Study of Umbelliferone Hydroxylation to Esculetin Catalyzed by Polyphenol Oxidase

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We characterize umbelliferone, a derivative of 2,4-dihydroxycoumaric acid, as a substrate of polyphenol oxidase. This enzyme hydroxylates umbelliferone to esculetin, its o-diphenol, and then oxidizes it to o-quinone. The findings show that umbelliferone, an intermediate in one of the coumarin biosynthesis pathways, may be transformed into its o-diphenol, esculetin, which is also an intermediate in the same pathway. The activity of the enzyme on umbelliferone was followed by measuring the consumption of oxygen, spectrophotometrically and by HPLC. Kinetic constants characterizing the hydroxylation process were: $k_{cat}=0.09\pm0.02s^{-1}$ and $K_m=0.17\pm0.06mM$. The o-diphenol, esculetin, was a better substrate and when its oxidation was followed spectrophotometrically, the kinetic constants were: $k_{cat}=1.31\pm0.25s^{-1}$ and $K_m=0.035\pm0.002mM$. Both compounds therefore can be considered as alternative substrates to L-tyrosine and 1,3,4-dihydroxyphenylalanine (1-DOPA), since both indirectly inhibit melanogenesis.

Key words tyrosinase; esculetin; umbelliferone; polyphenol oxidase

Tyrosinase or polyphenol oxidase (EC 1.14.18.1; PPO) is a binuclear copper cluster ubiquitously present in biological systems. It catalyzes two types of reactions in which molecular oxygen participates: (a) The orthohydroxylation of monophenols to o-diphenols (monophenolase activity) and (b) the oxidation of o-diphenols to their corresponding o-quinones (diphenolase activity).

Three enzymatic species participate in the catalytic cycle of PPO, Chart 1: met-PPO ($E_{met}$), deoxy-POO ($E_d$) and oxy-PPO ($E_{ox}$). Note that when the $E_{ox}$ form of the enzyme acts on a monophenol (M), the o-diphenol formed may be oxidized to o-quinone or be released, giving $E_d$ or $E_{met}$ respectively. To return to the hydroxylase catalytic cycle, the enzyme that has passed from $E_{ox}$ to $E_m$ must react with the o-diphenol (D). When o-quinones (e.g., o-dopaquinone), which are unstable, evolve to give o-diphenol in the medium, the enzyme shows a lag period until the steady state is reached, but when the o-quinone in its evolution does not accumulate o-diphenol the system shows hardly any activity. This last situation is evident when PPO acts on 4-tert-butylphenol.

In the hydroxycoumarin biosynthesis pathway starting from cinnamic acid, 7-hydroxycoumarin (umbelliferone), the common name of umbelliferone, is formed after a series of reactions, and from this compound, 6,7-dihydroxycoumarin (esculetin), 7-hydroxy-6-methoxycoumarin (scopeolin) and esculin are formed (Chart 2). The coumarins are especially abundant in the Umbelliferae and Rutaceae families. Some coumarins from natural sources have also been used as therapeutic agents for humans and their antibacterial, antifungal and anticancer activities make these compounds attractive for further derivatization and screening for use as therapeutic agents.

Esculetin (6) has been described as an inhibitor of lipoxygenase (LOX), and it has been demonstrated that the products resulting from the oxidation of 6 by peroxidase (POD) and PPO contribute to the defence against pathogens, increasing their fungitotoxicity 20-fold.

According to Chart 2, umbelliferone (5) would seem to be the precursor of 6, although previous studies have suggested that umbelliferone is hydroxylated by a P450 monooxygenase. In Arabidopsis thaliana it has been demonstrated that the genetic mutation of the gene encoding CYP98A3, reduces the biosynthesis of scopeolin (8) and scopolin (9), and it has been proposed that this enzyme may bring about the 3'-hydroxylation of p-coumaric acid. Furthermore, the same authors observed an increase in a derivative of 5 (umbelliferone glucoside). A detailed study of the biosynthetic pathways to hydroxycoumarins during post-harvest physiological deterioration in Cassava roots using stable isotope labelling pointed to

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two pathways emerging from \( p \)-coumaric acid: the main one
through caffeic and ferulic acids, giving rise to 8 and 9, and
the other through 5 giving rise to 6 and esculetin (7) (Chart
2). In the above pathways different hydroxylation steps were
proposed, but involving cytochrome P450 enzymes.5) Despite
the use of several experimental approaches, the possibility that
5 is hydroxylated to 6 by PPO was recently discarded when
it was seen that 5 behaves as a true competitive inhibitor of
PPO, with a \( K_i \) of 14 \( \mu \)M, when it is studied by measuring the
monophenolase activity with \( l \)-tyrosine as substrate.4)

Recently, coumarin-resveratrol hybrids have been synthet-
sized as potential inhibitors of PPO. For example, the com-
 pound 3-(3',4',5'-trihydroxyphenyl)-6,8-dihydroxy-coumarin,
when the tyrosinase activity was assayed with 3,4,5-trihy-
droxyphenylalanine (1-DOPA) as substrate, was seen to be
more potent than 5 with \( IC_{50} \) (mm) values of 0.27 and 0.42,
respectively.16) Based on the structure of coumarins new
compounds have been synthesized: for example, halogenated
derivatives of phenylcoumarins to increase the inhibitory power
between PPO, more specifically the 3-phenylcoumarins with
one atom of bromide.15) Coumarin derivatives with thiosemi-
carbazide moieties have also been described, the compound
2-(1-(coumarin-3-yl)ethylidene) hydrazinecarbothioamide
showing an \( IC_{50} \) value of 3.44 \( \mu \)M.16) A revision of the antioxi-
dant effects of these compounds (umbelliferone and esculetin)
have recently been published17) and neuroprotective effects have
been described in a mouse model of Parkinson’s disease.18)

The aim of this work was to study the reaction of PPO with
5 and 6, further clarifying the coumarin biosynthesis pathway.
We focus on the enzymatic mechanism depicted in Chart 1,
which underlines the need for \( o \)-diphenol to be present in
the reaction medium for the enzyme to make catalytic cycles
with the monophenols (to generate the \( oxy \) form from the \( met \)
form). In our case, the \( o \)-diphenol is 6 and the monophenol is
5. It is shown how the presence of a reductant (e.g., ascorbic
acid) or \( \text{H}_2\text{O}_2 \) has the same effect, that is, they encourage the
enzyme to act on the monophenols.

**MATERIALS AND METHODS**

**Enzyme Source** Mushroom polyphenol oxidase (4276 U/
mg) was purchased from Sigma (Madrid, Spain) and was puri-
fied as described in ref. 19, laccase from *Agaricus bisporus*
(5.1 U/mg) was purchased from Fluka, Madrid (Spain). The
protein concentration was determined by Bradford’s method20)
using bovine serum albumin as the standard.

**Reagents** \( l \)-Tyrosine, 1-DOPA, umbelliferone (5), escu-
letin (6) 4-tert-butylcatechol (TBC) and ABTS (2,2'-azino-
bi(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt)
were purchased from Sigma (Madrid, Spain). Stock solutions
of the reducing substrates were prepared in 0.15M phosphor-
ic acid to prevent autooxidation, except 5, which was prepared
in 2mM phosphate buffer pH=7.0.

**Spectrophotometric Assays** When the substrate was
TBC, the enzymatic reaction was followed by monitoring the
formation of 4-tert-butylquinone at 410 nm (\( e=1200 \text{m}^2\text{cm}^{-1}\)).21) When the substrate was \( l \)-tyrosine or 1-
DOPA the increase in absorbance at 475 nm (\( e=3500 \text{m}^2\text{cm}^{-1}\))
was monitored.21) The oxidation of 6 was monitored at 295 nm
by measuring the consumption of the ascorbic acid during the
oxidation of this acid by the \( o \)-quinone generated by the
enzyme.

The reaction of PPO with 5 was followed by measuring
spectrophotometrically, the \( o \)-quinone formed as a result of 6
oxidation by periodate in excess.

**Oxymetric Assays** Measurements of dissolved oxygen
concentration were made with a Hansatech (Kings Lynn,
Cambs, U.K.) oxygen unit controlled by a PC. The oxygraph
used a Clark-type silver/platinum electrode with a 12.5 \( \mu \text{m} \)
Teflon membrane. The sample was continuously stirred during
the experiments and its temperature was maintained at 25°C.
The zero oxygen level for calibration and experiments was
obtained by bubbling oxygen-free nitrogen through the sample
for at least 10 min. The oxygraph was calibrated as described
in ref. 22. The reaction of PPO with 5 was followed by mea-
suring, the consumption of oxygen oxymetrically.

**Chromatography** The reaction of PPO with 5 was fol-
lowed also measuring the formation of 6 in the presence of
ascorbic acid by HPLC. The enzyme was separated from
the reaction intermediates by centrifugation using 10000
MWCO filters. The reaction products were analyzed in a
high resolution liquid chromatograph (VWR-Hitachi, model
Elite LaChrom). Samples were introduced via a fixed volume
(20 \( \mu \text{L} \)). The compounds were separated in a Teknokroma
C-18 column (25 cm x 0.4, i.d. 5 \( \mu \text{m} \)) and eluted at a flow rate
of 1 mL/min with the mobile phase formed by the dissolu-
tions (A) Agua milli Q with 3% acetic acid and (B) HPLC
grade acetonitrile with 3% acetic acid. The following gradient
programme was used: 0 min, 94% A; 15 min, 82% A; 25 min,
67% A; 30 min, 94% A. Measurements were made at 259 nm,
324 nm and 359 nm.23)

**Kinetic Data Analysis of Monophenolase and Diphen-
olase Activities** Initial rate values \( V_0 \) were calculated from
triplicate measurements at each reducing substrate concen-
tration. The data were fitted by nonlinear regression to the
Michaelis–Menten equation, using the Sigma Plot 9.0 program
for Windows.8,24)
RESULTS AND DISCUSSION

When attempts are made to characterize a monophenol as possible substrate for PPO, problems may arise—for example, no enzymatic activity is detected or, if the monophenol is assayed by measuring the enzyme activity on l-tyrosine or l-DOPA and following the increase in absorbance caused by the formation of dopachrome at a wavelength of 475 nm, the dopachrome formation rate usually diminishes since most monophenols, including 5, give rise to o-quinones with low molar absorptivity coefficients and so, in its presence, the formation of dopachrome is slowed, and, consequently, such monophenols are usually classified as inhibitors.

When studying the action of 5 and 6 on PPO activity by measuring the monophenolase and diphenolase activities on l-tyrosine or l-DOPA, the effects described above normally lead to them being described as powerful inhibitors of PPO: 6 with an IC₉₀ = 0.043 mM when assayed with l-DOPA as substrate, and 5 with a Kᵢ = 0.014 mM when assayed with l-tyrosine as substrate. Moreover, the characterization of possible tyrosinase substrates is complicated by the fact that the reaction product (an o-quinone) is usually unstable.

In a previous work using a chronometric method, we characterized 6 not as a PPO inhibitor but as a true substrate of the same, a conclusion also reached by other authors. However, 5 has been classified as an inhibitor, a fact that can be explained if we take into consideration the enzyme’s action mechanism depicted in Chart 1 (see below).

Behaviour of 5 and 6 as Apparent Inhibitors of the Activity of PPO on l-Tyrosine and l-DOPA Table 1 shows the apparent % inhibition of PPO activity exercised by 5 and 6 on the monophenolase and diphenolase of PPO when measured with l-tyrosine or l-DOPA, respectively. When the inhibition of PPO by 6 is studied, this substrate apparently inhibits both the activity on l-tyrosine and l-DOPA more strongly, because it is a better substrate for the enzyme than 5. However, the inhibition of PPO by a monophenol, like 5, or by an o-diphenol, like 6, is greater in the case of the monophenolase activity of PPO on l-tyrosine, since in this case the enzyme is partly inhibited by the l-tyrosine itself (see Chart 1). Moreover, the binding of the monophenols to the met form is strong since it is facilitated by a base probably a histidine.

Physico-Chemical Characteristics of 5 and 6 The monophenol 5 has a pKₐ value of 6.89, which is low compared with other monophenols, and this is similarly the case with 6 (pKₐ=7.64). The existence of this pKₐ is confirmed in Fig. 1 and Fig. 1 inset, by the appearance of the isosbestic points. Furthermore, the high chemical shift values (δ) for the carbon atoms supporting the hydroxyl group (δ₋₁=158.1 ppm for 5 and δ₋₁=146.9 ppm and δ₋₁=144 ppm for 6) suggests that both are very bad substrates for PPO, as will be confirmed below.

Considerations on the PPO Action Mechanism The kinetic mechanism of PPO in its activity on monophenols and o-diphenols is described in Chart 1, where it can be seen that for PPO to act on a monophenol the presence of an o-diphenol is necessary. In the case of monophenols that give rise to unstable o-quinones (for example o-dopaquinone) that can accumulate o-diphenol in the medium as they evolve, the enzyme reaches the steady state after a lag period, which is the time the system needs to accumulate the o-diphenol concentration to reach the steady state.

Table 1. Percentage Inhibition of Monophenolase and Diphenolase Activities of Polyphenol Oxidase in the Presence of 5 or 6

<table>
<thead>
<tr>
<th>Substrates</th>
<th>Concentration (mm)</th>
<th>Monophenolase activity (%)</th>
<th>Diphenolase activity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>5</td>
<td>0.1</td>
<td>52.9</td>
<td>10.8</td>
</tr>
<tr>
<td></td>
<td>0.3</td>
<td>74.3</td>
<td>35.6</td>
</tr>
<tr>
<td></td>
<td>0.5</td>
<td>82.9</td>
<td>49.5</td>
</tr>
<tr>
<td>6</td>
<td>0.1</td>
<td>81.2</td>
<td>63.4</td>
</tr>
<tr>
<td></td>
<td>0.3</td>
<td>84.6</td>
<td>79.3</td>
</tr>
<tr>
<td></td>
<td>0.5</td>
<td>86.3</td>
<td>86.8</td>
</tr>
</tbody>
</table>

a) Monophenolase activity, the experimental conditions were: phosphate buffer 30 ms, pH=6.0 at 25°C, [E]₀=0.26 mm, [l-DOPA], 1-tyrosine]₀=0.046 mm. Umbelliferone (5) (mm): 0.1, 0.3 and 0.5. Esculetin (6) (mm): 0.1, 0.2 and 0.5. b) Diphenolase activity, the experimental conditions were: phosphate buffer 30 ms, pH=6.0 at 25°C, [E]₀=15 mm, [l-DOPA], 1-tyrosine=0.5 mm. Umbelliferone (5) and esculetin (6), the same concentrations as used for monophenolase activity.

To confirm that the product of PPO acting on 5 is 6 (Fig. 2), PPO was made to react with 5 in the presence of ascorbic acid in different conditions: Fig. 2a (●) with catalytic quantities of 6, Fig. 2b (▲) with catalytic quantities of TBC, and Fig. 2c (○) in the absence of o-diphenol. At given times, an aliquot was oxidized by periodate in excess and the absorbance corresponding to the o-quinone of 5 was monitored (see Fig. 2).

As can be seen, the absorbance increased with the incubation...
time. Note that the enzyme laccase cannot form 6, Fig. 2d (●).

It is known that mushroom PPO may be contaminated by laccase. However, Fig. 2 Inset curve (a) shows the activity of mushroom laccase on ABTS and Fig. 2 Inset curve (b) the absence of activity when the experiment is carried out at the concentration indicated in Fig. 2d (●). At time \( t=0 \), [esculetin] \( =0.01 \text{ mM} \) was added. (b) At time \( t=0 \), [TBC] \( =0.01 \text{ mM} \) was added. (c) No o-diphenol was added at \( t=0 \). (d) Mushroom laccase (0.15 mg/mL) was added instead of polyphenol oxidase. Inset. Spectrophotometric recording of the increase in absorbance at 414 nm, in phosphate buffer 30 mM, pH 6.0 at 25°C in the action of laccase on [ABTS] \( =2.4 \text{ mM} \). (b) Spectrophotometric recording of the increase in absorbance in the same experimental conditions as (a) but in the absence of laccase and a PPO concentration of 0.46 µM.

Fig. 4. HPLC Analysis of the Reaction of Polyphenol Oxidase with 5 in Different Experimental Conditions

5 (0.5 mM) was reacted with polyphenol oxidase (0.59 µM) in 30 mM phosphate buffer, pH=6.0 in the presence of 3 mM ascorbic acid, adding to the reaction medium 10 µM 6 (b) (A–C) or 10 µM TBC (D–F). At times \( t=0 \), \( t=2 \) h and \( t=3 \) h, an aliquot was taken, filtered to separate the enzyme and 20 µL were injected into the HPLC apparatus. The rest of the conditions are described in Materials and Methods.

Fig. 5. Variation in the Initial Rate of Polyphenol Oxidase on 6 vs. pH

Experimental conditions: [esculetin] \( =0.1 \text{ mM} \), [ascorbic acid] \( =300 \text{ µM} \) and [E] \( =62 \text{ nM} \), phosphate buffer 30 mM pH: 7.3; 7.1; 6.6; 6.0; 5.5 and 30 mM acetate buffer at pH 5.3; 4.5 and 4.0. Inset A. Overlapping of 6, ascorbic acid and PPO spectra. Spectrophotometric recordings: (a) [esculetin] \( =0.1 \text{ mM} \), (b) [ascorbic acid] \( =300 \text{ µM} \) and (c) [E] \( =62 \text{ nM} \). Inset B. Oxidation of 6. Spectrophotometric recordings of disappearance of ascorbic acid resulting from the reduction of the \( \alpha \)-quinone of 6, monitored at 295 nm. The experimental recordings were those described in Fig. 4 at the following pH-values: (a) 4.0, (b) 7.3 and (c) 6.0.

the concentration indicated in Fig. 2. The same experiments as in Fig. 2d (●), carried out with laccase, show no increase in absorbance so that laccase is incapable of attacking 5 and originating 6.

Another way to reveal the action of PPO on 5 consists in that this enzyme can act in its peroxygenase activity. In the presence of \( \text{H}_2\text{O}_2 \), the formation of the \( \alpha\chi\psi \) form of PPO (Chart 1) from \( E_{\text{in}} \) was followed, since it is capable of attack-
ing the 5, dehydroxylating and oxidizing it (Fig. 3). These experiments demonstrated the typical characteristics of this activity on monophenols, curve (a) representing a control experiment. It can be seen that as the concentration of enzyme increases, Fig. 3 curve (b), the rate increases and the lag period diminishes. When the substrate concentration decreases, so do the rate and lag period, Fig. 3, curve (c).

The clearest evidence of 5 being hydroxylated to 6 is obtained by using HPLC (Fig. 4). In this case, 5, ascorbic acid and PPO were reacted in the presence of catalytic quantities (10 µmol) of 6 or α-diphenol TBC. At the times indicated in Fig. 4, aliquots were taken and injected into the HPLC apparatus, giving the following results: Fig. 4A, at t = 0; Fig. 4B, at t = 2 h and Fig. 4C at t = 3 h, when 6 was added and Fig. 4D, at t = 0; Fig. 4E, at t = 2 h and Fig. 4F at t = 3 h, when TBC was added. Note the accumulation of 6 with time. Therefore, the enzyme hydroxylates 5 to 6 and then oxidizes this to α-quinone, which is reduced by ascorbic acid to form 6 again.

The present work takes another look at the oxidation of 6 by PPO (Fig. 5). Taking into account the spectra of ascorbic acid is reduced by ascorbic acid to form 6, the pH can be seen in Fig. 5. 32, 33) Note the bell shaped curve 34) giving the following results: Fig. 4A, at t = 0; aliquots were taken and injected into the HPLC apparatus, K low value of pδ, which agrees with the high PPO.

Concentration by nonlineal regression provides the values kcat and Km (not shown). Analysis of the initial rate versus 6 concentration by nonlinear regression provides the values kcat and Km (not shown). It is interesting that the kcat for diphenol 6 is greater than that for 5: kcat = 1.31 ± 0.25 s⁻¹ and 0.09 ± 0.02 s⁻¹, respectively.

CONCLUSION

In conclusion, we have characterized 5 as a substrate of PPO. 5 is seen to be a very bad substrate for polyphenol oxidase, which agrees with the high δ value of the carbon bearing the hydroxyl group. Furthermore, at physiological pH its hydroxyl group is highly deprotonated as a consequence of the low value of pKa. These results throw light on part of the coumarin biosynthesis pathway and help explain the participation of PPO in the pathway.

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