# Tyrosinase Catalyzes an Unusual Oxidative Decarboxylation of 3,4-Dihydroxymandelate†

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ABSTRACT: Tyrosinase usually catalyzes the conversion of monophenols to *o*-diphenols and oxidation of diphenols to the corresponding quinones. However, when 3,4-dihydroxymandelic acid was provided as the substrate, it catalyzed an unusual oxidative decarboxylation reaction generating 3,4-dihydroxybenzaldehyde as the sole product. The identity of the product was confirmed by high-performance liquid chromatography (HPLC) as well as ultraviolet and infrared spectral studies. None of the following enzymes tested catalyzed the new reaction: galactose oxidase, ceruloplasmin, superoxide dismutase, ascorbate oxidase, dopamine β-hydroxylase, and peroxidase. Phenol oxidase inhibitors such as phenylthiourea, potassium cyanide, and sodium azide inhibited the reaction drastically, suggesting the participation of the active site copper of the enzyme in the catalysis. Mimosine, a well-known competitive inhibitor of tyrosinase, competitively inhibited the new reaction also. 4-Hydroxymandelic acid and 3-methoxy-4-hydroxymandelic acid neither served as substrates nor inhibited the reaction. Putative intermediates such as 3,4-dihydroxybenzyl alcohol and (3,4-dihydroxybenzoyl)formic acid did not accumulate during the reaction. Oxidation to a quinone methide derivative rather than conventional quinone accounts for this unusual oxidative decarboxylation reaction. Earlier from this laboratory, we reported the conversion of 4-alkylcatechols to quinone methides catalyzed by a cuticular phenol oxidase [Sugumaran, M., & Lipke, H. (1983) *FEBS Lett. 155*, 65–68]. Present studies demonstrate that mushroom tyrosinase will also catalyze quinone methide production with the same active site copper if a suitable substrate such as 3,4-dihydroxymandelic acid is provided.

Tyrosinase (monophenol,dihydroxyphenylalanine:oxygen oxidoreductase, EC 1.14.18.1), also known as polyphenol oxidase, is a copper-containing monooxygenase widely distributed in nature (Lerch, 1983). It is responsible for the biosynthesis of melanin pigments in various organisms. Tyrosinase from the mammalian system hydroxylates L-tyrosine to L-3,4-dihydroxyphenylalanine (Dopa) and oxidizes the resultant catechol to its corresponding quinone. Dopa quinone, thus formed, undergoes a series of transformations to yield, finally, deeply colored melanin pigments. Apart from performing such general reactions, tyrosinase also catalyzes certain unique oxygenations such as hydroxylation of 3,4-dihydroxyphenylalanine to 5-hydroxy-Dopa (Hansson et al., 1980) and 2,4-dihydroxyphenylalanine to 6-hydroxy-Dopa (Morrison & Cohen, 1983) when ascorbic acid is provided as a cofactor.

In insects and other arthropods, it is widely recognized that the cuticular polyphenol oxidases generate highly reactive quinone intermediates from catecholamine derivatives and provide them for cross-linking structural proteins (Brunet, 1980; Lipke et al., 1983). Since *N*-acetyldopamine and *N*-β-alanyldopamine are considered to be universal sclerotizing precursors (Brunet, 1980; Hopkins et al., 1982), it is likely that their corresponding quinones play a dominant role in tanning of insect cuticle. Apart from the well-known quinone cross-links, evidence for the presence of an alternate type of cross-link, called β-cross-link, was provided by Andersen (1979). During his studies, his group isolated a number of side chain oxidized catecholamine derivatives from acid hydrolysates of the cuticle and attributed it to the presence of β-cross-links (Andersen & Roepstorff, 1978). Furthermore, he observed the liberation of tritium from the side chain tritiated *N*-acetyldopamine upon incubation with cuticular enzymes, confirming the presence of such cross-links (Andersen, 1974). Finally, his group isolated 1,2-dehydro-*N*-acetyldopamine from the sclerotized cuticle by alkali extraction and suggested that this compound could be the reactive species involved in β-sclerotization (Andersen & Roepstorff, 1982). On the other hand, we proposed a quinone methide intermediate for this process (Lipke et al., 1983). Recently, we synthesized the 1,2-dehydro-*N*-acetyldopamine and showed by radioactive trapping experiments that this compound is not involved in β-sclerotization and that quinone methides are responsible for this process (Sugumaran, 1986).

Our continued studies on the mechanisms of cuticular tanning resulted in characterization of a rather unusual phenol oxidase in *Sarcophaga bullata* cuticle, which catalyzes the formation of quinone methides from 4-alkylcatechols (Sugumaran & Lipke, 1983a,b). While comparing the properties of this cuticular enzyme with those of mushroom tyrosinase, we observed a novel oxidative decarboxylation of 3,4-dihydroxymandelic acid to 3,4-dihydroxybenzaldehyde common to both classes of enzymes. This paper details the study on the mushroom tyrosinase mediated oxidative decarboxylation of 3,4-dihydroxymandelate.

## Materials and Methods

Mushroom tyrosinase, ceruloplasmin, galactose oxidase, ascorbate oxidase, superoxide dismutase, dopamine β-hydroxylase, horseradish peroxidase, and mimosine were obtained from Sigma Chemical Co., St. Louis, MO. Aldrich Chemical Co., Milwaukee, WI, supplied all the catechol derivatives, 4-hydroxymandelic acid, and 3-methoxy-4-hydroxymandelic acid. Other chemicals and solvents were procured from commercial sources.

Spectral changes associated with enzymatic oxidation of catechols were monitored as follows. A reaction mixture (1 mL) containing 1 mM catecholic compounds and 18 μg of mushroom tyrosinase in 50 mM sodium phosphate buffer, pH 6.0, was incubated at room temperature. The reaction was

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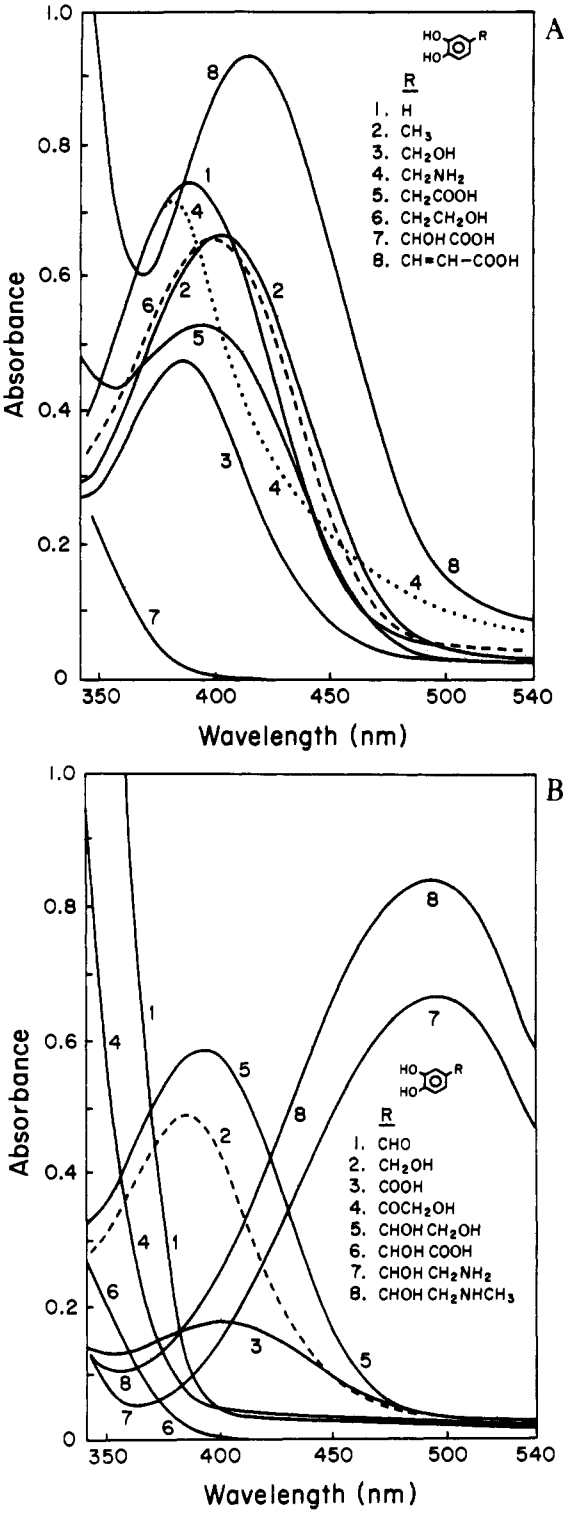


FIGURE 1: Visible spectra of products formed during the oxidation of various 4-substituted catechols by mushroom tyrosinase. A reaction mixture containing 1 $\mu$mol of the indicated catechol and 18 $\mu$g of mushroom tyrosinase in 1 mL of 50 mM sodium phosphate buffer, pH 6.0, was incubated at room temperature for 5 min, and the visible spectra of the reaction were recorded against a blank reaction mixture lacking the enzyme.

initiated by the addition of enzyme, and the visible spectral changes associated with the generation of quinones were recorded with a Gilford Model 250 spectrophotometer.

For high-performance liquid chromatographic (HPLC) studies, the amount of enzyme in the above reaction mixture was reduced to 9 $\mu$g. At zero time and 10- and 20-min intervals, aliquots of 10 $\mu$L were removed and subjected to

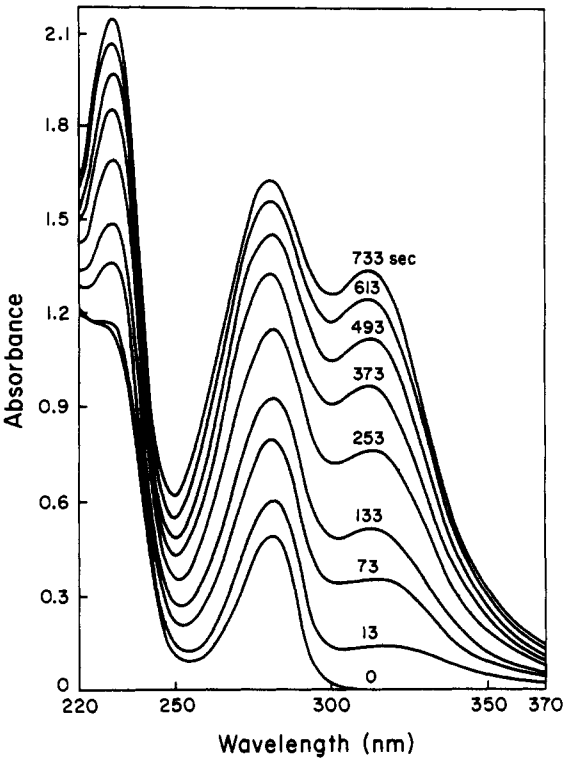


FIGURE 2: Ultraviolet spectral changes associated with the oxidation of 3,4-dihydroxymandelic acid catalyzed by mushroom tyrosinase. A reaction mixture containing 0.2 $\mu$mol of 3,4-dihydroxymandelic acid and 18 $\mu$g of mushroom tyrosinase in 50 mM sodium phosphate buffer, pH 6.0, was incubated at room temperature. After the reaction was started by the addition of enzyme, spectral changes associated with the oxidation were monitored at indicated time intervals.

HPLC analysis as outlined earlier (Sugumaran & Lipke, 1982). Isocratic elution of catechols was achieved with the solvent system 0.05 M acetic acid in methanol–water (1:4 v/v) containing 0.2 mM sodium octylsulfonate.

## Results and Discussion

In accordance with its wide substrate specificity, mushroom tyrosinase readily oxidized a number of *o*-diphenols such as catechol, 4-methylcatechol, 3,4-dihydroxybenzyl alcohol, 3,4-dihydroxybenzylamine, (3,4-dihydroxyphenyl)acetic acid, (3,4-dihydroxyphenyl)ethyl alcohol, caffeic acid, 3,4-dihydroxybenzoic acid, 3,4-dihydroxyphenyl ethylene glycol, (3,4-dihydroxyphenyl)propionic acid, chlorogenic acid, Dopa, dopamine, *N*-acetyldopamine, epinephrine, and norepinephrine to their corresponding quinone derivatives. Parts A and B of Figure 1, for instance, show the visible spectral changes associated with the enzymatic oxidation of a few representative catechols. Dopa and dopamine produced dopachrome, while norepinephrine produced adrenaline as the final oxidation product. During the oxidation of each of the other catechols, generation of corresponding quinones could be witnessed by their typical visible spectra. However, no such spectral changes accompanied the enzymatic oxidation of 3,4-dihydroxymandelate. Side chain oxygenated compounds such as 3,4-dihydroxybenzaldehyde and 3,4-dihydroxyacetophenone appear to be poor substrates for the enzyme (Figure 1B). Hence it is likely that 3,4-dihydroxymandelate may also be a poor substrate for this enzyme. That this was not the case is shown by the rapid spectral changes occurring in the UV region during the oxidation (Figure 2). The appearance of a peak at 312 nm during oxidation suggested the generation of a carbonyl-substituted catechol derivative. When the absorption changes reached a maximum, the spectrum corresponded to

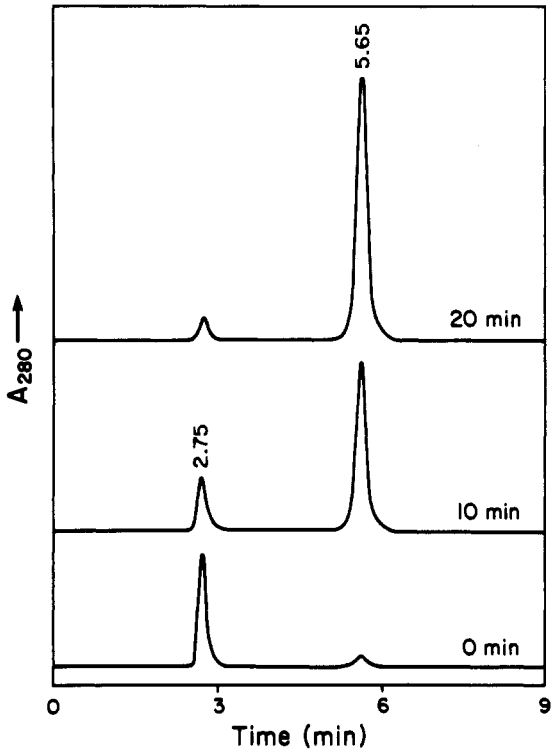


FIGURE 3: HPLC of the reaction mixture. A reaction mixture containing 1 μmol of 3,4-dihydroxymandelic acid and 9 μg of mushroom tyrosinase in 1 mL of 50 mM sodium phosphate buffer, pH 6.0, was incubated at room temperature. At the indicated time intervals, a 10-μL sample was withdrawn and subjected to HPLC as outlined by Sugumaran and Lipke (1982). The peak emerging at 2.75 min is 3,4-dihydroxymandelic acid and that eluting at 5.65 min is 3,4-dihydroxybenzaldehyde. No other compound is eluted up to 60 min.

that of 3,4-dihydroxybenzaldehyde. Further proof for such a conversion came from HPLC studies. HPLC analysis of reaction mixtures at various time intervals during the oxidation revealed the appearance of a product with retention time longer than that of 3,4-dihydroxymandelic acid (Figure 3). This new product had the same retention time as that of 3,4-dihydroxybenzaldehyde and cochromatographed with the latter compound as a single symmetrical peak in two different solvents on HPLC. In order to establish the structure of the product unequivocally, a large-scale reaction mixture was conducted and the product isolated was subjected to IR spectral studies. The IR spectrum of the isolated product (Figure 4A) and authentic 3,4-dihydroxybenzaldehyde (Figure 4B) matched peak to peak, confirming that they are one and the same compound.

Table I: Effect of Various Phenol Oxidase Inhibitors on the Oxidative Decarboxylation Reaction Catalyzed by Mushroom Tyrosinase

| compound (concn) | % inhibition |
|---|---|
| potassium cyanide (0.1 mM) | 21 |
| potassium cyanide (0.2 mM) | 59 |
| potassium cyanide (1 mM) | 100 |
| sodium azide (0.5 mM) | 41 |
| sodium azide (1 mM) | 67 |
| sodium azide (5 mM) | 88 |
| phenylthiourea (0.5 μM) | 26 |
| phenylthiourea (0.1 mM) | 100 |

Tyrosinase-catalyzed oxidative decarboxylation of 3,4-dihydroxymandelate showed a strict requirement of molecular oxygen. Heat-inactivated enzyme did not catalyze the above reaction. During the initial phase of the reaction a 1:1 stoichiometric relation between substrate utilization and product formation was observed. At the end of the reaction, due to the slow oxidation of the product, slightly less than 1 mol of product was formed. Other phenol oxidases such as hemolymph and cuticular phenol oxidases of *S. bullata* as well as *Manduca sexta* also catalyzed this unusual reaction. Various copper-containing proteins such as ceruloplasmin, superoxide dismutase, ascorbate oxidase, galactose oxidase, and dopamine β-hydroxylase and the iron-containing protein horseradish peroxidase did not catalyze the new reaction when tested under standard assay conditions. Thus it appears that the observed oxidative decarboxylation reaction is confined to the phenol oxidase type of enzymes only.

In order to check whether or not tyrosinase uses its regular active site to execute this new reaction, we performed some inhibition studies. Typical phenol oxidase inhibitors such as phenylthiourea, potassium cyanide, and sodium azide, which are known to react with the copper at the active site, inhibited the reaction drastically, demonstrating the participation of the active site copper in the catalytic process (Table I). Furthermore, mimosine, a well-known competitive inhibitor of tyrosinase (Hashiguchi & Takahasi, 1977), inhibited the oxidative decarboxylation reaction linear competitively with a $K_i$ value of 0.2 mM. It has been demonstrated by electron spin resonance (ESR) studies that this compound binds to the active site copper specifically (Winkler et al., 1981). Thus it appears that both the observed 3,4-dihydroxymandelate oxidation and the conventional quinone production are occurring at the same active site of tyrosinase.

Studies with substrate analogues provided the clue to the mechanism of this unusual reaction. Analogues such as 4-hydroxymandelic acid or 3-methoxy-4-hydroxymandelic acid

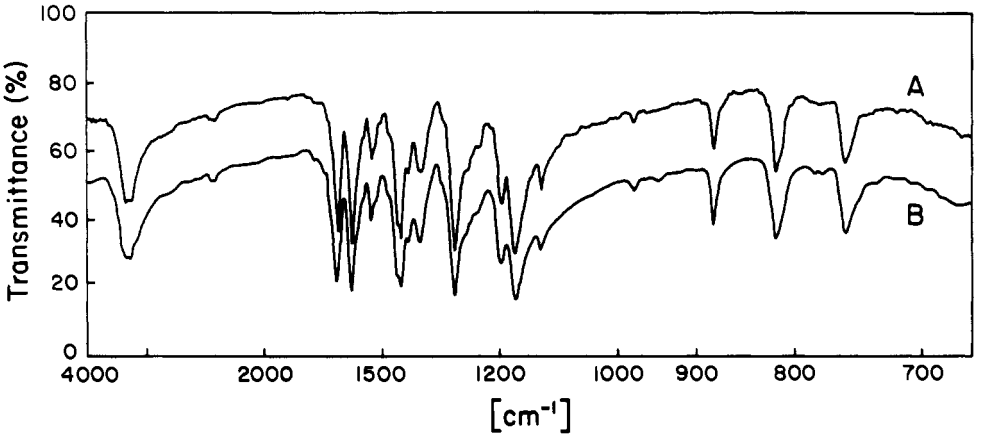


FIGURE 4: Infrared spectrum of (A) isolated products and (B) authentic 3,4-dihydroxybenzaldehyde.

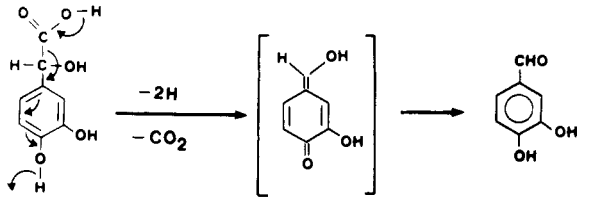


FIGURE 5: Proposed mechanism for the oxidative decarboxylation of 3,4-dihydroxymandelic acid catalyzed by mushroom tyrosinase.

neither inhibited the reaction nor served as substrates for the oxidative decarboxylation. Exogenously added 3,4-dihydroxybenzyl alcohol was converted to its corresponding quinone (Figure 1A) while (3,4-dihydroxybenzoyl)formate was a poor substrate like other ketocatechols and was not converted to 3,4-dihydroxybenzaldehyde. The above experiments together with the HPLC data (Figure 3) clearly indicate that neither (3,4-dihydroxybenzoyl)formate nor 3,4-dihydroxybenzyl alcohol is formed as an intermediate during the oxidative decarboxylation.

The possibility of any other stable intermediate formation during the reaction was totally ruled out on the basis of stoichiometric, spectral, and HPLC studies. First, the substrate conversion to the observed product followed a 1:1 stoichiometry during the initial phase of the reaction. Second, the transformation occurred without any detectable lag period. Third, spectral studies did not reveal the formation of any intermediates in either visible or UV regions. Fourth, no compound other than substrate and product could be detected in the reaction mixture, when it was directly subjected to HPLC analysis. On the basis of the above studies, we propose a simultaneous oxidative decarboxylation of the substrate as indicated in Figure 5 to account for this unusual reaction. The quinone methide product thus formed would rapidly isomerize to yield the more stable catechol form, viz., 3,4-dihydroxybenzaldehyde. Such a quinone methide intermediate formation is also consistent with the mechanism of tyrosinase action and the remarkable specificity of the reaction.

It appears if a suitable substrate such as 3,4-dihydroxymandelic acid is provided to phenol oxidases, which are known to produce only quinones as products, they will catalyze quinone methide production as well. Both quinone formation and quinone methide production from alkyl-substituted catechols involve a two-electron oxidation, but at different positions. Thus quinone methides are tautomers of 4-alkyl quinones. Therefore, with appropriate alterations at the active site, the same enzyme could catalyze the formation of both the products. Conversely, alterations in substrate structure can modify the course of the reaction from quinone to quinone methide and vice versa. Hence it is not surprising to observe this unusual oxidative decarboxylation of 3,4-dihydroxymandelate catalyzed by mushroom tyrosinase.

Bhat and Vaidyanathan (1976) have reported the conversion of 4-hydroxymandelic acid to 4-hydroxybenzaldehyde catalyzed by a bacterial flavoprotein containing $Mn^{2+}$. Although this reaction is catalyzed by a different type of oxidase and is accompanied by stoichiometric production of hydrogen peroxide, it might also involve a similar quinone methide intermediate formation.

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