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Direct spectrophotometric assay of monooxygenase and oxidase activities of mushroom tyrosinase in the presence of synthetic and natural substrates

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Abstract

Alternative substrates were synthesized to allow direct and continuous spectrophotometric assay of both monooxygenase (cresolase) and oxidase (catecholase) activities of mushroom tyrosinase (MT). Using diazo derivatives of phenol, 4-[(4-methoxybenzo)azo]-phenol, 4-[(4-methylphenyl)azo]-phenol, 4-(phenylazo)-phenol, and 4-[(4-hydroxyphenyl)azo]-benzenesulfonamide, and diazo derivatives of catechol 4-[(4-methylbenzo)azo]-1,2-benzenediol, 4-(phenylazo)-1,2-benzenediol, and 4-[(4-sulfonamido)azo]-1,2 benzenediol (SACat), as substrates allows measurement of the rates of the corresponding enzymatic reactions through recording of the depletion rates of substrates at their λ_{max}(s) with the least interference of the intermediates' or products' absorption. Parallel attempts using natural compounds, *p*-coumaric acid and caffeic acid, as substrates for assaying both activities of MT were comparable approaches. Based on the ensuing data, the electronic effect of the substituent on the substrate activity and the affinity of the enzyme for the substrate are reviewed. Kinetic parameters extracted from the corresponding Lineweaver–Burk plots and advantages of these substrates over the previously used substrates in similar assays of tyrosinases are also presented.

Keywords: Tyrosinase; Direct assay; Monooxygenase; Cresolase; Oxidase; Catecholase; Diazo derivatives; Phenol; Catechol; *p*-Coumaric acid; Caffeic acid; Kinetic parameters

Tyrosinase [EC 1.14.18.1] is a bifunctional enzyme responsible for the formation of the natural macromolecule pigment melanin in different species [1]. In its first reaction, monooxygenase (cresolase) activity, tyrosinase hydroxylates a phenolic substrate at an *ortho* position to the hydroxyl group. In its second reaction, oxidase (catecholase) activity, the produced *o*-dihydroxy compound is oxidized to the pertinent *o*-quinone derivative (Fig. 1). *o*-Quinone, in turn, takes part in a series of chemical and further enzymatic reactions and finally polymerizes to a macromolecule [2].

In vitro assay of tyrosinase activities has been the subject of numerous studies. Different methods based on chronometric [3], manometric [4], radiometric [5], oximetric [6], electrochemical [7] and spectrophotometric [8,9] techniques have been employed for this purpose. However, the spectrophotometric technique has attracted more attention mainly because it is convenient, sensitive, and inexpensive and allows the course of the reaction to be studied continuously [10,11].

Spectrophotometric methods for assaying tyrosinases are usually faced with notorious problems. Difficulties such as fast inactivation of the enzyme in the presence of the selected substrates, instability of the products, low extinction coefficients of the substrates, and interference of the intermediates' or the products' absorption with the absorption of the substrates [12] severely limit success of the introduced methods.

Neither cresolase nor catecholase activity of tyrosinase can be followed by the spectrophotometric technique through the formation of their products. Cresolase is succeeded by the catecholase reaction in such a way that the product of the former reaction,

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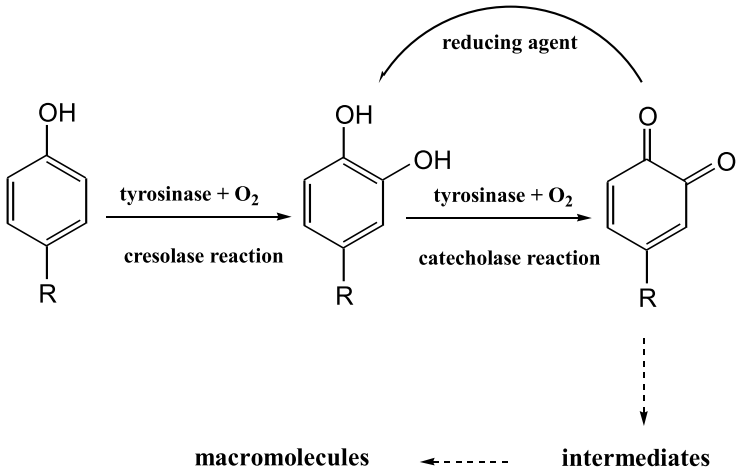

Fig. 1. One of the major driving forces toward the formation of natural macromolecules such as lignin and melanin is the enzymatic oxidation of phenolic compounds by tyrosinase. The substituent "R" could be different in different cells or experiments.

o-dihydroxy compound, is a substrate for the catecholase activity and the product of the later activity is a highly reactive molecule participating in further chemical reactions [2]. Consequently, assaying methods should be engineered on the basis of the reactant depletion rather than the product's formation.

Most of the substrates applied in assaying tyrosinase activities show increases at their λ_{max}(s) while reacting with the enzyme. The widely used substrates L-tyrosine, L-dopa, and catechol are good examples of this type. Therefore, the courses of reactions are usually studied either via the formation of intermediates, which are produced as a result of further chemical reactions of *o*-quinones, or via the depletion of an added reducing agent, which turns *o*-quinones back into the *o*-dihydroxy compounds (Fig. 1) [13]. Nevertheless, these resolutions engender indirect studies of the tyrosinase reactions.

To assay both activities of mushroom tyrosinase (MT)[1] directly through the depletion of substrates, some diazo derivatives of phenol and catechol (Fig. 2) were synthesized and applied in this work. The cresolase reaction of MT was studied in the presence of 4-[(4-methoxybenzo)azo]-phenol (MeOBAPh), 4-[(4-methylphenyl)azo]-phenol (MePAPh), 4-(phenylazo)-phenol (PAPh), 4-[(4-hydroxyphenyl)azo]-benzenesulfonamide (HPABS), and a natural substrate, *p*-coumaric acid (Fig. 2). The catecholase reaction of MT was studied in the presence of 4-[(4-methylbenzo)azo]-1,2-benzenediol (MeBACat), 4-(phenylazo)-1,2-benzenediol (PACat), 4-[(4-sulfonamido)azo]-1,2-benzenediol (SACat), and a natural substrate, caffeic acid. The results of these methods and their advantages over previously introduced spectrophotometric methods are presented and discussed.

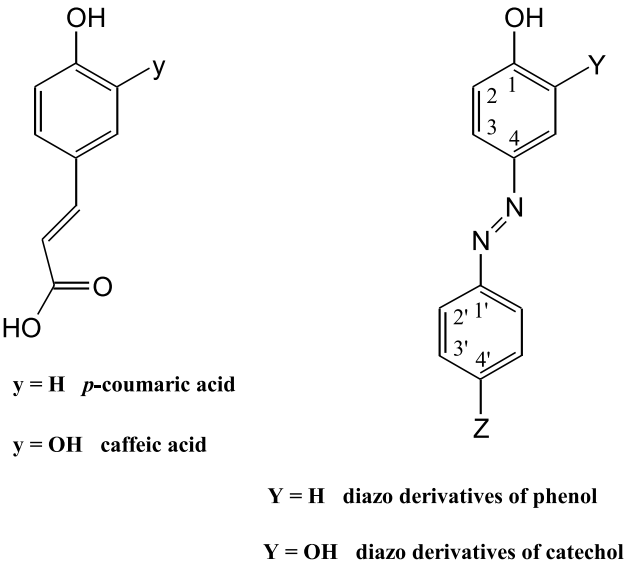

Fig. 2. General structures of the natural and synthetic substrates used in this work. "Z" for each substrate has been introduced in the text. See the introduction.

Materials and methods

MT was purchased from Sigma Chemical and Biochemical. Phenolic and catecholic substrates were synthesized as explained earlier [14]. Other chemicals used in this work were taken from authentic samples. Spectrophotometric measurements were carried out using a

[1] *Abbreviations used:* MT, mushroom tyrosinase; MeOBAPh, 4-[(4-methoxybenzo)azo]-phenol; MePAPh, 4-[(4-methylphenyl)azo]-phenol; PAPh, 4-(phenylazo)-phenol; HPABS, 4-[(4-hydroxyphenyl)azo]-benzenesulfonamide; MeBACat, 4-[(4-methylbenzo)azo]-1,2-benzenediol; PACat, 4-(phenylazo)-1,2-benzenediol, SACat, 4-[(4-sulfonamido)azo]-1,2-benzenediol; DHMA, 3,4-dihydroxymandelic acid; DHBA, 3,4 dihydroxybenzaldehyde; MBTH, 3-methyl-2-benzothiazolinone hydrazone.

Jasco Model V-550 UV-Vis-NIR spectrophotometer and the obtained results were analyzed by StatView SE + Graphics modified version 1994 and Jasco V-500 Series supporting software. Extinction coefficients of the substrates were obtained at their λ_{max}(s) in the selected buffer system at pH 6.8 and 20 °C.

Preparation of enzyme solution

Freshly prepared enzyme solutions were used in this work. Enzyme samples were weighed by a Mettler UMT2 balance with a readability of 0.1 μg and then dissolved in phosphate buffer solutions (0.01 M). Enzyme was dissolved in a volume of buffer solution to give the desired units[2] of activity in 100 μl of the resulting solution. Each 100 μl of enzyme solution contained 760 units of oxidase activity in the catecholase reactions and 56 units of monooxygenase activity in the cresolase reactions. The aforementioned units of enzyme were the selected results of a series of experiments on both activities of MT in the presence of constant concentration of substrates (50 μM). In these experiments, different concentrations of MT were applied on *p*-cresol and catechol in the cresolase and catecholase reactions, respectively. The best concentration of the enzyme, which produced linear and fast progress of the corresponding reaction, was selected (data not shown).

Preparation of substrate solutions

Due to the low solubility of the phenolic and catecholic diazo substrates in water, stock solutions of these compounds were made using 2-propanol as solvent. Diluting the dye solution with phosphate buffer solution (0.01 M) made the desired concentration of the substrate. The stability of the prepared substrate solutions was examined by measuring the change of absorption at the λ_{max} of each dye for 60 min. In all cases, no change in absorption of the dye solutions, due to precipitation or autooxidation, was observed at 20 °C.

The impact of the cosolvent, 2-propanol, on the rate of the catecholase reaction of MT was also studied in detail. This experiment was carried out in the presence of a constant concentration of L-dopa (50 μM) and the enzyme. Therefore, 100 μl of L-dopa solution (15×10^{-8} mol) was added into the UV cell (1 cm) and diluted to 2.9 ml by addition of phosphate buffer (pH 6.8); 100 μl of enzyme solution, 350 units of catecholase, was added to this solution and the rate of the reaction was measured at $\lambda_{max} = 475$ nm, using the dopachrome formation method [13], at 20 °C for 4 min. The experiment was repeated five more times but before dilution of L-dopa to 2.9 ml by buffer; 50, 100, 200, 400, and 600 μl of 2-propanol was also added into the UV cell. The reference cell in each experiment contained the corresponding volume of 2-propanol, equal to the amount of 2-propanol in the working cell, diluted to 3 ml by addition of phosphate buffer.

Although the rate of the enzymatic reaction was not seriously affected by 2-propanol concentrations lower than 10%, the amount of cosolvent did not exceed 7% of the reaction mixture in the experiments of this work.

Enzymatic reactions

All of the catecholase reactions were carried out in phosphate buffer solutions (0.01 M), containing 7% 2-propanol at most, at pH 6.8 at 20 °C. This temperature was chosen because a change in temperature from 17 to 21 °C had slighter impact on the rate of the catecholase activity than a similar change in temperature from 21 to 25 °C (Fig. 3). Therefore, any chance for a possible error caused by temperature change during the enzymatic reaction was minimized. Fig. 3 shows the results of a study on the rate of the catecholase reaction of MT at different temperatures.This experiment was carried out in the presence of a constant concentration of MeBACat (50 μM) and the enzyme. Therefore, 100 μl of the substrate solution (15×10^{-8} mol) was added into a UV cell and diluted to 2.9 ml with phosphate buffer (pH 6.8). Enzyme (100 μl = 350 units of catecholase) was added to this solution at 17 °C. The depletion rate of the substrate

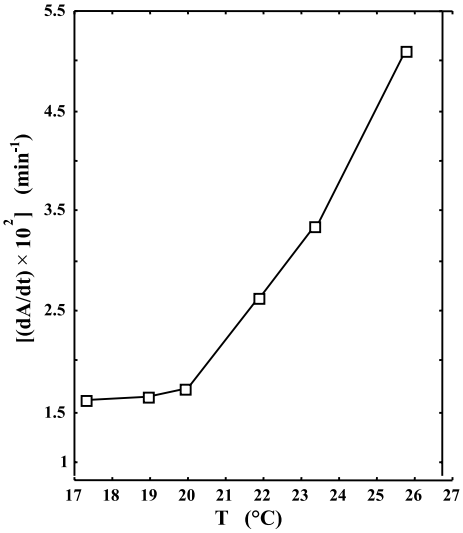

Fig. 3. Rate of the catecholase reaction of MT at different temperatures. Changes in temperature above 21 °C cause a jump in the rate of catecholase reaction, while any change between 17 and 21 °C does not create much change in the rate of the enzymatic reaction. Reactions were carried out in the presence of a constant concentration of MeBACat (50 μM) and the enzyme (350 units of catecholase) in phosphate buffer at pH 6.8. See Materials and methods for the enzyme and substrate preparations.

[2] The definitions of units described in the vendor's catalogue (Sigma, Biochemicals and Reagents for Life Science Research; T7755) were used in this work.

was measured at $\lambda_{max} = 364$ nm for 4 min. This reaction was repeated at 19, 20, 22, 24, and 26 °C. The rates of the reaction at different temperatures, under steady state conditions, were obtained from the corresponding plots (absorbance vs time) and the resulting data for the rates were plotted against temperature.

To reach comparable conclusions about both activities of MT, identical conditions of pH, solvent, and temperature were applied to the cresolase reactions. Hence, all the cresolase reactions were carried out in phosphate buffer (0.01 M) at pH 6.8 and 20 °C in the presence of a constant amount of MT (56 units of cresolase activity) and all the cresolase substrate solutions were prepared as described above.

Velocity curves

Velocity curves of each activity were obtained from the corresponding reactions carried out in the presence of a constant amount of the enzyme, as mentioned, and of different concentrations of substrates starting around 10 μM and ending at 100 μM.

Inactivation of MT

Inactivation of MT during the catecholase reaction was studied in the presence of the suggested substrate (MeBACat) and compared with catechol and L-dopa under the above-mentioned circumstances. Therefore, three fresh solutions of MT (760 units of catecholase) in phosphate buffer (0.01 M) at pH 6.8 were prepared. Each solution was incubated with 50 μM of MeBACat, catechol, and L-dopa, separately, at room temperature and the resulting mixtures were left for 24 h. Then, the catecholase activity of each mixture was assayed in the presence of MeBACat (50 μM) at 20 °C. To assay each mixture, the spectrophotometer was set at 364 nm and the absorbance was set at zero for each mixture prior to the MeBACat addition. The results of this study are compared to the results of the intact and fresh MT reaction with MeBACat under identical conditions (Fig. 4).

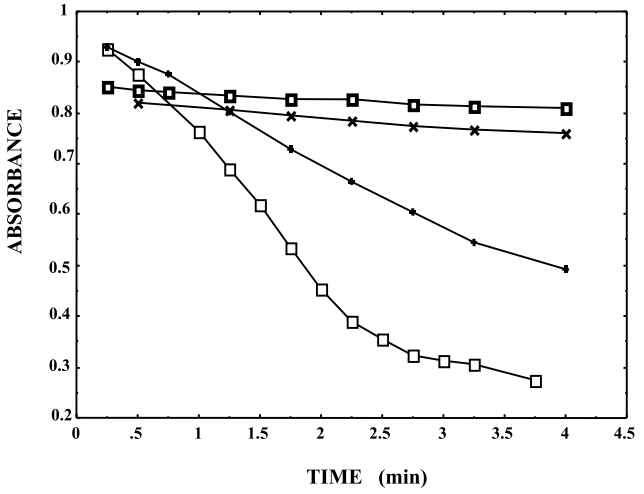

Fig. 4. Losing the catecholase ability (inactivation) of MT after reacting with MeBACat (+), catechol (■), and L-dopa (×). In each case, the fresh enzyme solution was first treated with 50 μM of each substrate and then the catecholase activity of the resulting mixture was measured in the presence of MeBACat (50 μM) after 24 h. The catecholase activity of the freshly prepared MT solution (□) with MeBACat discloses the amount of inactivation of MT in each case. All of these reactions were carried out in the presence of a constant amount of MT (760 units of catecholase activity) in phosphate buffer at pH 6.8 and 20 °C. See Materials and methods for the details.

Results and discussion

Assay of the catecholase activity of MT in the presence of synthetic substrates

The selected conditions of solvent, buffer, pH, temperature, and enzyme concentration were first applied for assaying the oxidase activity of MT according to the method introduced by El-Bayuomi and Frieden [15]. The calculated K_m of 1.65×10^{-4} M for catechol (data not shown) was in good agreement with the $K_m = 1.7 \times 10^{-4}$ M reported by El-Bayuomi and Frieden [15]. Hence, kinetics of the oxidase activity of MT were studied in the presence of MeBACat, PACat, SACat, and caffeic acid under the same conditions. Fig. 5 shows the corresponding Lineweaver–Burk plots and Table 1 contains the results of these studies and the relevant kinetic parameters.

Data in Table 1 indicate that all of the three diazo derivatives of catechol are capable substrates for the catecholase activity of MT; however, MeBACat has a smaller K_m and runs a faster reaction. MeBACat preparation involves a simple procedure as described [14]. This compound does not involve autooxidation in the optimum range of pH for the oxidase activity of MT. The high extinction coefficient of MeBACat at its λ_{max} at pH 6.8 guaranties the high sensitivity of the method.

Fig. 6 illustrates the overlapped spectra of MeBACat while reacting with MT. The spectra show that the optical density of MeBACat at the λ_{max}, 364 nm, decreases during catecholase reaction and produces two isosebstic points at 302 and 495 nm, which are far enough from the λ_{max}. Considering the regions of the isosebstic points, the dc/dt data in Table 1, the stoichiometry of the catecholase reaction [2], and the fact that the whole course of the assay in the presence of the catecholic diazo dyes does not take more than 5 min, it is understood that the amounts of the intermediates or products in an example reaction, after 3 min of the reaction, for MeBACat, PACat, and SACat are just 6.9, 4.9, and 2% of the starting substrate concentration (50 μM), respectively. Thus, if the absorbance of the product has any share in

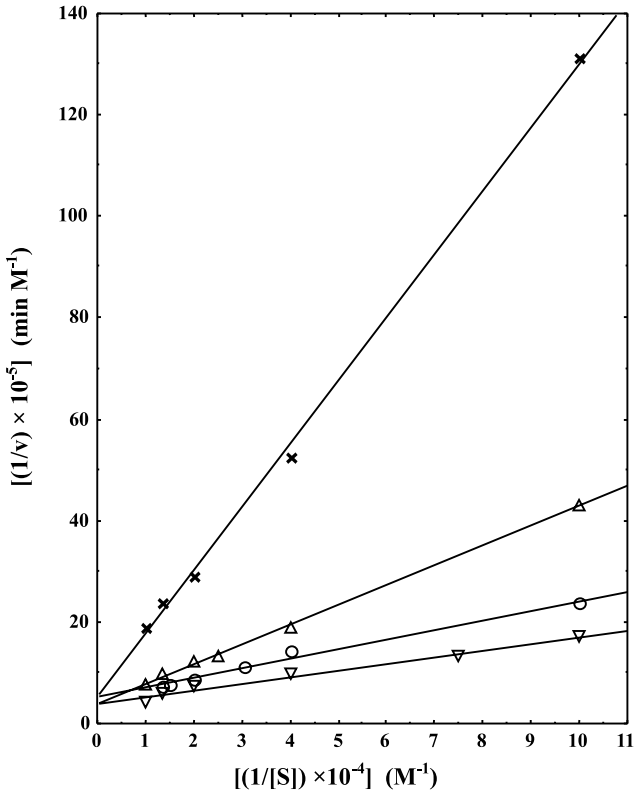

Fig. 5. Lineweaver–Burk plots of the catecholase reactions of caffeic acid (∇), MeBACat (○), PACat (Δ), and SACat (×) with MT. Catecholase reactions were carried out in the presence of a constant amount of MT (760 units of catecholase) in phosphate buffer (0.01 M) at pH 6.8 and 20 °C. Stock solutions of the substrates were prepared in 2-propanol and the desired concentration for each experiment was made by diluting the needed amount of the substrate by the above-mentioned buffer solution (see the text for details).

the region of the substrate peak, it would be less than 10% in the first 3 min of the reaction. Hence, the catecholase reaction of MT can be examined directly from the depletion of the substrate at its λ_{max}, which is the outstanding advantage of this method over the previously introduced spectrophotometric assay methods.

Methods based on utilizing catechol or L-dopa as substrate cannot offer direct measurement of the rate of the catecholase reaction since both compounds show increase instead of decrease at their λ_{max}(s) while reacting

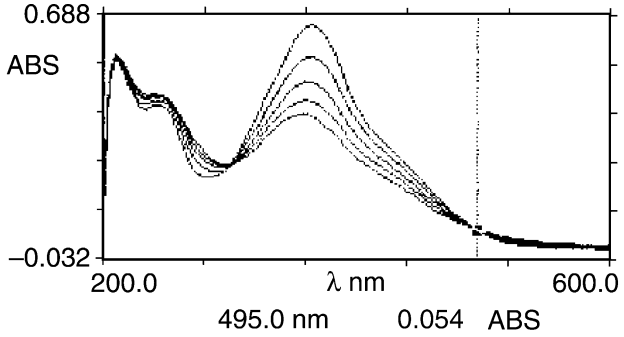

Fig. 6. Some overlaid spectra of MeBACat (50 μM) while reacting with MT (760 units of catecholase). See Materials and methods for the applied catecholase reaction conditions. A catecholase assay under these conditions does not take more than 5 min.

with the enzyme. Thus, the rate of the enzymatic reaction is measured through either the depletion of an added reducing agent, such as ascorbic acid, which reduces *o*-quinones back to their corresponding *o*-dihydroxy compounds, or the rates of the intermediates' formation in the further steps of *o*-quinones reactions [13]. In addition, in contrast to MeBACat, catechol and L-dopa inactivate MT remarkably fast [16], subsequently; the resultant data cannot be reliable. Experiments in this lab confirmed that MT lost almost all of its catecholase activity after a few minutes of its reaction with catechol or L-dopa while it maintained about 40% of its activity even after 24 h of its reaction with MeBACat (Fig. 4).

Garcia-Canovas et al. [17], introduced an assay method for the catecholase activity of MT based on using 3,4-dihydroxymandelic acid (DHMA) as substrate. Upon oxidation of DHMA with MT, its related *o*-quinone is produced. Because of instability, the quinonic compound goes into a decarboxylation reaction [18] and, hence, produces 3,4-dihydroxybenzaldehyde (DHBA). This compound is soluble in water with extinction coefficients of $\varepsilon = 9200\,cm^{-1}\,M^{-1}$ at $\lambda_{max} = 310$ nm at pH 3 and $\varepsilon = 15,200\,cm^{-1}\,M^{-1}$ at $\lambda_{max} = 350$ nm at pH 7.5. The λ_{max} of DHMA at 280 nm cannot be used for assaying purposes as it is overlaid by the strong absorption of DHBA. Therefore, the rate of the enzymatic reaction should be monitored through measuring DHBA formation at 350 nm and pH 7.5 [17].

Table 1
Results of the kinetic studies on the catecholase activity of MT

Substrate	ε ($M^{-1}\,cm^{-1}$)[a]	−dc/dt (μM min^{-1})[b]	K_m (μM)	V_{max} (μM min^{-1})
Caffeic acid	12,000 at 311 nm	1.37	33.20	2.55
MeBACat	15,400 at 364 nm	1.15	35.05	1.86
PACat	15,650 at 362 nm	0.82	97.50	2.51
SACat	15,730 at 375 nm	0.34	231.2	1.85

Enzymatic reactions were performed in the presence of a constant amount of MT (760 units) in phosphate buffer at pH 6.8 and 20 °C (see the text) and the ensuing results of the steady states were processed in double-reciprocal method (see Fig. 5).

[a] The mentioned λ_{max}(s) were selected for measuring the rate of substrate depletion during its enzymatic reaction.

[b] The result of a typical depletion rate measurement at 50 μM of the substrate.

There are three problems associated with the Garcia-Canovas et al. method. First, this method is indirect as DHBA is formed after a chemical reaction (decarboxylation). Second, DHBA is also a catechol derivative. Consequently, it can compete with DHMA, especially at higher concentrations. Thus, to minimize the inhibitory effect of DHBA, a higher concentration of DHMA should be used. Experiments in this lab confirmed the inhibitory effect of DHBA on the catecholase reaction of MT with L-dopa (data not shown). Third, the assay is carried out at pH 7.5 to have higher sensitivity, but this pH is out of the optimum range of the catecholase activity of MT. In addition, pH(s) higher than 7 enhance the chance of autooxidation for the catechol derivatives, especially those carrying electron-withdrawing groups such as DHBA [14].

Garcia-Canvas et al. [17] in a different attempt [19], introduced a continuous spectrophotometric method for assaying tyrosinase activities. The method is essentially based on the coupling reaction of 3-methyl-2-benzothiazolinone hydrazone (MBTH) with the quinonic product of the enzymatic reaction of tyrosinases; so the ensuing kinetic data embrace the results of the accompanying chemical reaction, adduct formation, also. In addition, the *o*-quinone turns back to the corresponding *o*-dihydroxy compound after coupling with MBTH [19], which is able to meddle in the steady state of the initial *o*-dihydroxy compound.

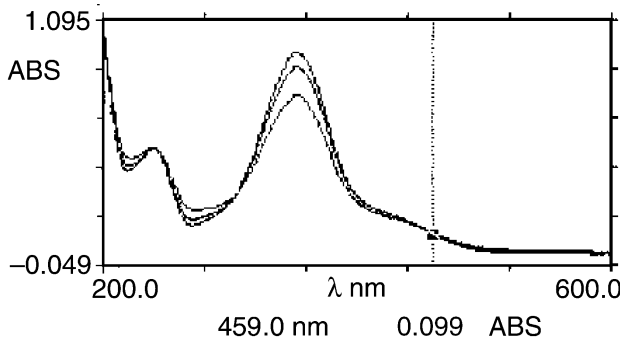

Fig. 7. Some overlaid spectra of MeBAPh (50 μM) while reacting with MT (56 units of cresolase). See Materials and methods for the applied cresolase reaction conditions. A cresolase assay under these conditions does not take more than 20 min.

Assay of the cresolase activity of MT in the presence of synthetic substrates

Similar to the catecholase, spectrophotometric assay of the cresolase activity of tyrosinase is hampered mainly because most of the cresolase substrates, such as cresol, phenol, 4-hydroxyanisol, and tyrosine, show increase instead of decrease at their maximum absorption. Consequently, the introduced spectrophotometric methods for assaying the cresolase activity have been indirect, so far [19,20]. In fact, a major volume of information available about the cresolase activity of tyrosinase comes from the experiments carried out by other techniques such as radiometry [21]. However, they are too cumbersome or expensive to be applied commonly.

Fig. 7 represents the overlaid spectra of MeBAPh, which show decrease in the optical density of substrate at its λ_{max}, 352 nm, and two isosebstic points at 300 and 459 nm. Upcoming species, intermediate or product, show λ_{max}(s) beyond the isosebstic points around 240 and 500 nm, far enough from the λ_{max}. Accordingly, this compound lets the whole course of the cresolase reaction be monitored directly via the substrate utilization with the least interference of the intermediates or products' absorption.

The kinetics of the cresolase activity of MT on MeOPAPh, PAPh, HPABS, and *p*-coumaric acid were also studied. HPABS had a reaction too slow to be worthy of kinetic analysis. Table 2 comprises the results of these studies along with the kinetic parameters obtained from the corresponding Lineweaver–Burk plots shown in Fig. 8.

Phenolic diazo compounds can be synthesized even easier than the catecholic diazo dyes [14]. Furthermore, the high extinction coefficients of these compounds promote the sensitivity of the assay method.

The cresolase reaction of tyrosinase, in contrast to its catecholase reaction, is slower and each reaction needs about 20 min to complete. Moreover, a lag time is observed in the cresolase reaction. However, it is possible to shorten the time of lag phase by adding a small

Table 2
Results of the kinetic studies on the cresolase activity of MT

Substrate	ε ($M^{-1}\,cm^{-1}$)[a]	$-dc/dt$ ($\mu M\,min^{-1}$)[b]	K_m (μM)	V_{max} ($\mu M\,min^{-1}$)
p-Coumaric acid	19,400 at 288 nm	0.86	97.47	4.6
MeOBAPh	21,950 at 360 nm	1.64	80.22	6.66
MePAPh	20,800 at 352 nm	1.13	64.42	3.74
PAPh	20,350 at 348 nm	0.60	51.03	1.88

Enzymatic reactions were performed in the presence of a constant amount of MT (56 units) in phosphate buffer at pH 6.8 and 20 °C (see the text) and the ensuing results of the steady states were processed in double-reciprocal method (see Fig. 8).

[a] The mentioned λ_{max}(s) were selected for measuring the rate of substrate depletion during its enzymatic reaction.

[b] The result of a typical depletion rate measurement at 50 μM of the substrate.

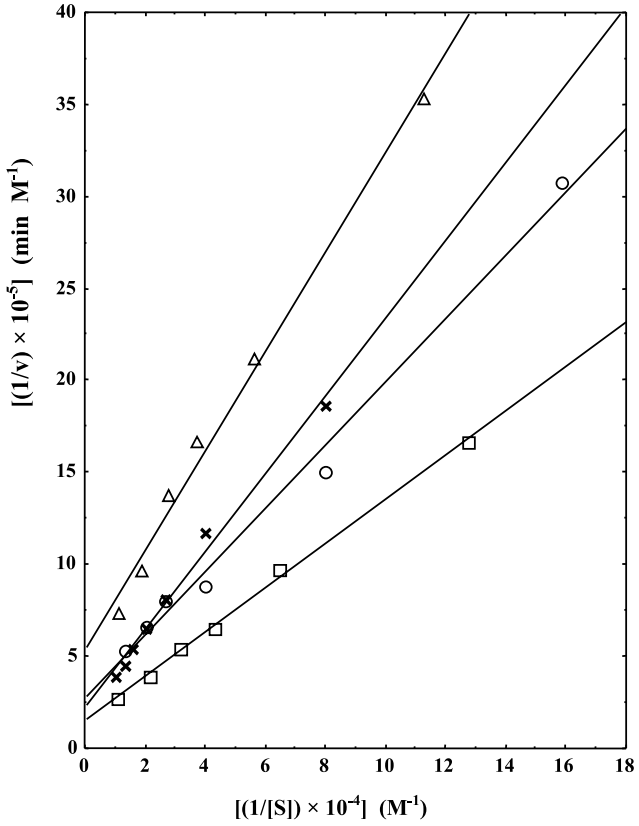

Fig. 8. Lineweaver–Burk plots of the cresolase reactions of *p*-coumaric acid (×), MeOBAPh (□), MePAPh (○), and PAPh (△) with MT. Cresolase reactions were carried out in the presence of a constant amount of MT (56 units of cresolase) in phosphate buffer (0.01 M) at pH 6.8 and 20 °C. Stock solutions of the substrates were prepared in 2-propanol and the desired concentration for each experiment was made by diluting the needed amount of the substrate by the above-mentioned buffer solution (see the text for details).

amount of a dihydroxy compound such as L-dopa [5]. Since no added compound was used in the assay method developed in this work, Fig. 8 and data in Table 2 corroborate that the dye carrying the stronger electron-donating group has not only a faster reaction but also a shorter lag time. The same effect of the substituent nature on the rate of reaction (dc/dt) is seen in the catecholase reaction. This conclusion is in agreement with the earlier reported results [22].

Assay of both activities of MT in the presence of natural substrates

p-Coumaric acid is the key intermediate in the shikimic acid metabolic pathway to lignin formation [23]. Caffeic acid is naturally synthesized from *p*-coumaric acid and known to be the precursor of phlorogenic acid in plants. It has also been shown in vitro that MT is able to convert *p*-coumaric acid into caffeic acid [24]. Literature review reveals that the reactions of epidermis tyrosinase with *p*-coumaric and caffeic acid have already been studied by spectrophotometric methods at pH 7,

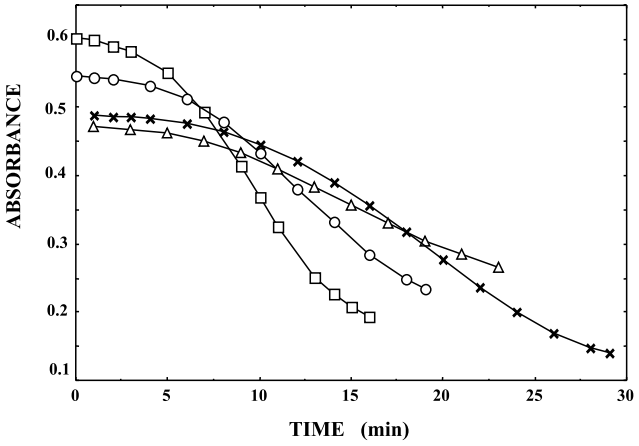

Fig. 9. Rate of cresolase reaction of MT (56 units of cresolase activity) with the constant concentration (50 μM) of MeOBAPh (□), MeBAPh (○), PAPh (△), and *p*-coumaric acid (×) under identical conditions. See Materials and methods for the applied conditions for the cresolase reaction.

and K_m values of 0.08 and 0.13 mM have been reported for them, respectively [25]. Although the reaction of the epidermis tyrosinase with caffeic acid had been studied at 310 nm, which is the λ_{max} of the substrate, the rate of the cresolase reaction had been measured at 334 nm, while *p*-coumaric acid has a λ_{max} at 288 nm and there was not any description for the choice.

p-Coumaric acid seems to be the only natural substrate for tyrosinase, which shows a decrease in absorbance at its λ_{max} during the course of the cresolase reaction. Its corresponding *o*-dihydroxy compound, caffeic acid, does also show a decrease at its λ_{max} during reaction with MT and its overlaid spectra produce three isosebstic points at 220, 265, and 445 nm. The isosebstic points for *p*-coumaric are at 270 and 323 nm. Assuming that the possible interference of the intermediates or products, in comparison with the initial concentration of the substrates, can be neglected, both activities of MT were studied in the presence of *p*-coumaric acid (at 288 nm) and caffeic acid (at 311 nm) under the same conditions applied for the synthetic substrates. Results of these studies are included in Tables 1 and 2, respectively. Table 1 indicates that caffeic acid has a smaller K_m in comparison with the synthetic diazo substrates and runs a faster catecholase reaction. However, its counterpart for the cresolase reaction, *p*-coumaric acid, not only runs a slower cresolase reaction but also has a longer lag time in comparison with MeOBAPh and MePAPh (Fig. 9).

Kinetic parameters

The maximum or limiting rate (V_{max}) values of different diazo derivatives of catechol and caffeic acid in Table 1 do not seem to be dissimilar. Since all the catecholase reactions, in this work, were carried out in the

presence of the same amount of MT and considering Eq. (1) below, it can be concluded that similar values of V_{max} for these compounds reflect similar k_{cat}(s). This means that the effect of the substituent at position 4′ of the dyes and the group on position 4 of the phenolic ring of caffeic acid (Fig. 2) do not influence the catalytic efficiency of MT. In other words, the product formation step in the catecholase reaction is not significantly affected by different substituents. A similar conclusion, that the activated MT by sodium dodecyl sulfate or protease had similar V_{max} even at different pH(s) while its K_m changed has recently been shown [26].

$$k_{cat} = \frac{V_{max}}{[Ei]}. \quad (1)$$

In contrast to the catecholase reaction, V_{max} values of the phenolic substrates in the cresolase reaction experience the effect of the substituent nature. These results are in agreement with the Solomon et al. [27] suggestion that the oxidation of *o*-diphenolic compounds by tyrosinase has fewer electronic requirements than the oxidation of monophenolic substrates.

It is also important to pay attention to the effect of the substituents' nature on the K_m values of the phenolic and catecholic substrates. While a stronger electron-donating substituent causes an increase in the K_m value of a phenolic substrate, it decreases the K_m value of the corresponding catecholic compound. To understand the opposite effect of a substituent on the K_m values of monooxygenase and oxidase substrates of tyrosinase, one must consider the mechanisms of these reactions.

In catecholase, tyrosinase mediates an electron transfer from the *o*-dihydroxy compound to the molecular oxygen to form water and a molecule of *o*-quinone [2]. The electron-donating group, at position 4′, of a catecholic substrate enhances the electron density at the phenolic oxygens. For this reason they can be considered softer Lewis bases in comparison with those carrying electron-withdrawing groups. On the other hand, copper, in the tyrosinase active site, can be considered a softer Lewis acid in comparison with group 13 or lower transition metal groups. As the activation energy for bond formation between a soft base and a soft acid is lower than that of the bond formation between a hard base and a soft acid, catecholic compounds with electron-donating groups show higher affinity for the tyrosinase active site.

On the other hand, based on an extended Huckel theory calculation for a tyrosinase active site model, it has been shown that the ionization of the hydroxyl group of the phenolic substrate is a crucial step in its interaction with the positively charged copper of the active site in the cresolase reaction [28]. Accordingly, it is assumed that an electron-withdrawing group decreases the K_m value of a phenolic substrate by increasing its acidity. It is known that electron-withdrawing groups increase acidity of phenolic compounds [14]. Thus, at a specific pH the presence of an electron-withdrawing group on the aromatic ring of a phenolic substrate increases its ionization and eases its interaction with the copper cation.

The K_m values of *p*-coumaric and caffeic acid suggest that the acidic residue of these compounds is in the ionized form at pH 6.8, and thus functions like an electron-donating group with opposite effects on the cresolase and catecholase reactions.

It has been shown that the specificity constant of a substrate, the ratio of k_{cat}/K_m, explains the ability of an enzyme to discriminate one substrate from another. Considering the aforementioned assumption that the obtained V_{max} values in these studies can be used as indicators of the k_{cat} values, estimated specificity constants of 0.083, 0.058, 0.037, and 0.047 for MeOBAPh, MePAPh, *p*-coumaric acid, and PAPh and those of 0.077, 0.053, 0.026, and 0.008 for caffeic acid, MeBACat, PACat, and SACat, respectively, were calculated. It is seen that, regardless of the smaller K_m values of PAPh, *p*-coumaric acid has a greater specificity constant than PAPh. This means that *p*-coumaric acid runs a faster cresolase reaction than PAPh. These data also suggest that the rate of hydroxylation for MePAPh and MeOBAPh by MT is about 22 and 76%, correspondingly, faster than the rate of the same reaction for *p*-coumaric acid. In contrast, the estimated values of the specificity constants illustrate that none of the catecholic diazo dyes can run a catecholase reaction as fast as can caffeic acid. The difference in the specificity constants of the phenolic and catecholic diazo dyes suggests that, in addition to the electronic effects of the substituents, the structural features of the substrates influence the progress of the reactions.

In the end it should be added that looking solely at the kinetic results in Tables 1 and 2 cannot be the best way for selecting the best substrates for in vitro assaying both activities of tyrosinases. However, this could be a guide to the best choice for the optional experiments. For example, according to the data in Table 1, SACat cannot be the selected substrate for the routine assay of the catecholase activity since it has the highest K_m in Table 1 and runs the slowest reaction. In addition, its counterpart for the cresolase reaction, HPABS, also runs its corresponding cresolase reaction too slow, but both SACat and HPABS may come in use as strong competitive inhibitors for the catecholase and cresolase activity of tyrosinases, respectively. On the other hand PACat and PAPh can be prepared easily and data in Tables 1 and 2 suggest that they can be good substrates, but not the best, compared with MeBACat and MePAPh. Finally, it seems that MeOBAPh has all the best parameters (Table 2) to be selected as the best choice for the cresolase assay. This is true when concomitant assay of the catecholase activity is not necessary.

How reliable are the obtained kinetic parameters?

The kinetic parameters presented in Tables 1 and 2 were calculated from the double-reciprocal form of the Michaelis–Menton equation, which is applicable to all hyperbolic velocity curves of enzymatic reactions [29]. It should be mentioned that direct assay of MT activities by the introduced natural or synthetic substrates creates reproducible data, although the corresponding velocity curves of these data might not be quite hyperbolic. The observed deviations can be explained on the basis of the real nature of the enzyme, which is beyond the scope of this article, but it is necessary to mention that repeated experiments revealed that the observed deviations were dependent not only on the structure of the substrate but also on its concentration. In view of that, it is possible to choose a proper range of concentration, which brings about a decent hyperbolic velocity curve. Inspection of Figs. 5 and 8 imparts the idea that the range of choice is around 10–100 μM for both activities of MT.

Conclusion

Although *p*-coumaric and caffeic acid can be used for direct kinetic studies of the MT activities, the following reasons have convinced the authors to introduce the analogue pair of MeBAPh and MeBACat for the regular direct assay of the cresolase and catecholase activities of MT, respectively: (a) they are cheaply and easily prepared, (b) they have high extinction coefficients, (c) they have simple and not busy UV–Vis spectra even while reacting with the enzyme, (d) they have fairly fast enzymatic reactions, (e) they are stable solutions at pH 6.8, (f) except for the cosolvent, there is no need for any other substances to be added to the reaction mixtures, and (g) MT does not suffer serious inactivation in the presence of these substrates.

Ultimately, it should be added that different diazo derivatives of phenol and catechol with different structures and substituents could be applied in direct kinetic studies on the MT activities with versatile objectives.

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