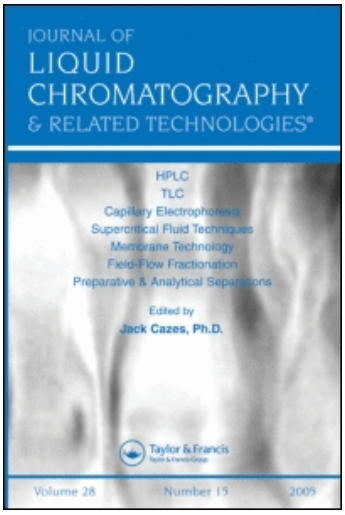

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ANALYSIS OF MONOPHENOL OXIDASE ACTIVITY USING HIGH PRESSURE LIQUID CHROMATOGRAPHY WITH ELECTROCHEMICAL DETECTION

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ABSTRACT

A very sensitive, specific and reliable quantitative assay was developed for measuring the rate of hydroxylation of tyrosine by mushroom tyrosinase using high pressure liquid chromatography with electrochemical detection (HPLC-ED). The assay employs N-acetyldopamine (NADA) as cofactor and ascorbate as a reducing agent. The product of the reaction, L-dopa (3,4-dihydroxyphenylalanine), was readily separated by HPLC-ED from the remaining interacting components. The reaction was linear with time and proportional to the amount of enzyme present. The amount of ascorbate gradually decreased during the hydroxylation of tyrosine, but as long as ascorbate was present in the reaction mixture the levels of L-dopa and NADA were not altered. The data

provide additional support for the proposal that ascorbate maintains the level of L-dopa produced during tyrosine hydroxylation by reducing dopaquinone back to the o-diphenol nonenzymatically. Comparative studies showed the sensitivity of the HPLC-ED assay was at least 100 times greater than a specific radioenzymatic assay which used [^{3}H]tyrosine. The applicability of the HPLC-ED assay was demonstrated by using microliter quantities of crude hemolymph from larvae of *Drosophila melanogaster.*

INTRODUCTION

Tyrosinase (monophenol, dihydroxyphenylalanine: oxygen oxidoreductase, EC 1.14.18.1), also known as polyphenol oxidase, is a copper-containing oxygenase widely distributed in nature, being responsible for melanization in animals and browning in plants. The enzyme exhibits a wide substrate specificity, and has been shown to catalyze two distinct types of O_2-dependent reactions: the ortho hydroxylation of tyrosine and other monophenols to o-diphenols (cresolase, monophenolase, or hydroxylase activity), and the oxidation of dopa and certain other o-diphenols to o-quinones (catecholase or diphenolase or diphenol oxidase activity). The mechanisms of action of various tyrosinases with both monophenol and diphenol substrates have been reviewed in considerable detail (1-4). Diphenol oxidase (DPO) activity is commonly assayed by monitoring the formation of dopachrome from L-dopa at 475 nm. Unfortunately, this spectrophotometric assay is fairly insensitive because of the low molar absorption coefficient ($E=3600 \cdot M^{-1} \cdot cm^{-1}$) of dopachrome (5). Recently, Li et al (6)

employed high pressure liquid chromatography with electrochemical detection to develop a sensitive diphenol oxidase (DPO) assay using 3,4-dihydroxymandelic acid as substrate for mushroom tyrosinase. Since the product of this reaction (3,4-dihydroxybenzaldehyde) is stable and does not undergo further oxidation (7) the DPO activity of tyrosinase can be accurately determined. Monophenol oxidase (MPO) activity has been more difficult to accurately ascertain than DPO activity because the product of tyrosine hydroxylation, L-dopa, is the diphenol substrate for the ensuing oxidation reaction by tyrosinase. This difficulty may be circumvented by using tritiated tyrosine and calculating the amount of ^3H_2O formed as a by product during the reaction (8). This radioenzymatic assay is very specific in terms of monitoring only the MPO activity of tyrosinase. The characteristic lag phase in MPO activity can be eliminated by adding a small amount of L-dopa to the reaction (8-10). However, this method of measuring the hydroxylation of tyrosine is cumbersome and very time consuming. Moreover, in addition to the concerns associated with the use of radioactive materials, the sensitivity of the radioenzymatic method is affected by high background counts resulting from the exchange of hydrogen protons, and by diluting factors caused by the ratio of labelled tyrosine vs cold tyrosine.

This paper describes a very sensitive and precise method for evaluating the MPO activity of mushroom tyrosinase using high pressure liquid chromatography with electrochemical detection (HPLC-ED) and incorporating ascorbate into the reaction mixture to prevent the oxidation of the o-diphenol (dopa) produced during the hydroxylation of tyrosine. We report on comparative studies of the

sensitivity of the HPLC-ED assay with a standard radiometric assay, and establish the suitability of the HPLC-ED method for measuring MPO activity in the blood (hemolymph) of larvae of *Drosophila melanogaster*.

MATERIALS AND METHODS

Chemicals and Reagents: Ascorbic acid, N-acetyldopamine (NADA), 3,4-dihydroxyphenenylacetic acid, L-dopa, dopamine, epinephrine, norepinephrine, caffeic acid, 3,4-dihydroxybenzioc acid, L-tyrosine, 3-(N-morpholino)propanesulfonic acid, octyl sulfate, 1-Phenyl-2-thiourea, and mushroom tyrosinase were purchased from Sigma Chemical Co. (St. Louis, Missouri). L-[3,5-^{3}H]tyrosine (56 Ci/mmol) was obtained from Amersham Corporation (Arlington Heights, Illinois).

Monophenol Oxidase Assay: To determine the optimal conditions for monitoring the MPO assay by HPLC-ED, the effects of various concentrations of ascorbate (0.1 to 4 mM) on the enzyme catalytic rate were investigated radioenzymatically using the method described by Townsend et al. (8). Following these determinations different concentrations of various diphenols were tested to determine their effect on eliminating the initial lag phase that characterizes the hydroxylation response of tyrosinase, a response that was observed by both radioenzymatic and HPLC-ED methods. After determining the effects of ascorbate and various cofactors on MPO activity, standard assay conditions were

established for all subsequent tests. All reaction mixtures, total volume 300 μL, were comprised of 100 μL enzyme preparation containing 0.3 μg mushroom tyrosinase protein in 50 μM MOPS buffer (pH 6.5), and 200 μL substrate preparation containing 0.42 μmol of L-tyrosine, 1.5 nmol N-acetyldopamine (NADA) and 0.12 μmol of ascorbate in 50 μM MOPS buffer. The reaction mixtures were incubated at 30°C. The reactions were stopped at 5, 10, 15 and 20 min by withdrawing 60 μL aliquots of the reaction mixture and adding an equal volume of ice-cold 1 M citric acid. The solutions were put on ice and centrifuged for 15 min at 15000 x g at 4° C in a microfuge. Five or 10 μL samples of the supernatant were injected for HPLC-ED analysis. The sensitivity of electrochemical detection for MPO activity was compared with a radioenzymatic assay. In these experiments the amount of mushroom tyrosinase in the reaction mixtures was reduced to 10, 3, 1.5, 0.6 ug protein by a serial dilution in a total volume of 60 ul and assayed under the conditions specified above for 5 min.

HPLC-ED analysis: The stopped reaction mixtures were analyzed by high pressure liquid chromatography with electrochemical detection (HPLC-ED). The HPLC-ED system consisted of a Bioanalytical Systems (West Lafayette, IN) LC-4B electrochemical detector equipped with a glassy carbon electrode maintained at a potential of +0.75 mV versus a Ag/AgCl reference electrode and a sensitivity range of either 20 or 100 nA full scale depending on the extent of enzyme activity. Separations were achieved at 40°C by a BAS Phase-II, 3-μm

ODS reverse phase column (3.2 mm I.D. x 10 cm). A Gilson (Madison, WI) 712 HPLC System Controller was used to integrate peak dimensions. The mobile phase consisted of 0.1 M citrate buffer (pH 3.2) containing 2.5% acetonitrile, 0.75 mM sodium octyl sulfate, and 0.5 mM Na_2EDTA. The flow rate was 0.8 ml/min. Attempts to identify unknown electroactive components in samples of hemolymph and in reaction mixtures were made by comparing retention times of these components with those of authentic standards. Product quantitation was based on comparisons of peak dimensions (height and/or area) produced during enzyme activity with those of standards curves established for each test and analyzed by linear regression. Standards were run before and after each set of assays in order to monitor changes in instrument sensitivity. Control incubation mixtures lacking enzyme were analyzed to determine the extent of nonenzymatic or autooxidation occurring during the time-course of each assay. One unit of MPO activity is defined as the amount of enzyme protein catalyzing the formation of 1 nmol of L-dopa per minute under the described conditions of the assay. Specific enzyme activity is expressed as units per mg protein.

MPO Activity of *Drosophila* Hemolymph: To evaluate the applicability of the MPO assay, hemolymph from third (last) instar larvae of *Drosophila melanogaster* was used as the enzyme preparation. The flies used in this study were raised on standard cornmeal and yeast medium at 25°C by previously described methods (11). Hemolymph issuing from a small incision made in the larval cuticle was collected with a microcapillary pipette. For analysis of enzyme

activity 0.1 to 0.2 μL of hemolymph collected in this manner from each host larva was placed into 100 μL of ice cold MOPS and frozen at -20°C and later used as the enzyme preparation.

RESULTS

L-dopa produced during the hydroxylation of tyrosine was easily resolved from ascorbate and NADA at the applied potential of +0.75 V (Fig. 1A). The same components were also separated from one another and from L-tyrosine at + 0.85 V, at which potential the substrate was also oxidized (Fig. 1B). Although quantitation of enzyme product could have been made at either of the two applied voltages, the electrochemical detector was maintained at +0.75 V for the standard MPO assay because the baseline at this voltage was more stable than that formed at the higher voltage. Under these analytical conditions the retention times for ascorbic acid, L-dopa and NADA were 0.88, 2.35 and 5.11 min, respectively. Electrochemical detector response, determined by measurements of peak dimensions, was proportional to the amount of L-dopa injected over a range of 2 to 70 pmol (Fig. 2), with a correlation coefficient of 0.99.

Ascorbate was found not to be a substrate for mushroom tyrosinase, since there was no oxidation of ascorbate when incubate alone with the enzyme. However, in initial experiments it was shown that the ascorbate-mediated hydroxylation of tyrosine by mushroom tyrosinase was characterized not only by elevated levels of L-dopa (Fig. 3), but also by a gradual decline in the level of the reducing agent during enzyme activity (Fig. 4). Thus, an initial concern was

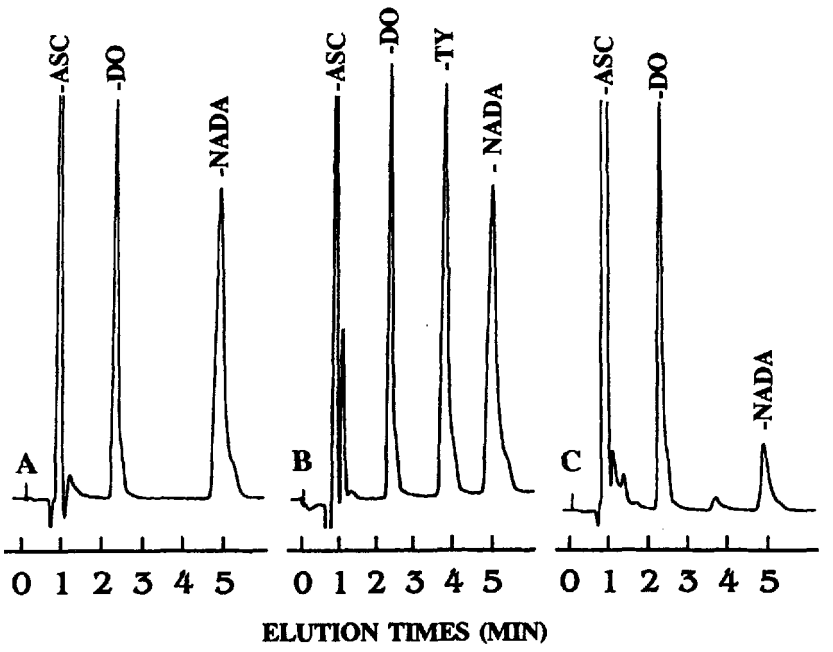

Figure 1. **(A) Chromatogram showing separation of 30 pmol of ascorbate (ASC), L-dopa (DO) and NADA by HPLC-ED at +0.75 V and a sensitivity of 100 nA full scale. (B) Separation of 0.4 nmol tyrosine (TY) at a +0.85 and 100 nA full scale. (C) L-dopa formed after incubating 0.2 μL hemolymph from a third stage larvae of *D. melanogaster* with 1.4 mM tyrosine, 0.4mM ascorbate, and 5 μM NADA for 15 min (+0.75 V, 100 nA).**

to establish the appropriate concentration of ascorbate that would be sufficient to prevent the oxidation of L-dopa during the entire period of the assay, and yet not alter the inherent activity of the enzyme with the monophenol substrate. Radioenzymatic analyses showed no significant alterations in the specific activity of mushroom tyrosinase when concentrations of ascorbate incorporated in the reactions mixtures ranged from zero to 4 mM (data not shown). Based on these

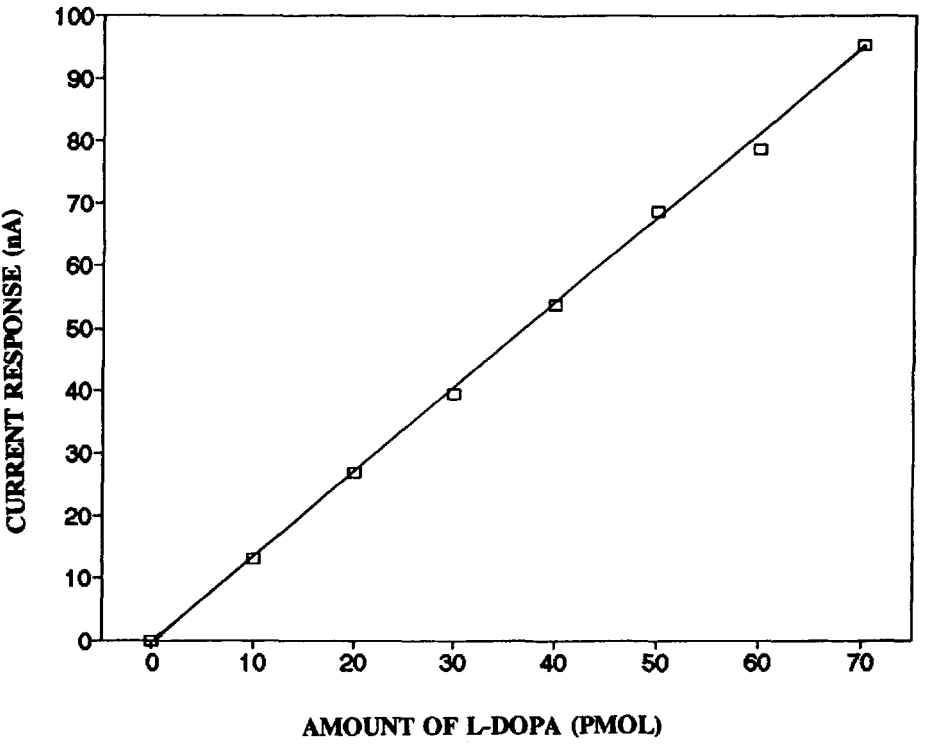

Figure 2. Electrochemical detector response as a function of L-dopa injected (+0.75 V, 100 nA full scale).

initial observations the final concentration of ascorbate used in all subsequent experiments was 0.4 mM. This level of ascorbate was found to be sufficient to maintain the level of L-dopa during 20 min incubations.

In reaction mixtures lacking a cofactor a lag phase in the rate of hydroxylation of tyrosine was clearly evident during the first five minutes of incubation (Fig. 5). NADA was selected as the single cofactor for the MPO

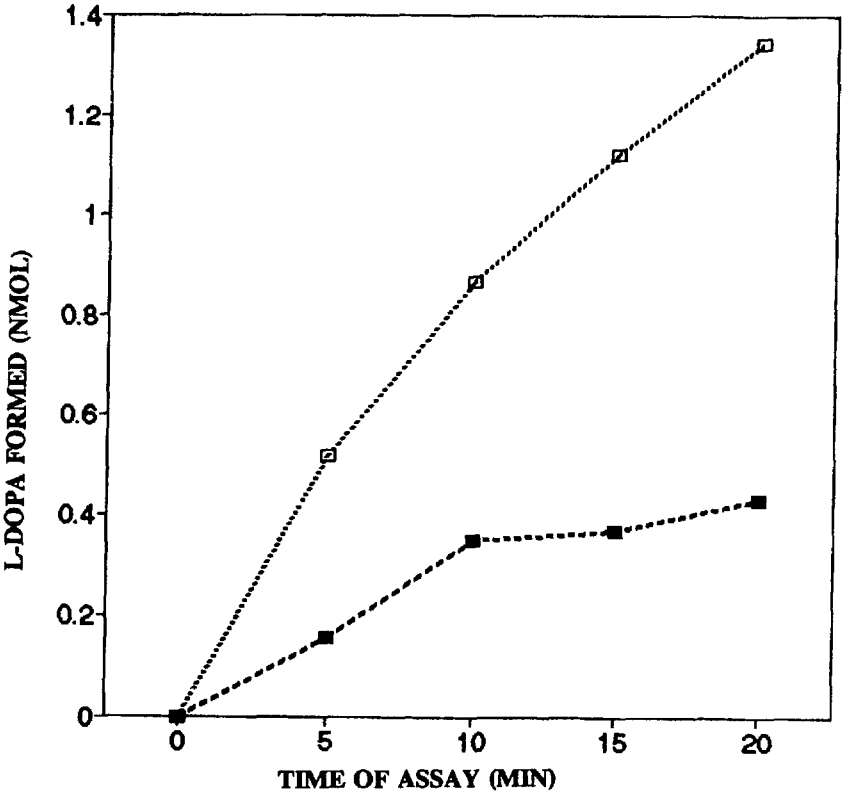

Figure 3. **Comparison of the levels of L-dopa detected during MPO activity in the presence of 0.4 mM ascorbate (open squares) and without ascorbate (solid squares). Both reaction mixtures (100 μL) contained 1.4 mM tyrosine and 0.25 mM NADA cofactor, and 1 μg/mL enzyme protein.**

assay from several other diphenols that were tested and found to be equally suitable including dopamine, norepinephrine, and epinephrine. L-dopa was not used as the cofactor for the assay since exogenous L-dopa could not be differentiated from that produced during MPO activity.

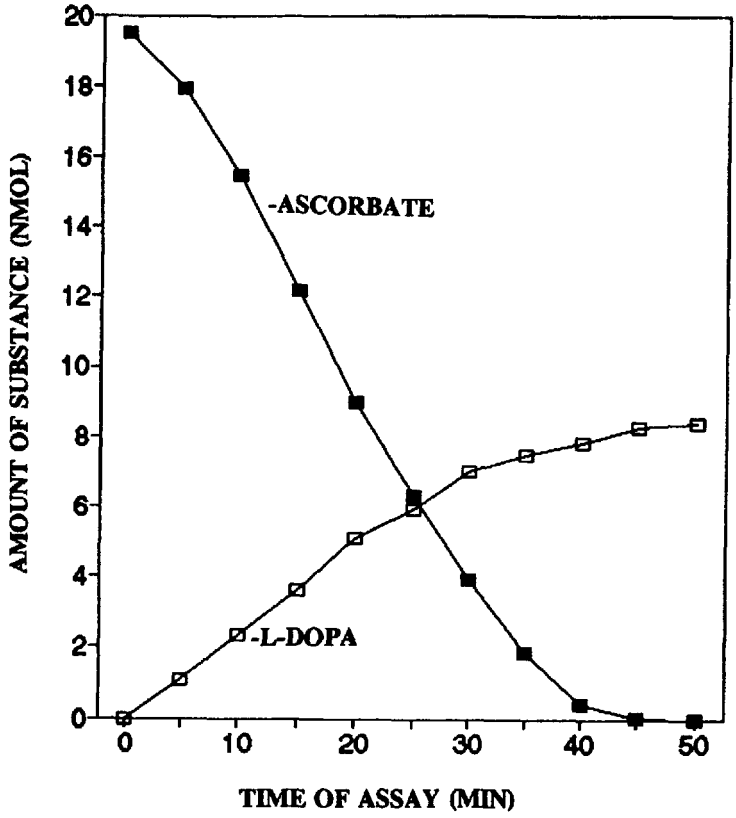

Figure 4. Production of L-dopa and concurrent oxidation of ascorbate during the hydroxylation of tyrosine by mushroom tyrosinase. The oxidation of L-dopa was not manifested until after the ascorbate was depleted from the reaction mixture. HPLC-ED analyses were based on 100 μl of reaction mixture containing 0.1 μg mushroom tyrosinase protein, 0.14 μmol of L-tyrosine, 20 nmol of ascorbic acid, and 0.5 nmol of NADA as cofactor.

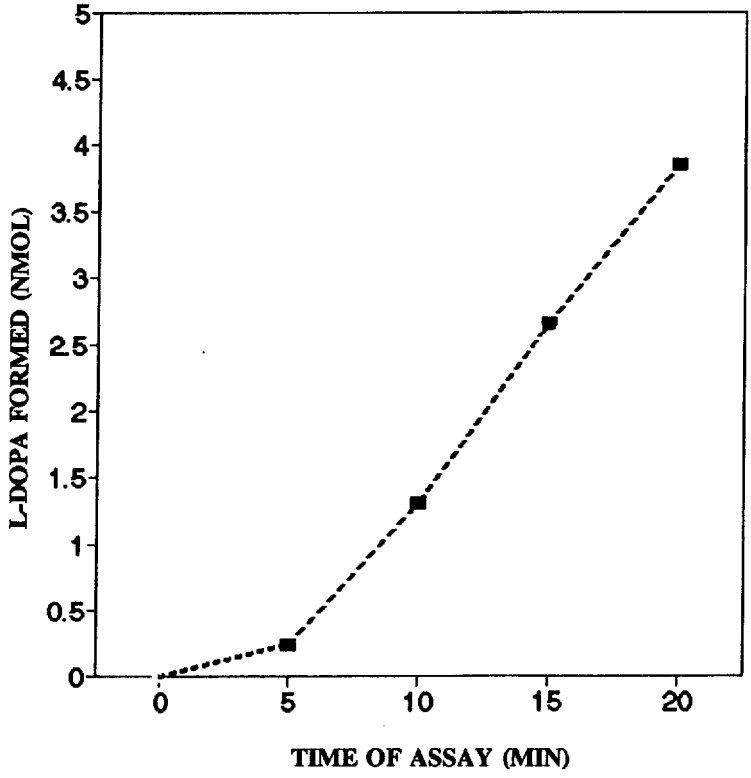

Figure 5. **An initial lag phase characterized MPO reactions that lacked a cofactor. The reaction mixture (100 μL) contained 1.4 mM tyrosine and 1 μg/mL enzyme protein.**

Various concentrations of NADA (0-500 μM) were incorporated into reactions mixtures containing 1.4 mM tyrosine and 0.4 mM ascorbate and incubated for 5 min (Fig. 6). Each concentration of cofactor tested was found to enhance MPO activity, but the highest activity was observed with 5 and 10 μM NADA. Concentrations of NADA above or below these two levels were less

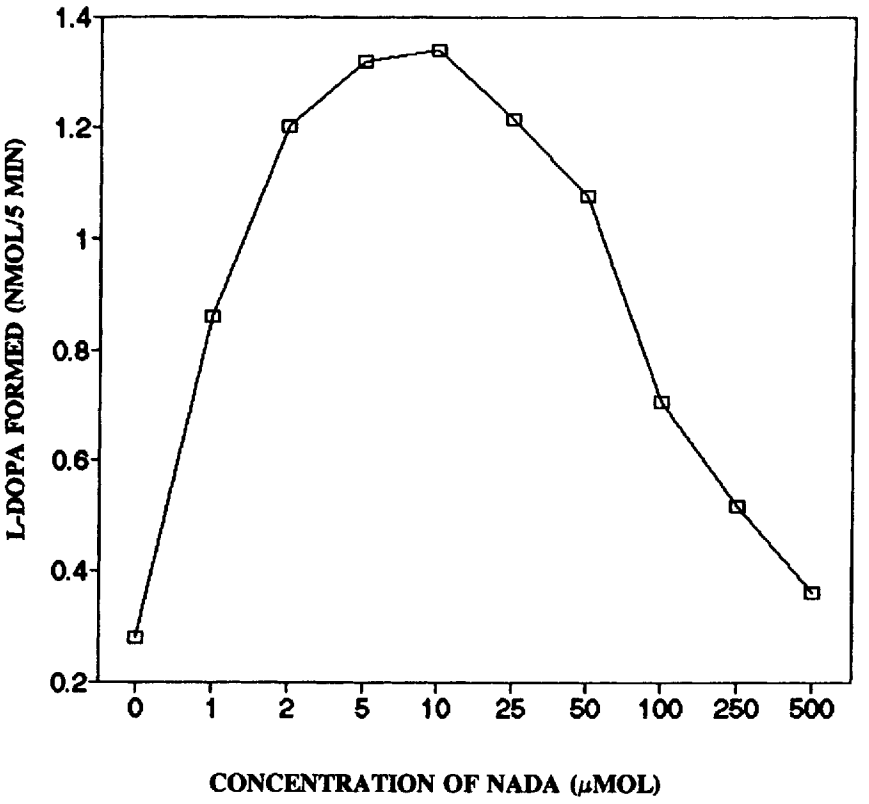

Figure 6. **Effects of various concentrations of NADA on MPO activity of mushroom tyrosinase. In addition to NADA the reaction mixture contained 1.4 mM L-tyrosine and 0.4 mM ascorbate.**

effective in reducing the lag phase in MPO function. When reaction mixtures with different concentrations of NADA were incubated for 20 min, a linear response for the duration of the assay was achieved with 5 μM NADA (Fig. 7).

The suitability of 5 μM NADA to serve as cofactor with two other concentrations of mushroom tyrosinase was ascertained in studies that showed

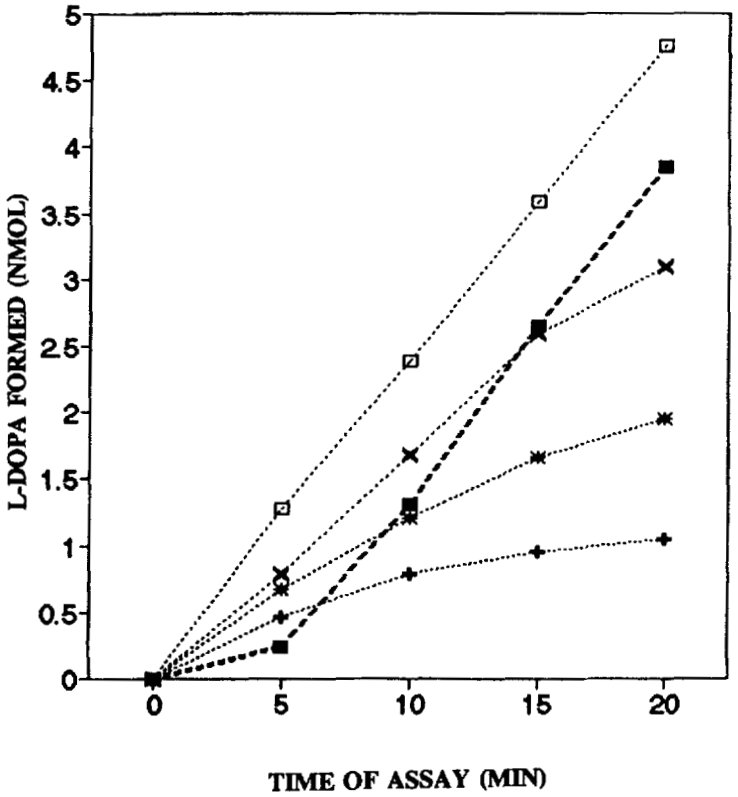

Figure 7. **Effects of various concentrations of NADA on MPO activity. Open squares = 5μM NADA; solid squares = no cofactor; X = 0.1 mM; * = 0.25 mM; + = 0.5 mM.**

MPO activity to be proportional both to reaction time and to concentration of enzyme protein (Fig. 8). The applicability of this enzyme assay for measuring MPO activity in crude biological samples was demonstrated using hemolymph of *Drosophila melanogaster* as a source of enzyme (Fig. 1C).

Comparative and quantitative studies were made to determine the level of sensitivity of the HPLC-ED. A series of reaction mixtures was prepared with

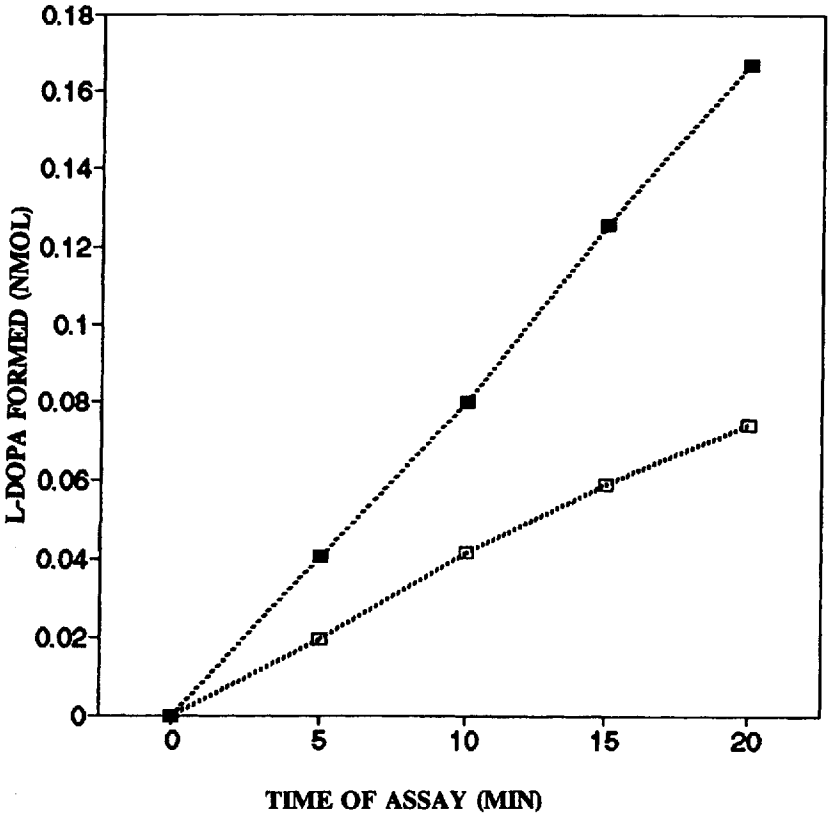

Figure 8. **MPO activity of mushroom tyrosinase as a function of time and protein concentration. The L-dopa formed was based on 100 μl reaction mixture containing either 2.5 ng (open square) or 5 ng enzyme protein (solid square), with a final concentration of L-tyrosine and NADA cofactor at 1.4 mM and 5 μM, respectively.**

various amounts of enzyme and then analyzed separately by both HPLC-ED and radioenzymatic methods. With the electrochemical detector maintained at 20 nA enzyme activity could be detected with as little as 0.6 ng enzyme protein incorporated into 60 μL of reaction mixture. The smallest amount of enzyme required to produce MPO activity that could be measured by the radioenzymatic method was 10 ng in a total volume of 60 μL. Considering that the volume of reaction mixture analyzed by HPLC-ED was only one-sixth the total volume of reaction mixture analyzed by the radioenzymatic method, the electrochemical method employed in this study was about 100 times more sensitive than the radioenzymatic assay.

DISCUSSION

The application of the MPO assay is dependent on NADA serving as a suitable cofactor and ascorbate retaining the L-dopa produced by the enzyme catalyzed hydroxylation of tyrosine. The molecular mechanism by means of which ascorbate functions in this capacity has not been described satisfactorily, but it is generally believed that ascorbate reduces and thus recycles o-quinones back to o-diphenols (2). This proposal does not mandate or preclude an altered catalytic activity, but merely accounts for an increase in the amount of detectable product, and thus a correspondingly higher estimate of the reaction rate. Our observations of elevated levels of L-dopa in ascorbate-mediated reactions suggest that ascorbate functions to retain this diphenol by reducing dopaquinone. Radioenzymatic studies showed that, with concentrations of 0.4 mM ascorbate and 5 μM NADA,

there was no detectable alteration in the catalytic property of mushroom tyrosinase with respect to MPO function, and HPLC-ED studies showed no detectable change in the level of NADA cofactor. During the reaction ascorbate levels decreased, and only when ascorbate was no longer present in the reaction mixtures was there any evidence of diphenol oxidation. Since there was no oxidation of ascorbate when it was incubated alone with tyrosinase, the two reducing equivalents required for MPO activity were likely derived from the diphenol product L-dopa and/or the diphenol cofactor NADA, and not directly from ascorbate Thus, it appears that the enzyme generates reducing equivalents for its MPO function by oxidizing diphenols to o-quinones, but in the presence of ascorbate these are recycled back to o-diphenols. Our studies with various amounts of NADA suggest that the higher the concentration of cofactor used, the greater the competition for available enzyme, the lower the rate of hydroxylation of tyrosine to L-dopa, and the more rapidly ascorbate is oxidized while reducing NADA quinone back to NADA.

We believe the assay provides a satisfactory method for measuring the MPO function of tyrosinase. Product separation and quantitation are accomplished in a single step. Maximum MPO activity is achieved with a concentration of cofactor (5 μM) that is not only sufficient to generate the reducing components that prime and enhance the reaction, but also low enough to render negligible any competition with the substrate for enzyme. The substrate is present in a saturated concentration (1.4 mM), with an adequate level of ascorbate to retain the L-dopa produced for quantitation. Based on the amount of L-dopa generated during a 20

min assay period, MPO activity is linear, and there is no indication that either ascorbate or NADA altered the inherent kinetic parameters of the enzyme relative to MPO function. Based on comparative and quantitative studies the level of sensitivity afforded by electrochemical detection is conservatively estimated to be 100 times greater than that of a specific radioenzymatic assay (8,9). However, if required, a four-fold higher increase in sensitivity easily can be attained merely by increasing the sensitivity of the electrochemical detector from the 20 nA level used throughout this study to 5 nA. Thus the MPO assay appears to be especially useful for dealing with small volumes of sample and/or low levels of enzyme activity. The suitability of the assay was convincingly demonstrated by measuring MPO activity in a sample of hemolymph form a third stage larva of *D. melanogaster*. The assay may be especially useful in distinguishing MPO from DPO activity in various insects and plants which possess three distinct phenol oxidases (12-18).

ACKNOWLEDGEMENTS

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