Hydrogen Peroxide Helps in the Identification of Monophenols as Possible Substrates of Tyrosinase

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Tyrosinase exists in three forms in the catalytic cycle depending on the oxidation state of the copper: met-(Emh), oxy- (Eox), and deoxy- (Ed). When O-quinones, products of the enzymatic reaction, evolve chemically to generate an O-diphenol in the reaction medium, the enzyme acts on a monophenol with O-diphenol as reductant, converting Emh to Eox. The binding of Ed to molecular oxygen gives Eox, which is active on monophenols, but when the O-quinone product does not generate O-diphenol through chemical evolution, the monophenol does not act as an enzyme substrate. The fact that Eox can be formed from Emh with hydrogen peroxide can be used to help identify whether a monophenol is a substrate of tyrosinase. The results obtained in this study confirm that compounds previously described as inhibitors of the enzyme are true substrates of it.

Key words: tyrosinase; monophenol; peroxygenase; monophenolase

Tyrosinase (EC 1.14.18.1), is a copper monoxygenase widely distributed in bacteria, fungi, plants, and animals. It is responsible for melanization in animals and browning in plants. It catalyzes two different reactions involving molecular oxygen: the hydroxylation of monophenols to O-diphenols and the oxidation of these to O-quinones.1,2 The substrates can be divided into different groups depending on the stability of the O-quinones generated by the enzyme acting on them.3

The stability of the O-quinone is important, since the activity of tyrosinase on a monophenol depends on a sequence of chemical reactions related to the evolution of its O-quinone and the probability that some of its corresponding O-diphenol is generated in the reaction medium.

This influence on enzymatic activity is better understood if we consider its action mechanism (scheme 1). As can be seen by its action on monophenols and O-diphenols, tyrosinase exists in three forms in the catalytic cycle: met-tyrosinase (Em), deoxy-tyrosinase (Ed), and oxy-tyrosinase (Eox). It is the step from Emh to Eox, that requires the presence of a reductant such as an O-diphenol.4,5 Hence, when the evolution of the O-quinones does not involve an accumulation of O-diphenol in the medium, the enzyme cannot act on monophenols (see scheme 1), but, the enzyme has no difficulty in acting on O-diphenols (scheme 2). When O-quinones are very unstable, alternative measurement methods can be used to solve these problems, such as

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measuring the consumption of oxygen, ascorbic acid, or NADH, or a chronometric method.\textsuperscript{31}

The intermediate \textit{oxy}-tyrosinase ($E_{ox}$) is capable of hydroxylating monophenols to \textit{O}-diphenols. $E_{ox}$ can be generated in two ways: through the reduction of $E_m$ by an \textit{O}-diphenol (scheme 1A), or by the reaction of $E_m$ with H$_2$O$_2$ (scheme 1B).\textsuperscript{6,8} Usually, the enzyme’s action on monophenols is studied by adding a catalytic quantity of the corresponding \textit{O}-diphenol. When working with monophenols for which there is no commercially available \textit{O}-diphenol, \textit{l-DOPA} is added.

The reaction of tyrosinase with H$_2$O$_2$ rapidly brings about the formation of $E_{ox}$,\textsuperscript{10} which can react with the monophenol to generate the \textit{O}-quinone and maintain the steady state.\textsuperscript{3–9} To the best of our knowledge, the peroxidase activity of the enzyme has been studied with monophenols that give rise to very stable \textit{O}-quinones such as 4-\textit{tert}-butylphenol (TBP)\textsuperscript{7} and \textit{N,N}-dimethylyramine,\textsuperscript{9} simply by measuring \textit{o}-quinone formation at a wavelength of 400 nm in the case of TBP and the formation of \textit{N,N}-dimethylydoliumolate at a wavelength of 290 nm. The peroxidase activity of tyrosinase can also be followed by the consumption of H$_2$O$_2$ using amperometric H$_2$O$_2$ sensors.\textsuperscript{9} Some enzymes, such as peroxidase and tyrosinase, when acted with H$_2$O$_2$ as unique substrate, show catalase activity, and at the same time undergo suicide inactivation.\textsuperscript{10,11}

The aim of this study was to determine whether a monophenol is a substrate of tyrosinase, taking advantage of the property of tyrosinase of reacting with H$_2$O$_2$ and generating the intermediate $E_{ox}$ which is capable of hydroxylating monophenols.

Materials and Methods

Reagents and materials. Mushroom tyrosinase (4276 U/mg) was purchased from Sigma (Madrid, Spain) and purified as described previously.\textsuperscript{12} The protein concentration was determined by Bradford’s method using bovine serum albumin as standard.\textsuperscript{10} Umbelliferone, (TBP), arbutin, hydroquinone, \textit{o}-naphthol, \textit{b}-naphthol, guaiacol, thymol, carvacrol, eugenol, isoeugenol (scheme 3), and MBTH (3-methyl-2-benzothiazolinone hydrazone hydrochloride) were also purchased from Sigma. Stock solutions of substrates were prepared in 0.15 mM phosphoric acid to prevent auto-oxidation, except for umbelliferone, which was prepared in 2 mM sodium phosphate buffer, pH 6.8.

Spectrophotometric assays. Spectrophotometric assays of the enzymatic activity of tyrosinase as its action on different monophenols were done at maximum absorbance in the visible spectrophotometric region of the \textit{O}-quinone corresponding to the monophenol under study.\textsuperscript{3} The figures show the measurement conditions for each substrates.

Results and Discussion

The action mechanism of tyrosinase on monophenols and \textit{O}-diphenols is shown in schemes 1 and 2, respectively.

In the case of \textit{O}-diphenols (scheme 2) the mechanism is linear, and the entire enzyme is acting on substrate D, but in the case of monophenols (scheme 1), part of the enzyme is inhibited in the form $E_m$ M. It is easy to measure the enzymatic activity on an \textit{O}-diphenolic substrate (D), where D may be \textit{O}-diphenol or a polyphenol, although the measurement of enzymatic activity is limited by the stability of \textit{O}-quinone, but, a series of methods have been designed to characterize the substrates that give rise to unstable \textit{O}-quinones.\textsuperscript{3} When the substrates under study are assayed in the presence of \textit{t}-tyrosine or \textit{l}-DOPA and dopachrome formation is followed at a wavelength of 475 nm, the accumulation rate diminishes (apparent inhibition), because these substrates behave as alternative substrates of tyrosinase and compete with tyrosine and \textit{l}-DOPA. This effect has been examined in several studies and reviews on tyrosinase inhibitors.\textsuperscript{14–16}

The situation is different in the case of monophenolase activity (see scheme 1) where the mechanism of

Scheme 3. Chemical Structures of the Compounds Studied.
(1) Hydroquinone, (2) 4-\textit{tert}-butylphenol, (3) umbelliferone, (4) \textit{a}-naphthol, (5) \textit{b}-naphthol, (6) arbutin, (7) carvacrol, (8) thymol, (9) guaiacol, (10) eugenol, (11) isoeugenol.
action of the enzyme is very complex. In the mechanism depicted in scheme 1, a reductant is necessary (represented here by an O-diphenolic substrate, D) for the enzyme to express its activity. Therefore, in the case of monophenols whose O-quinone does not chemically evolve to generate O-diphenol in the medium, the enzyme cannot act on them. In this sense, a large number of monophenols have been identified as inhibitors of the enzyme, including hydroquinone, umbelliferone, o-naphthol, p-naphthol, and arbutin. 

In this study, we identified the possibility that tyrosinase acts as catalyst in the presence of hydrogen peroxide and considered whether this might help to discriminate whether a compound is a substrate or an inhibitor of the enzyme. In scheme 1, the $E_m$ form can originate in two ways: by $E_m$ reacting with an O-diphenol or with $H_2O_2$ (scheme 1A and B). It is clear that depending on the medium in which it is found (the presence or absence of $H_2O_2$), the enzyme can act on the type of monophenol in question here. In the action of tyrosinase on monophenols (e.g., L-tyrosine) there is a lag period corresponding to the time necessary for this particular reaction network, including enzymatic and non-enzymatic reaction, to accumulate a given quantity of O-diphenol (e.g., 1-DOPA) in the reaction medium. 

The extension of this delay depends on the concentration of enzyme and substrate. For example, if the enzyme concentration is increased the lag time is shortened, and if the monophenol concentration is increased, the steady state rate increases and so does the lag period. This same behavior is seen in the action of tyrosinase on monophenols in the presence of $H_2O_2$, as described below.

**Effect of the enzyme concentration**

Figure 1A depicts the enzyme’s action on a constant concentration of hydroquinone and $H_2O_2$. When the concentration of tyrosinase was increased, the rate increased and the steady state was reached earlier. In this case, the absence of a lag period was due to further oxidation of the reaction product, 2-hydroxy-p-hydroquinone, by the oxygen in of the medium reaction.

Figure 1B shows the rate obtained for the action of the enzyme on hydroquinone when the reaction medium (hydroquinone, phosphate buffer, and $H_2O_2$) was preincubated at different times. Note that the rate of the enzyme’s action is the same, taking into account that at short times of measurement, the purely chemical oxidation of hydroquinone by $H_2O_2$ interfered with in the enzymatic oxidation.

**Effect of concentration of $H_2O_2$**

Figure 2 depicts the enzyme’s action on TBP under varying concentrations of $H_2O_2$. According to the mechanism of scheme 1, when the concentration of $H_2O_2$ is increased, the lag decreases and the steady state rate increases. In this case, since the O-diphenol is commercially available, the maximum wavelength is known, 400 nm.

![Fig. 1. Action of Tyrosinase on Hydroquinone.](image1)

**A.** Effects of varying the concentration of the enzyme. Spectrophotometric recordings of the action of tyrosinase on hydroquinone measured at a wavelength of 480 nm. The experimental conditions were 30 mM phosphate buffer (pH 7.0), initial hydroquinone concentration, [hydroquinone]: 0.5 mM, initial $H_2O_2$ concentration, [H$_2$O$_2$]: 2.5 mM and the initial enzyme concentrations, [E]$_0$, (nm) 0 (a), 10 (b), 35 (c), 55 (d), 75 (e), 90 (f), 115 (g), and 130 (h).

**B.** Action of tyrosinase on hydroquinone preincubating the reaction medium (hydroquinone, phosphate buffer, and $H_2O_2$) at the times indicated in the figure. The experimental conditions were [hydroquinone]: 0.5 mM, [H$_2$O$_2$]: 2.5 mM, 30 nM phosphate buffer (pH 7.0). At the indicated times, tyrosinase was added at a concentration of 135 nM and the increase in the absorbance at 480 nm is recording with the time.

![Fig. 2. Action of Tyrosinase on 4-tert-Butylphenol.](image2)

Effects of varying the concentration of hydrogen peroxide. Spectrophotometric recordings of the action of tyrosinase on TBP followed at a wavelength of 400 nm. The experimental conditions were 30 mM phosphate buffer (pH 7.0), initial TBP concentration, [TBP]: 0.5 mM, [E]$_0$: 3 nM, and [H$_2$O$_2$]: in mM 2.5 (a), 5 (b), 7.5 (c), and 10 (d).
Effect of concentration of monophenol

According to scheme 1, when the concentration of the monophenolic substrate is increased and the concentrations of enzyme and H$_2$O$_2$ are kept constant, the system takes longer to reach the steady state and the lag period is longer. Moreover, the steady state rate increases, as seen in Fig. 3 in the case of guaiacol. This behavior is similar to those shown by monophenols that are tyrosinase substrates in the absence of H$_2$O$_2$.11

Figures 1–3 depict the behavior of tyrosinase acting on monophenols. The form $E_{ox}$ of the enzyme is common to the monoxygenase activity of tyrosinase and peroxigenase activity, and is the only form capable of hydroxylating monophenols. In the experiments described below, it was found how monophenols whose O-quinone does not evolve to produce O-diphenol in the medium which have been described as inhibitors, in fact, they act as substrates of tyrosinase.

Description of the monophenols studied

The chemical structures of the various monophenols studied here are shown in scheme 3.

Hydroquinone

It has recently been described how hydroquinone, scheme 3 (1), is neither a tyrosinase substrate nor a suicide inactivator.17) It has also been proposed that the presence of the para-hydroxyl group prevents binding to the tyrosinase active site.17) However, the experiments described in Fig. 1 confirmed that hydroquinone acts as a substrate of tyrosinase, though the presence of H$_2$O$_2$ is required due to the difficulty involved in the formation of $E_{ox}$. We emphasize that our findings agree with those of others that hydroquinone stimulates tyrosinase activity through indirect formation over at long times of adducts that can act as secondary substrates.17)

4-tert-Butylphenol

The chemical structure of 4-tert-butylphenol is shown in scheme 3 (2). The action of tyrosinase on this compound in the absence of H$_2$O$_2$ was studied, because the stability of O-quinone implies that O-diphenol does not accumulate in the medium and that the enzyme shows almost no activity. Nevertheless, O-diphenol is released enzymatically to the medium, giving rise to anomalous kinetic behavior.18) When the peroxigenase activity of tyrosinase was tested, in the presence of H$_2$O$_2$, the enzyme showed high activity as to its action on TBP.7)

Umbelliferone

This compound (scheme 3 (3)) has been described as a powerful inhibitor of the monophenolase activity of tyrosinase, with a $K_I$ of 14 μM.19) However, Fig. 3 Inset (a) shows an accumulation of the O-quinone of esculetin (o-diphenol, corresponding to umbelliferone), and (b) indicates that as the enzyme concentration increases, the O-quinone formation rate increases and the lag period is shortened. This is typical behavior for the mechanism of tyrosinase acting as monophenolase, as can be seen when umbelliferone acts as substrate of tyrosinase.

α- and β-naphthol

Both compounds (scheme 3 (4) and (5)), have been described as inhibitors of tyrosinase,20) but as can be seen in Fig. 4A, which shows the action of the enzyme on α-naphthol, it acts as a substrate of tyrosinase. The acquired data against the reaction time allow one to

Fig. 3. Action of Tyrosinase on Guaiacol.

Effects of various guaiacol concentrations. Increase in absorbance followed at 337 nm due to the action of tyrosinase on guaiacol in the presence of [H$_2$O$_2$]$_0$ 10 mM in 30 mM phosphate buffer (pH 7.0). The experimental conditions were curve (a), initial guaiacol concentration, [guaiacol]$_0$ 1.75 mM, and [E]$_0$ 10 mM; curve (b), [guaiacol]$_0$ 1.5 mM and [E]$_0$ 85 mM; and curve (c) [guaiacol]$_0$ 7.5 mM and [E]$_0$ 85 mM. Inset, Action of tyrosinase on umbelliferone. (a) and (b) show the effects of varying the concentration of enzyme. The experimental conditions were [umbelliferone]$_0$ 0.5 mM, [H$_2$O$_2$]$_0$ 10 mM, curve (a) [E]$_0$ 0.1 μM, and curve (b) [E]$_0$ 0.2 μM.

select the wavelength at maximum absorbance, $\lambda_{max}$ (Fig. 4A). Regarding β-naphthol, a similar methodology was followed in selecting the corresponding $\lambda_{max}$ and registering the spectrophotometric data (Fig. 4B). In (b) and (c), shown in this graph, the substrate and the enzyme concentration are increased respectively with respect to those employed in (a). The shortening of the lag period, and the consequent increase in the O-quinone formation rate appear to indicate that α- and β-naphthol act as alternative substrates of tyrosinase and not as inhibitors.

Arbutin

This compound (scheme 3 (6)) has been proposed for use as a whitening agent in cosmetics,21) and the inhibition of tyrosinase showed an IC$_{50}$ value of 8.4 nM.22) It was also recently suggested that it acts as a tyrosinase substrate if catalytic quantities of L-Dopa are added.23) Figure 5 shows how the addition of H$_2$O$_2$ without L-Dopa makes it a substrate for the enzyme, in accord with the characteristics of monophenolase activity. In (b), the concentration of H$_2$O$_2$ was double that of (a). In (c) the concentration of enzyme was double that of (b), while the concentration of the rest of the reagents remained constant.

Carvacrol and thymol

The action of tyrosinase in the presence of H$_2$O$_2$ on these compounds (Scheme 3, (7) and (8) respectively) is depicted in Fig. 6 and Fig. 6 Inset. MBTH was added to highlight the spectrophotometric signal, since this compound forms adducts with quinones that show high molar absorbptivity. It has been suggested that both thymol and carvacrol inhibit the oxidation/reduction reaction between O-dopaquinone and leucodopachrome.24) Recently, it was found that they act as tyrosinase substrates when the catalytic quantities of
the corresponding O-diphenol are added. 25) The reaction observed in the presence of H$_2$O$_2$ clearly indicated that they are substrates of the enzyme. (b) indicates how an increase in substrate concentration compared with (a) increased the velocity of the reaction. The same effect was observed when the concentration of enzyme was increased (c) over the levels of (b).

Guaiacol, eugenol, and isoeugenol

Compounds guaiacol (9), eugenol (10), and isoeugenol (11) are shown in scheme 3. Guaiacol has been found to act as a tyrosinase substrate when the catalytic quantities of its corresponding O-diphenol are added. 25) By the method described here and transforming $E_m$ into...