

SOLUBLE TYROSINASES FROM PHARATE PUPAL INTEGUMENT OF THE TOBACCO HORNWORM, *MANDUCA SEXTA* (L.)

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Abstract—Two soluble phenoloxidases were partially purified from pharate pupal integument of *Manduca sexta* by gel filtration or sucrose density gradient centrifugation. Thiourea was used to retard the formation of higher molecular weight aggregates. Subsequent analysis by gel filtration HPLC showed that the apparent molecular weights were about 300 kD and >700 kD. The smaller phenoloxidase was further purified by anion exchange HPLC. In comparative studies with mushroom tyrosinase and a fungal laccase, the *Manduca* phenoloxidases were identified as tyrosinases, since they exhibited monophenol monooxygenase activity (EC 1.14.18.1) and catechol oxidase activity (EC 1.10.3.1). Both enzyme preparations catalyzed *ortho*-hydroxylation of tyrosine at a relatively slow rate, oxidized *o*-diphenols at a much faster rate than *p*-diphenols or monophenols, had broad pH optima from about pH 5.5–7.5, and were completely inhibited by 1 μ M phenylthiourea, *N*- β -alanyldopamine (NBAD) and *N*- β -alanyl norepinephrine (NBANE), which are both abundant in pupal cuticle, were oxidized to *o*-quinones by the tyrosinases, with the former catecholamine oxidizing at a 10-fold higher rate than the latter. NBANE was synthesized from NBAD, apparently by spontaneous tautomerization of the *o*-quinone to a *p*-quinone methide, which then reacted with water to form the β -hydroxylated product. The possible role of tyrosinase in insect integument is discussed.

Key Word Index: *Manduca sexta*, tyrosine, phenoloxidase, cuticle, integument, catecholamine, sclerotization, diphenol, quinone, DOPA, *N*- β -alanyldopamine, *N*- β -alanyl norepinephrine, β -hydroxylation, insect, tobacco hornworm

INTRODUCTION

Tyrosinases are widely distributed in nature, including many insect species (Brunet, 1980; Robb, 1984). They are multifunctional enzymes that catalyze both the hydroxylation of monophenols such as tyrosine to *o*-diphenols (EC 1.14.18.1, monophenol monooxygenase) and the oxidation of *o*-diphenols to *o*-

quinones (EC 1.10.3.1, catechol oxidase). Several functions for tyrosinases in insects have been suggested, including the generation of *o*-diphenols and quinones for use in pigmentation, wound healing, parasite encapsulation, and sclerotization, but the presence of other types of phenoloxidases such as the laccases and peroxidases makes the assignment of specific functions difficult (Andersen, 1985; Kramer and Hopkins, 1987; Sugumaran, 1988; Barrett, 1990; Hopkins and Kramer, 1990).

To better understand the enzymes that may be involved in cuticular tanning reactions, we have been focusing on the phenoloxidases in pharate pupal integument of the tobacco hornworm, *Manduca sexta*. Previously, we partially purified and characterized from the integument a mixture of soluble tyrosinases (Aso *et al.*, 1984) and a trypsin-solubilized laccase (Thomas *et al.*, 1989). In the present study, we have re-examined the soluble tyrosinases after preventing extensive aggregation by treatment with thiourea (Ashida and Yoshida, 1988). This treatment allows the partial purification of both low and high molecular weight *M. sexta* tyrosinases. We tested monophenolic, *o*-diphenolic, and *p*-diphenolic compounds as substrates for these enzymes and also for two commercially available fungal phenoloxidases, mushroom tyrosinase and *Pyricularia oryzae* laccase. The *Manduca* tyrosinases exhibit a high substrate preference for *N*- β -alanyldopamine (NBAD§), the

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§Abbreviations used: AMD, α -methyl DOPA; CO, carbon monoxide; CTAB, cetyl trimethylammonium bromide; DA, dopamine; DOPA, 3,4-dihydroxyphenylalanine; HPLC, high performance liquid chromatography; kD, kilodalton; MHQ, 2-methylhydroquinone; MOPS, morpholinopropane sulphonic acid; NADA, *N*-acetyl-dopamine; NBAD, *N*- β -alanyldopamine; NBANE, *N*- β -alanyl norepinephrine; NPD, 2,3-naphthalene diol; PHZ, phenylhydrazine; PTU, 1-phenyl-2-thiourea; SYR, syringaldazine or *N,N'*-bis-(3-5-dimethoxy-4-hydroxybenzylidene) hydrazine; Tris, 2-amino-2-hydroxymethyl-1,3-propanediol.

putative tanning precursor in pupal cuticle (Hopkins *et al.*, 1982, 1984; Morgan *et al.*, 1987). Oxidation of NBAD to its *o*-quinone can lead to the type of aromatic cross-link observed in pupal cuticle (Schaefer *et al.*, 1987). We also determined the amount of β -hydroxylation which occurs when NBAD is oxidized by tyrosinase.

MATERIALS AND METHODS

Insects and chemicals

M. sexta was reared as described by Bell and Joachim (1976) at 27°C with a photoperiod of 16 h light and 8 h dark. Chemicals were obtained commercially except for NBAD and NBANE, which were synthesized as described by Morgan *et al.* (1987). Mushroom tyrosinase and laccase from the fungus, *P. oryzae*, were obtained from Sigma Chemical Co. (St Louis, Mo.).

Enzyme purification

Integuments from brown bar stage pharate pupae were dissected and cleaned in 0.1 M Tris buffer containing 1 M sodium chloride, 20 mM calcium chloride, and 50 mM sodium ascorbate (pH 7.8), and then frozen at -70°C. Integuments (5.7 g) were ground finely in dry ice with a mortar and pestle and subsequently homogenized at 4°C in 75 ml of 0.1 M Tris buffer containing 1 M sodium chloride and 50 mM sodium ascorbate (pH 7.8). After centrifugation at 27,000 g for 20 min at 4°C, the supernatant was dialyzed against 12 mM thiourea in 0.1 M sodium phosphate buffer, pH 7. The dialysate was brought to 40% saturation with ammonium sulfate, and the resulting precipitate was dialyzed against 50 mM MOPS buffer containing 12 mM thiourea (pH 7). After centrifugation the supernatant (designated as supernatant A) was collected. The remaining precipitate was dialyzed against 0.1 M Tris buffer containing 1 M sodium chloride (pH 7.8), and this supernatant (designated as B) was collected after centrifugation.

Supernatant A was gel filtered on a column of Sephacryl S-400 (75 × 2.5 cm) in 50 mM MOPS (pH 7), and supernatant B was gel filtered in 0.1 M Tris buffer containing 1 M NaCl (pH 7.8). Aliquots of the fractions with the highest DOPA oxidase activity (see Enzyme assays section) were analyzed by density gradient centrifugation in 10–40% sucrose to determine the sedimentation coefficients. The DOPA oxidase that eluted in the void volume of the Sephacryl column was centrifuged with a Beckman SW 27 rotor at 26,000 rpm for 5 h at 4°C, and a later eluting DOPA oxidase fraction was centrifuged with a VTi 50 rotor at 50,000 rpm. Density gradient centrifugation was sometimes used as an alternative purification step to gel filtration for supernatant A. After storage at -20°C, the later-eluting fraction from the Sephacryl column was further purified by anion exchange HPLC using a Synchrom AX-300 column (4.6 mm × 25 cm) with a 0–0.5 M sodium chloride gradient in a 25 mM Tris buffer (pH 7.5).

Purity of the enzyme fractions was determined by non-denaturing polyacrylamide gel electrophoresis according to the procedure of Davis (1964) using a Bio-Rad Protean II slab gel apparatus. Gels were stained for protein with Coomassie blue R-250 or for enzymatic activity with substrate (5 mM DOPA). DOPA was dissolved in 0.1 M sodium phosphate buffer at pH 6, and 100 μ l was incubated with each gel. The active fractions from Sephacryl gel filtration were subjected to gel filtration HPLC to determine apparent molecular weights. A TSK 4000 column (7 mm × 50 cm) was used with 25 mM Tris buffer containing 0.1 M sodium chloride (pH 7.5). The protein standards were aprotinin, 6.5 kD, inclusion volume marker; bovine serum albumin, 66 kD; alcohol dehydrogenase, 150 kD; β -amylase, 200 kD; apoferritin, 443 kD; thyroglobulin 669 kD; and DNA, > 1000 kD, void volume marker.

Enzyme assays

Activity was assayed routinely in 0.1 M sodium phosphate buffer (pH 6) at 25°C with 0.3 mM substrate except for syringaldazine, which was used at 10 μ M because of low solubility. For the determination of pH optima, 0.1 M disodium phosphate was adjusted to the desired pH with 0.1 M citric acid. Enzyme kinetics were calculated by the Lineweaver–Burk method (1934) for Michaelis constant and maximum velocity determinations. Protein was determined by the Coomassie blue dye binding assay, using bovine serum albumin as the standard protein (Bradford, 1976). A Cary model 118 spectrophotometer was used for continuous monitoring of product formation. Products were quantified using extinction coefficients of 3600 M⁻¹ cm⁻¹ at 475 nm for dopachrome; 1100 at 390 nm for the *o*-quinones of NBAD, NBANE, and NADA; 65,000 at 525 nm for the *p*-quinone methide of syringaldazine (SYR); and 21,000 at 250 nm for methyl-*p*-benzoquinone. The quantities of dopachrome were multiplied by two for the calculation of specific activity with DOPA, since two sequential oxidations occur during conversion of DOPA to dopachrome (Korner and Pawelek, 1982).

HPLC was used for the quantitation of *o*-hydroxylation of tyrosine. Ten minutes after the addition of enzyme to 0.3 mM tyrosine containing either 0 or 60 μ M dopamine (DA), a 0.1 ml aliquot was added to 50 μ l of 0.9 M perchloric acid containing 0.5 nmol of α -methyl DOPA (AMD). The supernatant was analyzed by reversed phase HPLC with amperometric detection using a 26% acetonitrile mobile phase with dodecyl sulfate as the ion pair (Morgan *et al.*, 1987). The *o*-hydroxylated product, DOPA, was well resolved from AMD and the β -hydroxylated product of DA, norepinephrine, but care was needed to avoid poor resolution between DOPA and an unknown product of DA oxidation.

HPLC was used to quantify the consumption of NBAD and the amount of β -hydroxylation. After incubation of NBAD with the enzyme for 10 min, aliquots (10 μ l) were added to methanol (90 μ l) containing a reducing agent (50 mM ascorbic acid) or a nucleophile (0.8 M benzene sulfinate). Benzene sulfinate reacted with NBAD-*o*-quinone. The reaction of benzene sulfinate with NBAD-*o*-quinone prevented any spontaneous reduction of the quinone back to NBAD and allowed accurate quantification of NBAD (Morgan *et al.*, unpublished data). Ascorbic acid caused quantitative reduction of NBANE-*o*-quinone back to NBANE and provided an estimate of the total amount of β -hydroxylated product. The samples were left at 5°C for several hours and then 0.5 nmol of AMD was added as an internal standard in 50 μ l of 10 mM HCl. The *o*-diphenols were recovered from alumina essentially as described by Murdock and Omar (1981). HPLC was performed under the same conditions that were used for analysis of DOPA.

Inhibitors were added to the reaction mixtures just before addition of the enzyme except for phenylhydrazine (PHZ), a noncompetitive inhibitor, which was incubated with enzyme solution for 1 h prior to assay. 15 cm³ of CO was stirred with 5 ml of reaction mixture in a 20 ml glass vial sealed with a septum for 1 h prior to addition of enzyme. Nitrogen was used instead of CO for the control. The inhibitor constants were calculated using the Hunter–Downs method (Hunter and Downs, 1945; Webb, 1963).

RESULTS

Enzyme purification

The crude extract from prepupal integument of *M. sexta* was dialyzed against 12 mM thiourea and then subjected to ammonium sulfate fractionation. After further dialysis, the phenoloxidases were separated by gel filtration or by density gradient centrifugation. The peak DOPA oxidase activity fraction from

the gel filtration of supernatant A [Fig. 1(A), see Materials and Methods for details] sedimented as a clearly defined peak at about 14 Svedberg units during the density gradient centrifugation [Fig. 1(B)]. This preparation will be referred to as enzyme I. The peak DOPA oxidase activity fraction from gel filtration of supernatant B [Fig. 1(C)] was also subjected to density gradient centrifugation, but the oxidase activity was not well resolved, indicating that a mixture of different sized enzymes was present. However, most of the activity had sedimentation coefficients greater than 100 S. This preparation will be referred to as enzyme II.

The use of thiourea during dialysis of the crude integumental extract inhibited aggregation of the enzymes and allowed partial purification of enzyme I as well as the more aggregated enzyme II. Coomassie blue staining after nondenaturing electrophoresis of enzyme I revealed more than one band of protein, but staining with the substrate, DOPA, showed only one band of DOPA oxidase. Further purification of enzyme I by anion exchange HPLC resulted in a specific activity of $2940 \text{ nmol min}^{-1} \text{ mg}^{-1}$ for the oxidation of DOPA. This more highly purified preparation will be referred to as enzyme Ia. Nondenaturing electrophoresis of preparation II

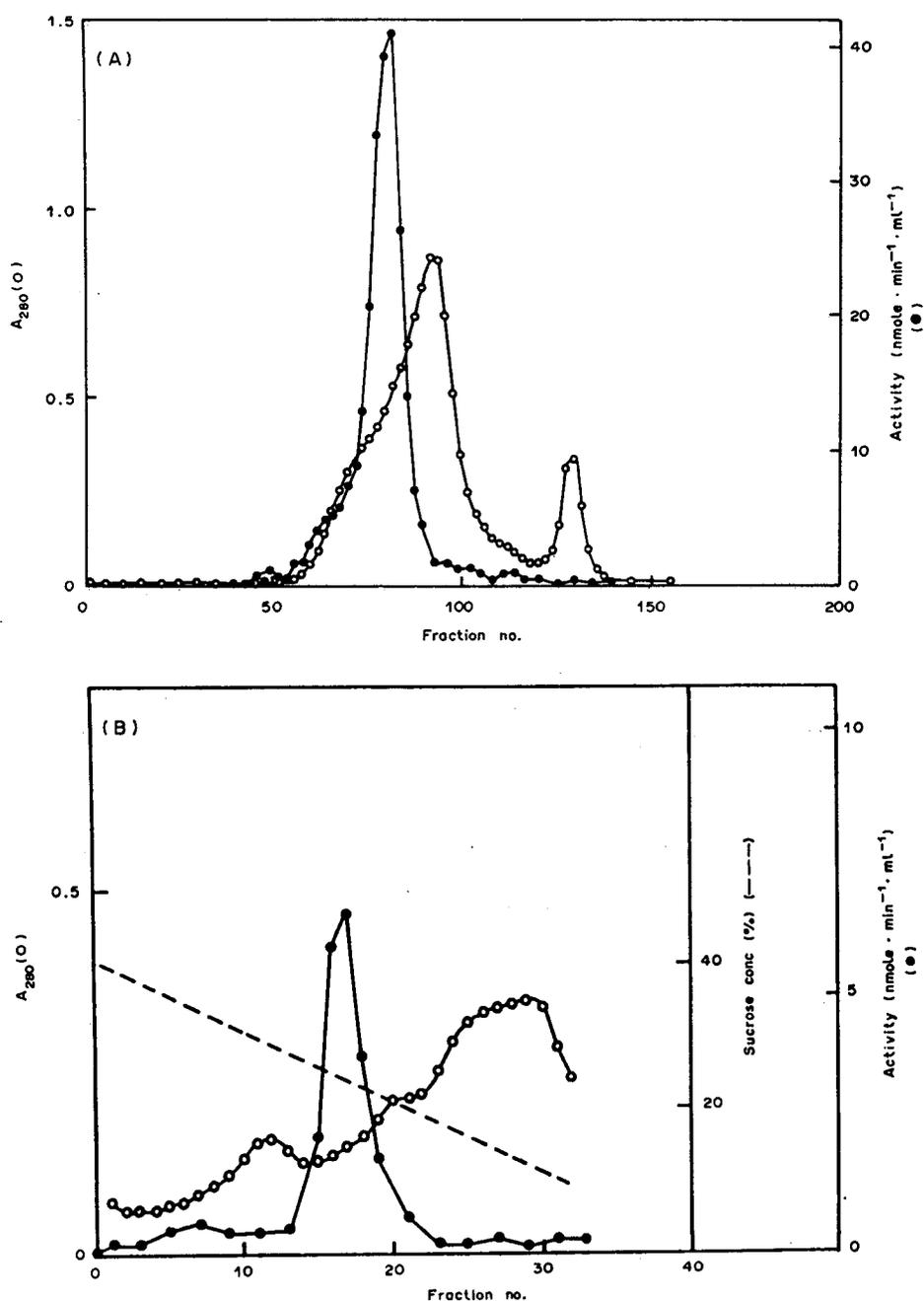


Fig. 1. (A) and (B) caption overleaf.

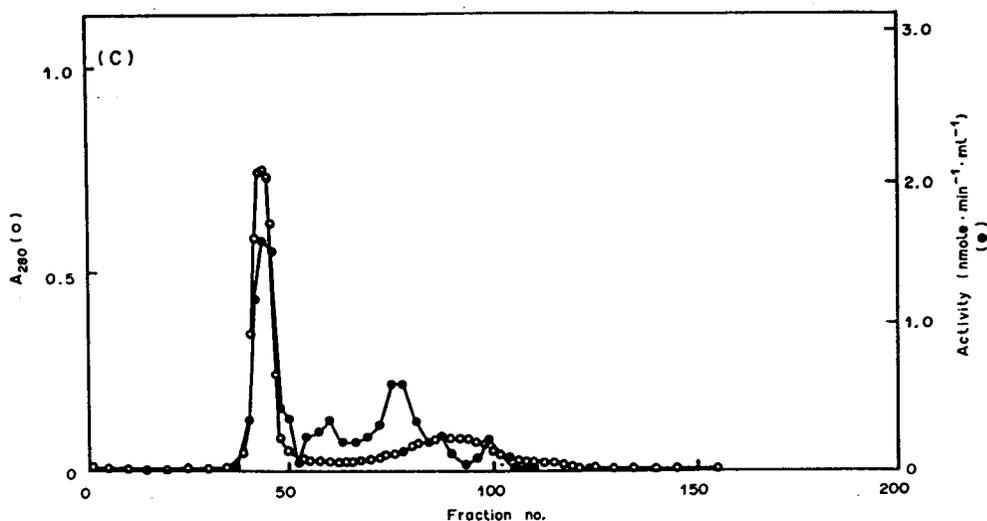


Fig. 1(C)

Fig. 1. Partial purification of tyrosinases from the pharate pupal integument of *Manduca sexta* by gel filtration and by ultracentrifugation. (A) Supernatant A (see Materials and Methods) was gel filtered on Sephacryl S-400 in 50 mM MOPS buffer (pH 7). (B) The fraction with maximum DOPA oxidase activity from the gel filtration of supernatant A was centrifuged in a 10–40% sucrose gradient giving a peak of activity with a sedimentation coefficient of about 14 Svedberg units. (C) Supernatant B was gel filtered on Sephacryl S-400 in 0.1 M Tris buffer with 1 M NaCl (pH 7.8). Solid circles represent oxidase activity ($\text{nmol min}^{-1} \text{ml}^{-1}$) with 3 mM DOPA as substrate. Open circles represent absorbance at 280 nm, which is an indication of protein concentration. The dashed line represents the sucrose gradient.

demonstrated that the more aggregated enzymes in this preparation did not penetrate the 5% polyacrylamide gel. Analysis of enzymes I and II by gel filtration HPLC indicated that the native molecular weights were $300 \pm 25 \text{ kD}$ and $> 700 \text{ kD}$, respectively.

pH dependence

The pH optima for the initial rate of oxidation of *N*-acetyldopamine (NADA) to NADA-*o*-quinone by *Manduca* enzymes I and II were very similar, with over 80% of the maximal activity present between pH 5.5 and 7.5 (Fig. 2). At pH 6, the initial rate of NADA-*o*-quinone production was $483 \pm 14 \text{ nmol min}^{-1} \text{mg}^{-1}$ for enzyme I and $573 \pm 46 \text{ nmol min}^{-1} \text{mg}^{-1}$ for enzyme II. The effect of pH on the oxidation of DOPA to dopachrome by the *Manduca* enzymes also was examined between pH 4 and 7, and over

60% of maximal activity occurred between pH 5 and 7. A pH of 6.0 was used for subsequent experiments.

Substrate specificity

The relative velocities of diphenol oxidation were determined for enzymes I, Ia, and II from *Manduca*, as well as for mushroom tyrosinase and a fungal laccase (Table 1). NBAD was preferred as a substrate over the other *o*-diphenols, NBANE and DOPA, by the phenoloxidases from both insects and fungi. With the *Manduca* enzymes, NBAD was oxidized at approximately a 10-fold higher rate than was NBANE or DOPA. Kinetic analysis of DOPA oxidation yielded values of 2.2 mM for the Michaelis constant (K_m) when using enzyme I and 1.0 mM when using enzyme II (Table 2). The V_{max}/K_m ratios for enzymes I and II were similar.

Syringaldazine (SYR) and methylhydroquinone (MHQ) were tested as substrates to determine whether the soluble phenoloxidases from *M. sexta* were laccases. SYR or *N,N'*-bis-(3,5-dimethoxy-4-hydroxybenzylidene) hydrazine is a compound containing two monophenol moieties. It is not oxidized by tyrosinases, but it is oxidized to a bis-*p*-quinone methide by most laccases and peroxidases (Harkin and Obst, 1973; Joel *et al.*, 1978). Enzymes I and II and mushroom tyrosinase did not oxidize SYR, but SYR was oxidized at a high rate by the fungal laccase (Table 1). MHQ, a *p*-diphenol, was oxidized at a relatively low rate by *Manduca* enzymes I, Ia and II and mushroom tyrosinase, whereas it was the best substrate tested for fungal laccase. The ratio of the velocities for the MHQ and DOPA oxidations ranged from 0.05 to 0.12 for the *Manduca* tyrosinases. *p*-Diphenols usually are not considered to be substrates for tyrosinases (Robb, 1984). Since tyrosinases

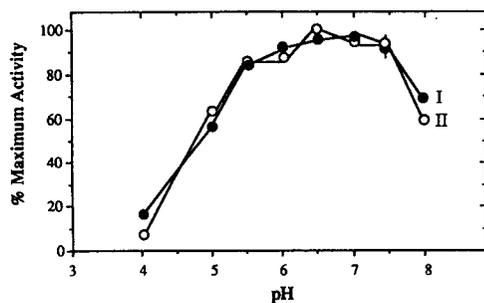


Fig. 2. The effect of pH on the oxidation of NADA to an *o*-quinone by tyrosinases I and II from *Manduca sexta* in 0.1 M citrate-phosphate buffer. Values are means \pm SEM ($n = 3$) for percent activity relative to the activity at the optimal pH.

Table 1. Specific activities of substrate oxidation (nmol min⁻¹ mg⁻¹) catalyzed by *Manduca* tyrosinases, mushroom tyrosinase, and fungal laccase*

Substrate	<i>M. sexta</i> tyrosinases			Mushroom tyrosinase	Fungal laccase
	I	Ia	II		
NBAD	1500 ± 104	ND	980 ± 9	10,200 ± 300	2100 ± 00
NBANE	155 ± 9	ND	107 ± 4	1669 ± 201	764 ± 38
DOPA	116 ± 6	2940 ± 148	92 ± 8	7200 ± 30	756 ± 30
SYR	0	ND	0	0	1150 ± 50
MHQ	6 ± 1	294 ± 15	11 ± 1	43 ± 2	4140 ± 230
TYR	<0.1	ND	0.1	772-885	<0.1
TYR + DA	6.4-7.2	ND	6.8-7.2	743-50	<0.1

*Mean values ± SEM ($n = 3$) or range of the values ($n = 2$). *o*-Quinones were measured for NBAD and NBANE, dopachrome for DOPA, *p*-quinone methide for SYR, *p*-quinone for MHQ, and DOPA for Tyr and Tyr + DA. The dopachrome values were multiplied by 2 to reflect the fact that two sequential oxidations occur during the conversion of DOPA to this product. Substrate concentration was 0.3 mM except for SYR, which was 10 μM. Tyr + DA contained 60 μM DA. See Introduction for list of abbreviations. ND, not determined.

and laccases contain copper(II) ions, which can act as an oxidizing agent even in the absence of an enzyme (Robb, 1984; Reinhammar, 1984; Miranda *et al.*, 1988), we compared the rates of enzymatic oxidation of MHQ and NBAD with the corresponding non-enzymatic rates of oxidation by copper(II). With 20 μM copper(II) ions, MHQ was oxidized at 0.4 nmol ml⁻¹ min⁻¹, and NBAD was oxidized at <0.1 nmol ml⁻¹ min⁻¹. These results show that MHQ is more easily oxidized than NBAD by copper and suggest that the preference of the *Manduca* enzymes and mushroom tyrosinase for NBAD over MHQ is a result of the preference of their active sites for *o*-diphenolic over *p*-diphenolic substrates.

Since the *Manduca* enzymes had substrate preferences that closely resembled that of mushroom tyrosinase, we examined their ability to hydroxylate tyrosine. Initially, sodium ascorbate was included in the incubations to preserve the initial product DOPA (which is also a substrate) by reducing DOPA-*o*-quinone back to DOPA. However, we found that DOPA synthesis occurred even in the absence of enzyme, when 50 mM ascorbate was present. Although the rate of nonenzymatic hydroxylation was slow (70 pmol ml⁻¹ min⁻¹ during the first 10 min), it nonetheless introduced a potential error that was unacceptable in our experiments because of the low hydroxylation rates of the *Manduca* enzymes. Little, if any, DOPA accumulated during incubation of tyrosine with the *Manduca* phenoloxidases in the absence of reducing agent during the first 10 min (or even the first hour), whereas about 800 nmol DOPA min⁻¹ mg⁻¹ accumulated during a 10 min incubation with mushroom tyrosinase (Table 1). However, when 6 μM DA was added as a reducing agent to an incubation of tyrosine with 15 μg of enzyme I per ml, 30-34 pmol of DOPA ml⁻¹ min⁻¹ accumulated, and 98-109 pmol ml⁻¹ min⁻¹ accumulated when 60 μM DA was added. An estimate of the hydroxylation rate with 600 μM DA present was 169 pmol ml⁻¹ min⁻¹,

but the higher level of DA made the quantitation of DOPA difficult. Enzymes I and II synthesized DOPA at similar rates in the presence of 60 μM DA, but these rates were about 100-fold lower than that of mushroom tyrosinase (Table 1). The presence of DA had little effect on DOPA accumulation during the 10 min incubation with mushroom tyrosinase. The fungal laccase failed to synthesize DOPA, regardless of the presence of DA, although DA was oxidized gradually. Chemical oxidation of DA by sodium periodate in the presence of tyrosine also did not result in DOPA synthesis. These results show that oxidation of DA does not cause nonenzymatic hydroxylation of tyrosine and that *o*-hydroxylation of tyrosine is a catalytic function of enzymes I and II. Since the *Manduca* enzymes hydroxylate tyrosine and oxidize *o*-diphenols but not *p*-diphenols at a high rate, they are classified as tyrosinases.

β-Hydroxylation of NBAD

β-Hydroxylation of NBAD was studied using phenoloxidase preparations having specific activities different from those used earlier to determine substrate specificity. After 10 min of incubation of the *Manduca* tyrosinases with NBAD, the yield of NBANE plus NBANE-*o*-quinone accounted for 16 and 23% of the consumption of NBAD by tyrosinases I and II, respectively (Table 3). With mushroom tyrosinase, total NBANE accounted for 9% of the substrate consumption, and 16% was accounted for by NBANE during the incubations with fungal laccase. These results show that β-hydroxylation is a quantitatively important pathway during the oxidation of catecholamines by tyrosinase or laccase, including the tyrosinases from *Manduca*.

Inhibition studies

Several compounds were tested as inhibitors because they had been reported to inhibit selectively

Table 2. Kinetic parameters for oxidation of DOPA by tyrosinases from the pharate pupal integument of *Manduca sexta**

Tyrosinase	K_m (μM)	V_{max} (nmol min ⁻¹ mg ⁻¹)	V_{max}/K_m	K_i for NPD (mM)	K_i for PTU (μM)
I	1020 ± 50	127 ± 9	0.17	29 ± 1	0.5 ± 0.1
II	2200 ± 300	667 ± 35	0.30	36 ± 2	0.5 ± 0.1

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

Table 3. Synthesis of *N*- β -alanyl norepinephrine during incubation of *N*- β -alanyldopamine with *Manduca* tyrosinases, mushroom tyrosinase, and fungal laccase*

Enzyme	Activity (nmol NBANE min ⁻¹ mg ⁻¹)	% NBAD turnover found as NBANE
Tyrosinase I	267 \pm 57	16 \pm 3
Tyrosinase II	336 \pm 7	23 \pm 1
Mushroom tyrosinase	543 \pm 33	9 \pm 1
Fungal laccase	28 \pm 2	16 \pm 3

*Mean values \pm SEM ($n = 3$ or 4). NBANE values are an estimate of total NBANE accumulation (NBANE plus NBANE-*o*-quinone).

either tyrosinases or laccases. Insect tyrosinases are more sensitive to inhibition by phenylthiourea (PTU) than are insect laccases (Barrett and Andersen, 1981; Barrett, 1987a,b). *Manduca* tyrosinases and mushroom tyrosinase were inhibited by PTU even at the 100 nM level, although fungal laccase was not inhibited by 10 μ M PTU (Table 4). The inhibition constants (K_i) for both *Manduca* tyrosinases were 0.5 nM (Table 2). 2,3-Naphthalene diol (NPD) is a competitive inhibitor of plant tyrosinases and phenylhydrazine (PHZ) is a noncompetitive inhibitor, but neither of those compounds inhibits plant laccases (Mayer and Harel, 1979). NPD and PHZ partially inhibited the *Manduca* tyrosinases and mushroom tyrosinase, but they had little effect on fungal laccase (Table 4). The K_i s for NPD with *Manduca* tyrosinases I and II by NPD were 29 and 36 mM, respectively (Table 2). Cetyl trimethylammonium bromide (CTAB) is a cationic detergent, which reportedly inhibits plant and fungal laccases but not tyrosinases (Walker, 1968; Walker and McCallion, 1980). The detergent had little effect on the *Manduca* tyrosinases at the concentration tested, although both types of fungal phenoloxidases were inhibited partially. Carbon monoxide is an inhibitor of tyrosinases but not laccases (Walker, 1968; Mayer and Harel, 1979; Robb, 1984). Although the mushroom tyrosinase was inhibited by CO, the *Manduca* tyrosinases were uninhibited or only slightly inhibited (Table 4). For the data present in Table 4, CO was preincubated with substrate, and the reaction was initiated by addition of enzyme. When CO was preincubated with *Manduca* tyrosinase I or mushroom tyrosinase and the reaction was started by addition of substrate, similar results were obtained. Other than the lack of an effect of CO, the effects of inhibitors on the *Manduca* phenoloxidases are consistent with their classification as tyrosinases.

DISCUSSION

Two phenoloxidase preparations that differ in molecular weight, tyrosinase I (MW_{app} \approx 300 kD) and tyrosinase II (> 700 kD), have been purified partially from pharate pupal integument of *M. sexta*. Previously, a partially purified tyrosinase preparation from this integument was characterized, but it consisted of a mixture of tyrosinases with a wide range of sizes (Aso *et al.*, 1984). One of the tyrosinases in that preparation was similar in electrophoretic mobility to tyrosinase I, whereas the more aggregated tyrosinases did not penetrate the polyacrylamide gel during electrophoresis, as we observed with tyrosinase II. *M. sexta* tyrosinase I is similar in size to *Musca domestica* phenoloxidase (Hara *et al.*, 1989).

In a previous study, we purified a protyrosinase (MW_{app} \approx 150 kD) from *M. sexta* hemolymph that, after activation, has properties similar to those of the integumental tyrosinases (Aso *et al.*, 1985). Whether the hemolymph proenzyme is the precursor for the integumental enzymes has not been determined. Insect protyrosinases have a common property, i.e. activation leads to formation of large aggregates (Karlson *et al.*, 1964; Ashida and Dohke, 1980). In a study by Aso *et al.* (1984), the larger tyrosinases increased in abundance as the sample aged prior to analysis by electrophoresis. The larger tyrosinase preparation II may consist of subunits of the smaller tyrosinase I, because the two preparations had similar pH optima, kinetic properties, substrate specificities, and sensitivities to inhibition. We used thiourea in this study to retard aggregation and increase the relative abundance of the smaller tyrosinase. Thiourea was used previously to prevent the aggregation of tyrosinase from *B. mori* (Ashida and Yoshida, 1988). Those authors suggested that the inhibition of enzyme aggregation resulted from chelation of the metal ion in the active site by thiourea. Since thiourea is also a strong nucleophile, inhibition also could involve reaction of thiourea with side chain residues of the enzyme, which are normally involved in the aggregation mechanism. After unbound thiourea was removed by dialysis, the *M. sexta* enzymes had catalytic properties typical of tyrosinases, and they were also similar, but not identical, to the tyrosinases from *Manduca* pharate pupal integument characterized by Aso *et al.* (1984), which had not been treated with thiourea. The K_m for DOPA was estimated to be 8 mM for the latter preparation, whereas K_m values were 1 and 2 mM for tyrosinases I and II, respectively. The pH optimum of tyrosinases I and II with NADA or DOPA as substrate was

Table 4. % Inhibition of dopachrome synthesis from DOPA by *Manduca* tyrosinases, mushroom tyrosinase, and fungal laccase*

Inhibitor	Conc. (μ M)	<i>Manduca</i> tyrosinases		Fungal enzymes	
		I	II	Tyrosinase	Laccase
PTU	0.1	100	55 \pm 1	68 \pm 5	0
	1	100	100	10	0
	10	100	100	100	0
NPD	100	38 \pm 1	42 \pm 3	61 \pm 5	0
PHZ	50	34 \pm 2	32 \pm 1	35 \pm 1	7 \pm 1
CTAB	100	0	4 \pm 1	17 \pm 1	25 \pm 1
CO	—	18 \pm 2	0	69 \pm 6	0

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

broad, ranging from pH 5.5 to 7.5, whereas that of the tyrosinase preparation of Aso *et al.* (1984) was narrower and had a maximum activity of pH 6 when DOPA was the substrate.

The substrate specificity of the soluble phenol-oxidases from the prepupal integument of *M. sexta* was consistent with their being classified as tyrosinases. They oxidized *o*-diphenols but not *p*-diphenols at a high rate and also hydroxylated tyrosine. A laccase that was solubilized from prepupal integument of *M. sexta* by treatment with trypsin also has been investigated in our laboratory (Thomas *et al.*, 1989). This enzyme oxidized both *p*-diphenols and *o*-diphenols at a high rate, but it did not hydroxylate tyrosine. The *Manduca* laccase also oxidized a specific laccase substrate, SYR, which was not oxidized by the *Manduca* tyrosinases.

DOPA accumulated at similar rates when tyrosine was incubated in the presence of DA with either tyrosinase I or II from *M. sexta*. This result differs from an earlier report that a highly aggregated tyrosinase from the hemolymph of *B. mori* had more tyrosine hydroxylase activity than did a less aggregated tyrosinase (Ashida and Dohke, 1980). Almost no DOPA accumulated in the first 10 min, when DA was not included in the incubations with tyrosinase I or II. A diphenol or some other reducing agent is required to reduce the copper ions of tyrosinase to the proper valence state for acceptance of the oxygen that is subsequently used for hydroxylation (Cabanes *et al.*, 1987). A delay or lag period is usually observed until sufficient diphenol is generated to reduce the metal ion. An exogenous reducing agent will shorten the lag period as will a high enzyme concentration, because the enzymatically produced diphenol accumulates more rapidly to an effective concentration in the latter case (Pau and Kelly, 1975; Cabanes *et al.*, 1987). Exogenous DA had little effect on the amount of DOPA present at 10 min in the incubations of tyrosine with mushroom tyrosinase, probably because of the high monophenolase activity of this enzyme. Previously, we had observed significant rates of tyrosine hydroxylation catalyzed by tyrosinase from *M. sexta* integument in the absence of exogenous diphenol, but at a substrate concentration 10-fold higher than used in the present study (Aso *et al.*, 1984). If the K_m for tyrosine is relatively high with the *Manduca* tyrosinases, then DOPA would be synthesized more rapidly at higher tyrosine concentrations. Therefore, hydroxylation of substrate would accelerate quickly under these conditions as catalytic amounts of DOPA were produced. Further study is needed to assess the full potential of the *Manduca* tyrosinases for tyrosine hydroxylation. Tyrosinases I and II were tested with 0.3 mM tyrosine, whereas tyrosine concentration in the hemolymph reaches about 20 mM in the prepupal stage (Ahmed *et al.*, 1983). The relatively low hydroxylating activity of the *Manduca* integumental tyrosinases relative to that of mushroom tyrosinase is consistent with the reported trend of higher hydroxylating activity by enzymes from more primitive organisms (Mayer and Harel, 1979).

In addition to substrate specificity, the tyrosinases from *M. sexta* were differentiated from a commercially available fungal laccase by their sensitivity

to inhibition by PTU, NPD and PHZ. PTU was the most potent inhibitor with a K_i of about 0.5 nM. PTU also is selective for the *Manduca* tyrosinases vs the trypsin-solubilized laccase from *Manduca* (Thomas *et al.*, 1989). In general, insect tyrosinases are more sensitive to PTU than are insect laccases (Barrett, 1987a).

NBAD was the preferred *o*-diphenolic substrate of those tested with the *Manduca* tyrosinases and surprisingly also for mushroom tyrosinases and fungal laccase. We also found NBAD to be the preferred substrate for the laccase in pharate pupal integument of *M. sexta* (Thomas *et al.*, 1989), and Hara *et al.* (1989) reported that it is the best substrate tested for house fly phenoloxidase. NBAD appears to be the most important precursor for tanning agents of *M. sexta* pupal cuticle (Hopkins *et al.*, 1982, 1984; Grun and Peter, 1984). NBAD and its cuticular metabolite, NBANE, are the major *o*-diphenols detected in methanolic and cold acidic extracts from cuticle that is just beginning to tan (Morgan *et al.*, 1987). However, NBAD is oxidized at a 10-fold higher rate than is NBANE by the tyrosinases from *Manduca* integument. This was also true for a trypsin-solubilized laccase from *M. sexta* integument (Thomas *et al.*, 1989). Large amounts of acid-extractable NBANE accumulate in pupal cuticle during the early stages of sclerotization (Morgan *et al.*, 1987), and this level could be influenced by the lower oxidation rate of NBANE relative to NBAD. However, much of the acid-extractable NBANE may be bound tightly or linked covalently to the cuticle and unavailable to the phenoloxidases (Morgan *et al.*, 1987). It is possible that the acid-extractable NBANE is derived hydrolytically from an acid-labile adduct, which consists of NBAD with a covalent bond between its β -carbon and a nucleophile other than water. NBANE and β -adducts of NBAD may be quantitatively important products of NBAD metabolism in the cuticle. Whether NBANE is important as a substrate for oxidation by phenoloxidases in cuticle and, therefore, involved in quinone tanning is unknown. However, NBANE, as well as its precursor NBAD, were found to stimulate pupal cuticle tanning *in vitro* to a much greater degree than other catecholamines, as scored by coloration changes (Roseland *et al.*, 1986).

Modification of the β -carbon of NBAD is an important step in the metabolism of NBAD in *M. sexta* pupal cuticle, as demonstrated by both *in vivo* and *in vitro* observations (Morgan *et al.*, 1987). In this study, we have observed that tyrosinase catalysis can cause NBANE synthesis. During incubation of NBAD with *Manduca* tyrosinases for 10 min, the synthesis of NBANE accounted for about 20% of the substrate turnover. Under the same conditions, however, NBANE accounted for over 40% of the NBAD turnover by *Manduca* laccase (Thomas *et al.*, 1989). This latter result corresponds well with the yield of NBANE that was obtained during incubations of NBAD with insoluble cuticular preparations from young pupae (Morgan *et al.*, 1987) and also suggests that the laccase may be more important physiologically than tyrosinase for side chain cross-linking (Thomas *et al.*, 1989).

Differential rates of β -hydroxylation of NADA by insect and mushroom tyrosinases have been reported previously (Peter, 1980). The yield of NANE was about 3% with mushroom tyrosinase, whereas a crude preparation of *M. sexta* tanning pupal cuticle yielded about 10% NANE under similar conditions. In the present study, approx. 9% of the NBAD turnover by mushroom tyrosinase was accounted for by NBANE (yield = 5%), whereas NBANE accounted for 20% of the substrate turnover by the *M. sexta* tyrosinases (yield = 14%). Therefore, these studies suggest that the insect integumental phenoloxidases may be more efficacious than the mushroom tyrosinase in generating side chain oxidation products from *N*-acyl catecholamines.

The *p*-quinone methide is an intermediate which may be involved in β -hydroxylation and also β -sclerotization in insect cuticle (Lipke *et al.*, 1983; Sugumaran and Lipke, 1983; Sugumaran, 1987, 1988). β -Hydroxylation of *N*-acyldopamines during incubation with phenoloxidases can result from tautomerization of the *o*-quinone to a *p*-quinone methide followed by addition of water to form a racemic mixture of the product (Peter, 1980; Peter and Vaupel, 1985). We have found that the chemical oxidation of NBAD by sodium periodate to form an *o*-quinone also results in β -hydroxylation, and nucleophiles such as methanol compete with water for reaction with the β -carbon (Morgan *et al.*, 1988 and unpublished data). β -Methoxy NBAD has been identified as a product of this reaction.

An *o*-quinone; *p*-quinone methide isomerase that catalyzes the conversion of NADA-*o*-quinone to NADA-*p*-quinone methide has been detected in pupal cuticle of *M. sexta* (Saul and Sugumaran, 1988). β -Hydroxylation could be the result of both enzymatic and non enzymatic conversions of *o*-quinones to *p*-quinone methides. It also has been proposed that mushroom tyrosinase and insect phenoloxidases catalyze novel oxidations that yield *p*-quinone methides directly from *o*-diphenolic substrates (Sugumaran and Lipke, 1983; Sugumaran, 1986; Sugumaran *et al.*, 1988). In contrast to that proposal, evidence has been obtained demonstrating that mushroom tyrosinase forms only *o*-quinone derivatives which, for certain substrates, are very unstable and subsequently undergo nonenzymatic reactions that generate novel products (Ortiz *et al.*, 1988; Cabanes *et al.*, 1988). Our results are consistent with the latter interpretation, in that only *o*-quinones are produced by both *M. sexta* and mushroom tyrosinases when catecholamine substrates are used and that subsequent nonenzymatic reactions lead to β -hydroxylated products.

Tyrosinases in general have several putative functions in insects. They may be involved in hydroxylation of tyrosine for the synthesis of catecholamines, but this has not been demonstrated yet *in vivo* (Kramer and Hopkins, 1987). Hemolymph contains a protyrosinase (Aso *et al.*, 1985; Saul and Sugumaran, 1986), and activated hemolymph tyrosinase may be responsible for the hydroxylation of tyrosine to form the DOPA, which is used not only for melanization reactions, but also for the synthesis of catecholamines and other diphenols that are transported into cuticle (Lackie, 1988; Hopkins and

Kramer, 1990). The tyrosinase present in larval cuticle of *Calpodex ethlius* has been implicated in wound healing, and preliminary evidence suggests that a proenzyme may be present in the larval cuticle of several species (Lai-Fook, 1966; Barrett 1984, 1987a, b, 1989; Andersen, 1985). Integumental tyrosinase may be involved in melanization of the cuticle, but this function also has not been adequately demonstrated. Dennell (1958) was able to prevent the formation of black stripes in the puparial cuticle of blowflies *in vivo* with doses of PTU that had no effect on cuticular sclerotization. Hiruma and Riddiford (1988) have isolated a phenoloxidase that is transported into specific regions of the larval cuticle and utilized for melanin synthesis. In some species of insects, such as *Schistocerca gregaria*, *Sarcophaga bullata*, and *Lucilia cuprina*, the time period in which laccase is active in the cuticle is more correlated with cuticular sclerotization than is the presence of tyrosinase (Andersen 1985; Barrett 1987a, b; Binnington and Barrett, 1988). However, we have found both tyrosinase and laccase in pharate pupal integument of *M. sexta* that is undergoing sclerotization (Aso *et al.*, 1984; Thomas *et al.*, 1989; this study). These phenoloxidases rapidly oxidize NBAD *in vitro*, and the products include NBAD-*o*-quinone and NBANE. NBAD-*o*-quinone and its tautomeric isomer, NBAD-*p*-quinone methide, which are putative sclerotization agents for the pupal cuticle of *M. sexta*. Further work is needed to clarify the localization within the integument and the physiological roles of these phenoloxidases.

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