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TIRON AS A SUBSTRATE FOR HORSERADISH PEROXIDASE*

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Key Word Index—Horseradish peroxidase; Tiron; yellow product.

Abstract—Tiron (4,5-dihydroxy-1,3-benzene disulphonic acid), in the presence of hydrogen peroxide, can serve as a hydrogen donor for horseradish peroxidase. The product(s) formed is yellow and is characterized by a peak at 435 nm.

The relationship between, and effect of, various concentrations of horseradish peroxidase, hydrogen peroxide and Tiron on the rate of oxidation of Tiron to the yellow product(s) are described.

Partial purification of the yellow product(s) was achieved by chromatography on Sephadex G-10, Sephadex G-25 and Sephadex G-50. The data suggest that the yellow product(s) formed is a low $-M_r$ polymer of Tiron-*o*-quinone.

INTRODUCTION

Peroxidase catalyses reactions in which a variety of hydrogen donors are oxidized in the presence of hydrogen peroxide [1–10]. Among the known hydrogen donors for this reaction are: guaiacol, benzidine, *o*-dianisidine, aromatic amines [2–5], monohydroxy-phenols such as tyrosine [6, 7], and dihydroxyphenols such as caffeic acid, ferulic acid, DOPA and catechol [8–10]. Peroxidase, in the absence of hydrogen peroxide but in the presence of oxygen, can also catalyse oxidatic and hydroxylation reactions [1].

Tiron is an *o*-dihydroxyphenol known as a scavenger of O_2^- [11], as well as of $OH^\cdot$ [12, 13]. We have shown recently that Tiron is also a substrate for mushroom tyrosinase [14]. Since *o*-dihydroxyphenols can serve as substrates for tyrosinase as well as for peroxidase (in the presence of hydrogen peroxide), it was of interest to determine if Tiron could also serve as a substrate for peroxidase.

The data presented in this paper provide evidence that Tiron, in the presence of hydrogen peroxide, can serve as a hydrogen donor for horseradish peroxidase (HRP).

RESULTS AND DISCUSSION

A solution of Tiron in water is colourless. Its absorbance spectrum is characterized by a high peak at 207–210 nm, a shoulder at 230 nm and a low peak at 285–290 nm. When HRP acts on Tiron in the presence of hydrogen peroxide, a yellow product(s) is formed at once. When Tiron and HRP are incubated in the absence of hydrogen peroxide, or when Tiron and hydrogen peroxide are incubated in the absence of HRP, very little yellow colour is formed. The visible spectrum of a reaction mixture containing 13.3 mM Tiron, 47 mM sodium phosphate buffer (pH 6.5), 3.3 mM hydrogen peroxide and 200 μg HRP is shown in Fig. 1. The yellow product(s) formed has a maximum absorbance at *ca* 425 nm after 1 min of incubation. This peak shifts to 430 nm and 435 nm after 4 and 10 min, respectively and remains at 435 nm thereafter.

Effect of different amounts of HRP on the rate of Tiron oxidation to the yellow product(s)

The initial rate of Tiron oxidation to the yellow product(s) in the presence of a fixed concentration of hydrogen peroxide is linearly related to HRP concentration in the range tested, as shown in the inset of Fig. 2. The absorbance at 435 nm attained after the reaction rate has reached a plateau is also directly related to HRP concentration (Fig. 2).

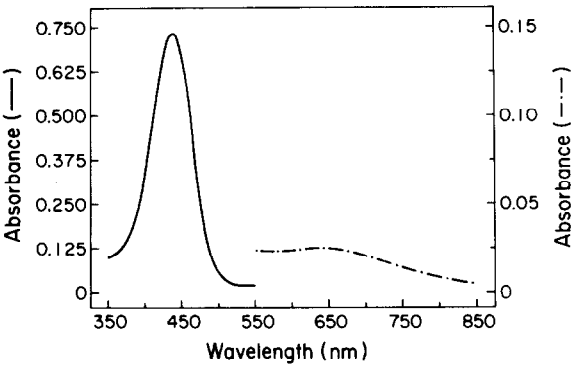

Fig. 1. Visible spectrum of a stable product(s) formed when Tiron is oxidized by the action of the HRP–H_2O_2 system. The reaction mixture included, in a total volume of 3 ml; 13.3 mM Tiron, 47 mM sodium phosphate buffer (pH 6.5), 3.3 mM H_2O_2 and 200 μg HRP (added last). The sample was diluted with water for scanning. The visible spectrum was scanned 20 hr after initiation of the reaction.

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Abbreviations: Tiron, 4,5-dihydroxy-1,3-benzene disulphonic acid; HRP, horseradish peroxidase (donor: hydrogen-peroxide oxidoreductase) (EC 1.11.1.7); tyrosinase, monophenol monooxygenase, phenolase (EC 1.10.3.1).

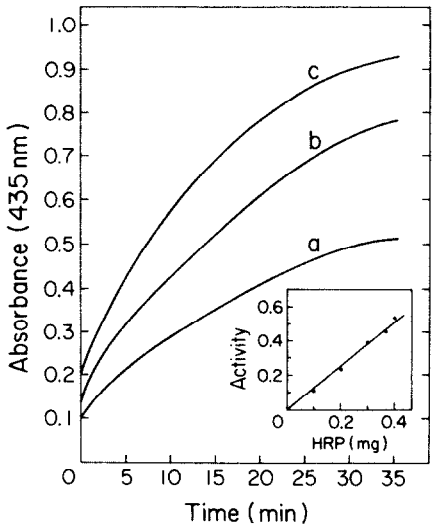

Fig. 2. Effect of different amounts of HRP on the rate of Tiron oxidation to the yellow product(s). The reaction mixture included, in a total volume of 3 ml: 6.7 mM Tiron, 47 mM sodium phosphate buffer (pH 6.5), 3.3 mM H_2O_2, and HRP (added last) as indicated. The inset shows peroxidase activity ($\Delta OD_{435\ nm}$/10 min) as computed from the initial portions of the kinetic plots obtained with various amounts of HRP (only some of the kinetic data are shown).

Effect of different concentrations of hydrogen peroxide on the rate of Tiron oxidation by HRP to the yellow product(s)

The effect of different concentrations of hydrogen peroxide on the rate of oxidation of 266 mM Tiron to the yellow product(s) by a fixed amount of HRP (20 μg) is shown in Fig. 3. There appears to be an optimal concentration of hydrogen peroxide which gives the largest increase in absorbance at 435 nm for a given HRP concentration. Increasing concentrations of Tiron in the range of up to 133 mM require increasing concentrations of hydrogen peroxide for maximal production of the 435 nm-absorbing product. Thus, for example, maximum product(s) formation from 33 mM Tiron occurs at 3 mM hydrogen peroxide, while maximum product(s) formation from 133 mM Tiron occurs at 18 mM hydrogen peroxide. Beyond 133 mM Tiron (tested up to 266 mM), the optimum concentration of hydrogen peroxide remains around 18 mM.

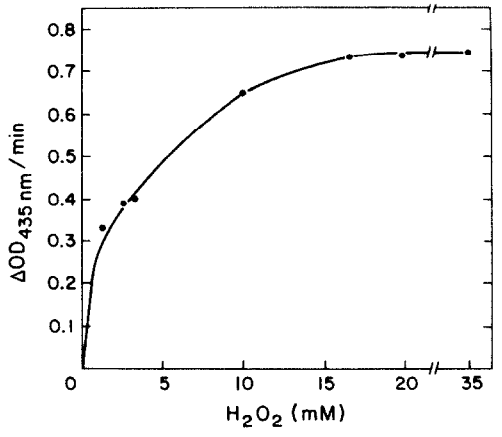

Fig. 3. Effect of different concentrations of H_2O_2 on the rate of Tiron oxidation by HRP to the yellow product(s). The reaction mixture included, in a total volume of 3 ml: 266 mM Tiron, 47 mM sodium phosphate buffer (pH 6.5), H_2O_2 as indicated, and 20 μg HRP (added last). The rate of Tiron oxidation was recorded at 435 nm and Δ_{OD} 435 nm/min (activity) was computed from the initial portions of the kinetic plots obtained (data not shown).

Hydrogen peroxide has been reported to inactivate HRP [15], and, as shown in Fig. 3, concentrations of hydrogen peroxide above 18 mM apparently inactivate the enzyme, as evidenced by no further increase in absorbance. It was also found that the yellow product(s) is bleached in the presence of high concentrations of hydrogen peroxide (data not shown).

Effect of Tiron concentration on the rate of its oxidation to the yellow product(s)

The data of Fig. 4 show that in the presence of 16.6 mM hydrogen peroxide (shown to be optimal for observing HRP action), 20 mg HRP and 47 mM sodium phosphate buffer (pH 6.5), the rate of Tiron oxidation ($\Delta OD_{435\ nm}$/min) varies non-linearly with Tiron concentration, with apparent saturation being reached between 400 and 500 mM Tiron. At Tiron concentrations above 560 mM, there is a decrease in the rate of Tiron oxidation (tested up to 700 mM Tiron), probably due to substrate inhibition. Because of the phenomenon of substrate inhibition and the observed non-hyperbolic saturation phenomenon, it is impossible to determine accurately the K_m of Tiron for HRP, but a value in the region of 200 mM can be estimated.

Partial characterization of the yellow product(s) obtained by the HRP–H_2O_2 system of Tiron

Of the several substrates that serve as hydrogen donors (AH_2) for peroxidase some have been shown to be converted to *o*-quinone [4, 8], while others polymerize to dimers, trimers and tetramers [1, 6, 7, 16–19].

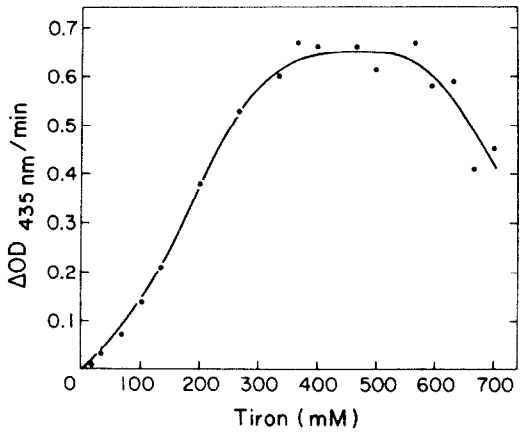

Fig. 4. The rate of oxidation of different concentrations of Tiron to the yellow product(s) by the HRP–H_2O_2 system. The reaction mixture included, in a total volume of 3 ml: Tiron, as indicated, 47 mM sodium phosphate buffer (pH 6.5), 16.6 mM H_2O_2 and 20 μg HRP (added last).

Partial purification of the yellow product(s) formed when Tiron is oxidized by the HRP–H_2O_2 system, and an estimate of its M_r were achieved by column chromatograph, as described below.

Chromatography on Sephadex G-25 column (2.5 × 60 *cm*). A reaction mixture of 3 ml, containing 6.7 mM Tiron, 47 mM sodium phosphate buffer (pH 6.5), 0.66 mM hydrogen peroxide and 20 μg HRP, was incubated at 24° for 24 hr. Two ml of the sample was then chromatographed on a column of Sephadex G-25 (2.5 × 60 cm) and the OD 280 nm and OD 435 nm of each fraction was measured (Fig. 5). The void volume of the column (V_o) was *ca* 128 ml (fraction 85). Immediately after elution, a yellow product(s) was detected in fractions 105–150 only (regions A and B), but 20 hr after elution the OD of the yellow product(s) in fractions 140–150 increased and an additional yellow product(s) appeared in fractions 156–170, with a flat peak around fraction 160 (region D, Fig. 5). The OD 280 nm material eluted late in the run and centred around fraction 165–170 was identified as Tiron.

As shown in Fig. 5, the OD's at 280 nm and 435 nm of fractions in region A are more or less parallel, but this is not the case in region D of the elution profile 20 hr after elution. The yellow product(s) in region D (OD 435 nm) which appears 20 hr after elution overlapped, in part, the region where Tiron (280 nm) is eluted.

The visible spectra of various yellow fractions eluted off the Sephadex G-25 column are characterized by a peak at 430–435 nm.

Chromatography on Sephadex G-50 column (2.5 × 60 *cm*) *followed by chromatography on a long Sephadex G-10 column* (2.5 × 150 *cm*). In order to get rid of HRP and in an attempt to separate better the yellow product(s) from untreated Tiron, a reaction mixture similar to that used in Fig. 5 was lyophilized and first chromatographed on Sephadex G-50 column (2.5 × 60 cm). Under these conditions (data not shown) HRP was eluted at V_o (135 ml) (fractions 40–45), while the yellow product(s) plus untreated Tiron were eluted at 280–360 ml (fractions 65–95). The latter fractions were pooled, lyophilized and then chromatographed on a long Sephadex G-10 column (2.5 × 150 cm) (Fig. 6) to separate the yellow product(s) from untreated Tiron. As seen in Fig. 6, under these conditions the mixture of yellow product(s) and untreated Tiron was well resolved. The yellow product(s) was eluted at the V_0 of the column (region A) and adjacent to it (region B), while untreated Tiron was eluted much later in the run in region D. Several of the latter fractions turned yellow 20 hr after elution (region D) (see below).

The visible spectrum of the yellow fractions (95–115) eluted off the long Sephadex G-10 column under the latter conditions (Fig. 6) is characterized by a peak at 430–435 nm in the visible range and can be taken to represent the purified yellow product(s).

We have shown recently that Tiron can be acted upon by mushroom tyrosinase and that a yellow product(s) is formed during such a reaction [14]. Our studies of the characterization of the product(s) formed when Tiron is oxidized by mushroom tyrosinase under chromatographic conditions identical to those described in Fig. 6 lead us to suggest that a relatively low $-M_r$ polymer of Tiron-*o*-quinone (yellow) is eluted in region A, a tetrameric Tiron-*o*-quinone (yellow) in region B, and a dimeric Tiron-*o*-quinone (yellow) in region C (V. Kahn, unpublished work).

As shown above, chromatography on Sephadex columns of product(s) formed when Tiron is acted upon by the HRP–H_2O_2 system gives fractions that are colourless when eluted off Sephadex G-25 (Fig. 5) or Sephadex G-10 (Fig. 6) in region D (where untreated Tiron was eluted), but which become yellow a few hours after elution. These must have contain some monomeric-Tiron-*o*-quinones (colourless) which slowly polymerizes to yield the yellow low $-M_r$ polymerized Tiron-*o*-quinone. We have shown recently [14] that during the initial

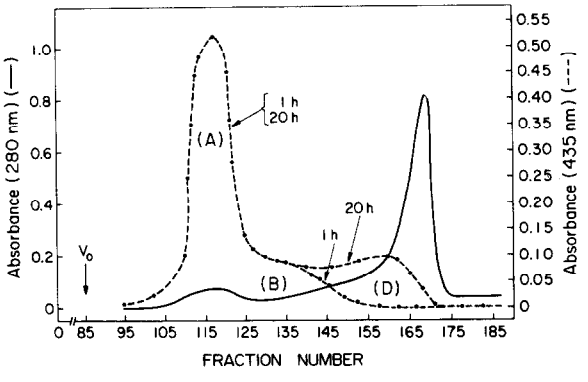

Fig. 5. Chromatography of the yellow product(s) on a Sephadex G-25 column (2.5 × 60 cm). A reaction mixture of 3 ml consisting of 6.7 mM Tiron, 47 mM sodium phosphate buffer (pH 6.5), 20 μg HRP and 0.66 mM H_2O_2 was incubated at 24° for 24 hr. Two-ml aliquots of the yellow reaction mixture obtained were applied to a column of Sephadex G-25 (2.5 × 60 cm) and the column was eluted with water. Fractions of 1.5 ml were collected and the OD at 280 nm was recorded. The absorbance at 435 nm of each fraction was measured 1 hr and 20 hr after elution. HRP was eluted at the V_o of the column.

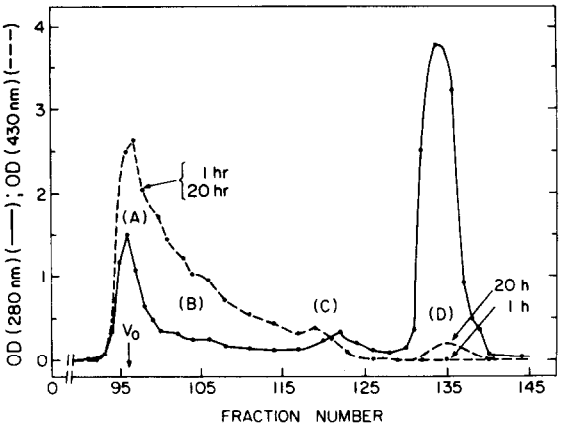

Fig. 6. Chromatography of the yellow product(s) on a Sephadex G-10 column (2.5 × 150 cm). A reaction mixture of 12 ml consisting of 6.7 mM Tiron, 47 mM sodium phosphate buffer (pH 6.5), 100 μg HRP and 0.06 mM H_2O_2 was incubated for 20 hr at 24°, lyophylized and a portion of it applied to a Sephadex G-10 column (2.5 × 150 cm). The column was eluted with water and fractions of 3.2 ml were collected. The OD at 430 nm was measured 1 hr and 20 hr after elution.

phase of Tiron oxidation by mushroom tyrosinase, an intermediate compound, probably Tiron-*o*-quinone, having a significant absorbance at 365 nm is detected and that hydrogen peroxide, at relatively low concentrations, accelerates the conversion of the intermediate compound to the final yellow products(s) (λ_{max} 435 nm).

It is thus reasonable to assume that hydrogen peroxide accelerates the conversion of Tiron-*o*-quinone to the low $-M_r$ -polymerized-Trion (probably directly and not via dimer-Tiron-*o*-quinone or tetramer-Trion-*o*-quinone). This would account for the observation that a relatively high amount of yellow product(s) is eluted in region A of the Sephadex G-10 column when Tiron is oxidized by the HRP–H_2O_2 systems (Fig. 6), while a relatively high amount of yellow product(s) is eluted in region C when Tiron is oxidized by mushroom tyrosinase. (V. Kahn, unpublished work).

EXPERIMENTAL

Peroxidase activity. The reaction mixture included, in a total vol of 3 ml, Tiron, NaPi buffer (pH 6.5), H_2O_2 and HRP (added last), as described in detail in the legends to the figures. The OD at 435 nm or at 440 nm was recorded as a function of time, and the activity ($\Delta OD_{435\,nm}$/min or 440 nm/min) was estimated from the initial linear portion of the curves obtained.

Chromatography was carried out as described in the legends to Figs 5 and 6. Fractions were collected in an ISCO No. 328 fraction collector attached to a UA-5 monitor and the absorbance at 280 nm was recorded. The absorbance at 435 nm was read separately immediately after elution and several hours afterwards.

H_2O_2 *concentration* was estimated at 240 nm using E_M (240 nm) of 43.6 $M^{-1}\,cm^{-1}$. *Materials.* Horseradish peroxidase (Type VI) and Tiron were obtained from Sigma, H_2O_2 from Merck, and Sephadex from Pharmacia. All other chemicals were reagent grade.

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REFERENCES

1. Whitaker, J. R. (1972) in *Principles of Enzymology for the Food Sciences*, pp. 591–605. Marcel Dekker, New York.
2. Josephy, P. D., Eling, T. and Mason, R. O. (1982) *J. Biol. Chem.* **257**, 3669.
3. Herzog, V. and Fahimi, H. D. (1973) *Anal. Biochem.* **55**, 554.
4. Danner, D. J., Brignac, P. J. Jr., Arceneux, D. and Patel, V. (1973) *Arch. Biochem. Biophys.* **156**, 759.
5. Sawahat, T. and Neal, R. A. (1982) *Biochem. Biophys. Res. Commun.* **109**, 988.
6. Gross, A. and Sizer, I. (1959) *J. Biol. Chem.* **234**, 1611.
7. Bays, G. S., Michaels, A. W. and Morrison, M. (1972) *Biochim. Biophys. Acta* **284**, 34.
8. Booth, H. and Saunders, B. J. (1956) *J. Chem. Soc.* 940.
9. Smith, P. E. and Swan, G. A. (1976) *Biochem. J.* **153**, 403.
10. Gibson, D. M. and Liu, E. H. (1978) *Ann. Bot.* **42**, 1075.
11. Greenstock, C. L. and Miller, R. W. (1975) *Biochim. Biophys. Acta* **396**, 11.
12. Bors, W., Saran, M. and Michel, C. (1979) *Biochim. Biophys. Acta* **582**, 537.
13. Van Ginkel, G. and Raison, J. K. (1980) *Phytochem. Phytobiol.* **32**, 793.
14. Kahn, V. and Miller, R. W. (1987) *Phytochemistry* **26**, 2459.
15. Weinryb, I. (1959) *Biochemistry* **5**, 2003.
16. Sawada, Y., Iyanagi, T. and Yamazaki, I. (1975) *Biochemistry* **14**, 3761.
17. Makinen, K. K. and Tenovou, J. (1982) *Anal. Biochem.* **126**, 100.
18. Papadimitriu, J. M., Van Duijn, P., Bredero, P. and Streefkert, J. G. (1976) *J. Histochem. Cytochem.* **24**, 82.
19. Hirsch, H. E. and Parks, M. E. (1982) *Anal. Biochem.* **122**, 79.