

# CHARACTERIZATION OF HAEMOLYMPH PROTYROSINASE AND A CUTICULAR ACTIVATOR FROM *MANDUCA SEXTA* (L.)

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(Received 7 March 1984)

**Abstract**—A protyrosinase has been isolated from fifth instar larval haemolymph of the tobacco hornworm, *Manduca sexta* (L.) by ammonium sulphate fractionation, hydroxylapatite chromatography and gel filtration. It exhibited a single band after polyacrylamide gel electrophoresis at pH 8.5 and two bands in the presence of sodium dodecyl sulphate with apparent molecular weights of  $7.7 \times 10^4$  and  $7.1 \times 10^4$ . The proenzyme is a metalloprotein containing 0.18% copper. The activating enzyme was partially purified from pharate pupal cuticle by ammonium sulphate precipitation and hydroxylapatite chromatography. The activation was inhibited by diisopropylphosphorofluoridate and had a pH optimum of 8.8. Chymotrypsin also activated the proenzyme. The cuticular activator is probably a serine protease. Activated protyrosinase exhibited physical, chemical and kinetic properties very similar to those of tyrosinase extracted from pharate pupal cuticle. Haemolymph protyrosinase may serve as a precursor for both haemolymph and cuticular tyrosinases that synthesize catecholamines and quinones used for wound healing, parasite encapsulation as well as for cuticular stabilization and pigmentation.

**Key Word Index:** Cuticle, haemolymph, tyrosinase, phenoloxidase, protease, tyrosine, catecholamine, proenzyme, copper, activation, enzyme, zymogen.

## INTRODUCTION

Tyrosinase is a multifunctional enzyme that may have several physiological roles during insect development such as cuticle sclerotization, melanization and wound healing (Brunet, 1980). A tyrosinase isolated from pharate pupal cuticle of *Manduca sexta* (L.) hydroxylates monophenols and dehydrogenates diphenols and dihydroxyindoles, giving rise to catechols, *o*-quinones, *p*-quinone imines and indole quinones (Aso *et al.*, 1984). In many species of insects a protyrosinase is found in the haemolymph that can be activated via a proteolytic process by an enzyme present in cuticle (Ohnishi, 1959; Karlson and Schweiger, 1961; Funatsu and Inaba, 1962; Karlson *et al.*, 1964; Sekeris and Mergenhagen, 1964; Evans, 1967; Ashida and Ohnishi, 1967; Ohnishi *et al.*, 1970; Thomson and Sin, 1970; Ashida, 1971; Dohke, 1973; Ashida *et al.*, 1974; Hughes and Price, 1975, 1976; Ashida and Dohke, 1980). It is still uncertain whether the endogenous tyrosinase in cuticle is derived from the haemolymph proenzyme.

We have purified and characterized a haemolymph protyrosinase from the tobacco hornworm, *M. sexta*, and found that it is activated by an activating enzyme from *M. sexta* pharate pupal cuticle. Our results show that the activated protyrosinase and the endogenous cuticular tyrosinase are very similar in

physical, chemical and kinetic properties, suggesting a relationship between the two.

## MATERIALS AND METHODS

### Chemicals

All chemicals were of highest purity commercially available or prepared as described previously (Kramer *et al.*, 1983).

### Insects

Eggs of *M. sexta* 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.

### Tyrosinase activity

Tyrosinase activity was determined by a continuous colorimetric assay in 0.1 M sodium phosphate, pH 6.0 (Duckworth and Coleman, 1970). Absorbance was monitored at 470 nm for reaction of 3,4-dihydroxyphenylalanine (DOPA), 430 nm for 3,4-dihydroxyphenethylamine (dopamine), 390 nm for *N*- $\beta$ -alanyldopamine (NBAD) and 540 nm for 5,6-dihydroxyindole (DHI). 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.

### Protyrosinase activating enzyme activity

Ten microliters (20-100  $\mu\text{g}$ ) of protyrosinase were incubated with 0.14 ml of 0.1 M *N*-morpholinoethanesulphonate (MOPS), pH 6.8 and 10  $\mu\text{l}$  (1-20  $\mu\text{g}$ ) of

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the protyrosinase activating enzyme fraction at 4°C for 5 min. The activation mixture (0.1 ml) was added to 0.1 ml of 0.1 M PIPES, pH 6.0 and 1 ml of 20 mM DOPA (or other catecholamines) in 0.1 M sodium potassium phosphate, pH 6.0. The absorbance tracing was monitored as described above.

#### Polyacrylamide gel electrophoresis

Electrophoresis was performed at pH 8.5 to assess purity under non-denaturing conditions (Davis, 1964) or denaturing conditions in 0.1% SDS buffer (Laemmli, 1970). After electrophoresis the gel was either stained for tyrosinase activity by soaking in 20 mM D,L-DOPA, 0.1 M sodium phosphate, pH 6 or for protein by fixing in 5% trichloroacetic acid and staining in 0.25% (w/v) Coomassie brilliant blue R-250 dissolved in 45% methanol-9% acetic acid. De-staining was achieved with several washes of 25% methanol-7.5% acetic acid.

#### 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 served as the standard protein.

#### Protyrosinase purification

Twelve grams of lyophilized haemolymph from fifth instar larvae were suspended in 60 ml of 50% saturated ammonium sulphate, 0.1 M Tris, 50 mM sodium ascorbate and 1 mM diisopropylphosphorofluoridate pH 7.8 at 4°C. After collection by centrifugation at 27,000 g for 20 min at 4°C the precipitate was resuspended in 30 ml of the same buffer except 30% saturated ammonium sulphate was used. The supernatant obtained after centrifugation at 27,000 g for 15 min at 4°C was dialyzed against 10 mM sodium potassium phosphate, 0.5 M sodium chloride, 10 mM sodium ascorbate, pH 6.8.

The dialyate was subjected to chromatography on hydroxylapatite (2.0 cm i.d. × 30 cm column) equilibrated with 10 mM sodium potassium phosphate, 0.5 M sodium chloride, pH 6.8. Adsorbed protein was eluted with a linear gradient of sodium potassium phosphate, pH 6.8 from 10 mM to 0.1 M with both reservoirs also containing 0.5 M sodium chloride. Active fractions were combined and dialyzed against column equilibration buffer and re-chromatographed on hydroxylapatite two more times, each time collecting a narrower pool of protyrosinase fractions. After concentration by ultrafiltration using an Amicon PM-10 membrane, the concentrate was subjected to gel

filtration on a column of Sephacryl S-300 (1.5 cm i.d. × 155 cm) equilibrated with 10 mM MOPS, 0.5 M sodium chloride, pH 6.8.

#### Partial purification of protyrosinase activating enzyme

Cuticles were dissected from pharate pupae about 0 to 24 hr before the bars on the metathoracic tergum tan, washed with 0.1 M Tris, 1 M sodium chloride, 20 mM calcium chloride, 60 mM sodium ascorbate, pH 7.8, frozen and stored at -78°C. Frozen ground pharate pupal cuticles were homogenized at 4°C in 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 for 15 min at 4°C, the supernatant was collected and brought to 50% saturation with ammonium sulphate. The precipitate was collected by centrifugation at 27,000 g for 15 min at 4°C, redissolved in and dialyzed against 10 mM sodium potassium phosphate, 0.5 M sodium chloride, pH 6.8. The dialyate was applied to a hydroxylapatite column (1.5 cm i.d. × 20 cm) equilibrated with the same buffer. The protyrosinase activating enzyme was eluted by a stepwise gradient of 0.1 M sodium potassium phosphate, 0.5 M sodium chloride, pH 6.8.

#### Copper analysis

Copper was determined using the graphite furnace atomic absorption method by the Kansas State University Emission Spectroscopy Laboratory. Approximately 0.1 mg protein was dissolved in 0.5 ml of 5% nitric acid for analysis. The solution phase detection limit was 0.5 ppb.

#### Amino acid analysis

Amino acids were determined by high performance liquid chromatographic analysis of their fluorescent *o*-phthalaldehyde derivatives (Lookhart *et al.*, 1982).

## RESULTS

#### Purification of protyrosinase

*M. sexta* protyrosinase was purified from fifth instar haemolymph utilizing ammonium sulphate fractionation followed by chromatography on hydroxylapatite and Sephacryl S-300 gels. The elution profile of the last hydroxylapatite chromatography at pH 6.8 is shown in Fig. 1. Proenzyme eluted at approx. 40 mM phosphate in fractions 57-63. Subsequent gel filtration on Sephacryl S-300 yielded a single symmetrical peak of protein centered at frac-

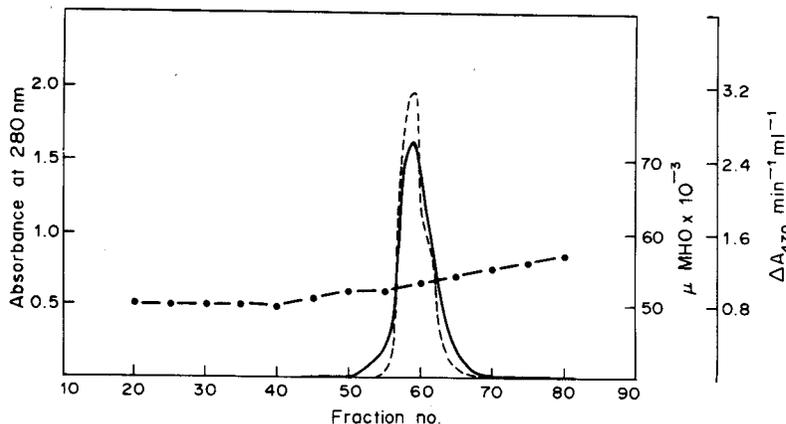


Fig. 1. Elution profile of *M. sexta* haemolymph protyrosinase from hydroxylapatite. Absorbance at 280 nm (—); tyrosinase activity after incubation of fraction with proenzyme activator (---); conductivity (●—●).

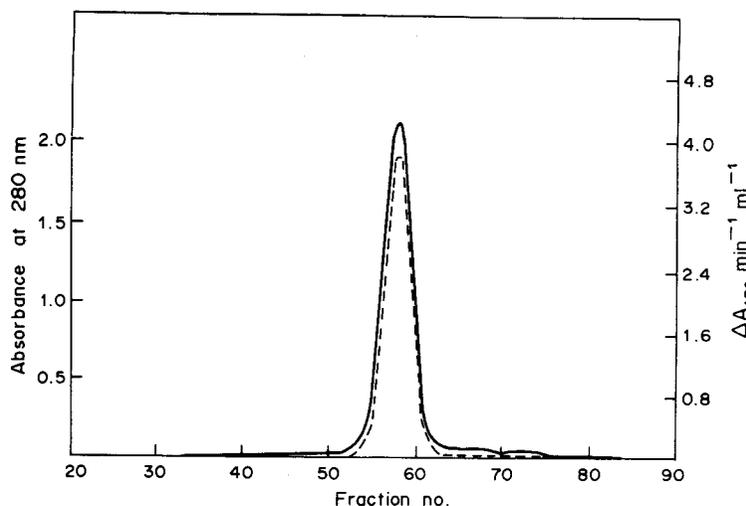


Fig. 2. Gel filtration profile of *M. sexta* protyrosinase from Sephacryl S-300. Absorbance at 280 nm (—); tyrosinase activity after incubation of fraction with proenzyme activating enzyme (---).

tion 58 (Fig. 2). This elution volume corresponded to a protein with an approximate molecular weight of  $1-1.5 \times 10^5$ . When subjected to polyacrylamide gel electrophoresis at pH 8.5, protyrosinase appeared as a single band using nondenaturing conditions (Fig. 3, lane I) and as two bands using the denaturing system containing sodium dodecyl sulphate (lane II). The apparent molecular weights of the two subunits were  $7.7 \times 10^4$  and  $7.1 \times 10^4$ . Thus, the proenzyme is probably a dimer composed of two nonidentical subunits.

Data on the purification procedure are presented in Table 1. Protyrosinase was purified approx. 100-fold with a recovery of about 35% relative to the activity present in the fraction obtained from the initial hydroxylapatite chromatography. There was an indication of an inhibitor of protyrosinase being present in the ammonium sulphate fraction because the total units of activity increased 35% after the first chromatography using hydroxylapatite. Prepupae of *Musca domestica* also contain a protein factor that inhibits the activator of prophenoloxidase (Namihiro *et al.*, 1979).

The amino acid composition of protyrosinase in mole percentage values is given in Table 2. Glycine, alanine and lysine were the most abundant residues with mole percentages of 10% or greater. Atomic absorption analysis revealed that copper was present at  $0.18 \pm 0.01\%$ , a level similar to those found in *Bombyx mori* (Ashida, 1971) and *Tenebrio molitor* proenzymes (Heyneman, 1965). Protyrosinase is thus a metalloprotein.

#### Partial purification of protyrosinase activating factor

Protyrosinase is converted to active enzyme by an activator in the cuticle. The activating factor was partially purified from the pharate pupal cuticle by ammonium sulphate fractionation and hydroxylapatite chromatography. Figure 4 shows the elution profile of activator from hydroxylapatite at pH 6.8 using a phosphate gradient. The activator eluted at approx. 30 mM phosphate in fractions 64-67. There was an overall 11-fold purification with a recovery of about 65% activity relative to that present in the

ammonium sulphate fraction (Table 3). The greater number of total units of activity present in the ammonium sulphate fraction indicated that an inhibitor of the activator may be present in the crude extract. Polyacrylamide gel electrophoresis of the hydroxylapatite fraction revealed the activator preparation to be highly heterogeneous in protein.

#### Protyrosinase activation

The activation of insect protyrosinases has previously been shown to be a proteolytic process (Ohnishi *et al.*, 1970; Ashida *et al.*, 1974). Both chymotrypsin and the partially purified protyrosinase activator from *M. sexta* cuticle activated the proenzyme. Boiling of the activator caused a loss of activity as did preincubation with millimolar diisopropylphosphorofluoridate. Therefore, the activator appears to be an enzyme with characteristics of a serine protease. It also exhibited an alkaline pH optimum at pH  $\sim 8.8$  (Fig. 5) which is typical of serine proteases (Walsh and Wilcox, 1970). The activator did not exhibit chymotrypsin-like or trypsin-like specificity because it did not hydrolyze benzoyl tyrosine ethyl ester or benzoyl arginine ethyl ester, typical low molecular weight ester substrates for those proteases. Figure 6 shows the activation of protyrosinase as a function of concentration of the activating factor. An upward curvature was observed which suggests that some type of positive cooperativity between the heterogeneous activator and the proenzyme occurred. There was a linear relationship between the concentration of proenzyme and the activity of the activated proenzyme. The absorbance trace at 470 nm for DOPA oxidation was linear for 10 min at both 4 and 25°C. The activation of protyrosinase was reversibly inhibited by high salt concentrations, i.e. 0.5 M sodium chloride. This property was taken advantage of during the proenzyme purification when most of the fractionation procedures were carried out under conditions of high ionic strength.

The activator was relatively unstable, being only 50% active after 10 min at 38°C. By contrast, the proenzyme was more stable; 50% of its activity

Table 1. Purification of protyrosinase from *M. sexta* haemolymph

Step	Total units*	Total protein (mg)	Specific activity	Overall yield	Overall purification
Crude extract	3264	1743	1.9	†	1
Ammonium sulphate fractionation	5616	1029	5.4	100	3
Hydroxylapatite chromatography					
1	7568	206	36.6	135	19
2	4352	70	90.7	113	47
3	4340	27	163	77	85
Gel filtration S-300	2670	14	195	48	102

\*Unit =  $\Delta A_{470} \cdot \text{min}^{-1}$ . †Due to the presence of sodium ascorbate in haemolymph and extraction buffer, activity could not be measured in crude extract. Recovery of activity in ammonium sulphate fractionation was assumed to be 100%.

Table 2. Amino acid composition of protyrosinase from *M. sexta* haemolymph

Amino acid	Mole percent*
Asp/asn	4.86 ± 3.23
Glu/gln	9.11 ± 3.09
Ser	9.08 ± 0.75
His	2.14 ± 0.30
Thr	4.80 ± 0.75
Gly	13.74 ± 1.25
Ala	11.13 ± 1.84
Tyr	2.92 ± 0.70
Arg	5.62 ± 0.27
Val	8.05 ± 1.87
Met	1.32 ± 0.16
Ile	5.60 ± 0.58
Leu	8.06 ± 0.66
Phe	5.24 ± 0.36
Lys	9.98 ± 0.84

\*Mean values ± SD from three analyses of protein hydrolyzed for 20 hr *in vacuo* in 6M HCl containing 0.01M phenol. Cys and trp were not stable to hydrolytic conditions. Proline was not quantitated by *o*-PTH-HPLC method.

remained after 10 min at 51°C. The activated protyrosinase was even more stable with 50% activity recovered after 10 min at 60°C.

#### Properties of activated protyrosinase

A comparison of properties of the activated haemolymph protyrosinase with those of the cuticular tyrosinase showed that the two enzymes were very similar. Polyacrylamide gel electrophoresis at pH 8.3 in 5% gels revealed that the relative mobility of the activated proenzyme was very close to that of the cuticular tyrosinase,  $R_m = 0.11$  vs 0.13, respectively (Aso *et al.*, 1984). Protein staining of the gels showed that the activated protyrosinase-activator mixture was very heterogeneous because of the impure preparation of activating enzyme used. Both cuticular tyrosinase and the activated proenzyme eluted in 0.2 M NaCl buffer at pH 6.8 during DEAE chromatography (Aso *et al.*, 1984). The pH optimum of the activated proenzyme ( $\sim$ pH 6) was the same as that of the cuticular tyrosinase.

Kinetically the activated haemolymph protyrosinase also behaved much like the cuticular enzyme. Figure 7A shows the absorbance increase at 290 nm caused by the oxidation of four catecholamines by activated protyrosinase. All of the diphenols underwent two two-electron oxidations together with an intermediate 1,4-Michael addition reaction (Kramer *et al.*, 1983). The dopamine and DOPA oxidation continued to generate an absorbance change after about 100 sec while NBAD and

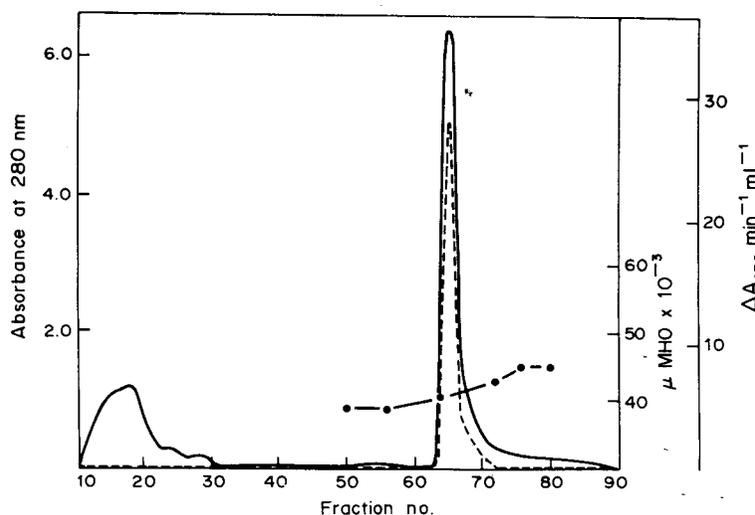


Fig. 4. Elution profile of *M. sexta* protyrosinase activating enzyme from hydroxylapatite. Absorbance at 280 nm (—); activator (---); conductivity (●—●).

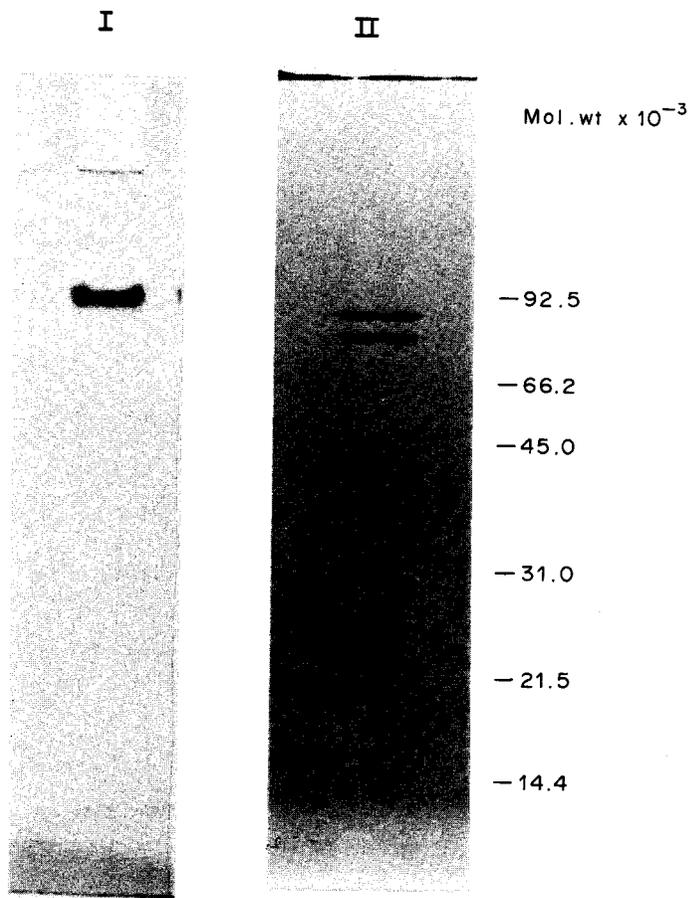


Fig. 3. Polyacrylamide gel electrophoresis of purified protyrosinase from *M. sexta* haemolymph. Lane I, 5% gel run under nondenaturing conditions at pH 8.5; lane II, 5% gel run under denaturing conditions containing 0.1% SDS.

Table 3. Partial purification of *M. sexta* protyrosinase activating enzyme

Step	Total units*	Total protein (mg)	Specific activity	Overall yield	Overall purification
Crude extract	199	342	0.6	100	1
Ammonium sulphate fractionation	296	156	1.9	150	3.3
Hydroxylapatite chromatography	196	31.3	6.3	98	10.8

\*Unit =  $\Delta A_{470} \cdot \text{min}^{-1}$ .

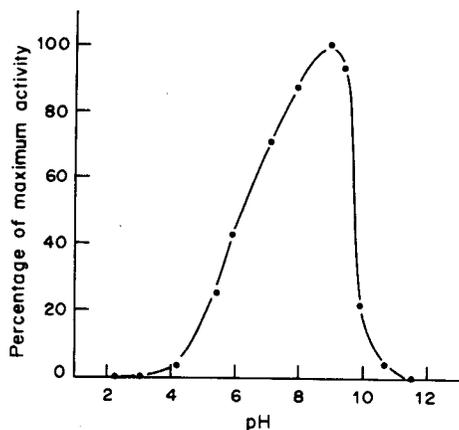


Fig. 5. Effect of pH on the activation of *M. sexta* protyrosinase from haemolymph with cuticular activating enzyme. Mean values  $\pm <10\%$  SD from three replicates.

NADA reached a plateau value. The plateau region was indicative of the *p*-quinone imine products from the *n*-acylated catecholamines which are relatively stable. The free catecholamines continued to react, yielding dihydroxyindole, indole quinone, and melanochrome products (Asó, *et al.*, 1984).

The hydroxylating activity of the activated protyrosinase is shown in Fig. 7B. Tyrosine and tyramine are converted to DOPA and dopamine, respectively, with the latter being more reactive. The addition of a catalytic amount of DOPA increased the rate of hydroxylation by more than 2-fold. A reducing agent

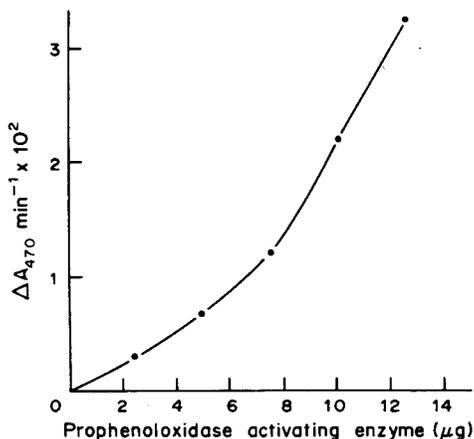


Fig. 6. Effect of activator fraction No. 65 from hydroxylapatite chromatography on activation of protyrosinase. Mean values  $\pm <10\%$  SD from two determinations.

such as an *o*-diphenol may be a cofactor that quantitatively converts the copper ion into the proper valence state before the enzyme is fully active.

Table 4 lists the Michaelis constants for the oxidation of dihydroxy aromatic substrates by *M. sexta* activated protyrosinase, together with the corresponding values for cuticular tyrosinase for comparison (Aso *et al.*, 1984). The lowest  $K_m$  was exhibited by 5,6-dihydroxyindole (DHI), an intermediate of tyrosine metabolism in the melanin biosynthetic pathway, followed by NBAD, dopamine and DOPA. The difference between the  $K_m$  values for the two enzymes is less than a factor of 2.

Activated protyrosinase was inhibited by compounds which also inactivated cuticular tyrosinase including  $10^{-4}$  M diethyldithiocarbamate,  $10^{-4}$  M phenylthiourea and  $10^{-3}$  M sodium cyanide, which apparently combine with the copper ion present in the enzyme.

DISCUSSION

The protyrosinase in the haemolymph of fifth stadia *M. sexta* has several properties in common with protyrosinase characterized from the haemolymph of Diptera (Karlson *et al.*, 1964; Hackman and Goldberg, 1967; Sin and Thompson, 1971; Pau and Kelly, 1975) and the silkworm, *Bombyx mori* (Ashida and Ohnishi, 1967; Ashida, 1971). In general they are copper-containing proteins made up of two or more subunits that catalyze the oxidation of both mono and diphenols after activation by cuticular proteases. The *Manduca* and *Bombyx* proenzymes are both

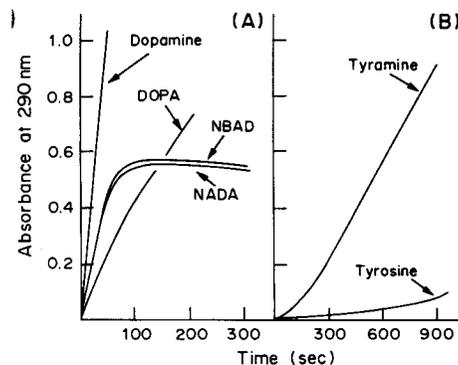


Fig. 7. Continuous spectrophotometric tracing of conversion of catecholamines (A) and monophenols (B) into oxidized and hydroxylated products, respectively, by *M. sexta* activated haemolymph protyrosinase. Dopamine, 1.37 mM in 0.1 M sodium phosphate buffer, pH 6.5; DOPA, 1.53 mM; NADA, 1.33 mM; NBAD, 1.67 mM; tyrosine, 1.43 mM; tyramine, 1.78 mM.

Table 4. Michaelis constants for the oxidation of substrates by activated haemolymph protyrosinase (I) and cuticular tyrosinase (II) from *M. sexta*\*

Substrate†	$K_m$ ( $M \times 10^3$ )‡	
	I	II§
DOPA (3,4-dihydroxyphenylalanine)	4.15 ± 0.37	8.40 ± 0.46
Dopamine (3,4-dihydroxyphenethylamine)	0.71 ± 0.05	0.91 ± 0.03
NBAD ( <i>N</i> -β-alanyldopamine)	0.42 ± 0.04	0.23 ± 0.02
DHI (5,6-dihydroxyindole)	0.03 ± 0.01	0.02 ± 0.01

\* $S_0 = 0.01$ – $15$  mM. Protein concentration =  $1$ – $76$  μg/ml. †Activity determined spectrophotometrically at 470 nm (DOPA), 430 nm (dopamine), 390 nm (NBAD) and 540 nm (DHI). Data were treated by Eadie-Hofstee analysis. ‡ ± SD ( $n = 2$ ). §Values from Aso *et al.* (1984).

dimers but the subunits of the former are nearly twice the molecular weight of the latter. The amino acid composition of the two proteins also differs somewhat but both protyrosinases have similar amounts of copper. The functional significance of insect haemolymph protyrosinases or phenoloxidases is not clearly known but they may be important for synthesis of haemolymph catecholamines involved in cuticular tanning, melanization, encapsulation of parasites and wound healing (reviewed by Brunet, 1980).

The physical, chemical and kinetic properties measured in this study of the haemolymph protyrosinase after activation by a cuticular enzyme are very similar to those of the tyrosinase extracted from pupal cuticle of *M. sexta* (Aso *et al.*, 1984). This suggests that the two enzymes are very closely related and possibly that the haemolymph proenzyme serves as a precursor for the cuticular tyrosinase. The zymogen which is probably utilized for generating a wound healing enzyme in response to injury may also provide the enzyme which functions in the sclerotization and melanization of cuticle.

Synthesis of an inactive enzyme precursor of tyrosinase as well as sequestration of the activating enzyme away from both the proenzyme and substrates are all components of the regulatory system for tyrosine metabolism in the tobacco hornworm. Tyrosinase is synthesized as a proenzyme that is soluble, stable and readily available in the haemolymph. However, once activated, the enzyme is quite unstable (Aso *et al.*, 1984), apparently catalyzing autoinactivation via oxidation of its tyrosyl residues or becoming crosslinked by quinones generated by substrate oxidation. Insect protyrosinases have been shown to aggregate and become insoluble after activation (Karlson *et al.*, 1964; Aso, Kramer and Hopkins, unpublished). The separate localizations of proenzyme in haemolymph, where substrates are plentiful, and activating enzyme in cuticle insures that premature oxidation of catechols does not occur in haemolymph. Also the presence of ascorbic acid in haemolymph may deter such oxidative metabolism and stabilize the catechols (Kramer *et al.*, 1981). Prottyrosinase is activated for sclerotization, melanization or wound healing only when the proenzyme, activating enzyme and substrates such as DOPA, dopamine, NBAD, NADA and oxygen come together in the cuticle or at the surface of damaged tissue. The products which result are most likely determined by the specific substrates available (Aso *et al.*, 1984). Melanin may be derived primarily from DOPA and dopamine while sclerotins

may be structural proteins stabilized by NBAD and NADA metabolites (Kramer *et al.*, 1984; Hopkins *et al.*, 1984).

*Acknowledgements*—Contribution No. 84-324-J. Cooperative investigation between ARS, USDA and the Kansas Agricultural Experiment Station. Mention of a proprietary product does not imply approval by the USDA to the exclusion of products that may also be suitable. Supported in part by National Science Foundation grant PCM-8003859-A01. Y.A. was on leave from the Laboratory of Sericultural Chemistry, Faculty of Agriculture, Kyushu University, Fukuoka 812, Japan.

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