

PROPERTIES OF TYROSINASE AND DOPA QUINONE IMINE CONVERSION FACTOR FROM PHARATE PUPAL CUTICLE OF *MANDUCA SEXTA* L.

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Abstract—Tyrosinase was partially purified from tobacco hornworm [*Manduca sexta* (L.)] pharate pupal cuticle by ammonium sulphate precipitation and anion-exchange chromatography. The enzyme hydroxylated tyrosine and tyramine to dihydroxyphenylalanine (DOPA) and dopamine respectively, and also oxidized DOPA, dopamine and other catecholamines including *N*-acetyldopamine (NADA) and *N*- β -alanyldopamine (NBAD) to *o*-quinone and *p*-quinone imine products. 5,6-Dihydroxyindole (DHI) was also dehydrogenated by tyrosinase to indole quinone. The order of substrate preference was NBAD > NADA > dopamine > DHI > DOPA. Maximum activity occurred at pH 6. Catalytic amounts of DOPA stimulated the hydroxylation of tyrosine and tyramine. Tyrosinase inhibitors were diethyl-dithiocarbamate, phenylthiourea, cyanide and benzoic acid. DOPA quinone imine conversion factor (QICF), a heat-labile and protease-susceptible substance that accelerates the decarboxylation of DOPA quinone imine to DHI, was partially purified from extracts of pharate pupal cuticle. Tyrosinase and QICF may play critical roles in generating catecholamine metabolites required for the sclerotization and melanization of insect cuticle.

Key Word Index: Tyrosinase, catecholamines, quinone, indole, sclerotization, melanization, tanning, cuticle, tyrosine, metabolism, dihydroxyindole, tobacco hornworm, *Manduca sexta*, quinone imine

INTRODUCTION

Tyrosinase is a multifunctional enzyme that catalyzes the orthohydroxylation of monophenols to diphenols and the subsequent dehydrogenation of *o*-diphenols to *o*-quinones (Dixon and Webb, 1979). The *o*-hydroxylase activity (EC 1.14.18.1) is stimulated by traces of diphenols which are themselves substrates for the catechol oxidase component (EC 1.10.3.1). Insect cuticular tyrosinases or phenoloxidases are involved in wound healing and repair of cuticle as well as sclerotization and melanization of newly formed exoskeleton (Hackman, 1974; Andersen, 1979; Brunet, 1980). Although the exact role played by tyrosinase is still uncertain, it appears that some and perhaps all of the tyrosinase-catalyzed and spontaneous reactions of the Mason-Raper pathway for melanin biosynthesis (Mason, 1948) occur during the sclerotization and melanization of cuticle.

Insect tyrosinases may occur as inactive pro-enzymes particularly in haemolymph and they are activated by a diversity of factors including proteolytic enzymes (Cottrell, 1964; Brunet, 1980; Aso and Kramer, unpublished). Recently some other factors regulating the melanin biosynthetic pathway in mice melanoma tumors have been identified including dopachrome conversion factor, dihydroxyindole

blocking factor and dihydroxyindole conversion factor (Pawelek *et al.*, 1980; Korner and Pawelek, 1980). The latter is probably a tyrosinase from the same tissue which catalyzes a third reaction in the melanin synthetic pathway, the dehydrogenation of 5,6-dihydroxyindole (DHI) to indole-5,6-quinone (Korner and Pawelek, 1982). The present study was undertaken to characterize the tyrosine and catechol oxidizing enzymes present in pharate pupal cuticle that may be catalyzing the synthesis of intermediates used for cuticle tanning and pigmentation. We have partially purified two proteins, a tyrosinase and a DOPA quinone imine conversion factor, from unsclerotized pharate pupal cuticle of *Manduca sexta* L. Some of the properties of these potential bio-regulators of cuticle stabilization and pigmentation are described in this report.

MATERIALS AND METHODS

Chemicals

Unless otherwise noted, all chemicals were of highest purity commercially available or prepared as described previously (Kramer *et al.*, 1983). Dopachrome was synthesized by oxidation of DL-DOPA (10 mg) with silver oxide (300 mg) in 20 ml of 0.1 M potassium phosphate pH 6.8 for several minutes at 4°C (Korner and Pawelek, 1980) followed by filtration through a Millex-PF 0.8 μ filter unit (Millipore Corp.). 5,6-Dihydroxyindole was prepared by oxidation of DL-DOPA with potassium ferricyanide and sublimation at 150–155° *in vacuo* according to Axelrod and Lerner (1963). HPLC was used to remove unreacted DL-DOPA from the preparation according to Pawelek *et al.* (1980).

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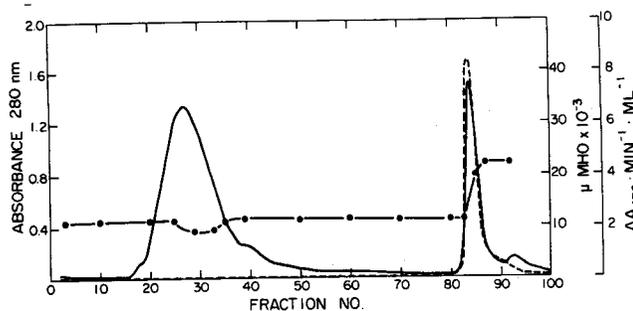


Fig. 1. Anion-exchange chromatography of *M. sexta* tyrosinase from pharate pupal cuticle. The precipitate from 40% saturation with ammonium sulphate was reconstituted in 10 mM Mops, 20 mM sodium chloride, pH 6.8 and passed over a 1.5×30 cm DEAE-BioGel column at 4°C. Tyrosinase was eluted with a stepwise sodium chloride gradient of 0.2 M. Absorbance at 280 nm (—); conductivity (●—●); enzyme activity(---).

Insects

Eggs of *M. sexta* (L.) were a gift from the Metabolism and Radiation Laboratory, Agricultural Research Service, U.S. Department of Agriculture, Fargo, North Dakota, U.S.A. Larvae were reared on a standard diet (Bell and Joachim, 1976) and kept at $28 \pm 2^\circ\text{C}$ during a 16 hr light–8 hr dark photoperiod.

Partial purification of tyrosinase

Pharate pupal cuticles from animals approximately at the time of dorsal metathoracic bar tanning were dissected and strapped clean of epidermis in 0.1 M Tris,* 1 M sodium chloride, 20 mM calcium chloride, 50 mM sodium ascorbate, pH 7.8 and immediately frozen to -70°C . Five cuticles (1.1 g) were finely ground using a mortar and pestle at -70°C and subsequently homogenized at 4°C in 30 ml of 0.1 M Tris, 1 M sodium chloride (to inhibit protyrosinase activating enzyme, Aso and Kramer, unpublished) and 50 mM sodium ascorbate, pH 7.8. After centrifugation at 27,000 g for 20 min at 4°C, the supernatant was collected and brought to 40% saturation with ammonium sulphate. The precipitate was collected by centrifugation at 27,000 g and 4°C for 10 min, redissolved in and dialyzed overnight against 7 l of 10 mM Mops, 20 mM sodium chloride, pH 6.8. The dialyze was applied to a DEAE-BioGel A column (1.5 cm i.d. \times 22 cm) equilibrated in 10 mM Mops, 0.1 M sodium chloride, pH 6.8. Tyrosinase was eluted using a linear gradient to 10 mM Mops, 0.2 M sodium chloride, pH 6.8.

Partial purification of quinone imine conversion factor

Twelve pharate pupal cuticles (2.6 g) obtained by the same method stated above were homogenized at 4°C in 60 ml of 0.1 M Tris, 1 M sodium chloride, 20 mM calcium chloride, 2 mM sodium diethyldithiocarbamate, pH 7.8. After centrifugation at 27,000 g and 4°C for 10 min, the supernatant was collected and brought to 50% saturation with ammonium sulphate. The precipitate was collected by centrifugation at 27,000 g and 4°C for 10 min, redissolved in and dialyzed overnight against 10 mM sodium potassium phosphate, 0.5 M sodium chloride, pH 6.8. The dialyze

was applied to a hydroxylapatite column (1.5 cm i.d. \times 20 cm) equilibrated with the same buffer. The quinone imine conversion factor was eluted by a linear gradient to 0.1 M sodium potassium phosphate, 0.5 M sodium chloride, pH 6.8.

Tyrosinase activity

Tyrosinase activity was determined by a continuous colorimetric assay in 0.1 M sodium phosphate, pH 6.0 using a molar extinction coefficient of 3715 for dopachrome and dopaminechrome (Mason, 1948; Duckworth and Coleman, 1970). Absorbance was monitored at 470 nm (DL-DOPA), 430 nm (dopamine), 390 nm (*N*-acetyldopamine and *N*- β -alanyldopamine) and 540 nm (dihydroxyindole). To calculate kinetic parameters, initial velocity data were treated by Eadie-Hofstee analysis (Zivin and Waud, 1982). The increase in absorbance caused by incubation of substrates with tyrosinase was a linear function of enzyme concentration and time over a period of several minutes.

Polyacrylamide gel electrophoresis

Electrophoresis was performed at pH 8.6 under non-denaturing conditions in 5% polyacrylamide gel (Davis, 1964). After electrophoresis the gel was either stained for tyrosinase activity by soaking in 20 mM DL-DOPA, 0.1 M sodium phosphate, pH 6 for 1 h or for protein by fixing in 5% (w/v) trichloroacetic acid and staining in 0.25% (w/v) Coomassie brilliant blue R-250 dissolved in 45% (v/v) methanol–9% acetic acid–48% water. Destaining was achieved with several washes of 25% (v/v) methanol–7.5% acetic acid–67.5% water.

Protein assay

Protein assays were performed using either the Bio-Rad assay method based on the Coomassie brilliant blue dye binding procedure of Bradford (1976) or the method of Lowry *et al.* (1951). Bovine serum albumin (Sigma) served as the standard protein.

RESULTS AND DISCUSSION

Tyrosinase preparation

Tyrosinase was partially purified from *M. sexta* pharate pupal cuticle by extraction in aqueous buffer which contained ascorbate and a high salt concentration, ammonium sulphate precipitation and anion-exchange chromatography (Table 1). The extraction buffer contained ascorbate and salt to stabilize tyrosinase and to inhibit protyrosinase activating enzyme, respectively (Aso and Kramer, unpublished). Enzyme activity was present in the precipitate from an extract brought to 40% saturation with ammo-

*Abbreviations used are Tris, 2-amino-2-hydroxymethyl-1,3-propanediol; Mops, morpholinopropane sulphonic acid; DEAE, diethylaminoethyl; DOPA, 3,4-dihydroxyphenylalanine; dopamine, 3,4-dihydroxyphenethylamine; NADA, *N*-acetyldopamine; NBAD, *N*- β -alanyldopamine; DHI, 5,6-dihydroxyindole; HPLC, high performance liquid chromatography; EDTA, ethylene diamine tetraacetic acid; DTC, diethyldithiocarbamate; QICF, DOPA quinone imine conversion factor; PTU, phenylthiourea.

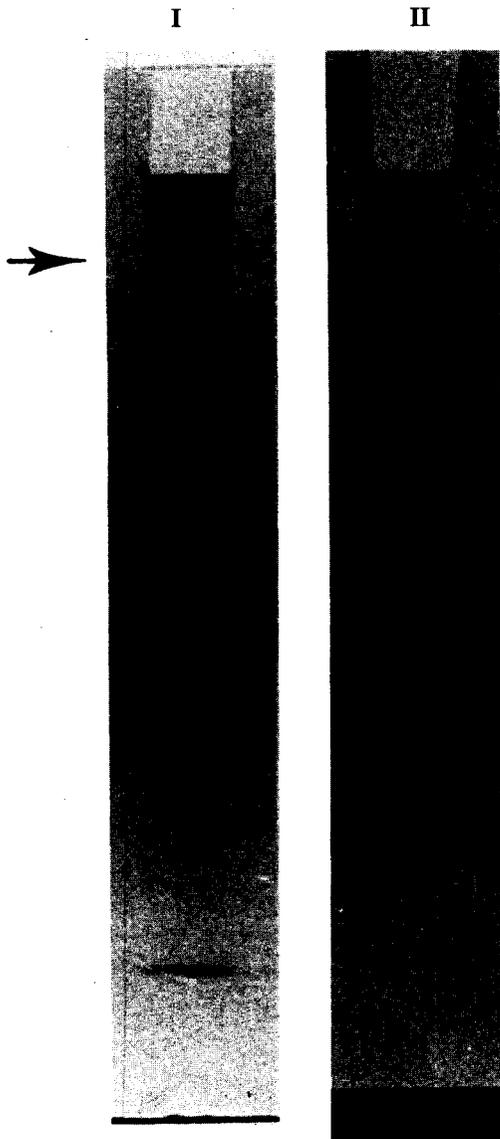


Fig. 2. Polyacrylamide gel electrophoresis of partially purified *M. sexta* cuticular tyrosinase. About 45 μg of protein per sample well was applied. (I) Protein stained with Coomassie brilliant blue; (II) enzyme activity revealed by incubation with 20 mM DOPA in 0.1 M sodium potassium phosphate, pH 6.0.

Table 1. Partial purification of cuticular tyrosinase from *M. sexta* pharate pupae.

Step	Total units*	Total protein (mg)	Specific activity	Overall yield	Overall purification
Crude extract	91.4	223	0.41	100	1
Ammonium sulphate fractionation	138.0	77.2	1.79	151	4.4
DEAE chromatography	80.8	17.8	4.55	88	11

*Unit = $\Delta A_{470} \text{ min}^{-1}$ using 3 mM DOPA as substrate at pH 6.0.

nium sulphate at pH 7.8 and was eluted from a DEAE-BioGel column in 0.2 M sodium chloride at pH 6.8 (Fig. 1). A homogeneous preparation of tyrosinase was not obtained. Attempts to fractionate the enzyme further by gel filtration chromatography or gradient elution from ion-exchange or hydroxylapatite gels were unsuccessful and resulted in very low yields probably due to the instability of highly purified preparations which occasionally precipitate upon standing or extensively adsorb to surfaces such as column packing material. *Bombyx mori* tyrosinase exhibited similar behaviour (Ashida and Dohke, 1980). Tyrosinase may catalyze its auto-inactivation where one molecule oxidizes tyrosyl residues present in another which ultimately leads to inter- and intramolecular cross-linking and polymerization of the enzyme itself.

Overall there was an 88% yield and 11-fold purification of tyrosinase from cuticle. An apparent reversible inhibition of enzyme activity occurred in the crude extract because the ammonium sulphate fractionation resulted in a 50% increase in total units. The chemical or physical nature of the inhibitor is unknown but it may be similar to a protein inhibitor of tyrosinase that has been found in human skin (Vijayan *et al.*, 1982). It is also possible that some protyrosinase may have activated during the ammonium sulphate precipitation step.

Polyacrylamide gel electrophoresis of the partially purified tyrosinase from *M. sexta* cuticle in 5% gels at pH 8.5 revealed that the preparation was quite heterogeneous in protein, but homogeneous in enzyme activity (Fig. 2). The lack of penetration of some of the enzyme in the sample was probably due to large molecular weight aggregates adsorbed to the leading edge of the gel. The aggregated form of tyrosinase increased in amount as the sample aged prior to electrophoresis. The specific activities of this preparation toward DL-DOPA and dopamine were nearly identical, 3.39 ± 0.31 and 2.97 ± 0.05 nmoles substrate oxidized $\text{min}^{-1} \text{ mg}^{-1}$, respectively.

Stability and pH effects.

pH rate studies of tyrosinase were performed using DL-DOPA as substrate over a pH range of 2 to 11 (Fig. 3). It is well known that DOPA is autooxidized at alkaline pH. Therefore, the initial velocity for spontaneous oxidation was subtracted from the rate determined in the presence of enzyme at pH values > 7 . Maximum activity occurred around pH 6 with 50% activity at approx pH 5.3 and 7.5 on the acid and alkaline sides, respectively. The dependence of activity on pH indicated that an ionized acidic group with $\text{pK} \sim 5.3$ and a protonated basic group with

$\text{pK} \sim 7.5$ are essential for enzymatic activity and/or native conformation.

The effect of pH on the stability of *M. sexta* tyrosinase was determined at constant ionic strength (0.1μ) over a pH range of 2.3 to 11.6 and an incubation period of 50 hr at 4°C . The enzyme was very stable between pH 7 and 8 and was generally more stable at alkaline pH values than at acidic ones. Eighty percent of the control activity was recovered at pH 10 and 6 after several days.

Tyrosinase was moderately thermostable. Full activity remained at pH 6 after a 10 min incubation at 45°C while 50% activity was recovered at 60°C and none at 75°C .

Kinetic properties

The time course for the absorbance change at 470 nm that occurred when tyrosine was incubated with tyrosinase is shown in Fig. 4. HPLC and cyclic voltammetric analyses of the time course of the reaction revealed that tyrosinase hydroxylates tyrosine to DOPA (Fig. 5, compound 1, $\text{R}_1 = \text{H}$ and $\text{R}_2 = \text{COO}^-$) and then dehydrogenates the latter to DOPA quinone (2) which spontaneously cyclizes to DOPA leucoaminochrome (3, Kramer *et al.*, 1983). The leucoaminochrome is subsequently oxidized by tyrosinase to DOPA quinone imine (4) whose absorbance maximum occurs at 470 nm (orange-red colour). An upward curvature was observed in the absorbance trace during the reaction which suggests that a mechanism takes place where either the hydroxylation and/or dehydrogenation steps are slower than the cyclization step or that one of the reaction intermediates formed is increasing the rate of an earlier step. Apparently, the first step (hydroxylation) is

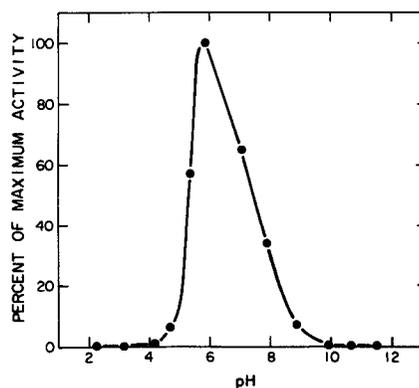


Fig. 3. Dependence of tyrosinase activity on pH for the catalyzed oxidation of DOPA. Mean values from two determinations with SD $< 10\%$ are shown.

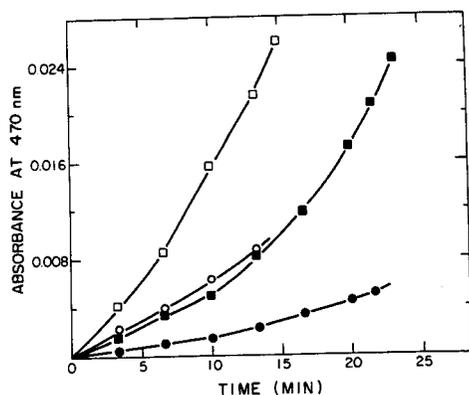


Fig. 4. Conversion of tyrosine to DOPA quinone imine catalyzed by *M. sexta* tyrosinase and effect of added DOPA. Mean values from two or three determinations with S.D. < 10% are shown. (a) 1.6 mM tyrosine in 0.1 M potassium phosphate pH 6.0 mixed with DEAE-BioGel tyrosinase fraction (●—●). (b) Same as (a) except 1.6 μM DOPA added (○—○). (c) Same as (a) except 3 mM tyrosine (■—■). (d) Same as (c) except 1.6 μM DOPA added (□—□).

rate-limiting because the cyclization of DOPA quinone is slower electrochemically than the dehydrogenation of DOPA or DOPA leucoaminochrome (Hawley *et al.*, 1967; Garcia-Carmona *et al.*, 1982; Kramer *et al.*, 1983). The addition of a catalytic amount of DOPA to tyrosine increased the rate of tyrosine hydroxylation by a factor > 2 (Fig. 4). Tyramine was hydroxylated to dopamine by tyrosinase in a similar manner. These results indicate that DOPA is a cofactor for the hydroxylation step and that when DOPA accumulates, hydroxylation is no longer rate-limiting. Mammalian and mushroom tyrosinases utilize DOPA as a cofactor as well (Lerner, 1953; Pomerantz, 1966; Duckworth and Coleman, 1970; Korner and Pawelek, 1982).

There are several catecholamines present in *M. sexta* that serve as substrates for tyrosinase including DOPA, dopamine, *N*-acetyldopamine and *N*-β-alanyldopamine (Hopkins *et al.*, 1982). Figure 6 shows the absorbance increase at 290 nm caused by the oxidation of the catecholamines by tyrosinase. For direct comparison, a u.v. wavelength was chosen instead of a visible one to monitor the reaction because chromophores with different visible absorbance spectra are generated from each catecholamine. All compounds undergo two two-electron oxidations and an intramolecular 1,4-Michael addition reaction (Fig. 5). The *o*-quinones from *N*-acetyldopamine and *N*-β-alanyldopamine cyclize 40 times more slowly than those from DOPA and dopamine (Kramer *et al.*, 1983). From the slope of the absorbance traces in Fig. 6, the most reactive catecholamine appeared to be dopamine. *N*-acetyldopamine and *N*-β-alanyldopamine reached similar plateau absorbance values after 100 sec corresponding to the quinone imine species. Dopamine and DOPA continued to generate other chromophores. The electron withdrawing properties of the acyl substituents on the nitrogen of the quinone imines derived from *N*-acetyldopamine and *N*-β-alanyldopamine ($\lambda_{\max} = 390$ nm) apparently prevented the occurrence of other reactions or electronic rearrangements such as in-

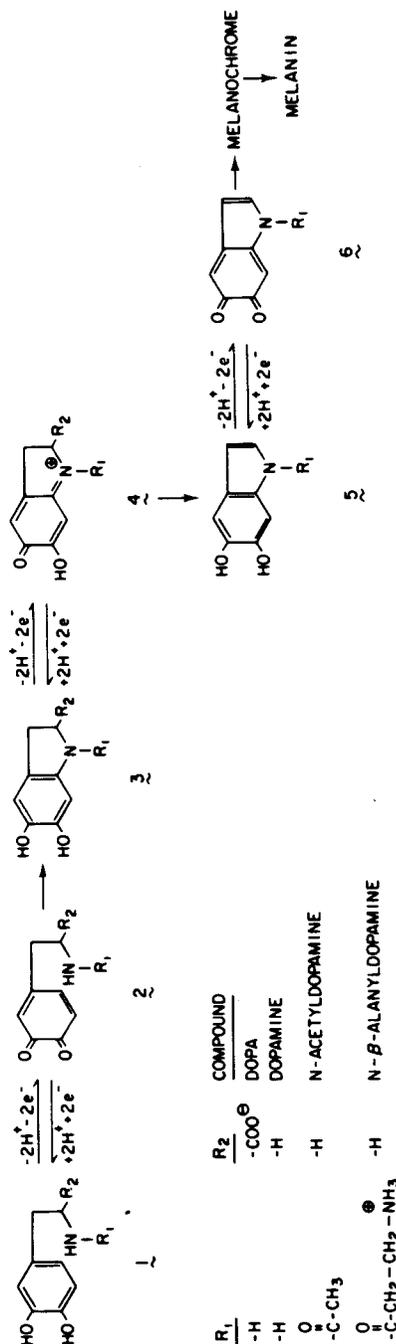


Fig. 5. The oxidation of catecholamines by tyrosinase (adapted from Mason, 1955).

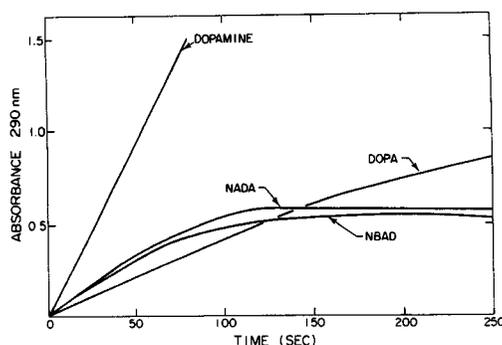


Fig. 6. Continuous absorbance trace of the conversion of catecholamines into oxidation products by *M. sexta* cuticular tyrosinase. Dopamine, 1.37 mM in 0.1 M sodium phosphate buffer, pH 6.5; DOPA, 1.53 mM; *N*-acetyldopamine, 1.33 mM; *N*- β -alanyldopamine, 1.67 mM. Protein concentration = 40 μ g/ml. Typical data from a single experiment are presented with SD < 5%.

dolization. In contrast DOPA and dopamine quinone imines ($\lambda_{\max} = 470$ and 430 nm, respectively) reacted further to yield 5,6-dihydroxyindole (Fig. 5, 5), a step that may be catalyzed by a quinone imine conversion factor (see next section). 5,6-Dihydroxyindole was oxidized by tyrosinase to indole quinone (Fig. 5, 6) which spontaneously gives rise to melanochrome ($\lambda_{\max} = 540$ nm) and melanin.

Table 2 lists the kinetic constants for the oxidation of dihydroxy aromatic substrates by *M. sexta* tyrosinase as determined by the Eadie-Hofstee technique (Zivin and Waud, 1982). DOPA and dopamine had the highest V_{\max} values followed by *N*-acetyldopamine and *N*- β -alanyldopamine. The lowest K_m was exhibited by 5,6-dihydroxyindole, an extremely unstable compound that readily oxidizes and polymerizes to a melanin polymer. To our knowledge DHI has not yet been detected in insect tissues but its presence has been inferred because of the production of melanin by insects (Raper, 1927; Brunet, 1980). Overall, the preferred substrates were *N*- β -alanyldopamine and *N*-acetyldopamine. The presence of a carboxylic acid in DOPA reduced the turnover number relative to dopamine by approximately one order of magnitude. Acylation with acetate to yield a neutral product or with β -alanine which retains an amino group in a rather bulky structure increased the turnover number by nearly a factor of three. The indole ring substrate DHI

exhibited a low turnover similar to DOPA. Since *N*- β -alanyldopamine and *N*-acetyldopamine are the major catecholamines at the time of pupal and larval tanning in *M. sexta* respectively, and are also the substrates preferred by tyrosinase from cuticle, these acylated catechols appear to be the major precursors for cross-linking agents in sclerotization reactions (Hopkins *et al.*, 1982, 1984). The two non-acylated catecholamines may primarily be precursors for polymerizing agents in melanization (Brunet, 1980).

Like mammalian tyrosinase, *M. sexta* tyrosinase catalyzes several distinct reactions that occur within a single biochemical pathway: the hydroxylation of monophenol to catechol, the dehydrogenation of catechol to *o*-quinone, and the dehydrogenation of dihydroxyindole to indole quinone (Korner and Pawelek, 1982). The latter reaction is also catalyzed by a dihydroxyindole conversion factor (which may actually be tyrosinase) detected in extracts from mouse melanoma cells (Pawelek *et al.*, 1980). Each of the tyrosinase-catalyzed reactions is distinct. The enzyme is very unusual in that it has the potential to regulate multiple steps in both sclerotization and melanization.

Inhibitors

The addition of sodium chloride to the tyrosinase assay system had a significant effect on enzyme activity. At 0.1 and 0.2 M salt, activity was only 30 and 10% of the control activity. However, the inhibition was reversible which allows the use of ionic strength changes in the purification steps. No inactivation occurred after incubation with sodium fluoride (10^{-3} M), disodium EDTA (10^{-3} M), *o*-benzylhydroxylamine ($\sim 10^{-4}$ M) and α, α' -bipyridyl ($\sim 10^{-4}$ M). A partial inhibition (25%) was caused by benzoic acid (10^{-4} M) which is probably a competitive inhibitor. The most potent inhibitors were diethyldithiocarbamate, phenylthiourea and sodium cyanide. Cyanide (10^{-3} M) caused 98% inhibition while phenylthiourea (10^{-4} M) completely inactivated the enzyme. The K_i for diethyldithiocarbamate was approx. 20 μ M. Apparently, cyanide binds to the oxygen binding site (Duckworth and Coleman, 1970) while the latter two compounds combine with copper present in tyrosinase (Heyneman, 1965; Korner and Pawelek, 1982). It is unknown whether *M. sexta* cuticular tyrosinase is a copper containing enzyme. However, the

Table 2. Kinetic parameters for oxidation of substrates by cuticular tyrosinase from *M. sexta* pharate pupae*

Substrate*	V_{\max} ($\Delta A \times \text{min}^{-1} \text{mg}^{-1}$)†	K_m ($\text{M} \times 10^3$)	V_{\max}/K_m
DOPA	12.22 \pm 1.13	8.42 \pm 0.46	1.45
Dopamine	10.68 \pm 0.17	0.91 \pm 0.03	11.74
<i>N</i> -acetyldopamine (NADA)	7.06 \pm 0.30	0.25 \pm 0.03	28.24
<i>N</i> - β -alanyldopamine (NBAD)	6.69 \pm 0.06	0.23 \pm 0.02	29.09
5,6-Dihydroxyindole (DHI)	0.05 \pm 0.01	0.02 \pm 0.01	2.67

*Protein concentration = 1–76 μ g/ml. $S_0 = 0.01$ –15.21 mm. †Activity determined spectrophotometrically at 470 nm (DOPA) 430 nm (dopamine), 390 nm (NADA and NBAD) and 540 nm (DHI). Data were treated by Eadie-Hofstee analysis and values are from three to five determinations \pm SD. For comparison of turnover numbers, negligible differences in molar extinction coefficients were assumed (Mason, 1948; Duckworth and Coleman, 1970).

haemolymph protyrosinase contains approx. 0.15% copper (Aso and Kramer, unpublished).

Tyrosinases have been characterized from haemolymph, cuticle or whole body of *Tenebrio molitor* (Heyneman, 1965) and *Calliphora vicina* (Munn and Bufton, 1973; Barrett and Andersen, 1981). Enzyme A from the latter species most resembles *M. sexta* tyrosinase in substrate preference (NBAD > NADA > dopamine), inhibitor susceptibility (DTC, PTU and cyanide being the most effective) and pH optimum (near neutrality).

DOPA quinone imine conversion factor

Besides tyrosinase there are other regulatory factors that function in melanin biosynthesis in higher animals. They are dopachrome conversion factor and indole blocking factor which are present in extracts of mouse melanoma cells (Korner and Pawelek, 1980). We have detected a factor in *M. sexta* cuticle, DOPA quinone imine conversion factor, that accelerates the conversion of DOPA quinone imine to dihydroxyindole. A fraction eluting from the hydroxylapatite chromatography of pharate pupal cuticle at 0.2 M sodium phosphate caused downward curvature of the absorbance trace at 470 nm of the tyrosinase-catalyzed oxidation of DOPA (Fig. 7A), suggesting that a factor was inhibiting DOPA quinone imine formation after about 40 sec of reaction time. However, the factor was actually converting DOPA quinone imine to dihydroxyindole. When incubated with silver oxide or enzyme-generated DOPA quinone imine ($\lambda_{\max} = 470$ nm), the factor increased the rate of dihydroxyindole formation significantly over the rate of spontaneous reaction. The absorbance trace at 470 nm of DOPA incubated with sequential additions of tyrosinase, diethyldithiocarbamate and DOPA quinone imine conversion factor is shown in Fig. 7B. HPLC analysis revealed that dihydroxyindole was indeed generated by the conversion factor. The factor was heat labile (Fig. 8A) and inactivated by digestion with pronase (Fig. 8B). When mixed with dopamine quinone imine, the factor did not facilitate conversion to dihydroxyindole and therefore appears to be specific for DOPA quinone imine which contains a carboxylic acid function at the 1-carbon position. When incubated with DOPA under similar conditions, it did not produce dopamine as monitored by HPLC analysis and therefore is not a DOPA decarboxylase. These results suggest that the conversion factor may be a DOPA quinone imine decarboxylase. A similar substance was first detected in mushrooms and several types of melanoma tumor cells but not in a variety of cell lines of non-melanocytic origin (Korner and Pawelek, 1980). The factor apparently stimulates production of melanin in a variety of organisms.

Concluding remarks

Figure 9 shows a proposed pathway for the metabolism of tyrosine and several catechols for the formation of sclerotin and melanin in insect cuticle. Sclerotin is defined as one or more structural proteins cross-linked by a quinone (sclerotin-I), quinone imine (sclerotin-II) or indole quinone (indole sclerotin) derivative. Melanin is defined as a homo- or heteropolymerisation product of quinone, quinone imine or

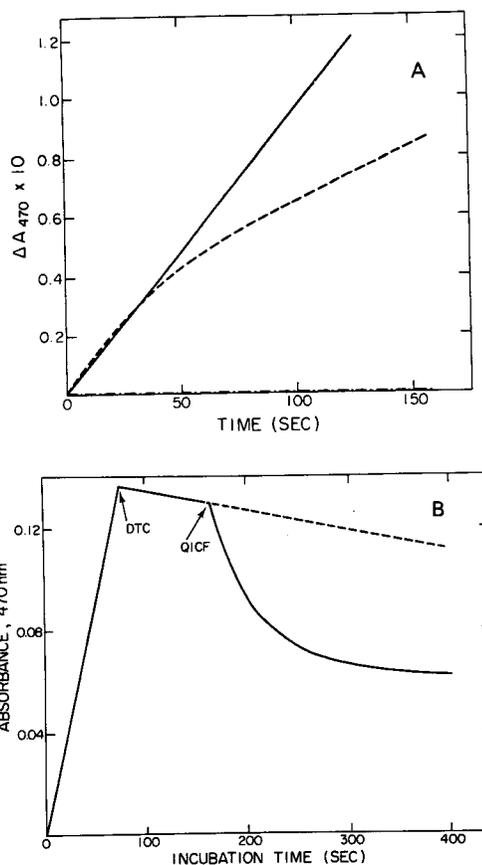


Fig. 7. Continuous absorbance trace of the oxidation of DOPA by *M. sexta* cuticular tyrosinase and the effect of DOPA quinone imine conversion factor. (A) DOPA (—); DOPA with DOPA quinone imine conversion factor added (---); the DOPA quinone imine fraction alone (· · ·). (B) Similar to A except that the tyrosinase inhibitor, diethyldithiocarbamate (DTC), added after 80 sec and DOPA quinone imine conversion factor (QICF) added after 170 sec. Dashed line indicates spontaneous conversion rate. Typical data from a single experiment with SD < 5% are shown.

indole quinone which may contain different combinations of the precursors shown (Hemple, 1966). The multifunctional enzyme tyrosinase (1) catalyzes many of the reactions of which there are two general types: the hydroxylation of a monophenol and the dehydrogenation of catechols, leucoaminochromes and dihydroxyindole. Other enzymes operating in the pathway include DOPA decarboxylase (2), *N*-acetyldopamine and *N*- β -alanyldopamine synthases (3 and 4, respectively) and DOPA quinone imine decarboxylase (5). The latter enzyme may facilitate melanin production in particular. However, since little or no melanin is apparently synthesized in pupal cuticle, any DHI produced may be used for indole quinone sclerotin or wound healing. The other reactions shown in Fig. 9 probably occur spontaneously, but they may also be regulated by other converting or blocking factors.

High titres of catecholamines are present in haemolymph (Hopkins *et al.*, 1982, 1984) and they are perhaps generated by enzymes outside the cuticle. The cross-linking and melanin-forming agents such

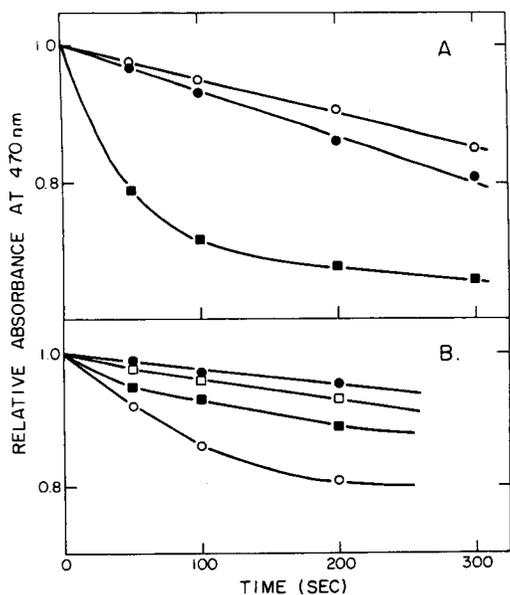


Fig. 8. Conversion of DOPA quinone imine to dihydroxyindole. Effect of DOPA quinone imine conversion factor on absorbance at 470 nm of DOPA quinone imine. DOPA quinone imine was made by oxidation of DOPA with Ag_2O (Korner and Pawelek, 1980). (A) Spontaneous reaction (●—●); 20 μg DOPA quinone imine conversion factor added (■—■); 20 μg DOPA quinone imine conversion factor boiled for 10 min added (○—○). (B) Effect of pronase digestion. Spontaneous reaction (●—●); 10 μg DOPA quinone imine conversion factor incubated with pronase for 10 min added (○—○); incubated with pronase for 25 min (■—■); for 35 min (□—□). Mean values from two determinations with SD < 10% are presented.

as *o*-quinones, *p*-quinone imines and indole quinones are probably derived from the catecholamines after the latter are transported into the integument where tyrosinase is localized. Whether the hydroxylation of tyrosine to DOPA by tyrosinase also occurs in cuticle *in vivo* is unknown.

The reactions shown in Fig. 9 depict primarily those which may occur in the ring oxidation mechanism for generating cuticle cross-linking agents (Andersen, 1979; Brunet, 1980). The aliphatic side chain mechanism or β-sclerotization pathway is not addressed here. We suggest that three different electrophilically activated ring systems may possibly be utilized in the ring oxidation pathway. Sclerotin-I would be cross-linked by a single ring *o*-quinone, sclerotin-II by a double ring *p*-quinone imine and indole sclerotin by indole-5,6-quinone. The availability of three different catecholamine precursors for cross-linking agents indicates that as many as seven different types of sclerotin may be generated by covalent bonding of proteins to aromatic rings. Because different types of structural proteins may also be utilized, a large variety of end products (sclerotins) are possible.

There are probably several other enzymes and regulatory agents that are still unidentified in the tyrosine-catecholamine oxidation pathway of insects.

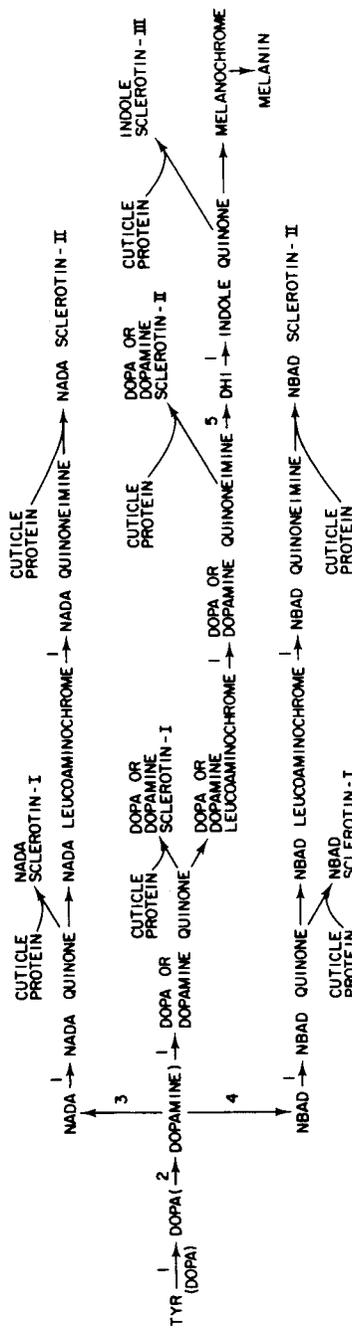


Fig. 9. Hypothetical pathway for metabolism of tyrosine and catecholamines to form sclerotin and melanin in insect cuticle. Numbered reactions catalyzed by enzymes: (1) tyrosinase; (2) DOPA decarboxylase (parentheses indicate DOPA may enter Mason-Raper pathway directly); (3) NADA synthase; (4) NBAD synthase; (5) DOPA quinone imine conversion factor. Other reactions probably occur spontaneously. Sclerotin-I, II and III denote quinone, quinone imine and indole quinone ring systems, respectively, used as protein cross-linking agents.

The mechanism which controls whether sclerotin or melanin is assembled is unknown, but is probably determined in part by the catecholamines available and the regulatory factors present. Sclerotin may be primarily derived from acylated catecholamines which do not readily form indole structures while melanin probably arises from non-acylated ones which form hydroxylated indoles. DOPA quinone imine conversion factor may accelerate melanin formation while a component similar to 5,6-dihydroxyindole blocking factor, which restricts melanogenesis in mammalian cells (Pawelek *et al.*, 1980), may prevent melanin formation in insects. Presently we are searching for other unidentified factors and metabolites which regulate cuticle sclerotization and pigmentation in insects and are also attempting to elucidate chemical structures so that their physiological roles can be better understood.

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