

# A Comparison of Mushroom Tyrosinase Dopaquinone and Dopachrome Assays Using Diode-Array Spectrophotometry: Dopachrome Formation vs Ascorbate-Linked Dopaquinone Reduction

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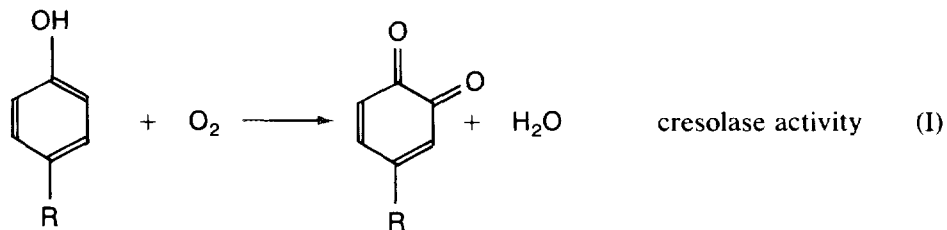
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Comparisons of two assay methods for the catecholase activity of mushroom tyrosinase (EC 1.14.18.1) are reported. Tyrosinase (or polyphenol oxidase) is a widely distributed copper-containing enzyme which possesses both monooxygenase (cresolase) and oxidase (catecholase) activities. In this report the substrate employed is L-3,4-dihydroxyphenylalanine and the dopachrome formation and ascorbate-linked dopaquinone reduction assay methods are compared using a photodiode array spectrophotometer. This instrument has an advantage over a conventional spectrometer for kinetic studies since it is able to carry out simultaneous multiwavelength kinetic measurements at a relatively fast rate. The use of this capability in performing the two assays is described. © 1993 Academic Press, Inc.

## INTRODUCTION

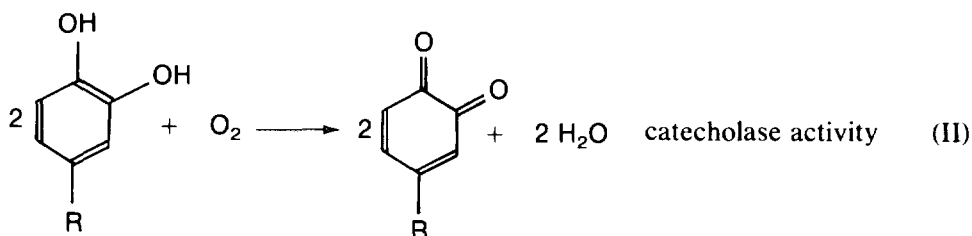
Upon injury to the tissue, mushrooms, bananas, apples, and many other plant products darken in color as the result of enzymatic oxidation of certain monohydric and *o*-dihydric phenols. The enzyme responsible for these oxidations is polyphenol oxidase/tyrosinase, a bifunctional enzyme (1-3). Using molecular oxygen, mushroom tyrosinase (EC 1.14.18.1, monophenol, *o*-diphenol: O<sub>2</sub> oxidoreductase) is capable of catalyzing two types of oxidation (4-6). One of these is the orthohydroxylation of phenols in which a hydroxyl group is introduced at the *ortho* position to the one already present in certain monohydric phenols (Reaction (I)). The other is the oxidation of certain *o*-dihydric phenols to their corresponding *o*-quinones (Reaction (II)). The oxygen atom inserted in the phenols during oxidation originates from molecular oxygen (7, 8).

*p*-Cresol and catechol have been most frequently employed as experimental substrates for studying the two types of oxidation. Consequently, the two activ-



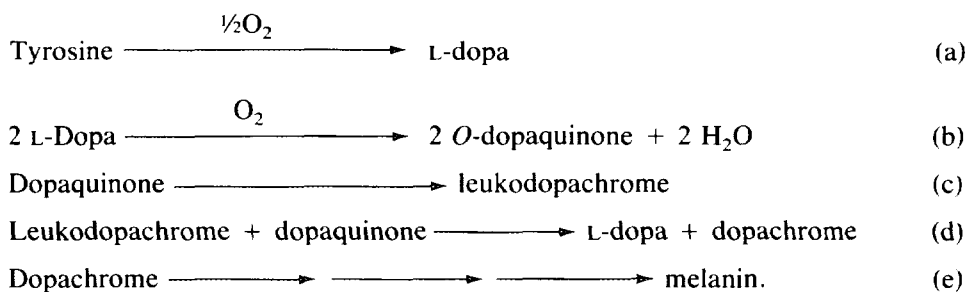
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ities of mushroom enzyme have come to be known as "cresolase" and "catecholase" activities, respectively (2, 9, 10).

The studies of Raper (11), Mason (7), and more recently of Jimenez *et al.* (12) and Garcia *et al.* (13, 14) indicate that tyrosinase catalyzes the conversion of L-tyrosine to L-3,4-dihydroxyphenylalanine (L-dopa) and then to melanin by the following reactions:



It is believed that reactions (a) and (b) are enzymatically catalyzed while reactions (c) and (d) may be spontaneous. Dopachrome is slowly converted to melanin through a series of reactions.

The catecholase activity of tyrosinase can be kinetically measured by the extent of dopachrome formation at 475 nm (using L-dopa as the substrate). A coupled assay can also be employed using the oxidation of ascorbic acid (measured at 265 nm) to determine the amount of dopaquinone formed from L-dopa. Because it is desirable to measure on a common basis the activity of different tyrosinase preparations, it seemed advisable to compare the catecholase activity of the enzyme by the two assay methods. For this purpose, a UV-visible photodiode array spectrophotometer (PDA) was used. There are a number of significant advantages associated with the use of the PDA spectrometer. Problems such as wavelength resettability and instrumental drift, common drawbacks of conventional spectrometers, are either reduced or eliminated (15). Fast spectral data acquisition, excellent wavelength reproducibility, and improved sensitivity through time and wavelength averaging are a few of the advantages. The availability of the entire spectrum, virtually free of wavelength resettability errors, allows the experimenter to choose the optimum wavelength for improved sensitivity. In addition, measurements at any wavelength, at peak maxima or on the side of the absorption band, can be made with essentially the same accuracy and precision (15, 16).

Another advantage of the PDA spectrophotometer is the capability of the instrument to make simultaneous multiwavelength measurements in a single sample. Although most conventional spectrophotometers are capable of multiwavelength measurements, they take time to move from one point in the spectrum to another. A PDA spectrophotometer, however, measures all wavelengths in the spectrum simultaneously and, as such, eliminates the need for multiple measurements. This capability of the instrument results in a significant increase in productivity and, in particular, makes it an excellent choice for kinetic measurements. The Milton-Roy 3000 photodiode array spectrometer used in this study is capable of collecting kinetic data at eight different wavelengths simultaneously at a rate of two points per second.

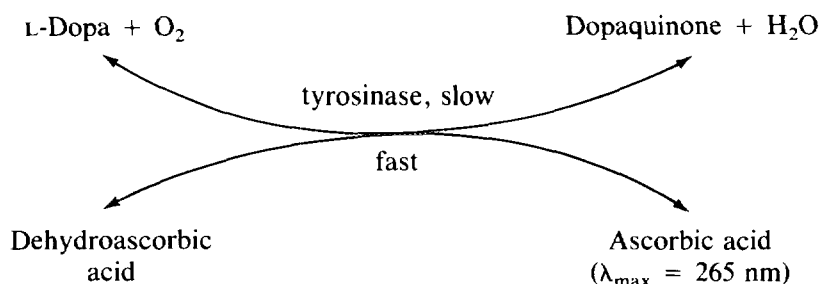
## MATERIALS AND METHODS

Mushroom tyrosinase was purchased from Sigma Chemical Co. and was used without further purification. L-Dopa was also from Sigma Chemical Co. L-Ascorbic acid was purchased from Fisher Scientific. All other reagents were of highest purity available. Distilled deionized water was used throughout for making all solutions. Typically, a stock solution of tyrosinase at 50  $\mu\text{g/ml}$  ( $\epsilon_{280}^{1\%} = 24.9$  (6)) in a 0.1 M potassium phosphate buffer at pH 7.0 was prepared and from that the aliquots were added to the assay mixtures. All kinetic runs were carried out in a 1-cm path length quartz cell on a Milton-Roy 3000 photodiode array spectrophotometer in the following ways.

*Molar absorptivities of ascorbic acid.* A stock solution of 1.0 mM ascorbic acid in a deoxygenated 0.1 M potassium phosphate buffer at pH 7.0 containing 3.0  $\mu\text{M}$  EDTA was prepared. A trace amount of dithiothriol (DTT) was added to the solution to help prevent ascorbic acid autooxidation. Proper dilutions were made from this solution to determine the extinction coefficient of the acid at the three wavelengths used for the coupled assays. Absorbances at 255, 265, and 275 nm of the solutions were recorded simultaneously using the "advanced scan" mode of the spectrophotometer. Using linear least-square analysis of the data, the molar absorptivities were determined to be  $(11.9 \pm 0.3) \times 10^3 \text{ M}^{-1} \text{ cm}^{-1}$  at 255 nm,  $(15.1 \pm 0.3) \times 10^3 \text{ M}^{-1} \text{ cm}^{-1}$  at 265 nm  $(13.2 \pm 0.4) \times 10^3 \text{ M}^{-1} \text{ cm}^{-1}$  at 275 nm. The value at 265 nm is essentially identical to  $15.3 \times 10^3 \text{ M}^{-1} \cdot \text{cm}^{-1}$  reported earlier (17).

*Measurement of dopachrome formation.* The oxidation of L-dopa was followed by measuring the initial rate of increase in absorbance at 475 nm, at which dopachrome has a molar absorptivity of  $3700 \text{ M}^{-1} \cdot \text{cm}^{-1}$  (7). The standard reaction mixture contained air-saturated potassium phosphate ( $\text{KPi}$ ), pH 7.0 (0.1 M), L-dopa (0.08 to 5.0 mM), and tyrosinase (2.5 to 5.0  $\mu\text{g}$ ) in a total volume of 3.0 ml. With these concentrations, a linear rate of increase in absorbance was achieved for the first 60 to 90 s and the rate was proportional to the amount of the enzyme.

*Measurement of dopaquinone formation.* The rate of formation of dopaquinone from L-dopa was determined in a coupled assay in which the fast nonenzymatic oxidation of ascorbic acid was coupled to the reduction of dopaquinone enzymatically formed from L-dopa.



It has been reported that ascorbic acid is not oxidized by the enzyme (17). Therefore, the rate of ascorbate oxidation by dopaquinone, measured by a decrease in absorbance at 265 nm, is directly proportional to the extent of L-dopa oxidized to dopaquinone. The usual experimental conditions for the assay were  $\text{KPi}$ , pH 7.0 (0.1 M), L-dopa (0.08 to 0.7 mM), enzyme (2.5 to 5  $\mu\text{g}$ ), ascorbic acid (0.02  $\text{mg} \cdot \text{ml}^{-1}$ ), and EDTA (2.0  $\mu\text{M}$ ) in a total volume of 3.0 ml. The ascorbic acid was added from a 0.42  $\text{mg} \cdot \text{ml}^{-1}$  stock solution in  $\text{KPi}$  buffer containing 0.1% (w/v) metaphosphoric acid. It has been reported that metaphosphoric acid stabilizes the ascorbic acid solution against autooxidation and it has no effect on the activity of the enzyme (18). No blank oxidation of ascorbic acid was observed in the presence of metaphosphoric acid. Under these conditions, the initial rate of ascorbic acid oxidation (after a 15- to 20-s lag) was maintained for 1 to 2 min following the initiation of the reaction and it was proportional to the amount of the enzyme.

*Data analysis.* Initial data analysis ( $v_0$  measurements) was done using the "application package software" of the Milton-Roy PDA spectrophotometer. Curve-fittings were done using "QUATRO.PRO Spreadsheet" from Borland on an IBM PC computer.

## RESULTS AND DISCUSSION

It has been reported that different preparations of tyrosinase could differ markedly in their activity toward catechol oxidation because of the inactivation of the enzyme during the catalytic reaction (1, 19). This means that catecholase activities based on rate measurements are influenced by the degree of enzyme inactivation. In lieu of this, it is apparent that such measurements can give reliable results only if they are based on the initial reaction velocity. A variety of assays have been used over the years to measure the activity of the enzyme (1, 20-23). The old Warburg manometer technique which is based on monitoring the course of oxygen uptake during oxidation of catechol suffered from the fact that activity measurements were made at some time after catechol had been added to the reaction mixture. As a result, rate measurements were influenced by what has happened to the enzyme during the time prior to measurements. Since absorbance can be made to be a linear function of concentration and it is easily measured, tyrosinase activities are now measured spectrophotometrically using L-dopa as the substrate.

In order to compare the catecholase activity of tyrosinase by dopachrome formation and dopaquinone reduction, a photodiode array spectrophotometer was

used. As explained earlier, since absorbance changes at several wavelengths can be measured simultaneously for a solution, the need for repeated measurements is, in part, eliminated. In monitoring the rate of dopaquinone formation in this study, absorbance changes at 255, 265, and 275 nm were simultaneously recorded. In other words, each run is in fact a collection of three identical single wavelength measurements. The rate of dopachrome formation was measured only at 475 nm since extinction coefficients at other wavelengths were not available. However, similar multiwavelength measurements of dopachrome can be easily made by first calculating the relative molar absorptivities at other absorbing wavelengths from the value of  $3700 M^{-1} \cdot \text{cm}^{-1}$  at 475 nm.

The initial velocity data from both assay methods were analyzed by Lineweaver–Burk, Eadie–Hofstee, and Hanes–Woolf methods. The Michaelis parameters obtained are shown in Table 1. Figure 1 shows the double-reciprocal plot of the rate data obtained at 475 nm (dopachrome, 5.0  $\mu\text{g}$  enzyme) and Fig. 2 displays the Hanes–Woolf plot of the rate data at 265 nm (dopaquinone, 5.9  $\mu\text{g}$  enzyme). The  $K_M$  values for L-dopa obtained from each assay method are nearly identical. An average value of  $(2.7 \pm 0.3) \times 10^{-4} M$  for  $K_M$  can be calculated from these results. This value compares favorably with  $(4\text{--}5) \times 10^{-4} M$  reported as the  $K_M$  value for the same substrate for hamster melanoma tyrosinase (17). To compare the maximal velocities from the two assay methods, the experimental  $V_{\text{max}}$  values in Table 1 were converted from  $\Delta A \cdot \text{min}^{-1}$  into  $\mu\text{mol} \cdot \text{min}^{-1}$  under assay conditions as follows:

$$\mu\text{mol} \cdot \text{min}^{-1} = \Delta A \cdot \text{min}^{-1} \times \frac{10^6}{\epsilon(\lambda_{\text{max}})}$$

Using molar absorptivities of  $3700 M^{-1} \cdot \text{cm}^{-1}$  (dopachrome) and  $15,100 M^{-1} \cdot \text{cm}^{-1}$  (ascorbate),  $V_{\text{max}}$  values of 33.6  $\mu\text{mol}$  dopaquinone per minute and 18.4  $\mu\text{mol}$  dopachrome per minute were calculated when an equal amount of the enzyme (5.0  $\mu\text{g}$ ) was used in the two assays. The results indicate that the maximal

TABLE I  
 $K_M$  and  $V_{\text{max}}$  Values for L-dopa Using the Dopachrome Formation and Ascorbate-Linked  
Dopaquinone Reduction Assays

	Method	$K_M$ (M)	$V_{\text{max}}$ ( $\Delta A \text{ min}^{-1}$ )
I	Dopaquinone	$(2.8 \pm 0.3) \times 10^{-4}$ (a)	$0.250 \pm 0.022$
	Reduction	$(2.5 \pm 0.2) \times 10^{-4}$ (b)	$0.234 \pm 0.007$
	(2.5 $\mu\text{g}$ protein)	$(2.5 \pm 0.2) \times 10^{-4}$ (c)	$0.231 \pm 0.008$
II	Dopaquinone	$(2.3 \pm 0.2) \times 10^{-4}$ (a)	$0.493 \pm 0.036$
	Reduction	$(2.4 \pm 0.2) \times 10^{-4}$ (b)	$0.503 \pm 0.017$
	(5.0 $\mu\text{g}$ protein)	$(2.7 \pm 0.2) \times 10^{-4}$ (c)	$0.506 \pm 0.020$
III	Dopachrome	$(3.2 \pm 0.2) \times 10^{-4}$ (a)	$0.069 \pm 0.004$
	Formation	$(3.1 \pm 0.1) \times 10^{-4}$ (b)	$0.068 \pm 0.002$
	(5.0 $\mu\text{g}$ protein)	$(3.1 \pm 0.2) \times 10^{-4}$ (c)	$0.068 \pm 0.001$
		Range = $(2.3\text{--}3.2) \times 10^{-4}$	

Note. See Materials and Methods section for assay conditions. (a) Lineweaver–Burk analysis, (b) Eadie–Hofstee analysis, (c) Hanes–Woolf analysis.

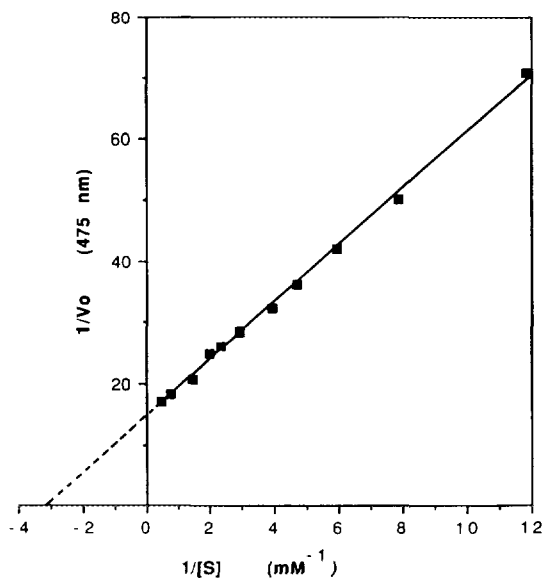


FIG. 1. Lineweaver-Burk plot of the dopachrome formation initial velocity data. Enzyme concentration:  $5.0 \mu\text{g}$  in 3.0 ml reaction mixture. Other conditions are given under Materials and Methods.

rate of L-dopa oxidation, measured by the extent of dopaquinone formation, is about twice the maximal rate of formation of dopachrome from L-dopa. This is in accord with the postulated mechanistic pathway of Raper (11) and Mason *et al.* (8) described earlier. From this pathway, the stoichiometry equation of 2 dopaqui-

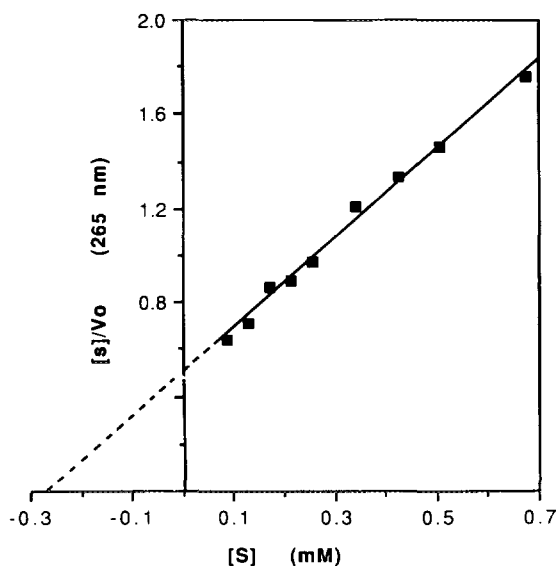


FIG. 2. Hanes-Woolf plot of the dopaquinone reduction initial velocity data. Enzyme concentration:  $5.0 \mu\text{g}$  in 3.0 ml reaction mixture. Other conditions are given under Materials and Methods.

none  $\rightarrow$  dopachrome + L-dopa is easily obtained by adding reactions (c) and (d). Knowing this, it can easily be shown that the following relationship exists:

$$\Delta A_{475} = \frac{\epsilon_{475}}{2\epsilon_{265}} \cdot \Delta A_{265},$$

where  $(\epsilon_{475})/(2\epsilon_{265}) = 0.123$ . This allows interconversion between the two assay methods.

In an effort to further compare the two assays, experiments were done to estimate the number of catecholase units of a tyrosinase solution from the initial velocity data. Since dopaquinone measurements cannot be performed at saturating concentrations of L-dopa due to a high absorbancy of L-dopa near 265 nm, both assays were carried out at 0.30 mM L-dopa ( $K_M$  value) to obtain half-maximal velocities. By converting the half-maximal velocities obtained in this way into catecholase units a direct comparison of the two assays is possible. The results are tabulated in Table 2. With the exception of fixing the concentration of L-dopa at 0.30 mM, other conditions were the same as those described under Materials and Methods. Figures 3 and 4 represent typical time courses obtained for dopachrome formation and dopaquinone reduction, respectively. The catecholase units in Table 2 were calculated by using a unit definition of catecholase activity as the

TABLE 2  
Calculation of Catecholase Units from Dopachrome Formation and Dopaquinone Reduction Assay Data

A. Dopachrome formation				
Enzyme ( $\mu\text{g}$ )	Dilution factor	$v_0$ ( $\Delta A \cdot \text{min}^{-1}$ ) <sup>a</sup>	$V_{\text{max}}$ ( $\mu\text{mol} \cdot \text{dopach} \cdot \text{min}^{-1}$ )	Units, total <sup>b</sup>
2.5	60	0.019	10.3	1488
5.0	30	0.037	20.4	1469
10.0	15	0.075	40.3	1459
				Ave. = 1472 $\pm$ 15
B. Dopaquinone reduction				
(nm)	Dilution factor	$v_0$ ( $\Delta A \cdot \text{min}^{-1}$ ) <sup>a</sup>	$V_{\text{max}}$ ( $\mu\text{mol} \cdot \text{dopaqu} \cdot \text{min}^{-1}$ )	Units, total <sup>c</sup>
255	60	0.103	17.4	1264
	30	0.213	36.0	1304
	15	0.438	73.8	1337
265	60	0.137	18.1	1309
	30	0.283	37.3	1352
	15	0.589	77.6	1406
275	60	0.113	17.0	1230
	30	0.238	35.7	1295
	15	0.492	73.9	1338
				Ave. = 1315 $\pm$ 50

<sup>a</sup> Each  $v_0$  represents an average of two measurements; data taken from Figs. 2 and 4.

<sup>b</sup> Units =  $V_{\text{max}}$  ( $\mu\text{mol} \cdot \text{dopach} \cdot \text{min}^{-1}$ )  $\times$  (1 catech. unit)/(0.414  $\mu\text{mol} \cdot \text{dopach} \cdot \text{min}^{-1}$ )  $\times$  D.F.

<sup>c</sup> Units =  $V_{\text{max}}$  ( $\mu\text{mol} \cdot \text{dopaqu} \cdot \text{min}^{-1}$ )  $\times$  (1 catech. unit)/(0.828  $\mu\text{mol} \cdot \text{dopaqu} \cdot \text{min}^{-1}$ )  $\times$  D.F.

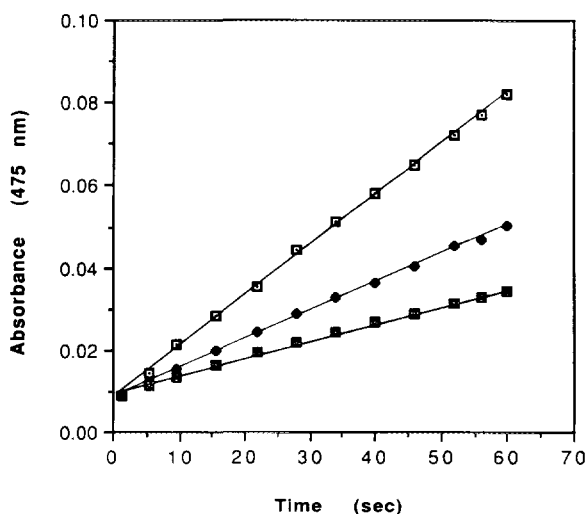


FIG. 3. Curves showing the linear production of dopachrome during the initial phase of oxidation of L-dopa by mushroom tyrosinase. Curves are corrected to start at the same initial absorbance. [L-dopa] = 0.30 mM. Tyrosinase (in 3.0 ml reaction volume): 2.5  $\mu\text{g}$  (■), 5.0  $\mu\text{g}$  (◆), 10  $\mu\text{g}$  (□).

amount of enzyme which causes an oxygen uptake of 10  $\mu\text{l}$  per minute (*l*). This is equivalent to formation of 0.828  $\mu\text{mol}$  dopaquinone or 0.414  $\mu\text{mol}$  dopachrome per minute. In the ascorbate-linked dopaquinone reduction method, simultaneous absorbance measurements were made at 255, 265, and 275 nm for the same solution. The data at the three wavelengths were then converted into catecholase

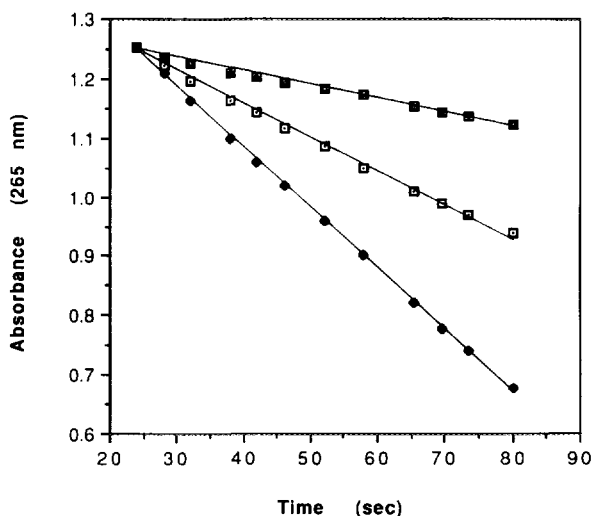


FIG. 4. Curves showing the linear disappearance of L-ascorbic acid (equivalent to dopaquinone reduction) during the initial phase of oxidation of L-dopa by mushroom tyrosinase. Curves are corrected to start at the same initial absorbance. [L-dopa] = 0.30 mM, [ascorbic acid] = 0.020  $\text{mg} \cdot \text{ml}^{-1}$ . Tyrosinase (in 3.0 ml reaction volume): 2.5  $\mu\text{g}$  (■); 5.0  $\mu\text{g}$  (□); 10  $\mu\text{g}$  (◆).



units by using absorptivities (determined as described under Materials and Methods) of 11.9, 15.1, and 13.3  $\text{mM}^{-1} \cdot \text{cm}^{-1}$  for 255, 265, and 275 nm, respectively. Average values of 1315 and 1472 catecholase units were calculated for the enzyme activity by the dopaquinone and dopachrome methods, respectively. An assay of the same enzyme solution by the dopachrome method at saturating concentration of L-dopa, 4.8 mM, gave a value of 1440 catecholase units for the solution, which is consistent with the numbers calculated above from the half-maximal velocities. The results clearly indicate that the two assay methods are complementary and that assays at the  $K_M$  concentration of L-dopa by either method can be used to obtain a reliable estimate of the number of catecholase units in a solution of the enzyme.

### SUMMARY

A thorough comparison of two assay methods for the enzyme mushroom tyrosinase has been presented. Under proper experimental conditions, the two assays are complementary and yield the same value for  $K_M$  when L-dopa is used as the substrate. Our data support the catalytic mechanism previously proposed for the enzyme. Assays at the  $K_M$  concentration of L-dopa can be used in conjunction with either method to estimate the catecholase activity of a tyrosinase solution. Data describing kinetic advantages associated with a photodiode-array spectrophotometer when compared to a conventional UV-vis instrument are also presented.

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