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TIRON AS A SUBSTRATE FOR MUSHROOM TYROSINASE

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Key Word Index—Mushroom; tyrosinase; Tiron; intermediate (λ_{max} 365 nm); yellow product (λ_{max} 435 nm).

Abstract—Tiron (4,5-dihydroxy-1,3-benzene disulphonic acid) can serve as a substrate for mushroom tyrosinase with a K_m value of 34 mM. The product(s) formed is yellow (λ_{max} 435 nm) and is stable with time. An intermediate product, having significant absorbance at 365 nm (probably Tiron-*o*-quinone), was detected and the kinetics of its conversion to the final yellow product(s) was studied. Hydrogen peroxide (at 0.03–1.3 mM) accelerated the conversion of the intermediate compound to the yellow product(s).

Tiron-semiquinone was detected by EPR spectroscopy during the initial phase of Tiron oxidation by mushroom tyrosinase. Maximum EPR signal intensity and steady state intensity due to Tiron-semiquinone and the time required to reach maximum intensity were dependent on the amount of mushroom tyrosinase but not on the presence or absence of hydrogen peroxide (3.3 mM).

In view of separate studies showing that the yellow product(s) is mainly a low M_r polymerized Tiron-quinone, suggestions as to possible pathways by which such a product(s) is formed are discussed.

INTRODUCTION

Evidence against the participation of the superoxide free radical (O_2^-) in the hydroxylation of monhydroxyphenols by mushroom tyrosinase was recently presented in ref. [1]. In the course of these studies, the effect of various O_2^- scavengers on the reaction, including that of Tiron, which is a scavenger of both O_2^- and OH^-, was studied. It was found that, at low concentrations, Tiron extends the lag period of tyrosine hydroxylation by mushroom tyrosinase, whereas at higher concentrations Tiron shortens the lag period [2]. Concomitantly, it was also noted that interaction of mushroom tyrosinase with relatively high concentrations of Tiron yielded a yellow product(s) with a peak around 435–440 nm.

This paper presents data showing that Tiron is a substrate for mushroom tyrosinase and that a stable yellow product(s) (λ_{max} 435 nm) is formed as a result of the reaction. Tiron-*o*-quinone will be shown to be a likely intermediate in the reaction leading to stable products absorbing maximally at 435 nm. The role of Tiron semiquinone as an additional intermediate in the reaction will be discussed.

RESULTS AND DISCUSSION

Tiron is highly soluble in water, relative to naturally occurring dihydroxyphenols. In aqueous solution, Tiron hydroquinone is colourless and gives a pH of approximately 3.0. The UV spectrum of Tiron is characterized by a strong absorbance maximum at 206 nm, a shoulder at 228 nm and a weak absorbance peak at 286 nm.

Incubation of 20 mM Tiron with 400 μg mushroom tyrosinase in the presence of 47 mM sodium phosphate buffer (pH 6.5) for about five min resulted in the formation of a stable yellow product(s). When the spectrum was scanned 10, 30 and 60 min and 2, 3, 8 and 20 hr after the initiation of the reaction, it was found that the position of the visible absorbance maximum did not change, indicating that the yellow product(s) (λ_{max} 435 nm) was the major final product and that it was stable.

Fig. 1 shows the visible spectrum of a reaction mixture consisting of Tiron and mushroom tyrosinase incubated for 20 hr at room temperature. The visible spectrum is

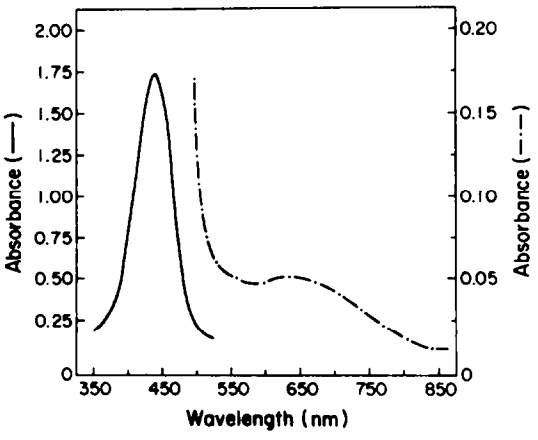

Fig. 1. Visible spectrum of stable product(s) formed when Tiron is oxidized by mushroom tyrosinase. The reaction mixture included, in a total volume of 3 ml, 33.3 mM Tiron (pH 6.5), 47 mM sodium phosphate buffer (pH 6.5) and 1.6 mg mushroom tyrosinase (added last). After 20 hr incubation at 20°, the sample was diluted with water 1:10 and scanned in the visible range.

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characterized by a very intense absorbance maximum at 435 nm and a small shoulder at 640–650 nm.

Controls showed that mushroom tyrosinase in the absence of Tiron had very little absorbance in the visible range of the spectrum but absorbed strongly below 300 nm. Additional controls showed that incubation of Tiron for a period of 3–4 hr did not produce any spectral changes compared with a spectrum of fresh Tiron. Incubation of a solution of Tiron (0.1 M) in the presence of sodium phosphate buffer (at pH 6.5) for more than 20 hr resulted in the slow appearance of a yellow colour, while a solution of Tiron (0.1 M, pH 3.1) in the absence of a buffer at pH 6.5 remained colourless throughout a period of at least six days.

Additional controls showed that whereas incubation and mushroom tyrosinase with Tiron in the presence of buffer (pH 6.5) resulted in the time-dependent formation of a yellow product(s) (λ_{max} 435 nm), incubation of the same amount of boiled mushroom tyrosinase with Tiron under the same conditions for up to 2–3 days did not yield any yellow product(s).

Effect of different amounts of mushroom tyrosinase on the rate of yellow product(s) formation

The data in Fig. 2 show the rate of formation of the yellow product(s) when 33.3 mM Tiron was acted on by 0.2–2.4 mg mushroom tyrosinase. Initially, there was a lag period in the formation of the yellow product(s) followed by a linear increase in the absorbance at 435 nm. The rate of Tiron oxidation, defined as activity (ΔOD at 435 nm/min), was estimated from the linear portions of the curves following the lag period (Fig. 2). As shown in the inset of Fig. 2, the activity of mushroom tyrosinase with 33.3 mM Tiron was linearly related to the amount of enzyme in the range tested (up to 2.4 mg enzyme). An explanation for the lag period in the rate of increase in absorbance at 435 nm formation will be dealt with further below.

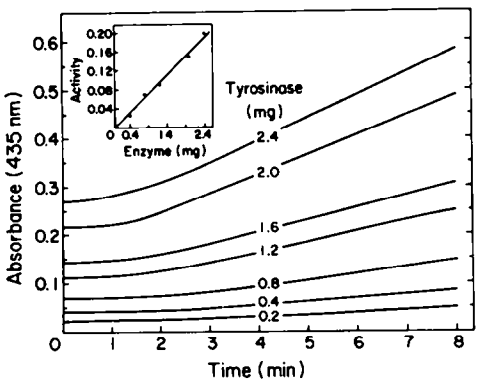

Fig. 2. Time course of Tiron oxidation to a yellow product(s) by different amounts of mushroom tyrosinase. The reaction mixture included, in a total volume of 3 ml; 33.3 mM Tiron, 47 mM sodium phosphate buffer (pH 6.5) and the indicated amounts of mushroom tyrosinase (added last). The absorbance at 435 nm was recorded as a function to time. Activity (ΔOD at 435 nm/5 min) was estimated from the rates observed after the initial lag period.

Estimate of the K_m of Tiron for mushroom tyrosinase

The rate of oxidation of different concentrations of Tiron to the yellow product(s) (λ_{max} 435 nm) by a fixed amount of mushroom tyrosinase was determined. Lineweaver–Burk plots [3] gave a K_m value for Tiron of approximately 34 mM. By comparison, we found that the K_m of mushroom tyrosinase for DL-DOPA was 0.48 mM and for 4-methyl catechol 0.08 mM. Duckworth and Coleman [4] reported a K_m of mushroom tyrosinase of 0.26 mM for L-DOPA and of 0.19 mM for pyrocatechol. Hence, Tiron is a less preferred substrate than non-sulphonated pyrocatechols, but still saturates the enzyme active site in the expected manner.

Oxidation of Tiron by mushroom tyrosinase to a yellow product(s) via an intermediate compound

Jimenez *et al.* [5] reported the simultaneous detection of two absorbance maxima (at 390 nm and at 480 nm) during the oxidation of dopamine by mushroom tyrosinase in sodium phosphate buffer (pH 6.1). However, when the oxidation was carried out at pH 7.0, only the maximum at 480 nm was detected [5]. These workers showed that the increase in absorbance of the intermediate product (λ_{max} 390 nm), identified as dopamine-*o*-quinone was initially rapid (first 40 sec) and then slowed down, while the formation of dopaminochrome (λ_{max} 480 nm) was characterized by an initial lag period (10–20 sec), after which the absorbance increased in a linear fashion (30–120 sec). The length of the lag period was not dependent on enzyme concentration, while the linear rates of dopaminochrome formation after the lag period were dependent on enzyme concentration [5]. Free radical forms of these substrates were not reported.

Using a spectrophotometer that scanned at high speeds (2 nm/sec), an intermediate compound with a yellow chromophore (λ_{max} at 395 nm) was detected during the oxidation of DOPA by periodic acid [6]. The transient yellow chromophore detected was attributed to dopaquinone [6]. On the other hand, during the oxidation of DOPA with mushroom tyrosinase, the dopa-quinone was not detected and it was suggested that in the enzymatic reaction, dopaquinone did not accumulate to sufficiently high concentrations to be detected [6].

In view of the reported stability of the oxidation products of Tiron relative to the oxidation products of naturally occurring catechols [7], it was reasonable to expect to detect intermediate compounds in the course of Tiron oxidation by mushroom tyrosinase.

The reaction was examined in a total volume of 3 ml using different concentrations of Tiron (33.3, 67 and 133 mM), 47 mM sodium phosphate buffer (pH 6.5), and different amounts of mushroom tyrosinase (20, 40 and 60 μg). The data obtained with 133 mM Tiron plus 60 μg mushroom tyrosinase best illustrate that transient intermediates are formed during the catalytic oxidation of Tiron by the enzyme (Fig. 3). As shown in Fig. 3, an intermediate product (probably Tiron-*o*-quinone) with an absorbance maximum at 365 nm was formed. The final product(s), characterized by a strong absorbance maximum at 435 nm, was seen clearly after 38 and 56 min incubation (Fig. 3B). Also, there was an immediate (< 1 min) increase in absorbance between 340 and 450 nm on mixing the reactants, indicating a rapid initial phase in Tiron oxidation followed by slower accumulation of 365 nm and 435 nm absorbing species.

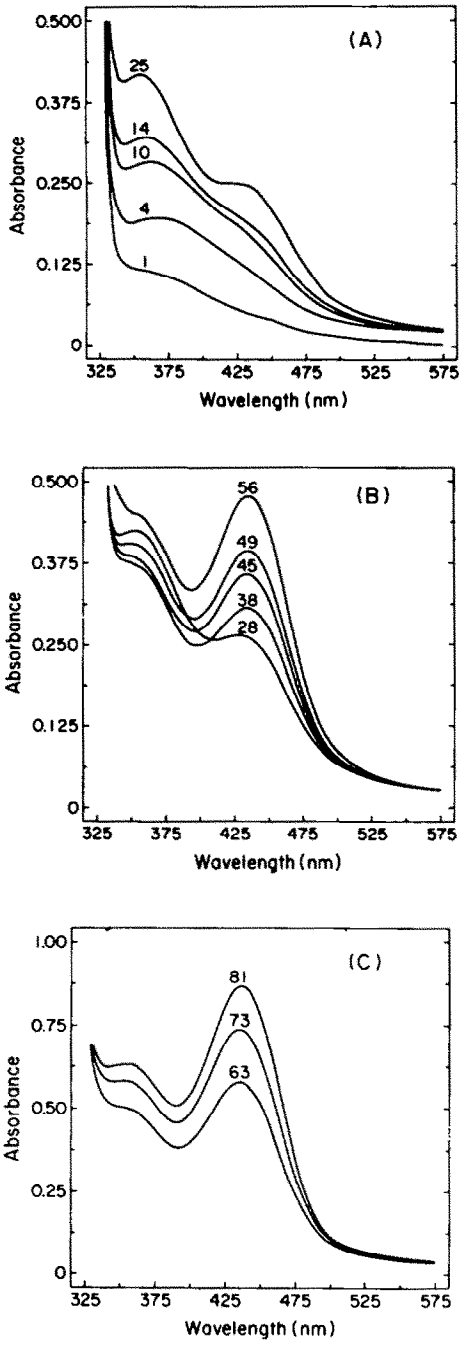

Fig. 3. Spectral changes occurring during the oxidaton of Tiron by mushroom tyrosinase. The spectral mixture included, in a total volume of 3 ml, 133 mM Tiron, 47 mM sodium phosphate buffer (pH 6.5) and 60 μg mushroom tyrosinase (added last). The spectrum was scanned at the indicated times against a blank containing 133 mM Tiron, plus 47 mM sodium phosphate buffer (pH 6.5). The number above each scan represents the reaction time in min.

From about one min after the initiation of the reacuion, the intermediate with a 365 nm absorbance maximum increased slowly as a function of incubation time (Figs 3B, 3C). Up to about 49 min of incubation, the absorbance at 365 nm was higher than that at 435 nm, but during additional incubation periods the absorbance at 435 nm (final product) became higher than that at 365 nm (Fig. 3C).

As will be shown below, Tiron-semiquinone was detected by EPR spectroscopy during the initial phase of Tiron oxidation by mushroom tyrosinase and the rate of Tiron-semiquinone formation (EPR signal) coincides well with an initial, rapid rate of increase in absorbance at 360 nm which occurred immediately after mixing (3–60 sec). However, since *o*-semiquinones usually have maximum absorbance at lower wavelengths, it is likely that initial rapid absorbance changes at 360 nm described below represent a mixture of quinone and semiquinone forms rather than Tiron-semiquinone alone. Also, evidence for the formation of Tiron-quinone with an absorbance maximum at 365 nm from the semiquinone, will be summarized below.

Effect of hydrogen peroxide on the disappearance of the intermediate compound with an absorbance maximum at 365 nm

The effect of hydrogen peroxide (H_2O_2) (in the range 0.03–1.3 mM) on the oxidation of Tiron by mushroom tyrosinase to a yellow product(s) (λ_{max} 435 nm) via an intermediate compound (λ_{max} 365 nm) was studied. As shown in Fig. 4, the formation of this compound was detected when 67 mM Tiron was incubated with 20 μg mushroom tyrosinase (Fig. 4). Hydrogen peroxide, at either 0.03 mM or at 1.3 mM, apparently accelerated the conversion of the intermediate compound (λ_{max} 365 nm) to the final yellow product(s) (λ_{max} 435 nm); the acceleration was less pronounced with 0.03 mM than with 1.3 mM H_2O_2 (Figs 4B and 4C, respectively).

The rate of oxidation of 67 mM Tiron to the intermediate compound (λ_{max} 365 nm) by 500 μg mushroom tyrosinase was linear with time for approximately 12 min (Fig. 5A). In the presence of H_2O_2 (0.66 mM), the apparent rate of accumulation of the intermediate compound was considerably lower. The rate of formation of the yellow product(s) (λ_{max} 435 nm) was characterized by a lag period of about 90 sec and it then proceeded at the rate of 0.1 ΔOD at 435 nm/10 min (Fig. 5B). H_2O_2, at 0.66 mM eliminated the lag period of the formation of the yellow product(s) and appreciably accelerated its formation (0.16 Δ0D nm/10 min) (Fig. 5B).

The data presented in Fig. 5 showing less 365 nm absorbing intermediate (part A) but more yellow (λ_{max} 435 nm) (part B) product(s) in the presence of H_2O_2, compared with its absence, give further support to the idea that H_2O_2 accelerates the conversion of the intermediate compound to the yellow product(s). We do not, as yet, have an explanation as to the mechanism by which H_2O_2 accelerates such a conversion.

The effect of various concentrations of H_2O_2 (up to 13.3 mM) on the rate of 6.7 mM Tiron oxidation by 400 μg mushroom tyrosinase to the yellow product(s) was examined further (Fig. 6). There was a lag period of about 6 min in Tiron oxidation to a stable yellow product(s) in the absence of H_2O_2 but no lag period was detected in the presence of 1.6–13.3 mM H_2O_2. Tiron oxidation was rapid initially, but then appeared to slow down considerably in this concentration range. The maximum absorbance value reached was inversely dependent on H_2O_2 concentration, while the time required to reach this maximum value was shorter at higher concentrations.

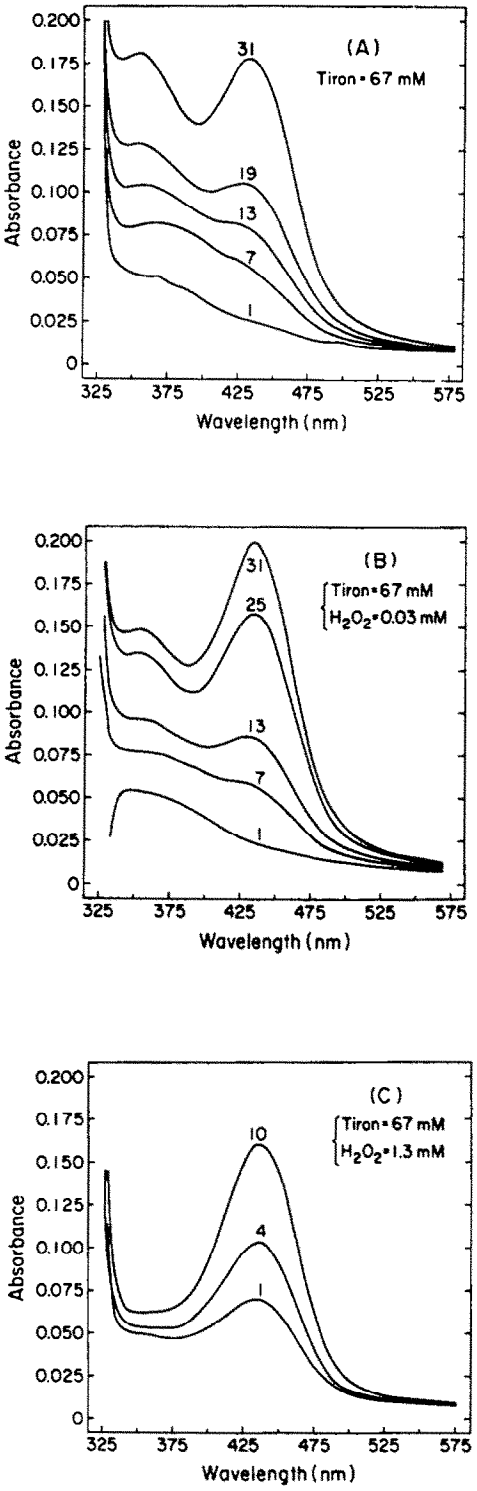

Fig. 4. Spectral changes occurring during the oxidation of Tiron by mushroom tyrosinase in the absence and presence of H_2O_2. Reaction mixture A, B and C included, in a total volume of 3 ml, 67 mM Tiron. 47 mM sodium phosphate buffer (pH 6.5), H_2O_2 as indicated and 20 μg mushroom tyrosinase (added last). The spectrum was scanned at the indicated times against a blank containing 67 mM Tiron and 47 mM sodium phosphate buffer (pH 6.5). The number above each scan represents the reaction time in min.

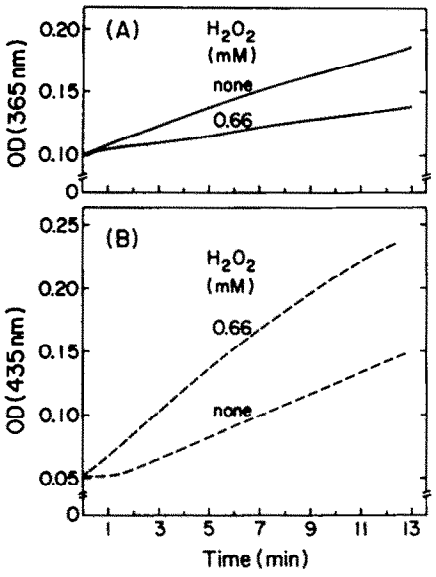

Fig. 5. Effect of H_2O_2 on the rate of formation of the intermediate compound (λ_{max} 365 nm) and the yellow product(s) (λ_{max} 435 nm) during the oxidation of Tiron by mushroom tyrosinase. The reaction mixture included, in a total volume of 3 ml, 67 mM Tiron (pH 6.0), 47 mM sodium phosphate buffer (pH 6.5), 500 μg mushroom tyrosinase (added last) and H_2O_2, as indicated. Absorbance was followed at either 365 nm (A) or at 435 nm (B) with identical reaction mixtures.

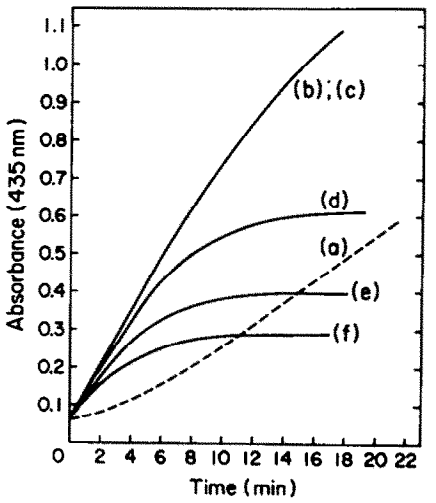

Fig. 6. Effect of H_2O_2 concentration on the time course of Tiron oxidation to a yellow product(s) by mushroom tyrosinase. The reaction mixture included, in a total volume of 3 ml, 6.7 mM Titon, 47 mM sodium phosphate buffer (pH 6.5), 400 μg mushroom tyrosinase (added last) and H_2O_2 (in mM) as follows: a = none; b = 1.6; c = 3.3; d = 6.7; e = 10; f = 13.3.

It can thus be concluded that H_2O_2 (1.6–13.3 mM) eliminates much of the lag period and increases the initial rate of Tiron oxidation of mushroom tyrosinase to the yellow product(s). However, H_2O_2 also suppresses the overall accumulation of yellow product(s), the decrease being due to the bleaching effect of relatively high concentrations of H_2O_2 on the yellow product(s) (data

now shown) and to the fact that these relatively high concentrations of H_2O_2 inactive mushroom tyrosinase [8].

Is H_2O_2 generated during the lag period of Tiron oxidation by mushroom tyrosinase to the yellow product(s)?

The observation that exogenously added H_2O_2 eliminates the lag period of Tiron oxidation to the yellow product(s) (λ_{max} 435 nm) by mushroom tyrosinase (Figs 5,6), prompted us to test the possibility that when Tiron is acted upon by mushroom tyrosinase, H_2O_2 is somehow generated and that the H_2O_2 thus generated is responsible for the faster subsequent rate of Tiron oxidation to the yellow product(s) occurring after the lag period. This possibility was tested by including catalase in a reaction mixture consisting of Tiron, sodium phosphate buffer (pH 6.5) and mushroom tyrosinase. If the above hypothesis were correct, then there should have been only a slower rate of Tiron oxidation to the yellow product(s) in the presence of catalase. However, excess catalase (540 or 1080 μg) had no effect on the rate of oxidation of 6.7 mM Tiron to the yellow product(s) (data not shown), suggesting that any H_2O_2 that might be generated is not important in the reaction.

Effect of ascorbate on Tiron oxidation to yellow product(s) by mushroom tyrosinase

Ascorbate is an effective reductant of *o*-quinone [9]. When included in a reaction mixture in which a naturally occurring *o*-dihydroxyphenol such as DOPA is oxidized by mushroom tyrosinase, an initial lag period in the formation of dopachrome (λ_{max} 475 nm) is observed, since the ascorbate reduced the dopaquinone back to DOPA.

Ascorbate was found to extend the initial lag period of Tiron oxidation to the yellow product(s), with the length of the lag period increasing with increasing ascorbate concentration (tested up to 120 μM). Following the lag period, the rate of formation of the yellow product(s) (ΔOD at 435 nm/min) was linear with time. The rate of Tiron oxidation following the lag period was the same in the presence or absence of relatively low concentrations of ascorbate (tested up to 112 μM).

The above results support the idea that Tiron-*o*-quinone is formed as an intermediate product when mushroom tyrosinase acts on Tiron, and that the Tiron-*o*-quinone formed can be reduced by ascorbate, thereby delaying the conversion of Tiron-*o*-quinone to the stable yellow product(s) (λ_{max} 435 nm).

Effect of hydroxylamine on the spectrum of product(s) obtained when Tiron is oxidized by mushroom tyrosinase

It has been shown recently that addition of hydroxylamine to a reaction mixture consisting of *o*-dihydroxyphenols, sodium phosphate buffer (pH 6.5) and mushroom tyrosinase results in products having an absorbance spectrum different from that obtained in the absence of hydroxylamine [10]. The explanation for the above observation was that *o*-quinones, formed when *o*-dihydroxyphenols are oxidized by mushroom tyrosinase, react with hydroxylamine to form either monooxime or dioxime derivatives [10].

When Tiron was acted upon by mushroom tyrosinase in the presence of hydroxylamine, the product(s) lacked the peak at 435 nm, but instead was characterized by a peak at 385 nm. This occurred, for example, when 6.7 mM Tiron, in the presence of 47 mM sodium phosphate buffer (pH 6.5) and 100 μg mushroom tyrosinase, was incubated with 3.3, 6.7, 10, 13.3, 16.6 or 20 mM hydroxylamine. Figure 7 shows the data obtained with 6.7 mM hydroxylamine. Identical spectra were obtained in the presence of 3.3–20 mM hydroxylamine. These data indicate that Tiron-*o*-quinone is trapped in the form of an oxime, thus preventing the production of the final yellow product(s).

Formation of Tiron-semiquinone during the initial phase of Tiron oxidation by mushroom tyrosinase

Previous investigators detected Tiron-semiquinone when Tiron was oxidized by either O_2^- or $OH^\cdot$ [11–14], The EPR signal of this free radical has a characteristic hyperfine structure which allows its positive identification [7].

It was of interest of determine if Tiron-semiquinone can also be detected during the oxidation of Tiron by mushroom tyrosinase. EPR spectra of a reaction mixture containing, in a total volume of 0.6 ml, 40 mM sodium phosphate buffer (pH 6.5), either 50 μg, 100 μg or 200 μg mushroom tyrosinase, and 133.3 mM Tiron (pH 6.5) (added last), revealed that Tiron-semiquinone was formed immediately upon the initiation of the reaction (Fig. 8) In the presence of 50 μg mushroom tyrosinase, the level of Tiron-semiquinone continued to build up for a few seconds followed by a decrease to a steady state concentration, while in the presence of 100 μg mushroom tyrosinase, the maximum level of Tiron-*o*-semiquinone was detected during the first eight sec of the reaction following an initial 15 sec mixing period. In all cases, the free radical signal declined to a steady state level following the initial rapid increase. This was in contrast to previously reported results on the oxidation of pyrocatechol in the presence of tyrosinase [15], where no steady state

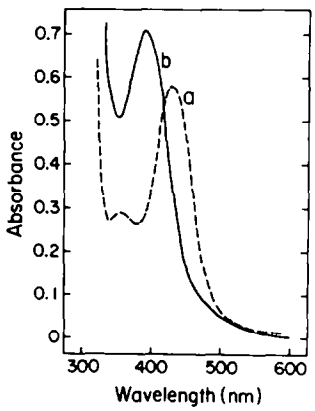

Fig. 7. Effect of hydroxylamine on the spectrum of product(s) formed when Tiron is oxidized by mushroom tyrosinase. The reaction mixture included, in a total volume of 3 ml, 6.7 mM Tiron, 47 mM sodium phosphate buffer (pH 6.5). 100 μg mushroom tyrosinase (added last) and hydroxylamine, as follows: a = none; b = 6.7 mM. The spectrum was scanned 120 min after the initiation of the reaction.

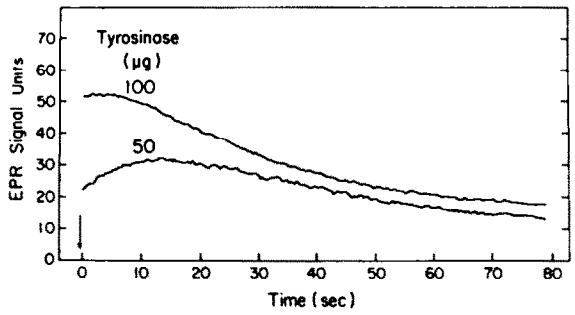

Fig. 8. Time dependence of Tiron-semiquinone EPR signal at two concentrations of mushroom tyrosinase. The reaction mixture included, in a total volume of 0.6 ml, 133.3 mM Tiron (pH 6.0), 40 mM sodium phosphate buffer (pH 6.5) and either 50 or 100 μg mushroom tyrosinase (added last) (indicated by the arrow). EPR signal is expressed in arbitrary units.

level of *o*-benzosemiquinone by ESR spectroscopy was observed.

Due to the accuracy of the magnetic field control and frequency lock systems of the spectrometer, and the fact that the flat sample cell was held rigidly in tuned position during aspiration and mixing of samples, recorded signal intensities were a realible indicator of free radical concentration as a function of time after mixing. The arbitrary EPR scale units used correspond to 8.0 μM spins per 10 units as determined with a 10 μM 4-oxo-2,2,6,6-tetramethyl-piperidine-*N*-oxyl free radical standard under conditions identical to those employed for observation of Tiron-semiquinone signals (see Experimental section). Maximum free radical concentrations in our reaction mixture were 39 ± 6 μM, while steady state levels were near 11 ± 1.2 μM with 100 μg of tyrosinase and 133 mM Tiron (initial concentration).

It was shown above (Figs 4,5) that H_2O_2 accelerates the conversion of the intermediate *o*-quinone (λ_{max} 365 nm) to the final yellow product(s) (λ_{max} 435 nm). Using a fixed concentration of Tiron, the relationship between EPR signal vs different amounts of mushroom tyrosinase and different concentrations of H_2O_2 was therefore studied as a function of time. The kinetic data obtained are summarized in Table 1. These data show that maximum EPR signal intensty and the steady state signal intensity due to Tiron-semiquinone as well as the time required to reach maximum intensity were dependent on the amount of mushroom tyrosinase but not on the presence or absence of 3.3 mM H_2O_2. Plots of steady state semiquinone levels obtained at 60 sec against enzyme concentration were non-linear but data were not sufficient to determine if there was a proportionality with the square root of enzyme concentration. The Tiron-semiquinone signal reached maximum intensity within the indicated times and it then declined to a steady state level approximately one-third that of the maximum level. Rates of decline in EPR signal intensity were also enzyme-dependent, but were accelerated in the presence of H_2O_2.

The accelerated rate of Tiron-semiquinone decay in the presence of H_2O_2, compared with its absence (Table 1), is consistent with our observation (Figs 5, 6) that H_2O_2 accelerates the conversion of the intermediate *o*-quinone compound to the final yellow product(s). Removal of the quinone would be expected to pull the reaction towards the stable yellow product(s). Concomitant with the EPR studies (Fig. 8), kinetic measurements were made with a recording spectrophotometer at 340 nm, 365 nm and 435 nm, since it was already found (Fig. 3) that intermediate compounds are formed during the early phase of Tiron oxidation by mushroom tyrosinase.

The data in Fig. 9 show that during the initial, rapid phase of Tiron oxidation by mushroom tyrosinase there is a close correspondence between the rate of formation of Tiron-semiquinone (EPR) and the initial, rapid change (< 1 min) in absorbance at 365 nm. A similar correlation was also observed at 340 nm, although background absorbance was higher at this wavelength (data not shown). This initial change correlates well with EPR signal intensity. The somewhat slower appearance of the putative *o*-quinone intermediate (λ_{max} 365 nm) does not correlate with an increase in EPR signal intensity but occurs *after* the EPR signal has reached a steady state level. After 20 sec of reaction, the correspondence between absorbance at 365 nm and EPR signal intensity became less pronounced, primarily because the Tiron-semiquinone free radical is the only free radical signal source in the reaction mixture, whereas changes in absorbance near 365 nm due to formation of semiquinone, *o*-quinone and polymer overlap in this region. It was noted that the initial absorbance at 365 nm increased to a temporary maximum level at the same time as the EPR signal was at a maximum. Between 25 and 60 sec of reaction time, the absorbance at 365 nm actually declined slightly before increasing due to quinone accumulation. Increase in absorbance at 435 nm began subsequent to the

Table 1. Effect of mushroom tyrosinase and of H_2O_2 on the formation and decay of Tiron-semiquinone (EPR signal)

Mushroom tyrosinase (μg)	H_2O_2 (mM)	Time to max. EPR signal (sec)	Height of EPR signal (units)	Initial rate of decay of EPR signal (units/10 sec)
50	—	14	34.0	4.0
50	3.3	14	42.5	7.1
100	—	7	53.0	9.0
100	3.3	1.5	64.5	13.0

The reaction mixture contained, in a total volume of 0.6 ml, 133 mM Tiron (pH 6.0), 40 mM sodium phosphate buffer (pH 6.5), mushroom tyrosinase and H_2O_2 as indicated. EPR signal strength is expressed in arbitrary units. Spectrometer gain = 1×10^4, modulation amplitude = 0.63 gauss. Time measured subsequent to mixing time (15 sec).

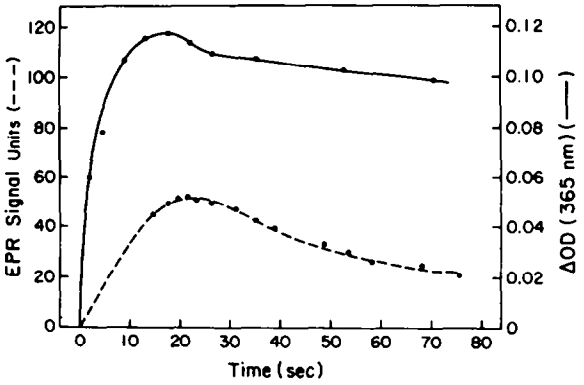

Fig. 9. Comparison between the time course of EPR signal strength and rapid absorbance change at 365 nm during the oxidation of Tiron by mushroom tyrosinase. The reaction mixture for EPR measurements included, in a total volume of 0.6 ml, 40 mM sodium phosphate buffer (pH 6.5), 50 μg mushroom tyrosinase and 133.3 mM Tiron (pH 6.0) (added last). The reaction mixture for spectrophotometric measurements included, in a total volume of 3 ml, 40 mM sodium phosphate buffer (pH 6.5), 200 μg mushroom tyrosinase and 133.3 mM Tiron (pH 6.0) (added last). The EPR signal is expresed in arbitrary units.

initial lag phase and continued linearly for at least 10–12 min.

As pointed out above, several previous studies were carried out on the interaction between Tiron and O_2^-, as well as on that between Tiron and $OH^·$ [11–14]. In these studies, both EPR spectroscopy and kinetic near-UV spectroscopy were employed. Greenstock and Miller [11] demonstrated the appearance of the Tiron-semiquinone. Concomitantly, they detected some build up of absorbance at 400 nm and attributed it to Tiron-semiquinone free radical (TH) [11]. The absorbance at 400 nm reported in ref. [11] is more likely to be due to a yellow stable oxidation product(s) similar to that found in the present work.

Using the method of pulse radiolysis and kinetic spectroscopy, Bors *et al.* [14] compared the products formed when Tiron interacts with O_2^- with those formed on reaction with $OH^·$. When Tiron interacted with $OH^·$, they detected an absorbance shoulder near 340 nm which was attributed to a Tiron–$OH^·$ adduct. They suggested that elimination of water from the Tiron–$OH^·$ adduct resulted in the appearance of Tiron-semiquinone absorbance maxima at 240 nm and at 295–300 nm. The Tiron-semiquinone, although relatively stable compared with other semiquinones, was reported to be converted to Tiron-*o*-quinone, which was characterized by an absorbance maximum at 260 nm and shoulders near 354 nm [14]. When Tiron interacted with O_2^-, Tiron-semiquinone was detected, which was characterized by absorbance maxima at 240–250 nm and at 290–300 nm. The appearance of a weak absorption at 440 nm was attributed to an unidentified photoproduct, but no experimental findings were presented to verify this assumption. The small absorbance maximum at 400 nm detected when Tiron interacts with O_2^- in ref. [11], was not observed in ref. [14].

It is likely that the slower absorbance increase that we detected in the near-UV region is not due to Tiron-semiquinone accumulation, in spite of the good correlation between rapid initial changes in EPR signal intensity and initial changes in absorbance at 365 nm during the early phase of Tiron oxidation by mushroom tyrosinase (Fig. 9). The Tiron-semiquinone is apparently converted very rapidly to Tiron-*o*-quinone and related oxidation products that have an absorbance peak at 365 nm. Absorbance in this region due to the semiquinone alone would be expected to be weak, since the absorbance maximum of the latter should occur in the UV region near 280 nm [14], which was unavoidably obscured in our experiments by protein absorbance.

The observation of steady state levels of Tiron-semiquinone and the observed non-linear dependence of maximum and steady state level EPR signal strength on tyrosinase concentration appear to represent a significant difference in the tyrosinase-catalysed oxidation of Tiron as compared to that of tyrosinase-catalysed oxidation of pyrocatechol, reported in ref. [15]. The semiquinone form most probably acts as an intermediate in Tiron oxidation by tyrosinase especially in view of the relatively slow appearance of absorbance due to *o*-quinone and polymeric reaction products during Tiron oxidation by the enzyme. The rate of oxidation of Tiron is slower than that of pyrocatechol [15] allowing better time resolution of the process.

As will be reported separately, the final yellow product(s) formed when Tiron is oxidized by mushroom tyrosinase is mainly a low M_r polymer of Tiron-*o*-quinone The latter can be formed as follows: two molecules of Tiron-*o*-semiquinone may disproportionate to form Tiron-*o*-quinone plus Tiron. Tiron-*o*-quinone may further react and polymerize to give a higher M_r, oxidized yellow product(s). The participation of charge transfer complexes between substrate and product intermediates in Tiron oxidation cannot be ruled out. The presence of long wavelength absorbing products in 20 hr incubation mixtures suggests that such polymeric complexes may be stabilized.

EXPERIMENTAL

Materials. Mushroom tyrosinase (grade III), catalase (C-100) from bovine liver, Tiron, ascorbate and NH_2OH were obtained from Sigma Chemical Co.; H_2O_2 was from Merck Chemical Co. Spectrophotometric measurements of the slower changes in absorbance at 350–650 nm were carried out at 24° using a Varian DMS 90 spectrophotometer equipped with a recorder.

For measurement of free radical concentrations, mushroom tyrosinase (50–400 μg) was mixed with Tiron (133 μm) in a final vol. of 0.6 ml 40 mM NaPi buffer (pH 6.5). The reaction mixture was immediately aspirated into a flat quartz sample cell fixed in the EPR spectrometer cavity. The spectrometer was pretuned and the magnetic field preset to a value corresponding to the positive resonance peak position of the low field signal of Tiron semiquinone [7]. Mixing time was estimated to be 10–15 sec. Spin concentrations were estimated by comparison of EPR signal magnitude with that of known concentrations of 4-oxo-2,2,6,6-tetramethyl-piperidine-*N*-oxyl in NaPi buffer (pH 6.5) in the same flat sample cell. A Bruker 200 spectrometer was used for these determinations. Modulation amplitude was 0.63 gauss and microwave power was 20 mW. Reaction temperature was 24°.

Parallel experiments were carried out in a Beckman Du-7 spectrophotometer. Tiron held on a plastic plunger was mixed with the other reaction mixture components for 2–3 sec and the absorbance of the products was recorded as a function of time at

340, 365 or 435 nm. H_2O_2 concentration was determined spectrophotometrically at 240 nm using E_m (240 nm) of $43.6\,M^{-1}\,cm^{-1}$.

o-Dihydroxyphenolase activity of mushroom tyrosinase with either DL-DOPA or 4-methyl catechol was measured in a total vol. of 3 ml containing different concentrations of either substrate, 47 mM NaPi buffer (pH 6.5) and 100 μg enzyme (added last). The rate of oxidation of DL-DOPA was followed at 475 nm and of 4-methyl catechol at 410 nm. *o*-Dihydroxyphenolase activity for either substrate was estimated from the initial linear portions of the absorbance vs time curves obtained and the K_m was estimated from a Lineveaver–Burke plot as described by Webb [3].

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