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MULTIPLE EFFECT OF HYDROXYLAMINE ON MUSHROOM TYROSINASE*

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Key Word Index—Mushroom tyrosinase; hydroxylamine; multiple effect.

Abstract—Mushroom tyrosinase is affected by hydroxylamine (NH_2OH) in several ways. At relatively low concentrations (up to 33 mM) NH_2OH shortens the lag period of tyrosine hydroxylation. The *o*-dihydroxyphenolase activity of mushroom tyrosinase is slightly stimulated by short exposure to relatively low concentrations of NH_2OH (1.5 mM). Relatively high concentrations of NH_2OH (above 20 mM) inhibit the *o*-dihydroxyphenolase activity of the enzyme and lowers the extent of final pigment production. Preincubation of mushroom tyrosinase with different concentrations of NH_2OH for different times results in the inactivation of the enzyme. The rate of inactivation occurred much faster under anaerobic than under aerobic conditions. It was also found that NH_2OH changes the spectra of *o*-quinones prepared chemically or of products formed during the oxidation of *o*-dihydroxyphenols by mushroom tyrosinase. These spectral changes were attributed to the formation of oximes (mono- or dioximes) as a result of an interaction between *o*-quinones and NH_2OH. The apparent inhibition exerted by NH_2OH on the *o*-dihydroxyphenolase activity of mushroom tyrosinase is, in part, due to spectral changes in pigmented product formation and, in part, due to the inactivation of the enzyme by NH_2OH.

INTRODUCTION

Tyrosinase (EC 1.14.18.1) carries out two different biochemical reactions:

(A) monophenol + O_2 + AH_2 → *o*-dihydroxyphenol + H_2O + A

(B) 2*o*-dihydroxyphenol + O_2 → 2*o*-quinone + $2H_2O$

where AH_2 represents a reductant.

In the absence of an exogenous reductant (AH_2), the hydroxylation of monohydroxyphenol by mushroom tyrosinase (reaction A) is characterized by an initial lag period [1–6]. The lag period is overcome when sufficient *o*-dihydroxyphenol is formed endogenously, probably by a non-enzymatic reaction [1, 2]. However, in the presence of exogenously added reductants, such as ascorbate [3–5] or DOPA analogues [6], the lag period is shortened or abolished.

According to recent ideas [2, 7–10], tyrosinase [Cu(II) Cu(II) (mettyrosinase, previously referred to as T-resting] has to be reduced to deoxytyrosinase [Cu(I) Cu(I)] by endogenously formed *o*-dihydroxyphenols or by exogenously added reductants. *o*-Dihydroxyphenol is thought to be the most efficient reductant for this reaction [11]. Deoxytyrosinase then interacts with oxygen to form oxytyrosinase [Cu(II) Cu(II) O_2] and the latter is the active species with which the substrate (mono- or *o*-dihydroxyphenol) interacts [7–10]. Jolley *et al.* [2] detected the spectrum of oxytyrosinase (strong absorbance maximum at 345 nm and a broad weak band at 600 nm) when they incubated mushroom tyrosinase with H_2O_2 under aerobic conditions. Schoot Uiterkamp *et al.* [7] studied the circular dichroism spectrum of mushroom tyrosinase (T-resting) and of the enzyme treated with NH_2OH or H_2O_2 and found that aerobic treatment of T-resting with H_2O_2 or NH_2OH resulted in the formation of oxytyrosinase.

Although oxytyrosinase is detected when tyrosinase is treated with either NH_2OH or H_2O_2, the mechanism by which oxytyrosinase is formed by these two chemicals is thought to be different. The formation of oxytyrosinase as a result of an interaction of mettyrosinase with H_2O_2 under aerobic conditions is thought to occur by the direct interaction of mettyrosinase with H_2O_2 while the formation of oxytyrosinase as a result of interaction of mettyrosinase with NH_2OH under aerobic conditions is thought to occur via deoxytyrosinase [2, 7–10].

We have recently shown that H_2O_2 has a multiple effect on tyrosinase of avocado [12]. It was found that H_2O_2, at relatively low concentrations, shortened the lag period of tyrosine hydroxylation and stimulated the *o*-dihydroxyphenolase activity of the enzyme. Relatively high concentrations of H_2O_2 inactivated avocado [12] and mushroom tyrosinase [13].

To the best of our knowledge, the effect of NH_2OH on the rate of mono- and *o*-dihydroxyphenolase activities of tyrosinase and on the stability of the enzyme has not been studied previously. Therefore, in view of the chemical analogy between H_2O_2 and NH_2OH, we decided to study in some detail the effect of NH_2OH on the rate of mono- and *o*-dihydroxyphenolase activities of mushroom tyrosinase, in an attempt to see if the effects of H_2O_2 and NH_2OH on the enzyme are similar.

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RESULTS

Effect of NH_2OH *on the rate of tyrosine hydroxylation by mushroom tyrosinase*

It was found that the addition of relatively small concentrations of NH_2OH shortened the lag period of tyrosine hydroxylation by mushroom tyrosinase, as demonstrated in the inset to Fig. 1. The lag periods in the presence and absence of different concentrations of NH_2OH were estimated from the kinetic data and the results are summarized in Fig. 1. The data show that relatively small concentrations of NH_2OH (up to 8 mM) have a relatively more pronounced effect on the lag period than relatively larger concentrations of NH_2OH (8–33 mM). When the lag period in the absence of NH_2OH is taken as 100%, then a 50% shortening in the lag period occurs at 5 mM NH_2OH, while at a concentration of 33 mM NH_2OH the lag period was shortened by only 90%.

Comparison among NH_2OH, H_2O_2, *ascorbate and DOPA for their ability to shorten the lag period of tyrosine hydroxylation by mushroom tyrosinase*

As pointed out above, *o*-dihydroxyphenols, ascorbate and NH_2OH are known as reductants for the hydroxylation of monohydroxyphenols by tyrosinase [1, 3–5, 11]. The reducing property of NH_2OH has been well documented (see references in [14, 15]). H_2O_2 can also shorten the lag period of mono hydroxyphenol hydroxylation [12] probably due to its ability to interact directly with tyrosinase to form oxytyrosinase [2, 8–10].

It was interesting to compare the effectiveness of NH_2OH, ascorbate, DOPA and H_2O_2 to shorten the lag period of tyrosine hydroxylation by mushroom tyrosinase. This was tested, and an apparent K_m was estimated from the kinetic data obtained (data not shown), as suggested by Pomerantz and Warner [16]. DL-DOPA shortens the lag period most effectively with a K_m of 3.0×10^{-7}; ascorbate is about ten times less effective, with a K_m of 2.0×10^{-6} M. H_2O_2 and NH_2OH are much less effective than DOPA or ascorbate, with a K_m of 4.0×10^{-4} M and 2.5×10^{-3} M, respectively. Of the latter two, H_2O_2 is about ten times more effective than NH_2OH in shortening the lag period of tyrosine hydroxylation by mushroom tyrosinase.

Effect of NH_2OH *on the o-dihydroxyphenolase activity of mushroom tyrosinase*

The effect of various concentrations of NH_2OH on the *o*-dihydroxyphenolase activity of mushroom tyrosinase was tested using various *o*-dihydroxyphenols as the substrate. It was found that NH_2OH has a multiple effect on the *o*-dihydroxyphenolase activity of the enzyme. The data in Fig. 2 show the multiple effect of NH_2OH on DL-DOPA as the substrate. Relatively small concentrations of NH_2OH (up to 8 mM) stimulate the rate of dopachrome formation compared with the control (in the absence of NH_2OH), with maximum stimulation occurring at 1.5 mM, but at relatively large concentrations of NH_2OH the rate of dopachrome formation is inhibited (Fig. 2). Furthermore, the data in Fig. 2 show that in the presence of small or large concentrations of NH_2OH, the rate of dopachrome formation increases linearly with time only during the initial 80–100 sec of the reaction, but thereafter reaches a plateau; the *A* 475 nm at the plateau is lower, the larger the concentration of NH_2OH.

The observation that the oxidation of an *o*-dihydroxyphenol by mushroom tyrosinase is linear for only 80–100 sec and then reaches a plateau, is usually attributed to the formation of *o*-quinones, which inactivate the enzyme (suicide-type inactivation). The data presented above (Fig. 2) show that NH_2OH, at relatively large concentrations (150 mM), inhibits the rate of DL-DOPA oxidation by mushroom tyrosinase; they also show that the higher the NH_2OH concentration, the smaller is the amount of *o*-quinone (or other pigmented products) formed. Thus, the plateau reached at smaller *A* with higher NH_2OH concentrations is not due to a larger

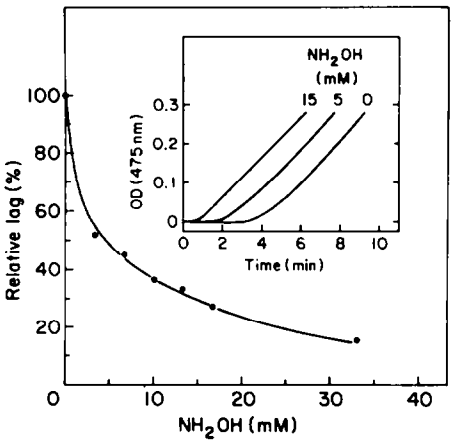


Fig. 1. Effect of NH_2OH on the rate of tyrosine hydroxylation by mushroom tyrosinase. The reaction mixture included, in a total volume of 3 ml: 3.3 mM L-tyrosine, 47 mM sodium phosphate buffer (pH 6.5), NH_2OH as indicated and 33 μg mushroom tyrosinase (added last).

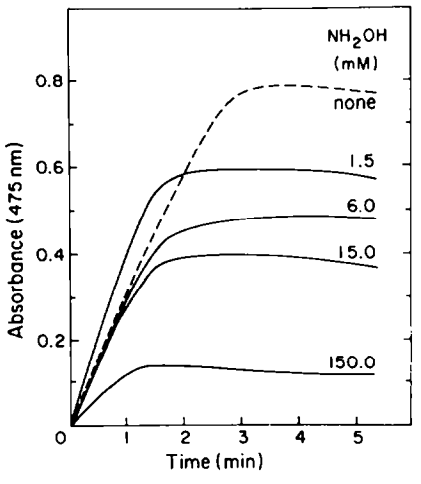


Fig. 2. Effect of different concentrations of NH_2OH on the rate of DL-DOPA oxidation by mushroom tyrosinase. The reaction mixture included, in a total volume of 3 ml: 1.66 mM DL-DOPA, 47 mM sodium phosphate buffer (pH 6.5), 25 μg mushroom tyrosinase (added last) and NH_2OH as indicated.

concentration of *o*-quinones, but rather, the data suggest that NH_2OH has an effect on the extent of final pigment formation, and/or that exposure of the enzyme to NH_2OH for a relatively long time results in the inactivation of the enzyme. These two possibilities will be dealt with further below.

The effect of NH_2OH on the *o*-dihydroxyphenolase activity of mushroom tyrosinase described above is similar to our findings on the effect of H_2O_2 on avocado tyrosinase [12]. We have shown that in a reaction mixture of 3 ml, H_2O_2 at 3.3–13.2 mM slightly stimulates the oxidation of 6.7 mM DL-DOPA by avocado tyrosinase (50 μg) while relatively larger concentrations of H_2O_2 (66 mM and up) decreased both the rate and the final level of dopachrome formation [12]. Mushroom tyrosinase (75 μg) was stimulated by H_2O_2 up to 6 mM, with maximum stimulation occurring at 2 mM H_2O_2 (in a total volume of 3 ml) (data not shown).

Effect of NH_2OH *on the extent of final pigment formed by the action of mushroom tyrosinase on o-dihydroxyphenols*

In the course of our studies we noted that when dopaquinone and dopamine quinone (formed enzymatically) were mixed with excess NH_2OH, polymerization to melanin (precipitate formation) did not occur, whereas it did occur in controls (without NH_2OH). It was also noted that in some cases the colour of products formed when *o*-dihydroxyphenol was oxidized by mushroom tyrosinase in the presence of NH_2OH was different from that obtained in its absence. The data in Table 1 illustrate the differences in colours noted after incubating various *o*-dihydroxyphenols (for 1 and 20 hr) with mushroom tyrosinase in the presence or absence of NH_2OH. Moreover, we prepared the *o*-quinones of several *o*-dihydroxyphenols by oxidizing the latter with Ag_2O [17] and immediately mixing them with either water or NH_2OH. The colours seen 1 and 20 hr later in the absence of NH_2OH were different from those seen in its presence (Table 2).

The effect of NH_2OH on the visible spectrum of products obtained by the action of mushroom tyrosinase on different *o*-dihydroxyphenols was studied in some detail, using DL-DOPA, dopamine, catechol and 4-methylcatechol as the substrates for the enzyme. In all cases, it was found that the spectrum of products formed in the presence of NH_2OH was different from that observed in its absence (control) (data not shown).

Table 1. Comparison between the colour of products formed when *o*-dihydroxyphenols were oxidized by mushroom tyrosinase in the presence and absence of NH_2OH

Substrate	Colour seen after interaction for 1 hr with: H_2O	NH_2OH	Colour seen after interaction for 20 hr with: H_2O	NH_2OH
DL-DOPA	red	orange-yellow	dark red	orange
Dopamine	violet-red	orange-yellow	violet-black ppt colourless supernatant	orange
Catechol	beige-violet	pink	black-violet	orange
4-Methylcatechol	yellow-pink	orange-yellow	brown-red	orange
4-Nitrocatechol	orange	yellow	orange	yellow
Protocatechuic acid	yellow	colorless	light violet	light yellow

Each reaction mixture included, in a total volume of 15 ml: 13.3 mM *o*-dihydroxyphenol, 67 mM NaPi buffer (pH 6.5) and 100 μg mushroom tyrosinase (added last). After 5 min incubation at 24°, two samples of 5 ml each were withdrawn and added to 2 ml containing either water or 2 mmol NH_2OH (yielding final NH_2OH concentration of 285 mM). Colour seen 1 hr and 20 hr afterwards is shown above.

Table 2. Comparison between the colour of products formed when *o*-dihydroxyphenols were oxidized by Ag_2O in the presence and absence of NH_2OH

Substrate	Colour seen after interaction for 1 hr with: H_2O	NH_2OH	Colour seen after interaction for 20 hr with: H_2O	NH_2OH
DL-DOPA	pink	yellow	violet-pink	yellow
Dopamine	pink	pink	violet ppt colourless supernatant	yellow
Catechol	yellow	light yellow	light pink some ppt	light yellow
4-Methylcatechol	light yellow	light yellow	pink	orange
4-Nitrocatechol	green (opaque)	yellow-green (opaque)	yellow-green (opaque)	beige ppt yellow supernatant
Protocatechuic acid	light green	beige	beige	colourless

Each reaction mixture included, in a total volume of 15 ml: 13.3 mM *o*-dihydroxyphenol and 100 mg Ag_2O. Two samples of 3-ml aliquots were withdrawn after 5 min and added to either 1 ml of H_2O or 1 ml containing 1 mmol NH_2OH (yielding final NH_2OH of 250 mM).

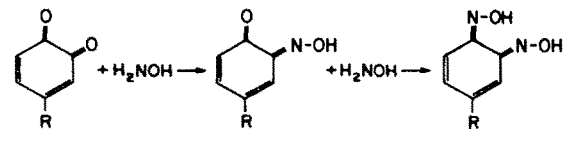
The effect of NH_2OH on the spectrum of products formed by the action of mushroom tyrosinase on *o*-dihydroxyphenols is most likely a result of an interaction of *o*-quinones with NH_2OH yielding oximes (mono- or dioximes). The chemistry of this reaction was presented in a summary form by Finley [18]. By analogy with Finley's scheme, showing that an interaction of 1,4-benzoquinone with NH_2OH yields either monoxime or dioxime, the interaction between *o*-quinone and NH_2OH can be depicted as in Scheme 1.

In view of the changes that occur in the spectrum of products formed when *o*-dihydroxyphenols are oxidized by mushroom tyrosinase in the presence of NH_2OH, it is important to be cautious when studying the effect of NH_2OH on the rate of *o*-quinones formed enzymatically by mushroom tyrosinase or by peroxidase. An observed inhibition by NH_2OH may actually be only due to the fact that during the first few seconds of the reaction, the peak wavelength of the product formed changes in the presence of NH_2OH, and therefore monitoring the rate of the reaction at a fixed wavelength does not reflect the actual rate of the reaction. Therefore, our interpretation that NH_2OH inhibits *o*-dihydroxyphenolase activity of mushroom tyrosinase based on the data of Fig. 2 is probably erroneous and reflects an apparent inhibition due to the effect of NH_2OH on the extent of final pigment formation.

Inactivation of mushroom tyrosinase by NH_2OH

As pointed out above, the data presented in Fig. 2 could, in part, be also due to an inactivation of mushroom tyrosinase by exposure to NH_2OH. This possibility was tested, and it was found that preincubation of mushroom tyrosinase with different concentrations of NH_2OH for different lengths of time resulted in the inactivation of the enzyme. A plot of the percent remaining activity as a function of preincubation time demonstrated that the rate of inactivation was very rapid during the first few minutes of exposing the enzyme to NH_2OH, followed by slow rate thereafter (data not shown). The data in Fig. 3 were obtained by preincubating mushroom tyrosinase for 60 min in the presence of the indicated concentrations of NH_2OH, dialysing the sample exhaustively, and then assaying the dialysed samples for *o*-dihydroxyphenolase activity. As seen in Fig. 3, the extent of inactivation of the enzyme was dependent on NH_2OH concentration, in a non-linear fashion, i.e. 28% and 87% of the activity was lost by preincubating the enzyme for 60 min in the presence of 5 mM and 100 mM NH_2OH, respectively.

The rate of inactivation of mushroom tyrosinase by NH_2OH under anaerobic conditions was extremely fast compared with the rate observed under aerobic conditions. For example, under anaerobic conditions, 92% activity was lost within the first few minutes of preincubation in the presence of 250 mM NH_2OH, while under



Scheme 1.

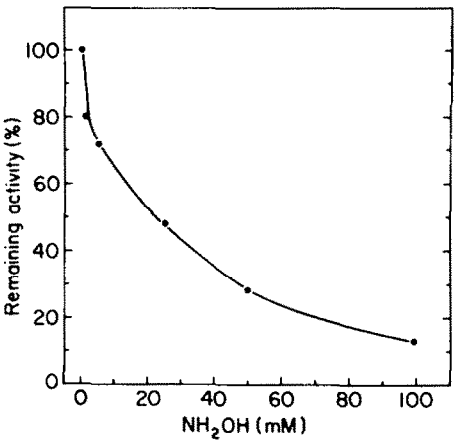


Fig. 3. Inactivation of mushroom tyrosinase by NH_2OH. The preincubation mixture contained, in a total volume of 26 ml, 70 mM sodium phosphate (NaPi) buffer (pH 6.5), 650 μg mushroom tyrosinase (added last) and various concentrations of NH_2OH as indicated. After 60 min of preincubation, each mixture was dialysed overnight against 0.01 M NaPi buffer (pH 6.5) with four changes of the same buffer. Aliquots (2 ml) of each dialysed mixture were then withdrawn and assayed for *o*-dihydroxyphenolase activity in the presence of 6.7 mM DL-DOPA (in a total volume of 3 ml). The *o*-dihydroxyphenolase activity of the control sample (without NH_2OH) was 0.55 ΔA 475 nm/min and was taken as 100%.

aerobic conditions 80% activity was lost after 90 min preincubation (Table 3).

By comparison, under conditions identical to those described in Fig. 3, we found that 50% inactivation of mushroom tyrosinase occurred at 4 μM H_2O_2. Thus, NH_2OH is 5500-fold less effective than H_2O_2 as an inactivator of mushroom tyrosinase.

We have recently offered an explanation for the inactivation of mushroom tyrosinase by H_2O_2 which can account for the observation that the rate of inactivation is faster under anaerobic than under aerobic conditions [13]. We do not yet have an explanation for the inactivation of mushroom tyrosinase by NH_2OH. One possibility is that the inactivation occurs via H_2O_2, NH_2O-radical or O_2^- that can be generated when NH_2OH interacts with a metal [19]. An alternative possibility is that NH_2OH effectively reduces the Cu^{2+} at the active site of the enzyme and that Cu^+, in a reduced state, is easily released from the enzyme, with a consequent loss of activity.

EXPERIMENTAL

Mushroom tyrosinase (grade III), L-tyrosine, DL-DOPA, dopamine, catechol, 4-methyl catechol and NH_2OH were from Sigma. H_2O_2 was from Merck.

Unless otherwise indicated, monohydroxyphenolase activity was assayed in a reaction mixture of 3 ml that included: 3.3 mM L-tyrosine, 47 mM NaPi buffer (pH 6.5), and mushroom tyrosinase as indicated. Unless otherwise indicated, *o*-dihydroxyphenolase activity was assayed in a reaction mixture of 3 ml that included: 6.7 mM DL-DOPA, 47 mM NaPi buffer (pH 6.5), and mushroom tyrosinase as indicated. In both cases, the rate of formation of dopachrome as a function of time was followed at 475 nm in a

Table 3. Comparison between the rate of mushroom tyrosinase inactivation by NH_2OH in air and under nitrogen

Part	Conditions	NH_2OH (mM)	Time (min)	ΔA 475 nm/min	Activity (%)
A	air	none	1	0.166	100
			9	0.183	110
			15	0.191	115
			30	0.183	110
			93	0.170	102
	air	250	1	0.17	100
			3	0.137	80
			10	0.112	66
			15	0.096	56
			25	0.083	49
			40	0.066	39
			80	0.037	21
B	nitrogen	none	1	0.187	100
			3	0.196	104
			10	0.191	102
			26	0.21	112
			70	0.2	106
		250	1	0	0

The reaction mixture included, in a total volume of 26 ml: 70 mM NaPi buffer (pH 6.5), in the presence or absence of 250 mM NH_2OH, as indicated. Air was bubbled into reaction mixture A while purified nitrogen (99.99%) (passed through an oxygen trap) was bubbled into reaction mixture B. After 60 min bubbling of either air (A) or nitrogen (B), a 0.1-ml aliquot containing 260 μg mushroom tyrosinase was added to each (time zero). Thereafter, in both parts A and B, 2-ml aliquots were withdrawn at various times and *o*-dihydroxyphenolase activity was assayed aerobically in the presence of 6.7 mM DL-DOPA and 47 mM NaPi buffer (pH 6.5), in a total volume of 3 ml immediately after mixing the components well.

Varian 635 Spectrophotometer equipped with a recorder. The lag period of tyrosine hydroxylation was estimated by extrapolation of each curve to the *x*-axis, as suggested in ref. [16]. *o*-Dihydroxyphenolase activity was computed from the initial linear portion of each curve and expressed as ΔA 475 nm/min. *o*-Quinones were prepared by oxidizing *o*-dihydroxyphenols with Ag_2O as described in ref. [17].

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