylation of the substrate's aliphatic side chain occurred at a rate of 293 ± 41 nmol min⁻¹ mg⁻¹ during the first 10 min. This rate was approx 3-fold lower than the initial rate of NBAD-*o*-quinone formation. The amount of NBANE and NBANE *o*-quinone formed accounted for 43 ± 5% of the consumption of NBAD during this time period. This extent of *β*-hydroxylation was high in comparison to the 16 ± 3% production of *β*-hydroxylated metabolites obtained with the fungal laccase under the same conditions. The results showed that, in addition to the expected NBAD-*o*-quinone product, NBANE was a major product when NBAD was oxidized by the *Manduca* laccase.

Inhibitor studies

Phenylthiourea (PTU), a tyrosinase inhibitor (Andersen, 1979; Barrett and Andersen, 1981; Aso *et al.*, 1985; Barrett, 1987 a,b), did not inhibit fungal laccase oxidation of DOPA and MHQ or the *Manduca* laccase catalyzed oxidation of MHQ when tested at up to 10 μM concentration (Table 2). The mushroom tyrosinase and, to a lesser extent, the *Manduca* laccase were inhibited by PTU when DOPA was the substrate. Inhibition of mushroom tyrosinase was approximately six times that of the *Manduca* laccase at the lower inhibitor concentration (0.1 μM). Therefore, *Manduca* laccase displayed a tyrosinase-like inhibition behavior when DOPA was the substrate, but a laccase-like immunity to PTU when MHQ was the substrate.

Naphthalene diol (NPD), a competitive inhibitor of plant tyrosinases (Mayer *et al.*, 1964; Mayer and Harel, 1968), inhibited the *o*-diphenol oxidizing activity of *Manduca* laccase and mushroom tyrosinase to the same extent, but not that of fungal laccase (Table 2). This further suggested that the *Manduca* laccase may have some tyrosinase-like character, unlike the fungal laccase. The *p*-diphenol oxidizing

activity of both laccases was not inhibited by NPD when MHQ was the substrate. However, when SYR was the substrate, the enzymes were completely inhibited. Use of a 30-fold lower concentration of SYR relative to the other *p*-diphenolic substrate MHQ, as necessitated by the low solubility of SYR, may have been a factor in the differential inhibition of oxidation of the two substrates by NPD. There was substantially less SYR than MHQ present to compete with the inhibitor for the active site of the enzyme.

Phenylhydrazine (PHZ), a noncompetitive inhibitor of plant tyrosinases (Lerner *et al.*, 1971), yielded a pattern of inhibition similar to that observed with NPD (Table 2). With DOPA as the substrate, the *Manduca* laccase was most sensitive to this inhibitor, followed by mushroom tyrosinase, whereas very little inhibition of the fungal laccase occurred. Neither the insect nor the fungal laccase was inhibited when MHQ was utilized as a substrate at 0.3 mM, but as was observed with NPD, both enzymes were inhibited by PHZ when the substrate SYR was used at 10 μM. The relatively low concentration of SYR was probably a contributing factor to the inhibition by PHZ.

The *Manduca* laccase was only weakly inhibited by carbon monoxide (CO), a fungal tyrosinase inhibitor (Walker, 1968), except when SYR was used as the substrate, whereas the fungal laccase was not inhibited by CO when using any of the substrates (Table 2). Mushroom tyrosinase as expected, was strongly inhibited by CO. Thus, depending on the substrate and inhibitor used, the *Manduca* laccase differed in its pattern of CO inhibition from either mushroom tyrosinase or fungal laccase.

Cetyl trimethylammonium bromide (CTAB), a cationic detergent fungal laccase inhibitor (Walker, 1968), was not effective against *Manduca* laccase, but partially inhibited fungal laccase when either DOPA or MHQ was the substrate (Table 2). However, the

Table 2. % Inhibition of *Manduca sexta* laccase, fungal laccase and mushroom tyrosinase by laccase and tyrosinase inhibitors*

Inhibitor	Substrate	<i>M. sexta</i> laccase	Fungal laccase	Mushroom tyrosinase	
					% Inhibition
PTU	DOPA	1 μM	36 ± 2	0	100 ± 5
		0.1 μM	11 ± 5	0	68 ± 5
	MHQ	10 μM	0	0	—
		0.1 μM	0	0	—
NPD	DOPA	0.1 mM	53 ± 6	0	61 ± 5
				0	—
				100 ± 5	—
PHZ	DOPA	50 μM	76 ± 4	7 ± 1	35 ± 1
				0	—
				22 ± 1	—
CO	~ saturated	DOPA	15 ± 1	0	69 ± 6
		MHQ	12 ± 1	0	—
		SYR	100 ± 5	0	—
CTAB	0.1 mM	DOPA	0	25 ± 1	17 ± 1
		MHQ	0	62 ± 4	—
		SYR	17 ± 1	0	—

*Substrate concentration was 0.3 mM except SYR which was 10 μM. Mean values ± SEM (n = 3). See Introduction section for abbreviations.

SYR oxidizing activity of *Manduca* laccase was weakly inhibited by CTAB, whereas the fungal laccase showed no inhibition.

In general, effects of inhibitors on the fungal laccase and mushroom tyrosinase were different, whereas the *Manduca* laccase inhibition in most cases resembled that of the fungal laccase when the *p*-diphenolic substrates were used. However, in some cases it more closely resembled mushroom tyrosinase when the *o*-diphenolic substrates were used. Therefore, the insect laccase appears to have more of a tyrosinase-like character than does the fungal laccase.

Enzyme kinetics

A comparison of the Michaelis constants (K_m) determined for the *Manduca* laccase with the *o*-diphenol DOPA and the *p*-diphenol MHQ indicated that the affinity for the latter substrate is more than 400 times greater than that for the former (Table 3). The maximum velocity (V_{max}) or turnover rate of reaction with the *p*-diphenol was over five times that of the *o*-diphenol turnover rate. The V_{max}/K_m ratio revealed that the MHQ oxidation is approx. 2000 times more catalytically efficient than the DOPA reaction.

Inhibition constants (K_i) were obtained for NPD and PTU when DOPA was used as the substrate (Table 3). The K_i for NPD was relatively high (42 mM), and the inhibition pattern indicated a mixed inhibition behavior at the higher concentrations of NPD. Although PTU also exhibited a mixed inhibition behavior at high concentrations, its low K_i (0.2 μ M) appears to make it more suitable for use as a specific tyrosinase inhibitor in physiological studies when both a laccase and a tyrosinase are present in tissues, such as the integument of *Manduca*.

Laccase activity in the intact cuticle

M. sexta pupal forewing cuticle, treated *in situ* with SYR, developed an intense pink colour characteristic of the SYR *p*-quinone methide in the cuticle, and most rapidly with the younger, least sclerotized cuticle. The color was stable for several hours. This result indicated that laccase is present in the intact, newly formed, pupal cuticle.

DISCUSSION

Trypsin treatment is a proven technique for solubilizing insoluble cuticular phenoloxidases (Yamazaki, 1972; Andersen, 1978). We have used this procedure to release a laccase-like enzyme from the pupal integument of *M. sexta*. Although trypsin treatment may alter the catalytic properties of these

enzymes (Andersen, 1978), we have also observed laccase activity *in situ* in newly ecdysed pupal cuticle by incubating the cuticle with the *p*-diphenolic substrate SYR. Insect and fungal tyrosinases are unable to oxidize this substrate (Morgan *et al.*, unpublished data). These results confirmed that a laccase-type phenoloxidase occurs as an insoluble enzyme in *M. sexta* pupal cuticle undergoing sclerotization.

Although there was apparent protein heterogeneity in the solubilized laccase preparation, multiple bands of enzyme activity were not observed in electrophoretic gels using either DOPA or SYR as a substrate for staining. The purified enzyme yielded a single coincident SYR- and DOPA-oxidizing band upon substrate incubation in non-denaturing gels, indicating the presence of only one oxidase.

The use of SYR as a substrate stain for laccase in electrophoresis gels has not been described previously, but it proved useful as a diagnostic substrate for detecting laccases in non-denaturing PAGE. An advantage of SYR for enzyme assays and gel staining is its relatively high specificity for laccases and the highly visible pink chromophore produced by oxidation (Harkin and Obst, 1973a,b). It was originally synthesized as a substrate for fungal lignin-degrading enzymes and its structure is similar to that of phenolic β -1 linked lignin-like compounds (Kawai *et al.*, 1988). Our use of SYR in staining insect tissue *in vivo* is analogous to the staining of intact plant tissue for which the substrate was originally employed (Harkin and Obst, 1973a,b; Leonowicz and Grzywnowicz, 1981).

The primary catalytic activities of a laccase (EC 1.10.3.2, *p*-diphenol: O₂ oxidoreductase) are the oxidation of *para*- as well as *ortho*-diphenols in the absence of hydrogen peroxide (Webb, 1984). A laccase will not hydroxylate monophenols. By this definition, the trypsin-solubilized *Manduca* cuticular phenoloxidase behaves primarily as a laccase. It oxidizes *p*-diphenolic compounds as well as *o*-diphenols, but does not hydroxylate monophenols. Enzyme kinetic analysis showed that the *Manduca* laccase has a relatively high affinity for *p*-diphenols as substrates and that the catalytic efficiency of the oxidation of MHQ is 2000 times greater than that of DOPA, an *o*-diphenol. However, the natural substrate of the laccase *in vivo* is most likely an *o*-diphenol, since to our knowledge no *p*-diphenol has been unambiguously detected in insect cuticle.

NBAD and NBANE are the most abundant *o*-diphenols during the early stages of pupal cuticle sclerotization in *M. sexta* (Morgan *et al.*, 1987) and, therefore, are likely substrates for the laccase. However, the 10-fold higher rate of NBAD oxidation relative to that of NBANE oxidation by the *Manduca* laccase may indicate greater importance for NBAD as a precursor of sclerotizing agents. This trend in *o*-diphenol susceptibility was also observed with soluble tyrosinases that were partially purified from *Manduca* integument (Morgan *et al.*, 1988).

β -Hydroxylation occurred when the laccases were incubated with NBAD. However, when using the same assay conditions, the yield of NBANE from NBAD oxidation by *Manduca* laccase (~40%) was more than double that catalyzed by the fungal laccase. β -Hydroxylation of NADA during incubation

Table 3. Kinetic parameters for oxidation of *o*- and *p*-diphenolic substrates by *Manduca sexta* laccase*

Substrate	Kinetic constants			NPD K_i (mM)	PTU K_i (nM)
	K_m (μ M)	V_m (nmol min ⁻¹ mg ⁻¹)	V_m/K_m		
DOPA	8700 \pm 500	2600 \pm 200	0.3	42 \pm 3	198 \pm 11
MHQ	21 \pm 1	13800 \pm 400	648	—	—

*See Introduction section for list of abbreviations. Mean values \pm SEM ($n = 3$).

with cuticular phenoloxidases was first reported by Peter (1980), who proposed that a *p*-quinone methide may be an intermediate because of the racemic *N*-acetyl-norepinephrine product formed. *p*-Quinone methides have since been proposed as tanning agents of insect cuticle based on other types of indirect evidence (Lipke *et al.* 1983; Sugumaran, 1987). In previous studies using partially purified insect laccases, the amount of tritium released from the β -carbon of NADA was relatively small when compared to the amount released during incubation with whole cuticle (Andersen, 1978; Barrett and Anderson, 1981). In this study, however, direct product analysis revealed that about 40% of the turnover of NBAD by *Manduca* laccase was accounted for by NBANE synthesis. This was approximately the same yield of NBANE that was obtained during incubation of NBAD with cuticular homogenates (Morgan *et al.*, 1987). The relatively low yield of NBANE that was obtained from the fungal laccase catalyzed oxidation of NBAD was similar to the yields obtained from reactions catalyzed by tyrosinases from *Manduca* integument (Morgan *et al.*, 1988 and unpublished data).

In a recent study, Saul and Sugumaran (1988) found that NADA-*o*-quinone was consumed and that NANE was produced by a cuticular residue from *M. sexta* pharate pupae. They asserted that these activities are due to a novel enzyme, a "quinone methide isomerase", which converts the *o*-quinone to a *p*-quinone methide. We found a high rate of synthesis of NBANE from NBAD during incubation with a laccase that was solubilized by trypsin treatment of *M. sexta* integument. Since the initial rate of synthesis of NBAD-*o*-quinone by the laccase appears to be comparable to that obtained using tyrosinases or sodium periodate (Morgan *et al.*, unpublished data), it is possible that *Manduca* laccase enhances the rate of tautomerization of the *o*-quinone to a *p*-quinone methide. Direct evidence for an "isomerase" function of the laccase will be difficult to obtain for several reasons. Spontaneous tautomerization of the *o*-quinone to a *p*-quinone methide appears to occur at a significant rate (Morgan *et al.*, 1988 and unpublished data; Cabanes *et al.*, 1988; Ortiz *et al.*, 1988). In addition to tautomerization, consumption of quinone can occur due to reaction with nucleophiles. We have also found that spontaneous reduction of some of the quinone occurs at pH 6, resulting in regeneration of the diphenol which may be reoxidized by the enzyme (Morgan *et al.*, 1988 and unpublished data). Further experiments are required to elucidate the catalytic function of *Manduca* laccase regarding production of an intermediate with an electrophilic β -carbon.

To help characterize the *M. sexta* cuticular oxidase, a series of phenoloxidase inhibitors was tested based on a survey of the literature about inhibition of plant and insect tyrosinases and laccases (Walker and McCallon, 1980; Barrett, 1987a,b). Depending on the substrate and inhibitor used, a range of inhibitory activity was observed that related to how well the inhibitor competed with the substrate and to the relative concentrations tested. Most of these inhibitors behaved as expected with *Manduca* laccase, that is, they differentiated tyrosinase activity from

laccase activity. The inhibitor studies show that the *o*-diphenol oxidizing activity for the *Manduca* enzyme is inhibited in a manner similar to that of the plant tyrosinases, whereas the *p*-diphenol oxidizing activity is inhibited like that of the plant laccases. It is possible that the *Manduca* laccase has more than one active site, one for *o*-diphenols and the other for *p*-diphenols, each with a different sensitivity to inhibitors. However, preliminary experiments showed that 10 μ M MHQ inhibited the laccase catalyzed oxidation of DOPA, indicating that there is only one active site. The *Manduca* laccase was relatively insensitive to CO when MHQ was used as the substrate, as were the laccases from *Bombyx mori* and *Drosophila virilis* when hydroquinone was used as the substrate (Yamazaki, 1969, 1972). In a companion study, we examined the inhibition of tyrosinases obtained from the prepupal integument of *Manduca* (Morgan *et al.*, unpublished data). The principal difference between inhibition of *Manduca* laccase- and tyrosinase-catalyzed *o*-diphenol oxidations was the relative insensitivity of the former enzyme to 1-phenyl-2-thiourea (PTU). This property is characteristic of all the insect laccases that have been tested for PTU inhibition (Barrett, 1987a,b).

Laccase-like enzymes have been detected in cuticles from a wide variety of insects. Table 4 lists 16 species in which laccase activity has been demonstrated, including eight species of Diptera, three of Lepidoptera, two of Orthoptera and Coleoptera, and one of Hemiptera. Laccases were found in cuticles from larval, pupal and adult stages of development. The *Manduca* laccase exhibited a high specific activity relative to those determined for some of the other preparations. For example, the final specific activity of the most purified *M. sexta* laccase fraction was almost five times that obtained by Andersen (1978) for locust cuticular laccase under similar reaction conditions. It was calculated that the specific activity of the *Manduca* laccase of 18 μ mol 2-methyl *p*-benzoquinone formed $\text{min}^{-1}\text{mg}^{-1}$ is equivalent to 463 absorbance units $\text{min}^{-1}\text{mg}^{-1}$ as defined by Andersen (1978), whereas the locust preparation had a specific activity of 96 absorbance units $\text{min}^{-1}\text{mg}^{-1}$.

Manduca laccase exhibited a rather broad pH optimum for NADA oxidation centered around pH 7, which was similar to that of *Bombyx mori* laccase with hydroquinone as the substrate (Yamazaki, 1972). Laccases from other species so far examined have pH optima in the range of 4.5–7. The Michaelis constants obtained for *Manduca* laccase were substantially different than those determined for other insect laccases. The K_m for MHQ with the *Manduca* enzyme was only 0.02 mM, which is an order of magnitude lower than the values reported for laccases from *Schistocerca* and *Calliphora* (see Table 4). The K_m for DOPA with the *Manduca* laccase was about 9 mM, whereas this value was 1 mM for the *B. mori* laccase. Laccases from Lepidoptera, Coleoptera, Hemiptera and Orthoptera apparently exist as tightly bound enzymes in the cuticle, whereas most of the cuticular laccases from Diptera are more readily solubilized. The localization of laccases in the matrix of the insect cuticle undergoing sclerotization points to a critical role for those enzymes in the tanning process. Laccase may

Table 4. Properties of insect cuticular laccases*

Species (developmental stage)	Physical state	MW† (kDa)	Substrate	Optimum pH	Specific activity† (units mg ⁻¹)	K _m (mM)	Reference	
<i>Bombyx mori</i> (unsclerotized pupae)	trypsin solubilized	ND	HQ	5.5	24 (a)	0.244	Yamazaki, 1972	
<i>Calliphora vicina</i> (larvae)	soluble	90	DOPA	ND	ND	1.33	Barrett and Andersen, 1981	
			MHQ	4.5	110 (b)	0.2		
			CAT	ND	ND	1.33		
			MC	ND	147 (b)	0.22		
			DOPAC	ND	ND	1.67		
			HQ	ND	ND	0.88		
			DA	ND	8	ND		
<i>Diptera punctata</i> (unsclerotized adults)	insoluble	ND	MHQ	ND	ND	ND	Barrett, 1987b	
<i>Drosophila melanogaster</i> (larvae)	soluble	ND	MHQ	ND	ND	ND	Andersen, 1985	
<i>Drosophila virilis</i> (unsclerotized pupae)	insoluble	ND	HQ	7.5	ND	ND	Yamazaki, 1969	
<i>Lucilia cuprina</i> (wandering larvae)	soluble	90	MHQ	4.5	132 (b)	ND	Barrett, 1987a	
<i>Lucilia sericata</i> (larvae)	soluble	ND	MHQ	5	ND	ND	Barrett 1987b and personal commun.	
<i>Manduca sexta</i> (unsclerotized pupae)	trypsin solubilized	250	MHQ	6	9220 (d)	0.021	This study	
	solubilized	70-90	DOPA	ND	230 (d)	8.7		
			NBAD	ND	4350 (d)	ND		
			NBANE	ND	465 (d)	ND		
			SYR	ND	155 (d)	ND		
			NBADG	ND	NR	ND		
			MHQ	ND	ND	ND		
<i>Musca domestica</i> (larvae)	soluble	ND	MHQ	ND	ND	ND	Barrett, 1987b	
<i>Oryctes rhinoceros</i> (larvae)	soluble	ND	MHQ	ND	ND	ND	Jeyaraj <i>et al.</i> , 1986	
			MC	7	ND	ND		
			HQ	ND	ND	ND		
			DA	ND	ND	ND		
			DOPA	ND	ND	ND		
			MHQ	ND	ND	ND		
<i>Papilio xuthus</i> (unsclerotized pupae)	insoluble	ND	MHQ	ND	ND	ND	Yamazaki, 1969	
<i>Phormia regina</i> (wandering larvae)	soluble	ND	MHQ	ND	ND	ND	Barrett, 1987b	
<i>Rhodnius prolixus</i> (larvae)	insoluble	ND	MHQ	ND	ND	ND	Barrett, 1987b	
<i>Sarcophaga bullata</i> (wandering larvae)	soluble	ND	MHQ	4.5	19 (b)	ND	Barrett, 1987b	
			DA	ND	6 (b)	ND		
			MC	ND	18 (b)	ND		
			MHQ	5	962 (c)	0.19		
			CAT	ND	ND	1.2		
			MC	ND	541 (b)	0.17		
<i>Schistocerca gregaria</i> (unsclerotized adult)	trypsin solubilized	90-100	DA	ND	ND	2.7	Andersen, 1978	
			HQ	ND	ND	0.93		
			NADA	ND	ND	1.3		
			NADA	6	ND	1.7		
	insoluble	ND	ND	MHQ	ND	ND	1.3	Andersen, 1979
				MHQ	ND	ND	1.7	
				MHQ	ND	ND	1.3	
				MHQ	ND	ND	ND	
<i>Tenebrio molitor</i> (larvae)	insoluble	ND	MHQ	ND	ND	ND	Barrett, 1987b	

*See Introduction section for list of abbreviations.

†Units for specific activity defined as: (a) $\mu\text{l oxygen h}^{-1}$, (b) 1.0 AU (265 nm) 10 min⁻¹, (c) 1.0 AU (250 nm) 10 min⁻¹ at 40 C, (d) nmol product formed min⁻¹.‡Determined by SDS-PAGE except for native molecular weight of *M. sexta* laccase determined by gel filtration HPLC.

catalyze the oxidation of catecholamines in cuticle to *o*-quinones and *p*-quinone methides which then react with nucleophilic groups of proteins and chitin to form covalent cross-links that lead to sclerotization.

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