Improvement of a Continuous Spectrophotometric Method for Determining the Monophenolase and Diphenolase Activities of Mushroom Polyphenol Oxidase

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A spectrophotometric method for determining the monophenolase and diphenolase activities of mushroom polyphenol oxidase (PPO) at pH 6.8 has been improved. The method is based on the coupling reaction between the nucleophile 3-methyl-2-benzothiazolinone hydrazone (MBTH) and the quinone products of the oxidation of monophenols and *o*-diphenols in the presence of polyphenol oxidase. MBTH−quinone adduct is further oxidized by another molecule of *o*-quinone. Different *o*-diphenols were assayed: L-dopa, dopamine, catechol, 4-methylcatechol, 3,4-dihydroxyphenylacetic acid (DHPAA), and 3,4-dihydroxyphenylpropionic acid (DHPPA) (and their corresponding monophenols). The PHPPA (*p*-hydroxyphenylpropionic acid)/DHPPA pair was chosen as the best pair from those assayed thanks to its kinetic features, molar absorptivity (ϵ), and solubility. All the MBTH−*o*-quinone adducts from the above substrates evolved at pH 6.8. A reaction mechanism for explaining the evolution of the MBTH−*o*-quinone adduct of DHPPA has been proposed and kinetically studied for the first time. The wavelength where the MBTH−*o*-quinone adduct of DHPPA showed an isosbestic point ($\lambda_i = 466$ nm) was chosen for spectrophotometrically recording the action of PPO on the PHPPA/DHPPA pair. This method could be useful for determining microquantities of PPO in problem samples.

Keywords: *3,4-Dihydroxyphenylpropionic acid; diphenols; enzyme kinetics; p-hydroxyphenylpropionic acid; MBTH; monophenols; mushroom; polyphenol oxidase; spectrophotometry; tyrosinase*

INTRODUCTION

Polyphenol oxidase (EC 1.14.18.1) (PPO) as present in plant tissues plays an important role in fruit and vegetable processing and during storage of the processed foods. Prevention of browning of foods, enzymatic or nonenzymatic, has long been the concern of food scientist (Matheis, 1987). PPO is a copper enzyme, which in the presence of oxygen catalyzes two different reactions: the hydroxylation of monophenols to *o*-diphenols (monophenolase activity) and the oxidation of *o*-diphenols to *o*-quinones (diphenolase activity) which, in turn, are polymerized to brown, red, or black pigments (Mason, 1955; Prota, 1988). Moreover, the enzyme is of central importance in vertebrate pigmentation. It is directly responsible for the conversion of the amino acid tyrosine to one of several types of melanin pigments (Robb, 1984).

Due to the poor specificity of PPO for the phenolic substrates, several assay methods have been developed: oximetric methods (Mayer and Harel, 1979); spectrophotometric methods which can measure the appearance of the *o*-quinones (Waite, 1976; Cabanes et al., 1987) or aminechrome (Mason, 1948; García-Moreno et al., 1991; Rodríguez-López et al., 1992a). Moreover, using 3,4-dihydroxymandelic acid as substrate, 3,4-dihydroxybenzaldheyde production has been monitored by HPLC (Czapla et al., 1991) and by spectrophotometry (Rodríguez-López et al., 1991).

Most of the above described methods are focused on the diphenolase activity of this enzyme. However, there are only a few works on monophenolase activity (Rodríguez-López et al., 1992a; Ros et al., 1994; Espín et al., 1995b) because the enzyme's kinetic mechanism action on monophenols has been recently clarified (Rodríguez-López et al., 1992a; Ros et al., 1994). This has led us to develop continuous methods to follow this activity.

Recently we developed a continuous spectrophotometric method to determine the monophenolase and diphenolase activities of mushroom PPO, using L-dopa and L-tyrosine as substrates and MBTH as coupled reagent. (Rodríguez-López et al., 1994).

In a further attempt to improve and apply this method to measure PPO activity from several fruits and vegetables, different substrates (including L-dopa, dopamine, catechol, 4-methylcatechol, and DHPPA) were used for apple PPO (Espín et al., 1995a). In order to determine the molar absorptivities (ϵ) for their corresponding MBTH−*o*-quinone adducts, the substrates were depleted in the presence of MBTH and a high PPO concentration. When the experiments were carried out at acidic pH (∼5), the adducts were soluble and stable, whereas at the optimum pH for mushroom PPO, 6.8, the adducts evolved showing isosbestic points (Espín et al., 1995a). This latter fact may not be very significant when diphenolase activities were assayed at 60−200 s per recording but may be much more significant when the monophenolase activity is assayed, which usually requires more than 15 min of assay time.

The aim of this paper is to determine the best substrate for mushroom PPO, to propose a reaction mechanism to explain the evolution of its corresponding

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adduct, and to carry out the kinetic characterization of the whole pathway in order to characterize quantitatively the enzymatic activity. These approaches will improve the continuous spectrophotometric method for assaying both monophenolase and diphenolase activities of PPO previously described (Rodríguez-López et al., 1994; Espín et al., 1995a). This improvement will make it possible to apply the method to studying enzymes with an optimum pH of near neutrality, such as mushroom PPO.

MATERIALS AND METHODS

Reagents. L-Dopa, dopamine, 4-methylcatechol, MBTH, PHPPA, DHPPA, and DHPAA were purchased from Sigma (St. Louis, MO) and catechol from Merck (Darmstadt, Germany). All other chemicals were of analytical grade and supplied by Fluka (Madrid, Spain). Stock solution of the phenolic substrates were prepared in 0.15 mM phosphoric acid to prevent autoxidation. Several buffers were used: 50 mM sodium acetate (pH 3.0−5.5), 50 mM sodium phosphate (pH 5.8−7.0). The acidic character of MBTH requires the use of 50 mM buffer in the assay medium. To dissolve the MBTH−quinone adducts 2% (by volume) *N,N′*-dimethylformamide (DMF) was added to the assay medium (Winder and Harris, 1991; Rodríguez-López et al., 1994; Espín et al., 1995a,b).

Enzyme Source. Mushroom (*Agaricus bisporus*) tyrosinase (3900 units/mg) was purchased from Sigma and purified by the procedure of Duckworth and Coleman (1970). Protein content was determined by a modified Lowry method (Hartree, 1972). The different isoenzymes were not separated, and therefore our kinetic constants may be a conglomeration of kinetics from different isoenzymes.

Spectrophotometric Assays. Absorption spectra were recorded in an ultraviolet−visible Perkin-Elmer Lambda-2 spectrophotometer (Überlingen, Germany), on-line interfaced with an Amstrad PC2086 microcomputer (Madrid, Spain), with 60 nm/s scanning speed. Temperature was controlled at 25 °C using a Haake D1G circulating bath (Berlin, Germany) with a heater/cooler and checked using a precision of ±0.1 °C. Kinetic assays were also carried out with the above instruments by measuring the appearance of the products in the reaction medium. Reference cuvettes contained all the components except the substrate, with a final volume of 1 mL. Other conditions and reagents are detailed in the text, in the legends to the figures, and in the footnotes to the tables.

One unit of enzyme was taken as the amount that produced 1 μmol of the adduct/min when DHPPA was assayed.

Oxymetric Assays. Oxygen consumption was followed by a Hansatech DW oxymeter (Norfolk, U.K.), based on the Clark electrode covered with a Teflon membrane, equipped with an Amel 863 digital X/Y recorder (Milan, Italy). Calibration was made by the 4-*tert*-butylcatechol/tyrosinase method (Rodríguez-López et al., 1992b).

Kinetic Data Analysis. The values of K_m and V_{max} on different substrates were calculated from triplicate measurements of the steady state rate, V_{ss}, for each initial substrate concentration, $[S]_0$. The reciprocals of the variances of V_{ss} were used as weighting factors in the nonlinear regression fitting of V_{ss} vs $[S]_0$ data to the Michaelis equation (Wilkinson, 1961; Endrenyi, 1981). The fitting was carried out by using a Gauss−Newton algorithm (Wilkinson, 1961) implemented in a BASIC program (Duggleby, 1981). Initial estimations of K_m and V_{max} were obtained from the Hanes−Woolf equation, a linear transformation of the Michaelis equation (Wilkinson, 1961).

The sensitivity of the method was tested with triplicate determinations of V_{ss} for each initial PPO concentration, $[PPO]_0$. The reciprocals of the variances of V_{ss} were used as weighting factors in the linear regression fitting of V_{ss} vs $[PPO]_0$ (Wilkinson, 1961; Endrenyi, 1981). The precision of the method was checked by means of 10 assays of V_{ss} at each one of three $[PPO]_0$ values, whose coefficients of variation were calculated. The sensitivity of the method was characterized from 10 measurements of the blank, leading to the evaluation of the corresponding detection and quantitation limits (ACS, 1980; Espín et al., 1995a,b).

Table 1. Properties of MBTH Adducts from Several *o*-Diphenols

o-diphenol	λ_{max}[a]	ϵ[a,c,d] (M^{-1} cm^{-1})	λ_i[b]	ϵ[b,c] (M^{-1} cm^{-1})
L-dopa	507	38 000	484	22 300
dopamine	503	42 500	476	20 700
catechol	500	32 500	459	17 500
4-methylcatechol	494	32 500		
DHPPA	500	40 000	466	20 000
DHPAA	503	42 500	471	23 000

[a] Assay medium: 50 mM acetate buffer, pH 5, 2% DMF. [b] Assay medium: 50 mM phosphate buffer, pH 6.8, 2% DMF. [c] The values of ϵ for the MBTH adducts were determined by fast oxidation of low concentration of *o*-diphenols in the presence of high concentratons of mushroom PPO with saturating MBTH. [d] Espín et al. (1995a).

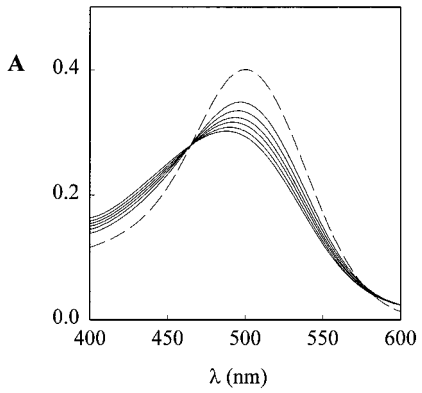


Figure 1. Scan spectra for the oxidation of DHPPA by mushroom PPO in the presence of MBTH. Conditions were 2% DMF, 10 μM DHPPA, and 6.5 units/mL PPO: (dashed trace) 50 mM acetate buffer, pH 5, 1 mM MBTH; (solid trace) 50 mM phosphate buffer, pH 6.8, 5 mM MBTH. Time between recordings, 300 s.

RESULTS AND DISCUSSION

Diphenolase Activity. (1) Formation and Properties of the MBTH−Quinone Adducts. MBTH is a potent nucleophile through its amino group, which is in different degrees of protonation−deprotonation, depending on the pH. The ionization constant for this group has been determined by both potentiometric and spectrophotometric methods, $pK_a = 5.8 \pm 0.4$, (Rodríguez-López et al., 1994). To study the characteristics of these adducts, the oxidation of various *o*-diphenols by O_2 catalyzed by mushroom PPO was carried out in the presence of MBTH. The pigment formed with L-dopa, dopamine, catechol, 4-methylcatechol, DHPAA, or DHPPA was dark pink and had an absorbance maximum ranging from 494 to 507 nm (Table 1). The pH affected the solubility and stability of the adducts. The solubilization of these compounds was achieved by adding 2% (by volume) DMF to the reaction medium (Winder and Harris, 1991; Rodríguez-López et al., 1994; Espín et al., 1995a,b). Under these conditions, the adducts formed by the addition of MBTH to the *o*-quinone from different *o*-diphenols were soluble at every pH studied (from 3.5 to 7). At values of pH lower than 5, the adducts were soluble and stable (Figure 1, dashed trace), whereas at higher values the adducts were unstable but showed an isosbestic point (Figure 1, solid trace). The exceptions were the *o*-diphenols catechol and 4-methylcatechol (results not shown). The MBTH−benzoquinone adduct showed an isosbestic point at pH 6.8, although it was broken after long assay times

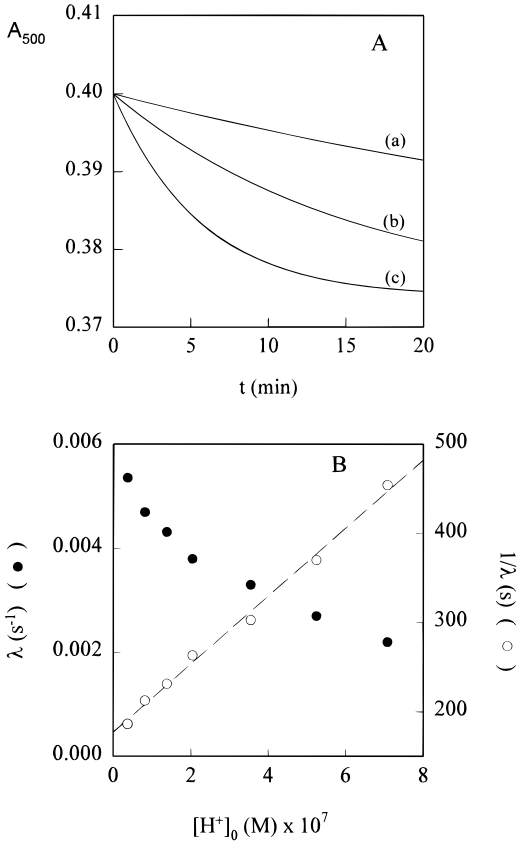


Figure 2. (A) Spectrophotometric recordings of the evolution of the adduct at different pHs. Conditions were 10 μM DHPPA, 2% DMF, 1 mM MBTH, 0.1 M PB, 2.3 units/mL PPO: pH (a) 6.1; (b) 6.8; (c) 7.4. (B) Dependence of the apparent constant of the adduct's evolution on pH.

(results not shown). The MBTH−4-methylbenzoquinone adduct was the only one that did not show an isosbestic point at pH 6.8 (results not shown). At acidic pH, both adducts were slightly insoluble, which meant that it was only possible to determine the diphenolase activity in rapid kinetic assays.

(2) Effect of pH on the Kinetic Evolution of the Adduct. Taking into account that the adducts were stable at acidic pH and unstable at higher pH (Figure 1), the influence of pH on this evolution was studied (Figure 2A). The evolution followed a first-order kinetics and the apparent constant increased when pH rose (Figure 2B).

(3) Effect of the Oxygen Concentration. In some experiments, the adduct was quickly generated by the O_2 oxidation of DHPPA catalyzed by a high PPO concentration. Then, $HClO_4$ was added to inactivate PPO and stop the reaction. No enzymatic activity was detected after addition of $HClO_4$. The resultant mixture at pH 1.5 was centrifuged to isolate the chromophoric adduct in the supernatant (Winder, 1994).

The adduct was submitted to a sudden increase of pH with 0.1 M phosphate buffer, pH 6.25. The adduct remained stable when N_2 was bubbled in the medium to remove O_2 (Figure 3a). In these conditions, with no enzymatic activity, the presence of saturating O_2 concentration (0.26 mM) in the medium caused the adduct to evolve with time (Figure 3b). Thus, the nonenzymatic oxidation of the adduct by O_2 appeared to be of some importance and increased when the pH rose.

(4) Effect of the Enzyme Concentration. An increase in enzyme concentration in the assay medium, at constant pH, resulted in an increase of the transient

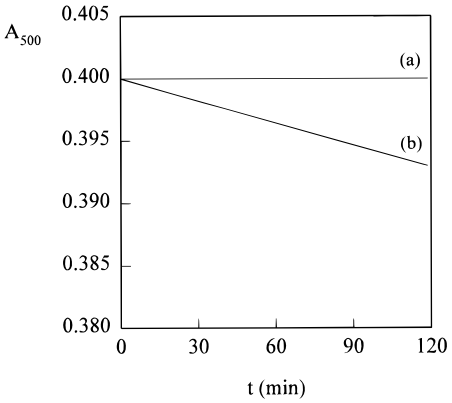


Figure 3. Spectrophotometric recordings of the nonenzymatic evolution of the adduct in the absence and presence of O_2. Conditions were 10 μM DHPPA, 2% DMF, 1 mM MBTH, and 0.1 M PB, pH 6.25: (a) $[O_2]_0 = 0$; (b) $[O_2]_0 = 0.26$ mM.

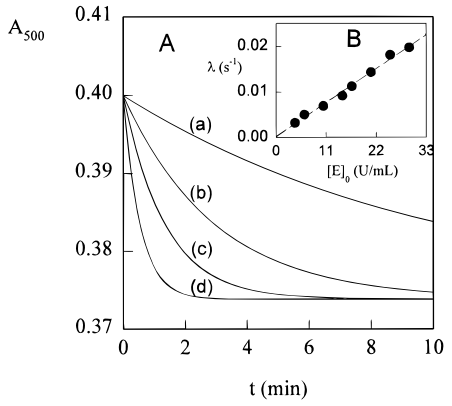
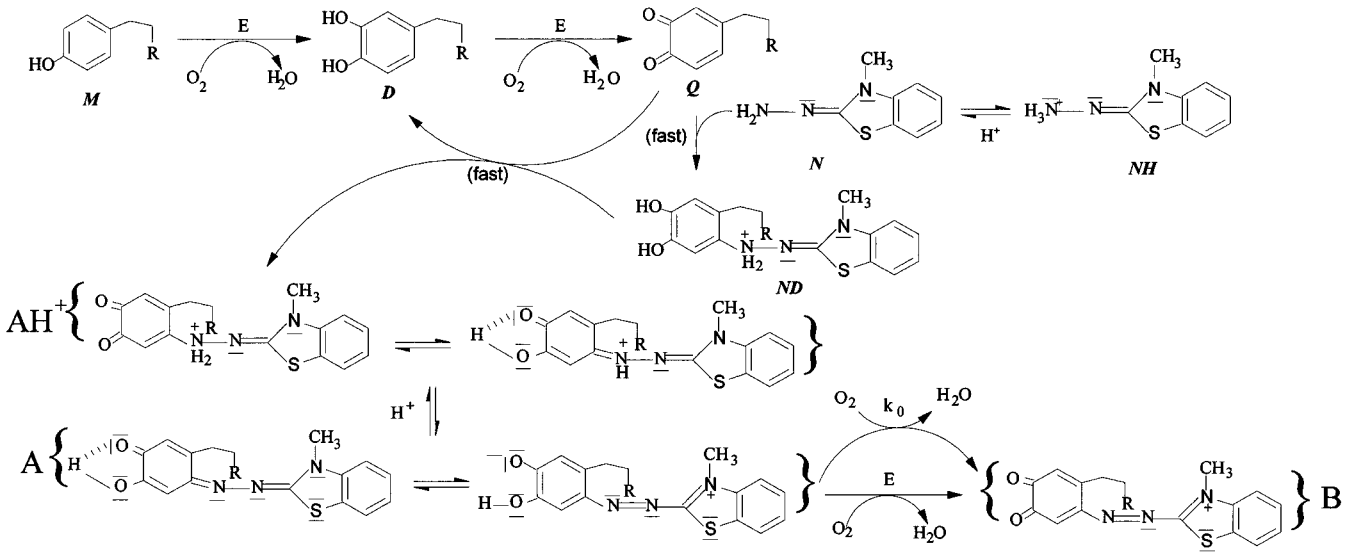


Figure 4. (A) Spectrophotometric recordings of the evolution of the adduct generated with different enzyme concentrations. Conditions were 10 μM DHPPA, 2% DMF, 1 mM MBTH, 0.1 M PB pH 6.25, and (a) 3.3 units/mL PPO; (b) 6.7 units/mL PPO; (c) 13.3 units/mL PPO, and (d) 26.7 units/mL PPO. (B) Dependence of the apparent constant (λ) of the evolution of the adduct on PPO concentration.

apparent constant (Figure 4A). This dependence showed a linear pattern (Figure 4B). In a further experiment, the enzyme was inactivated by acidification using $HClO_4$. The pH was later returned to its original value, after which the evolution of the adduct was slower than when the enzyme was active. This indicated that the oxygen oxidized the adduct and caused its evolution in the absence of enzyme. This reaction increased when the pH rose.

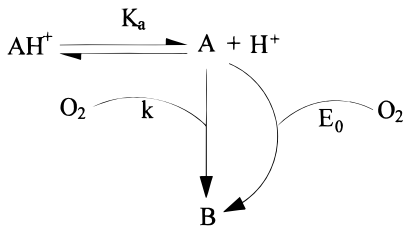
Based on the data obtained and taking into account the spectrophotometric recordings of the evolution of the adduct in which λ_{max} moved toward ultraviolet and ϵ decreased, a reaction mechanism was proposed. In this mechanism, the oxidized adduct (*o*-quinone) suffers a tautomery evolving to a more stable *p*-quinoid structure (AH^+). The deprotonation of this *p*-quinone yields an oxidizable product (A), which renders a yellow *o*-quinone by oxidation (B) (Scheme 1). The species measured is AH^+ and B. In the beginning, the species measured is AH^+. When the adduct evolves, the species detected are AH^+ and B. Both species have the same molar absorptivity at the isosbestic point. At the λ_{max}, the molar absorptivity of AH^+ is higher than that of B. Therefore is not appropriate to measure at λ_{max} because, with time, the species B is increasing and AH^+ decreasing in the medium and the absorbance would change (Figure 1). At the isosbestic point, this does not occur; the absorbance is the same.

Scheme 1. Sequence of Reactions for the Monophenolase and Diphenolase Activities of Mushroom PPO in the Presence of a Chromogenic Nucleophile (MBTH): Evolution of the MBTH−Quinone Adduct[a]



[a] Key: *M*, monophenol; *D*, *o*-diphenol; *Q*, o-quinone; *N*, deprotonated nucleophile; *NH*, protonated nucleophile; *ND*, nucleophile−diphenol colorless adduct; *AH*, protonated *p*-quinoid adduct; *A*, deprotonated *p*-quinoid adduct; *B*, nucleophile−*o*-quinone adduct.

(5) Kinetic Analysis. The above described reaction mechanism can be summarized in the following scheme:



where AH^+ represents the protonated *p*-quinone adduct, A the deprotonated *p*-quinone adduct, and B the *o*-quinone adduct. If the sum of AH^+ and A are considered in rapid equilibrium as the species X, its evolution can be described as follows:

$$-\frac{d[X]}{dt} = \left(\frac{V_{max}}{K_m + f_A[X]} + k_0[O_2]_0\right) f_A[X] \quad (1)$$

$$f_A = \frac{K_a}{K_a + [H^+]_0} \quad (2)$$

because the adduct concentration is very low

$$f_A[X] \ll K_m \quad (3)$$

so eq 1 becomes

$$-\frac{dx}{dt} = \left(\frac{V_{max}}{K_m} + k_0[O_2]_0\right) f_A[X] \quad (4)$$

integrating eq 4 gives

$$[X] = [X]_0 \exp\left\{-\left(\frac{V_{max}}{K_m} + k_0[O_2]_0\right)\frac{K_a}{K_a + [H^+]_0} t\right\} \quad (5)$$

Thus, the transformation of the adduct (red *p*-quinone) into yellow *o*-quinone fulfills a first-order kinetics (Figure 2B). The apparent constant of this transformation becomes

$$\lambda = \left(\frac{V_{max}}{K_m} + k_0[O_2]_0\right)\frac{K_a}{K_a + [H^+]_0} \quad (6)$$

Taking the inverses in eq 6, gives

$$\frac{1}{\lambda} = \frac{K_m[H^+]_0}{(V_{max} + k[O_2]_0 K_m)K_a} + \frac{K_m}{V_{max} + k_0[O_2]_0 K_m} \quad (7)$$

Linear regression of $1/\lambda$ vs $[H^+]_0$ (Figure 2B) gives $K_a = (4.64 \pm 0.2) \times 10^{-7}$ M. From this datum, the pK_a value can be calculated (6.33 ± 0.02).

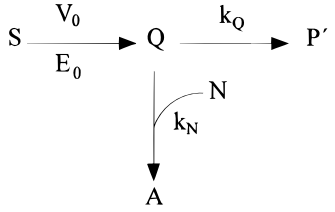
From eq 6, the effect of the enzyme concentration at constant pH can be represented as (Figure 4A)

$$\lambda = \frac{k_{cat}K_a}{(K_a + [H^+]_0)K_m}[E]_0 + \frac{k_0[O_2]_0 K_a}{(K_a + [H^+]_0)} \quad (8)$$

The expression of the ordinate of eq 8 can lead to low values that make it difficult to accurately evaluate k_0 (Figure 4B). A more appropriate method for determining k_0 is based on assays involving adduct oxidation in the absence of enzyme (Figure 3B), which gives a value of $k_0 = 2.45 \pm 0.15$ M^{-1} s^{-1}.

Once the adducts and isosbestic points of the different *o*-diphenols were characterized, attempts were made to improve the enzymatic assay.

(6) Improvement of the Enzymatic Assay. The enzymatic reaction under study gives rise to a product that becomes unstable through a first- or pseudo-first-order reaction.



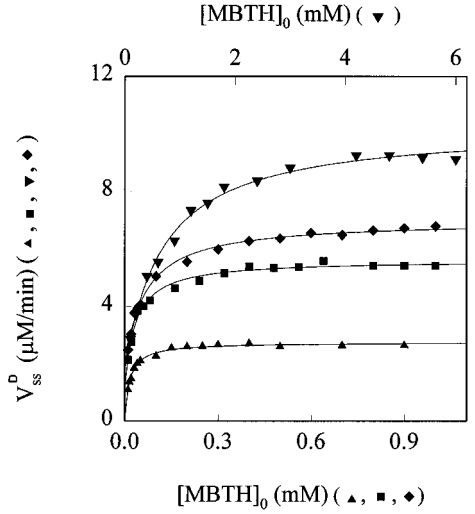


Figure 5. Determination of the $[MBTH]_{sat}$, at different pHs, in the oxidation of L-dopa by mushroom PPO. Conditions were 2.1 mM L-dopa and 0.17 unit/mL PPO. The pHs were (▲) 5.0, (■) 5.4, (◆) 5.8, and (▼) 6.8.

The accumulation of A with time is described by the equation

$$[A] = \frac{V_0 k_N[N]}{(k_Q + k_N[N])}t - \frac{V_0 k_N[N]}{(k_Q + k_N[N])^2}[1 - e^{-(k_Q + k_N[N])t}] \quad (9)$$

From eq 9, if the condition $k_N[N] \gg k_Q$ is accomplished, the enzyme's entire activity can be followed. This condition may be reached with an arbitrary enzyme and substrate concentration because competition is established between the two first-order kinetics.

If the product becomes unstable through a first-order apparent constant that depends on pH, as in the case of catecholamines, (L-dopa, dopamine), the saturating MBTH concentration ($[MBTH]_{sat}$) must be determined at a pH where the apparent constant (k_Q) reaches its highest value until satisfying the condition $k_N[N] \gg k_Q$ (Figure 5). An experimental plot of the rate vs MBTH concentration (Figure 5) increases in a hyperbolic way with the MBTH concentration until it reaches the maximum value, in which $V = V_0$. The results from the experiments carried out on dopamine at several substrate and enzyme concentrations confirm the above (Figure 6). Note that $[MBTH]_{sat}$ does not depend on $[S]_0$ or $[E]_0$ (Figure 6). The same result was obtained when the other substrates were used following the same procedure.

The $[MBTH]_{sat}$ for the monophenol was the same as that previously determined for the *o*-diphenols because $V_{max}^M \cong 0.1 V_{max}^D$ in mushroom PPO.

(7) Improvement of the Wavelength for the Enzymatic Assays. As previously described, the adducts evolved with time to show an isosbestic point. This evolution could be followed spectrophotometrically at both wavelengths, λ_{max} and isosbestic point (λ_i). Taking into account that the monophenolase activity required long kinetic assays, this reaction should always be followed at the λ where the isosbestic point appears (λ_i). If the monophenolase activity is not followed at λ_i, the steady state rate obtained could give rise to erroneous kinetic determinations. For the purpose of uniformity in the assay conditions, the diphenolase activity was also followed at the isosbestic point.

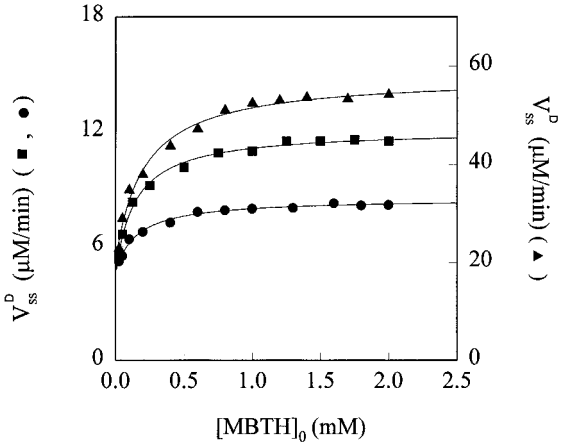


Figure 6. Determination of the $[MBTH]_{sat}$, at constant pH, and different substrate and mushroom PPO concentrations: (●) 5 mM dopamine, 0.07 unit/mL PPO; (■) 12 mM dopamine, 0.07 unit/mL PPO; (▲) 12 mM dopamine, 0.04 unit/mL PPO.

Table 2. Values of the Kinetic Constants, V_{max} and K_m, for the Oxidation of Various *o*-Diphenols Catalyzed by Mushroom Polyphenol Oxidase[a]

o-diphenol	$[MBTH]_{sat}$ (mM)	K_m^D (mM)	V_{max}^D (μM/min)	V_{max}^D/K_m^D (min^{-1})	ratio of V_{max}^D
L-dopa	5	0.76	24.5	0.032	1
dopamine	2	2.20	38.3	0.017	1.15
catechol	1	0.44	288.0	0.654	11.75
4-methylcatechol	2.5	0.51	465.4	1.058	19
DHPPA	5	1.36	126.0	0.093	5.14
DHPAA	5	5.0	144.0	0.03	5.88

[a] Conditions were 2% DMF, PB 50 mM, pH 6.8, and mushroom PPO 0.13 unit/mL, with $[MBTH]_{sat}$ for each *o*-diphenol.

Enzymatic Activity. (1) Effect of $[D]_0$ and $[M]_0$. The effect of the initial concentration of the different *o*-diphenols on their rate of PPO-catalyzed oxidation, in the presence of MBTH, was kinetically characterized.

A hyperbolic dependence of the rate on the different *o*-diphenols was obtained (data not shown). These data were fitted by nonlinear regression to the Michaelis equation, giving K_m and V_{max} values which are summarized in Table 2.

From these V_{max} values, a relation among the substrates tested could be inferred: 4-methylcatechol > catechol > DHPAA ≅ DHPPA > dopamine > L-dopa. Catechol and 4-methylcatechol were not good substrates due to the solubility problems previously described for their adducts, especially taking into account the long kinetic assay for the monophenolase activity of their corresponding monophenols. The DHPAA/PHPAA pair was not chosen as the best pair because both kinetic parameters, V_{max} and K_m, proved to be worse for PHPAA (V_{max} = 10.16 μM/min; K_m = 1.9 mM) than for PHPPA (V_{max} = 19.3 μM/min; K_m = 0.44 mM). In the diphenolase activity, the V_{max} value for DHPAA was slightly better than that for DHPPA, but K_m and catalytic power, V_{max}/K_m, were better for DHPPA (0.093 min^{-1}) than for DHPAA (0.03 min^{-1}) (Table 2). Thus, DHPPA was chosen as the best substrate from those assayed.

(2) Effect of $[PPO]_0$. Linearity was obtained between diphenolase and monophenolase activities and PPO concentration (Figure 7), which might also be useful as a linear calibration curve for the determination of microquantities of PPO in problem samples. The method was applied in triplicate assays, and a linear regression fitting was obtained (Figure 7).

The precision of the method was checked by repeating the estimation of V_{ss}^D 10 times for three samples at

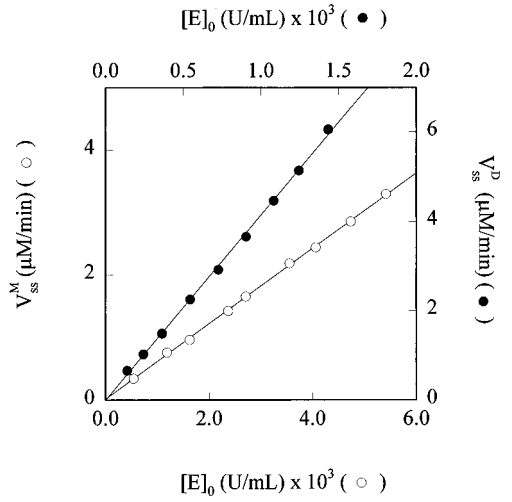


Figure 7. Calibration curve for the determination of mushroom PPO. Conditions were 2% DMF, 5 mM MBTH, 50 mM PB, pH 6.8, and (●) 16 mM DHPPA and (○) 4 mM PHPPA.

three levels of $[PPO]_0$ 1.3×10^{-4}, 6.9×10^{-4}, and 1.4×10^{-3} unit/mL, the corresponding coefficients of variation being 9.4, 2.6, and 2.1%. The same procedure was applied for the monophenolase activity, by repeating the estimation of V_{ss}^{M} 10 times for three samples at three levels of $[PPO]_0$ 5.1×10^{-4}, 2.6×10^{-3}, and 5.2×10^{-3} unit/mL, the corresponding coefficients of variation being 5, 1.5, and 0.7%.

The sensitivity of the method (ACS, 1980; Espín et al., 1995a; 1995b) was characterized by determining the limit of detection (LOD^D) and the limit of quantitation (LOQ^D). Thus, $LOD^D = 2.5 \times 10^{-5}$ unit/mL and $LOQ^D = 2.9 \times 10^{-5}$ unit/mL, as well as $LOD^M = 3 \times 10^{-5}$ unit/mL and $LOQ^M = 7.2 \times 10^{-5}$ unit/mL were obtained for the diphenolase and monophenolase activities, respectively. It should be noted that the LOD and LOQ for both monophenolase and diphenolase activities were not very different due to the high spontaneous oxidation of DHPPA at pH 6.8. However, its corresponding monophenol (PHPPA) did not show significant nonenzymatic oxidation.

MBTH Method. In a previous work (Rodríguez-López et al., 1994), we proposed a method based on the formation of adducts between the nucleophilic reagent MBTH and the *o*-quinones generated by mushroom PPO from L-tyrosine/L-dopa and L-tyramine/L-dopamine. That paper was mainly focused on the advantage of the MBTH method with regard to the method that measures the *o*-quinone formation thanks to the high molar absorptivities of the MBTH−*o*-quinone adducts.

In the present paper, several diphenolic substrates of PPO have been assayed (Tables 1 and 2). The monophenolase activity of mushroom PPO was assayed with the monophenols corresponding to the *o*-diphenols tested. When L-tyrosine ($V_{max} = 1.8$ μM/min; $K_m = 0.2$ mM) and L-tyramine ($V_{max} = 2.4$ μM/min; $K_m = 0.68$ mM) were assayed, 11 and 8 times less activity was detected with respect to PHPPA, respectively. Thus, PHPPA was chosen for characterizing the monophenolase activity of mushroom PPO. When the results obtained from the different substrates were compared, the PHPPA/DHPPA pair was chosen as the optimum pair for measuring the activity of mushroom PPO. The improvement of the assay of the diphenolase activity using DHPPA as substrate was 5.1 and 4.6 times higher that when L-dopa and dopamine were assayed, respectively.

As previously stated, if the kinetics is not followed at λ_i, this could give rise to the determination of erroneous kinetic constant. V_{max} and K_m values for PHPPA when the kinetics was followed at λ_{max} were 8.5 μM/min and 0.13 mM, respectively. These erroneous determinations were due to the fall in the absorbance provoked by the adduct's evolution during the kinetic assays. Moreover, the resulting Michaelis kinetics did not show the typical hyperbolic profile but a shaped-bell profile. This latter occurred because at higher monophenol concentration (with higher lag period and therefore longer kinetic assays) the adduct's evolution was more significant. In the case of high enzyme concentration, low monophenol concentration and short lag period the adduct's evolution may not be very significant (Rodríguez-López et al., 1994). But these conditions are not always present in our assays. This method is also applicable to other enzymes with optimum neutral pH. In many crude extracts of PPO from fruits and vegetables, the monophenolase activity is very low and the kinetic characterization is carried out under conditions where the adduct's evolution may be significant.

The improved method proposed here for the determination of the monophenolase and diphenolase activity of mushroom PPO has several advantages over other continuous spectrophotometric methods:

(a) The substrates, PHPPA and DHPPA, showed no solubility problems.

(b) The MBTH−quinone adduct was unstable at the optimum pH for mushroom PPO but showed an isosbestic point with high molar absorptivity. A sequence of reactions for explaining the evolution of the adduct has been proposed and kinetically characterized.

(c) The method was more sensitive than when other substrates were used, which may be mainly due to the high value of the catalytic constant of mushroom PPO toward DHPPA.

(d) The method has been extended to assay enzymes with optimum pHs near neutrality, such as mushroom PPO.

ABBREVIATIONS USED

A, deprotonated nucleophile−*p*-quinoid adduct; AB, acetate buffer; AH^+, protonated nucleophile−*p*-quinoid adduct; B, nucleophile-*o*-quinone adduct; DHPAA, 3,4-dihydroxyphenylacetic acid; DHPPA, 3,4-dihydroxyphenylpropionic acid; DMF, *N*,*N*′-dimethylformamide; ϵ, molar absorptivity; $[H^+]_0$, initial proton concentration; k_{cat}^{D}, catalytic constant of PPO toward *o*-diphenols; k_{cat}^{M}, catalytic constant of PPO toward monophenols; k_0, apparent rate constant for the nonenzymatic oxidation of A to B; k_Q, apparent rate constant for the evolution of Q to P; k_N, apparent rate constant for the addition of N to Q to yield A; K_a, dissociation constant of the acid−base equilibrium: $AH^+ \rightleftharpoons A + H^+$; K_m^D, Michaelis constant of PPO toward *o*-diphenols; K_m^M, apparent Michaelis constant of PPO toward monophenols; LOD, limit of detection; LOD^D, limit of detection of the diphenolase activity; LOD^M, limit of detection of the monophenolase activity; LOQ, limit of quantitation; LOQ^D, limit of quantitation of the diphenolase activity; LOQ^M, limit of quantitation of the monophenolase activity; λ, apparent constant of the uniexponential evolution of the MBTH−quinone adduct; λ_i, wavelength in the isosbestic point; λ_{max}, wavelength in the maximum of the spectrum; MBTH, 3-methyl-2-benzothiazolinone hydrazone; $[MBTH]_{sat}$, saturating MBTH con-

centration; PB, phosphate buffer; PHPAA, *p*-hydroxyphenylacetic acid; PHPPA, *p*-hydroxyphenylpropionic acid; PPO, (mushroom) polyphenol oxidase; $[PPO]_0$, initial PPO concentration; $[S]_0$, initial substrate concentration; V^D_{max}, maximum steady state rate of PPO toward *o*-diphenol; V^M_{max}, maximum steady state rate of PPO toward monophenol; V_{ss}, steady state rate; V^D_{ss}, steady state rate of PPO toward *o*-diphenol; V^M_{ss}, steady state rate of PPO toward monophenol.

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