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EFFECT OF TIRON ON MONOHYDROXY AND *o*-DIHYDROXYPHENOLASE ACTIVITY OF MUSHROOM TYROSINASE

VARDA KAHN and A. ANDRAWIS

Department of Food Science, Agricultural Research Organization, The Volcani Center, Bet Dagan, Israel

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Key Word Index—Mushroom; tyrosinase; Tiron.

Abstract—Tiron has a multiple effect on mushroom tyrosinase. At relatively low concentrations (up to 3.3 mM), Tiron extended the lag period of tyrosine hydroxylation appreciably, while at concentrations between 3.3 and 8.3 mM the lag period was shortened and approached that of the control. At concentrations above 10 mM, Tiron shortened the lag period of tyrosine hydroxylation compared with that of the control.

Tiron, at relatively high concentrations (above 266 mM), inhibited the initial rate of DL-DOPA oxidation by mushroom tyrosinase and lowered the final level of dopachrome formed. Preincubation of mushroom tyrosinase with Tiron resulted in the inactivation of the enzyme, with 50% inactivation of 650 μg enzyme occurring in the presence of 400 mM Tiron.

INTRODUCTION

Tiron is known as an efficient scavenger on O_2^- [1, 2]. In some cases, Tiron is a better scavenger of O_2^- than SOD since, due to its much smaller M_r, Tiron is more easily accessible to O_2^- produced at an active site of an enzyme [3]. Recent experiments [4, 5] have demonstrated that Tiron is not only a scavenger of O_2^- but also of $OH^\cdot$ and that with either O_2^- or $OH^\cdot$, Tiron is oxidized to a semiquinone.

We recently studied the possible participation of O_2^- in the hydroxylation of tyrosine by mushroom tyrosinase but found that SOD, as well as low M_r scavengers of O_2^- (such as copper salicylate and nitro blue tetrazolium), had no direct effect on the hydroxylation reaction [6]. The effect of Tiron on the hydroxylation of tyrosine by mushroom tyrosinase was also studied and since it was found that Tiron has a multiple effect on the hydroxylation reaction, as well as an effect on the *o*-dihydroxyphenolase activity of mushroom tyrosinase, we decided to present the data in some detail, in this paper.

While studying the effect of Tiron on the monohydroxy and *o*-dihydroxy phenolase activity of mushroom tyrosinase, we found that relatively large amounts of mushroom tyrosinase can oxidize Tiron to a yellow product(s) characterized by a major peak at 435 nm and a minor peak at 650 nm. These data will be presented in detail in a separate paper [7].

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Abbreviations—AH_2, reductant; O_2^-, superoxide ion; $OH^\cdot$, hydroxyl radical; Tiron = 4,5-dihydroxy-1,3-benzene disulphonic acid; tyrosinase, monophenol monooxygenase, phenolase (EC 1.10.3.1).

RESULTS AND DISCUSSION

Effects of Tiron on the rate of tyrosine hydroxylation by mushroom tyrosinase

The hydroxylation of monohydroxyphenols by tyrosinase is carried out as follows:

$$2 \text{ monohydroxyphenol} + O_2 + AH_2 \rightarrow$$
$$2 \text{ } o\text{-dihydroxyphenol} + H_2O + A$$

where AH_2 represents a reductant. When exogenous AH_2 is not added, the hydroxylation reaction is characterized by a lag period. The lag period is overcome when enough *o*-dihydroxyphenol is somehow formed in the course of the reaction [8]. Reductants, such as ascorbate, hydroxylamine and hydroquinone, when added exogenously, can also shorten the lag period but relatively less effectively than o-dihydroxyphenols [9]. Tyrosinase is pictured as having different loci within the active site; one for the substrate (*o*-dihydroxyphenol), and another for the reductant (AH_2) (*o*-dihydroxyphenol or exogenously added AH_2, such as ascorbate [10].

The effect of Tiron on the rate of tyrosine hydroxylation was studied and some of the kinetic data (*A* 475 nm vs time) obtained are illustrated in Fig. 1A. At certain concentrations, Tiron affected the lag period of tyrosine hydroxylation, but had little effect on the rate of hydroxylation following the lag period. As the concentration of Tiron increased to 3.3 mM, the lag period of tyrosine hydroxylation was lengthened by approximately 280%. At a Tiron concentration in the range from 3.3 to 8.3 mM, the lag period was shortened and approached that of the control (without Tiron). Increasing Tiron beyond 10 mM shortened the lag period of tyrosine hydroxylation considerably, compared with the control, so that at 33.3 mM Tiron, the lag period was only 30% that of the control.

Control showed that incubation of various concentrations of Tiron (in the range of up to 35 mM) and 33 μg

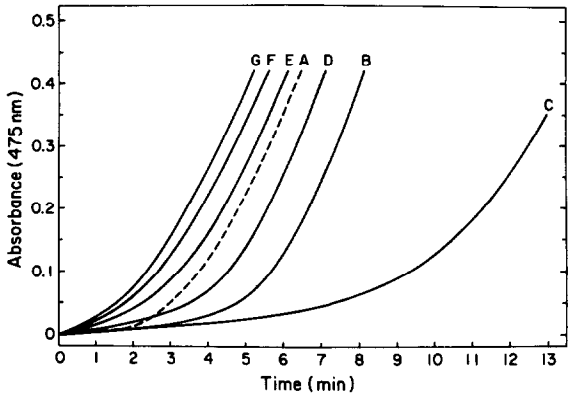

Fig. 1A. Effect of Tiron on the rate of tyrosine hydroxylation by mushroom tyrosinase. The reaction mixture included in a total volume of 3 ml, 3.3 mM L-tyrosine, 47 mM sodium phosphate buffer (pH 6.5), 100 μg mushroom tyrosinase (added last) and Tiron (in mM), as follows: A, none; B, 1.6; C, 3.3; D, 8.3; E, 16.6; F, 26.6; G, 33.3.

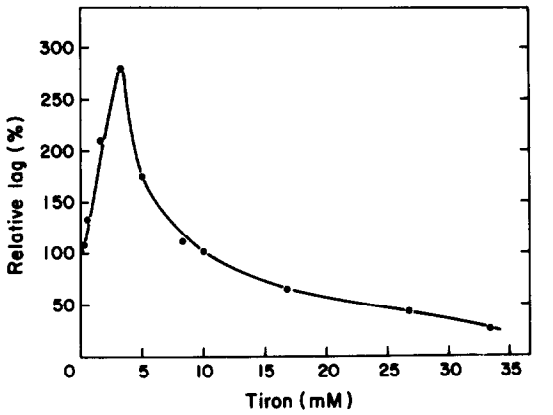

Fig. 1B. Summary of the effect of Tiron on the lag period of tyrosine hydroxylation by mushroom tyrosinase. The lag periods of tyrosine hydroxylation were computed from the curves presented in Fig. 1A and plotted as a function of Tiron concentration.

mushroom tyrosinase produced a negligible increase in A at 475 nm. The multiple effect of Tiron on the lag period of tyrosine hydroxylation can be seen clearly in Fig. 1B, which is a summary of the kinetic data presented in Fig. 1A.

It is important to note that in different experiments there was some variability in the extent to which a low concentration of Tiron lengthened the lag period of tyrosine hydroxylation; we do not have an explanation for such variabilities.

The results demonstrating that certain low concentrations of Tiron lengthen the lag period of tyrosine hydroxylation but not the rate of subsequent dopachrome formation (Fig. 1A), imply at first sight the requirements for O_2^- and/or $OH^\cdot$ for the hydroxylation reaction since, as pointed out above, Tiron is an efficient scavenger of these two radicals [5]. However, we have recently reported that O_2^- does not participate directly in the hydroxylation of tyrosine by mushroom tyrosinase [6]. Moreover, we have also demonstrated that $OH^\cdot$ scavengers, such as histidine (at 6.6, 20 and 30 mM), ethanol (at 3.2, 6.4 and 32 mM) and mannitol (at 32 and 160 mM), have no effect on the lag period of tyrosine hydroxylation [6]. Also, if O_2^- or $OH^\cdot$ were, indeed, required for the hydroxylation of tyrosine by mushroom tyrosinase, then it is difficult to explain the observation that high concentrations of Tiron no longer extend the lag period but rather shorten it (Figs 1A and 1B). In view of the above rationale against the implication that O_2^- and/or $OH^\cdot$ are required for tyrosine hydroxylation by mushroom tyrosinase, we would like to offer another explanation for the data presented in Figs 1A and 1B.

The dual effect of Tiron on tyrosine hydroxylation by mushroom tyrosinase could, perhaps, be explained as follows: at high concentrations, but not at low concentrations, Tiron can effectively reduce dopaquinone nonenzymatically to DOPA. The thus formed DOPA would then shorten the lag period as an effective AH_2, rather than Tiron itself.

o-Quinones can be reduced to *o*-dihydroxyphenols by reductants such as ascorbic acid [8]. The ability of a compound to reduce an *o*-quinone can best be tested in a reaction mixture that contains low concentrations of DL-DOPA, sodium phosphate buffer (pH 6.5) and mushroom tyrosinase. In the absence of a reductant, the oxidation of DOPA to dopachrome (A 475 nm) via dopaquinone proceeds very fast and in a linear fashion during the first 60–120 sec of the reaction. On the other hand, in the presence of a reductant of *o*-quinone, there is an initial lag period in dopachrome formation. The lag period is overcome when the reductant is exhausted due to its being oxidized when it reduces the *o*-quinone.

A comparison between the ability of Tiron vs ascorbate to reduce dopaquinone was tested and the results are shown in Table 1. A lag period in dopachrome formation was not observed when 33.0 or 333 mM Tiron was added to a reaction mixture containing 0.6–3.0 mM DL-DOPA and 8 μg mushroom tyrosinase. On the other hand, in the presence of 1.2 mM DL-DOPA and 4 μg mushroom tyrosinase, a lag period in dopachrome formation was detected in the presence of 18 μM ascorbate (Table I).

Based on the data presented in Table 1, it can be concluded that Tiron, even at high concentrations, does not reduce dopaquinone and therefore the above suggestion that Tiron reduces dopaquinone cannot explain the finding that Tiron shortens the lag period of tyrosine hydroxylation. An alternative explanation for the dual effect of Tiron on the lag period of tyrosine hydroxylation summarized in Fig. 1B can be as follows: Tiron is an *o*-dihydroxyphenol and, as shown in a separate paper [7], can serve as a substrate for mushroom tyrosinase but, relative to naturally occurring *o*-dihydroxyphenols, Tiron has a very high K_m for the enzyme.

Tiron, at low concentrations, can prevent the access to the enzyme of the few molecules of DOPA endogenously formed during tyrosine hydroxylation, that are required as AH_2 for hydroxylation reaction [11]. Thus, low concentrations of Tiron would extend the lag period of tyrosine hydroxylation. Tiron itself must be pictured as being a relatively weak AH_2 compared with DOPA, thus being incapable of shortening the lag period efficiently when present at low concentrations. However, at higher

Table 1. Comparison between the ability of ascorbate and of Tiron to reduce dopaquinone

DOPA (mM)	Mushroom tyrosinase (μg)	Tiron (mM)	Ascorbate (mM)	Lag period (sec)
1.2	4	—	—	none
1.2	4	—	0.003	none
1.2	4	—	0.018	60
1.2	4	—	0.030	120
1.2	4	—	0.300	360
0.6	8	—	—	none
0.6	8	333.0	—	none
1.2	8	—	—	none
1.2	8	33.3	—	none
3.0	8	—	—	none
3.0	8	33.3	—	none

The reaction mixture included, in a total volume of 3 ml, 47 mM sodium phosphate buffer (pH 6.5), DL-DOPA, Tiron, ascorbate and mushroom tyrosinase, as indicated. Absorbance at 475 mm was recorded as a function of time (data not shown) and the lag period was estimated from the curves obtained as described in ref. [10].

concentrations, Tiron can act as an efficient AH_2, thus shortening the lag period of tyrosine hydroxylation.

The following provides support to this suggestion: the lag period of tyrosine hydroxylation by tyrosinase is known to be shorter at low, rather than at high, concentrations of tyrosine [12]. This phenomenon is attributed to the ability of tyrosine to compete for the AH_2 site of the enzyme, since higher concentrations of tyrosine increase the concentration of DOPA (AH_2) required for an equal shortening of the lag period [12]. Indeed, we observed that Tiron extends the lag period of tyrosine hydroxylation to a much greater extent at high concentrations of tyrosine than at low concentrations (data not shown).

Tiron apparently does not compete effectively with DOPA on the substrate binding locus of the active site of the enzyme as judged by the observation described below that it has relatively little effect on the rate of DOPA oxidation by mushroom tyrosinase (Fig. 2).

Effect of Tiron on the o-dihydroxyphenolase activity of mushroom tyrosinase

The effect of Tiron on the *o*-dihydroxyphenolase activity of mushroom tyrosinase was tested using DL-DOPA as the substrate. It was found that in a reaction mixture consisting of 6.7 mM DL-DOPA, 47 mM sodium phosphate buffer (pH 6.5) and 33 μg mushroom tyrosinase, Tiron, in the range of 7–40 mM, has no effect on the rate of DL-DOPA oxidation to dopachrome (data not shown).

When the effect of Tiron on mushroom tyrosinase was tested in the presence of only 1.33 mM DL-DOPA and of very much higher concentrations of Tiron (up to 650 mM), it was found (Fig. 2) that Tiron at 53–133 mM had hardly any effect on the initial rate of DL-DOPA oxidation; at 266 mM Tiron had a very small effect, while at 530 mM it inhibited the initial rate of DL-DOPA oxidation by approximately 60%, indicating that Tiron competes with DL-DOPA.

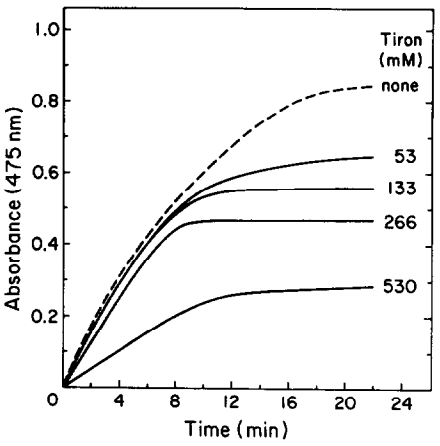

Fig. 2. Effect of Tiron on the rate of DOPA oxidation by mushroom tyrosinase. The reaction mixture included in a total volume of 3 ml, 1.3 mM DL-DOPA, 47 mM sodium phosphate buffer (pH 6.5), 20 μg mushroom tyrosinase (added last), and Tiron as indicated.

In the absence of Tiron (control), the rate of oxidation of 1.33 mM DL-DOPA was linear for approximately 16 min and at *A* (475 nm) of 0.8, it reached a plateau. However, in the presence of Tiron, the plateau was reached earlier and at a lower *A* (475 nm) value. As seen in Fig. 2, the higher the Tiron concentration, the lower the *A* (475 nm) at which the plateau was reached. The appearance of the plateau suggest that the enzyme undergoes inactivation. Indeed, preincubation of 650 μg mushroom tyrosinase in the presence of 400 mM Tiron for 1 hr resulted in 50% inactivation of the enzyme (data not shown). As shown in a separate paper [7], Tiron is oxidized by mushroom tyrosinase to a yellow product(s) (λ_{max} 435 nm) via Tiron-semiquinone. It is likely that the thus formed Tiron-semiquinones inactivate the enzyme in a suicide-type reaction [13].

EXPERIMENTAL

Materials Mushroom tyrosinase (grade III), L-tyrosine, DL-DOPA and Tiron were obtained from Sigma.

Monohydroxyphenolase activity. This was assayed in a reaction mixture that included L-tyrosine, NaPi buffer (pH 6.5) and mushroom tyrosinase (added last) in the absence and presence of Tiron as indicated; details are given in the legends. The rate of formation of dopachrome as a function of time was followed at 475 nm and the lag period of tyrosine hydroxylation was estimated by extrapolation of each curve to the x-axis, as suggested in ref. [10].

o-*Dihydroxyphenolase activity.* This was assayed in a reaction mixture that included DL-DOPA, NaPi buffer (pH 6.5) and mushroom tyrosinase (added last) in the absence or presence of Tiron as indicated in the legends. The rate of formation of dopachrome as a function of time was followed at 475 nm and activity (ΔA 475 nm/min) was computed from the initial linear portions of each curve obtained. The spectrophotometric measurements were conducted on a Varian model DMS-90 spectrophotometer equipped with a recorder.

REFERENCES

1. Greenstock, C. L. and Miller, R. W. (1975) *Biochim. Biophys. Acta* **396**, 11.
2. Rajogopalan, K. V., Fridovich, I. and Handler, P. (1962) *J. Biol. Chem.* **237**, 922.
3. Miller, R. W. and McDowall, F. D. H. (1975) *Biochim. Biophys. Acta* **387**, 176.
4. Bors, W., Saran, M. and Michel, C. (1979) *Biochim. Biophys. Acta* **582**, 537.
5. Van Ginkel, G. and Raison, J. K. (1980) *Phytochem. Photobiol.* **32**, 793.
6. Kahn, V., Golan-Goldhirsh, A. and Whitaker, J. R. (1983) *Phytochemistry* **22**, 1875.
7. Kahn, V. and Miller, R. W. (1987) *Phytochemistry* **26**, 2459.
8. Vanneste, W. H. and Zuberbuhler, A. (1974) in *Molecular Mechanisms of Oxygen Activation* (Hayaishi, O., ed.). Academic Press, New York.
9. Halliwell, B. (1977) in *Superoxide and Superoxide Dismutases* (Michelson, A. M., McCord, J. M. and Fridovich, I., eds). Academic Press, New York.
10. Pomerantz, S. H. and Warner, M. C. (1976) *J. Biol. Chem.* **242**, 5308.
11. Jolley, R. L. Jr., Evans, L. H., Makino, N. and Mason, H. S. (1974) *J. Biol. Chem.* **249**, 335.
12. Coleman, J. E. (1974) in *Advances in Chemistry Series: Food Related Enzymes* (Coleman, J. E. and Whitaker, J. R., eds) pp. 136, 267.
13. Wood, B. J. B. and Ingraham, L. L. (1965) *Nature* **205**, 291.