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Irreversibly inhibitory kinetics of 3,5-dihydroxyphenyl decanoate on mushroom (*Agaricus bisporus*) tyrosinase

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Abstract—3,5-Dihydroxyphenyl decanoate (DPD) is found to inhibit the diphenolase activity of tyrosinase from mushroom (*Agaricus bisporus*). The effects of DPD on the diphenolase activity of mushroom tyrosinase have been studied. The results show that the enzyme activity decreases very slowly with an increase in DPD concentrations at lower concentrations of DPD (between 5 and 60 μM). But at higher concentrations of DPD, DPD can strongly inhibit the diphenolase activity of the enzyme and the inhibition is irreversible. The IC_{50} value was estimated to be 96.5 μM. The inhibition mechanism of DPD has been investigated and the results show that DPD can bind to the free enzyme molecule and enzyme–substrate complex and lose the enzyme activity completely. The inhibition kinetics has been studied in detail by using the kinetic method of the substrate reaction described by Tsou. The microscopic rate constants of the enzyme inhibited by DPD at higher concentrations have been determined.

1. Introduction

Tyrosinase (EC 1.14.18.1) is a copper-containing enzyme that is widely distributed in nature. The enzymatic oxidation of L-tyrosine and L-3,4-dihydroxyphenylalanine (DOPA) to melanin is of considerable importance because melanin has many functions and alterations in melanin synthesis occurred in many disease states.[1] For example, melanoma-specific anticarcinogenic activity is linked with tyrosinase activity.[2] Melanin pigments are also found in the mammalian brain. Tyrosinase may play a role in neuromelanin formation in the human brain, particularly in the substantia nigra. It is also of central importance in processes such as vertebrate pigmentation and the browning of fruits and vegetables.[3] This browning can cause deleterious changes in the organoleptic properties of food product with the loss of fruit and vegetable qualities.[4–6]

Tyrosinase inhibitors should have broad applications, so much effort has been put into searching for feasible and effective tyrosinase inhibitors. Although a large number of naturally occurring tyrosinase inhibitors have already been reported,[7] their individual activity is not potent enough to be put into practical use. In addition, the safety regulations of food additives limit their applications in vivo. We are left to rely on the laboratory synthesis or extraction from plants[8] to resolve the problems. In our previous paper, CPC,[9] cupferron,[10] flavonoids,[11] hexylresorcinol,[12] dodecylresorcinol[12] and alkylbenzaldehydes[13] were shown to inhibit the enzymatic oxidation of DOPA and the inhibitory kinetic study was performed in detail. Recently, 3,5-dihydroxyphenyl decanoate (DPD) was found to inhibit the enzyme. The aim of the research reported in this paper is, therefore, to carry out a kinetic study of the inhibition of the enzyme. The inhibition mechanism was investigated. The results show that inhibition of the enzyme by DPD is irreversible, and the microscopic rate constants for the reaction of this inhibitor with free enzyme and the enzyme–substrate complex were determined and compared.

2. Results

2.1. Determination of the kinetic parameters of mushroom tyrosinase

The kinetics behavior of the enzyme during the oxidation of DOPA has been studied. Under the condition

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employed in the present investigation, the oxidation of DOPA by mushroom tyrosinase follows Michaelis–Menten kinetics. The kinetic parameters of the enzyme for the oxidation of DOPA were determined by the plot of Lineweaver–Burk. The values of K_m and V_m are equal to 0.663 ± 0.012 mM and 40.25 ± 0.025 μM/min, respectively.

2.2. Effect of DPD on the activity of mushroom tyrosinase

The effects of 3,5-dihydroxyphenyl decanoate (DPD) (see Fig. 1A for structure)[14] on the oxidation of DOPA catalyzed by mushroom tyrosinase were first studied. The activity of the enzyme was inhibited by DPD depending on the concentrations as shown in Figure 2A. When the concentration of DPD was lower than 60 μM, the enzyme was inhibited very weakly, and the activity was lost by 7.4% at 60 μM DPD. After that, the remaining enzyme activity was rapidly decreased as the concentration of DPD increased. When the concentration of DPD reached 160 μM, the enzyme was completely suppressed. The inhibitor's concentration (IC_{50}) leading to 50% activity loss was estimated to be 96.5 ± 0.5 μM. However, nonyl-3,5-dihydroxybenzoate (see Fig. 1B for structure)[14] has no effects on the enzyme activity (Fig. 2B). When the concentration of nonyl-3,5-dihydroxybenzoate reached 200 μM, the enzyme activity remained unchanged. Although the structure of nonyl-3,5-dihydroxybenzoate is similar to 3,5-dihydroxyphenyl decanoate, their inhibitory effects are very different. The reason may be that the group of benzoate absorbs electrons and reduces the electron cloud density of the benzene, which weakens and prevents the inhibition of 3,5-dihydroxy benzene on the enzyme for oxidation of DOPA.

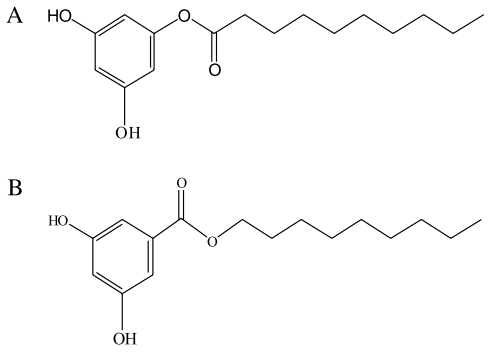


Figure 1. Chemical structures of (A) 3,5-dihydroxyphenyl decanoate (DPD) and (B) nonyl-3,5-dihydroxybenzoate.

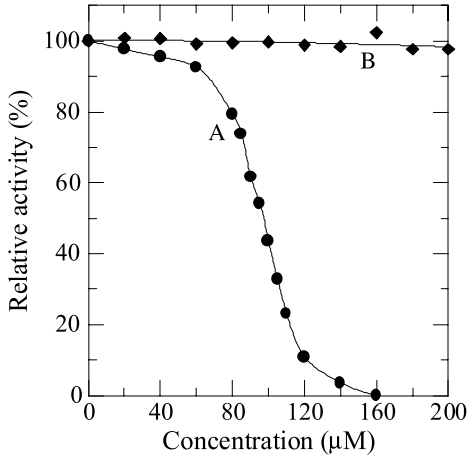


Figure 2. Inhibitory effects of (A) 3,5-dihydroxyphenyl decanoate and (B) nonyl-3,5-dihydroxybenzoate on the activity of mushroom tyrosinase for the catalysis of DOPA at 30 °C. Assay conditions were 3 ml reaction system containing 0.05 M phosphate sodium buffer, pH 6.8, 0.5 mM DOPA, and different concentrations of effector. Final concentration of tyrosinase and DMSO was 6.67 μg/ml and 3.3%, respectively.

2.3. Inhibition mechanism of DPD on the enzyme

The inhibition mechanism of the enzyme by DPD during the oxidation of DOPA was first studied. The relationship of enzyme activity with its concentration in the presence of different concentrations of DPD was determined. The plots of the remaining enzyme activity versus the concentrations of enzyme at different concentrations of DPD gave a family of parallel straight lines with the same slopes and different abscissa intercepts (Fig. 3), indicating that the inhibition of DPD on the enzyme was an irreversible reaction course at higher than 70 μM. The enzyme molecules are combined with DPD and then irreversibly inhibited. The increase of the abscissa intercept indicated that the amount of the efficient enzyme was brought down because of the irreversible inhibition. The inhibitory kinetics of DPD on mushroom tyrosinase for the oxidation of DOPA has been studied. Under the conditions employed in the present investigation, the oxidation reaction of DOPA by mushroom tyrosinase follows Michaelis–Menten kinetics. In the presence of DPD, the kinetic studies of

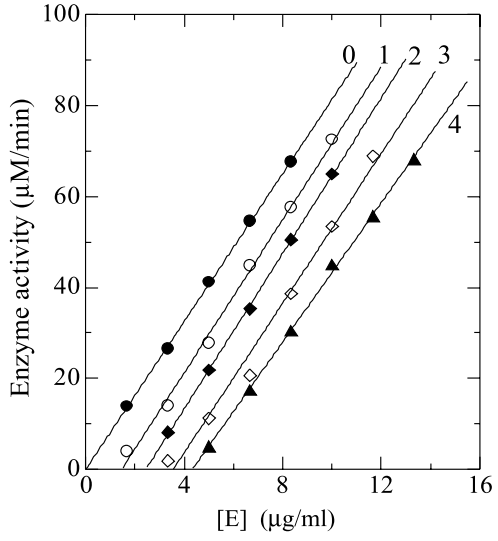


Figure 3. The effect of concentrations of 3,5-dihydroxyphenyl decanoate (DPD) on the activity of mushroom tyrosinase for the catalysis of DOPA at 30 °C. Assay conditions were as described in Figure 2 except that the final concentration of enzyme was variational. Concentrations of 3,5-dihydroxyphenyl decanoate for curves 0–4 were 0, 70, 80, 90, and 100 μM, respectively.

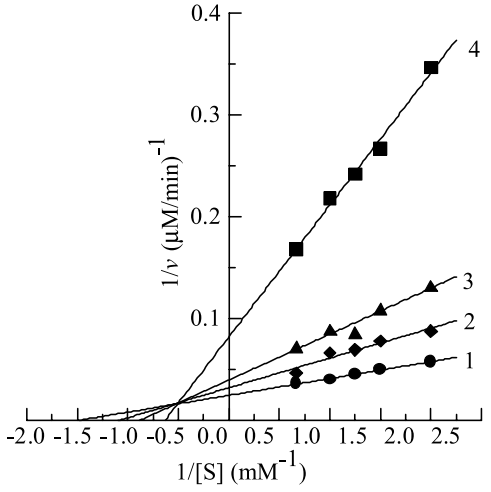


Figure 4. Lineweaver–Burk plots for inhibition of 3,5-dihydroxyphenyl decanoate (DPD) on the oxidation of DOPA by mushroom tyrosinase. Concentrations of DPD for curves 1–4 were 0, 70, 80, and 90 μM, respectively.

the enzyme by the plot of Lineweaver–Burk are shown in Figure 4. The results illustrated in Figure 4 show that the plots of Lineweaver–Burk yield a family of straight lines intersecting at the 2nd quadrant. Both K_m and V_m are affected and the value of K_m increased while the value of V_m decreased with an increase in DPD concentration. The results showed that DPD was a competitive and uncompetitive mixed-type inhibitor.

2.4. Kinetic course of the substrate reaction in the presence of different concentrations of DPD

The temporal variation of the product concentration during the oxidation of DOPA catalyzed by mushroom tyrosinase in the presence of different concentrations of DPD are shown in Figure 5. At each concentration of DPD, the rate decreased with increase in time until a straight line ran parallel with the X-axis, which indicated that the enzyme activity was fully lost. At given concentrations of DPD, when the reaction time goes beyond 900 s, the concentration of the product, [P], approached a constant final value $[P]_\infty$, which decreased with the increasing DPD concentrations. The results showed that the enzyme bound with DPD and underwent an irreversible inhibition.

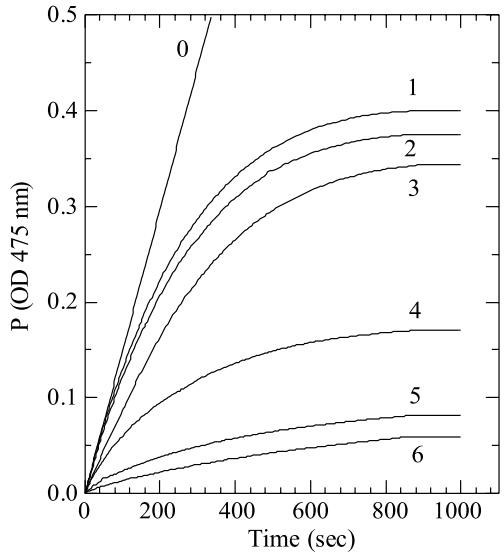
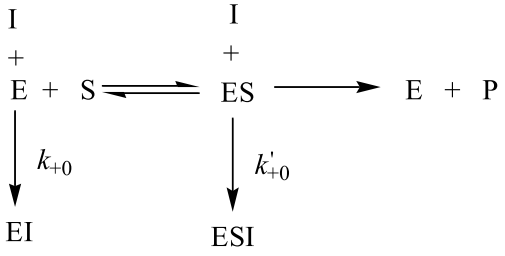


Figure 5. Courses for the substrate reaction of mushroom tyrosinase inhibited by different concentrations of 3,5-dihydroxyphenyl decanoate (DPD) with 1.0 mM of DOPA. Conditions were as in Figure 2 except that the concentration of DOPA was 1.0 mM. Concentrations of DPD for curves 0–6 were 0, 60, 65, 70, 80, 90, and 100 μM, respectively.

From the results of Figure 5, we can see that when the reaction time is sufficiently large, the product concentration, [P], approaches a constant final value $[P]_\infty$, which decreases with increase in concentrations of DPD. The results show that the inhibition of mushroom tyrosinase by DPD is an irreversible reaction without residual activity. This reaction scheme can be written as follows:



where S, I, P, and E are the substrate, inhibitor (DPD), product, and native enzyme, respectively. ES is enzyme–substrate complex. EI and ESI are inhibited enzyme complexes. The k_{+0} and k'_{+0} are the microscopic rate constants of free enzyme and enzyme–substrate complex inhibited by DPD, respectively. The inhibition reaction of DPD with the enzyme is irreversible. As is usually the case, $[S] \gg [E_0]$ and $[I] \gg [E_0]$ and the inhibition reactions are relatively slow compared with the setup of the steady state of the enzymatic reaction:

$$[E] = \frac{K_m}{K_m + [S]}[E_T],$$

$$[ES] = \frac{[S]}{K_m + [S]}[E_T],$$

where $[E_T] = [E] + [ES]$, $[E_T^*] = [EI] + [ESI]$, are, respectively, the total concentration of the active and inhibited enzymes, $[E_0] = [E_T] + [E_T^*]$; K_m is Michaelis constant. The rate of decrease of $[E_T]$ can be given by the following reaction:

$$-\frac{d[E_T]}{dt} = \frac{d[E_T^*]}{dt} = k_{+0}[E] + k'_{+0}[ES] = \frac{k_{+0}K_m + k'_{+0}[S]}{K_m + [S]}[E_T] = A[E_T].$$

$$\therefore \quad \frac{[E_T]}{[E_0]} = Ae^{-At},$$

A is the apparent rate constant for the inhibition.

$$\frac{v}{v_0} = \frac{[E_T]}{[E_0]}, v = v_0\frac{[E_T]}{[E_0]} = \frac{V_m[S]}{K_m + [S]}Ae^{-At},$$

$$\frac{d[P]}{dt} = v = \frac{V_m[S]}{K_m + [S]}Ae^{-At} = \frac{V_m[S]}{k_{+0}K_m + k'_{+0}[S]}e^{-At}.$$

The product concentration can be written as:

$$[P]_t = \frac{V_m[S]}{k_{+0}K_m + k'_{+0}[S]}(1 - e^{-At}) \quad (1)$$

and

$$A = \frac{k_{+0}K_m + k'_{+0}[S]}{K_m + [S]} \quad (2)$$

where $[P]_t$ is the concentration of the product formed at time t, which is the reaction time; A is the apparent rate constant of inactivation; [S] is the concentration of the substrate; K_m and V_m are the Michaelis constant and maximum velocity constant in the absence of DPD, respectively.

When the reaction time is sufficiently large, the product concentration, [P], approaches a constant final value $[P]_\infty$,

$$[P]_\infty = \frac{V_m[S]}{k_{+0}K_m + k'_{+0}[S]}. \quad (3)$$

and

$$\frac{1}{[P]_\infty} = \frac{k_{+0}K_m}{V_m}\frac{1}{[S]} + \frac{k'_{+0}}{V_m}. \quad (4)$$

Plots of $1/[P]_\infty$ against 1/[S] give a straight line with a slope of $k_{+0}K_m/V_m$ and an intercept of k'_{+0}/V_m. As K_m and V_m are known quantities, the microscopic rate constants k_{+0} and k'_{+0} can be calculated.

2.5. Determination of the microscopic rate constants of inhibition of the enzyme by DPD

The kinetic courses for the substrate oxidation in the presence of different DOPA concentrations during inactivation at 70 μM of DPD are shown in Figure 6. It can be seen that when the reaction time is sufficiently large, the concentration of the product, [P], approaches a constant final value $[P]_\infty$, which increases with the increasing substrate concentrations. According to Eq. 4, plot of $1/[P]_\infty$ versus 1/[S] (Fig. 6B) give a straight line with $k_{+0}K_m/V_m$ and k'_{+0}/V_m as the a slope and an intercept of the straight line, respectively. As K_m and V_m are known quantities from the measurement of the substrate reaction in the absence of inactivator (DPD) at different substrate concentrations, the apparent inactivation rate constants k_{+0} and k'_{+0} can be obtained from the slope and intercept of the straight line, respectively. The results obtained are listed in Table 1. For the other concentrations of DPD, plots of $1/[P]_\infty$ versus 1/[S] give a straight line with $k_{+0}K_m/V_m$ and k'_{+0}/V_m as the slope and intercept of the straight line, respectively. The apparent inactivation rate constants k_{+0} and k'_{+0} obtained are also summarized in Table 1.

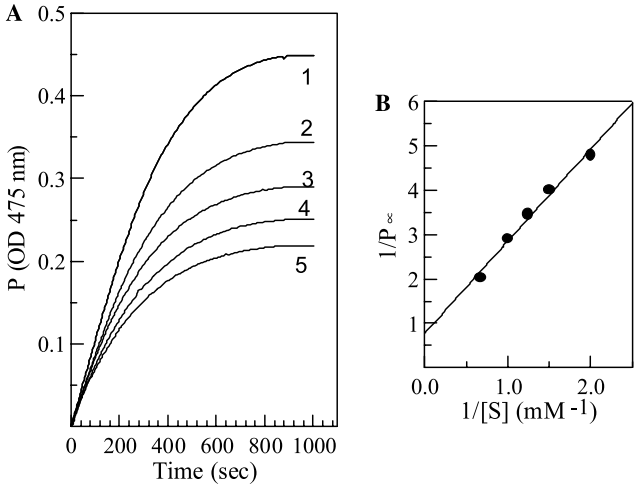


Figure 6. (A) Courses for the substrate reaction of mushroom tyrosinase inhibited by 70 μM 3,5-dihydroxyphenyl decanoate with different concentrations of substrate (DOPA). Experimental conditions were as in Figure 5 except the presence of substrate at different concentrations. For curves 1–5, the substrate concentrations were 1.5, 1.0, 0.80, 0.67, and 0.50 mM, respectively. (B) The plot of $1/[P]_\infty$ against 1/[S]. Final concentration of the enzyme was 6.67 μg/ml.

Table 1. Rate constants for inhibition of mushroom tyrosinase by 3,5-dihydroxyphenyl decanoate

3,5-Dihydroxyphenyl decanoate (μM)	$\frac{k_{+0}}{k'_{+0}}$	Rate constants ($\times 10^{-3}$ s^{-1})	
		k_{+0}	k'_{+0}
70	4.02	7.760	1.931
80	4.04	15.609	3.867
90	4.01	31.715	7.912

3. Discussion

Tyrosinase may play a role in neuromelanin formation in the human brain, particularly in the substantia nigra. This mixed function oxidase could be central to dopamine neurotoxicity as well as contribute to the neurodegeneration associated with Parkinson's disease.[15] Tyrosinase inhibitors have become increasingly important in medicinal[16] and cosmetic[17] products, primarily in relation to hyperpigmentation. It was reported that mushroom tyrosinase can be inhibited by quercetin[18] reversibly and the inhibition mechanism was shown to be competitive. Cetylpyridinium chloride (CPC) was found to inactivate the enzyme activity and induce the enzyme conformation changes.[9]

Tyrosinase catalyzes two distinct reactions of melanin synthesis, the hydroxylation of monophenol to *o*-diphenol (monophenolase activity) and the oxidation of *o*-diphenol to the corresponding *o*-quinone (diphenolase activity).[13] Quinones are easily polymerized spontaneously to form high-molecular weight compounds or

brown pigments (melanins).[19] It can also react with amino acids or proteins to form the brown color products. In the previous studies,[12] we found that hexylresorcinol and dodecylresorcinol are reversible inhibitors of mushroom tyrosinase, and the inhibition type is competitive. In this study, we found that the inhibition mechanism of 3,5-dihydroxyphenyl decanoate (DPD) was irreversible to the enzyme and the inhibition belongs to a mixed type, indicating that the inhibition behavior is completely different.

In our investigation, combining DPD with the enzyme molecule induced the enzyme activity loss. By the reaction scheme, the enzyme undergoes an irreversible inhibition course. The inhibition rate is limited by the EI and ESI concentrations. The inhibition reaction is a single molecule reaction and the apparent rate constant is dependent on DPD concentration. The experimental results can be fit very well to the predicted curves and the microkinetic constants have been determined.

The substrate reaction kinetic method described by Tsou[22] has been widely used in studies of inactivation of various enzymes and different types of inactivation or inhibition. However, complex inhibition kinetics is well established and most studies are focused on competitive reactions in which the enzyme will lose its activity and substrate binding ability after binding with the inhibitor. Here, we report on the irreversible inhibition of DPD with free enzymes and enzyme–substrate complex molecules. The inhibition rate constant of free enzyme (k_{+0}) is about four times as much as that of the enzyme–substrate complex (k'_{+0}). The inhibition rate constant of mushroom tyrosinase also is not linear with DPD concentration, indicating that the inhibition belongs to a complex type. For this irreversible inhibition, the traditional method has the disadvantage of possible dissociation of the complex because of dilution especially in the presence of the substrate and possible continued inactivation during enzyme activity assay.

4. Materials and methods

4.1. Materials

Mushroom tyrosinase (EC 1.14.18.1) was purchased from Sigma (USA). 3,5-Dihydroxyphenyl decanoate (DPD), nonyl-3,5-dihydroxybenzoate, L-3,4-dihydroxyphenylalanine (DOPA), and dimethyl sulfoxide (DMSO) were the products of Aldrich (USA). All other reagents were of analytical grade. The water in use was re-distillated and ion free.

4.2. Enzyme assays

Tyrosinase catalyzes a reaction between two substrates, a phenolic compound and oxygen, but the assay was carried out in air-saturated aqueous solutions. Tyrosinase catalyzes the oxidation of DOPA to *o*-DOPAquinone, which is characterized by an absorbance peak at 475 nm. In this investigation, DOPA was used as the substrate for the enzyme activity assay as previously described.[20] Enzyme activity was determined at 30 °C by following the increase in absorbance at 475 nm accompanying the oxidation of the substrate with the molar absorption coefficient of 3700 (M^{-1} cm^{-1}).[21] The enzymatic activity is defined as the oxidation reaction rate of DOPA (μM/min). The progress-of-substrate reaction method described by Tsou[22] was used for the study of the inhibition kinetics of mushroom tyrosinase. In this method, the mushroom tyrosinase (1.0 mg/ml in 0.1 M phosphate buffer, pH 6.8) was first diluted with water 50 times, and then 50 μl of the solution was added to 200 μl of an assay substrate solution with 25 μl DMSO containing different concentrations of DPD. The substrate reaction progress curve was analyzed to obtain the reaction rate constants. The reaction was carried out at a constant temperature of 30 °C. Absorption measurements were recorded using a Spectra MAX plus Microplate spectrophotometer.

Acknowledgments

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