Inhibitory Effects of Hexylresorcinol and Dodecylresorcinol on Mushroom (Agaricus bisporus) Tyrosinase

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The effects of hexylresorcinol and dodecylresorcinol on the monophenolase and diphenolase activity of mushroom tyrosinase have been studied. The results show that hexylresorcinol and dodecylresorcinol can inhibit both monophenolase and diphenolase activity of the enzyme. The lag period of the enzyme was obviously lengthened, and the steady-state activity of the enzyme decreased sharply. Two µM of hexylresorcinol and dodecylresorcinol can lengthen the lag period from 98 s to 260 and 275 s, respectively. Both hexylresorcinol and dodecylresorcinol can lead to reversible inhibition of the enzyme. The IC50 values of hexylresorcinol and dodecylresorcinol were estimated as 1.24 and 1.15 µM for monophenolase and as 0.85 and 0.80 µM for diphenolase, respectively. A kinetic analysis shows that hexylresorcinol and dodecylresorcinol are competitive inhibitors. The apparent inhibition constant for hexylresorcinol and dodecylresorcinol binding with free enzyme has been determined to be 0.443 and 0.405 µM for diphenolase, respectively.

KEY WORDS: Diphenolase; dodecylresorcinol; hexylresorcinol; inhibition; monophenolase; mushroom tyrosinase.

1. INTRODUCTION

Enzymatic browning in fruits and vegetables is predominantly catalyzed by a copper-containing enzyme, tyrosinase (EC.1.14.18.1) (Martínez and Whitaker, 1995; Mayer, 1987). This enzyme is widely distributed in microorganisms, animals, and plants. It catalyzes two distinct reactions of melanin synthesis, the hydroxylation of monophenol to o-diphenol (monophenolase activity) and the conversion of o-diphenol to the corresponding o-quinone (diphenolase activity) (Robb, 1984). Quinones are easily polymerized spontaneously to form high-molecular-weight compounds or brown pigments (melanins) (Prota, 1988). It also can react with amino acids or proteins to form the brown-color products. This unfavorable browning can cause deleterious changes in the appearance and organoleptic properties of raw fruits, vegetables, and beverage products and also decreased market-value. The browning becomes a major problem in the food industry and one of the main causes of quality loss during postharvest handling and processing. Therefore, the control of tyrosinase activity is of importance in preventing the synthesis of melanin in the browning of plants and animals.

It is well-known that tyrosinase can be inhibited by aromatic aldehydes, aromatic acids (Jiménez et al., 2001; Robit et al., 1997), tropolone (Espín and Wichers, 1999), and kojic acid (Kahn et al., 1997). 4-Hexylresorcinol has been known to be effective in preventing shrimp and

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Abbreviations: DMSO, dimethyl sulfoxide; l-DOPA, l-3,4-dihydroxyphenylalanine; Na2HPO4–NaH2PO4, disodium hydrogen phosphate–sodium dihydrogen phosphate buffer; vss, the steady-state rate; IC50, the inhibitor concentration leading to 50% activity lost; Km, Michaelis–Menten constant; Kmapp, apparent Michaelis–Menten constant.
frozen crab melanosis, and it has been recognized as safe for use in the browning control of fresh and hot-air-dried apple slices as well as potatoes, avocados, and grape juices (McEvily et al., 1991). In order to clarify this, the inhibitory kinetic study of hexylresorcinol and dodecylresorcinol on tyrosinase was performed in detail, as their kinetic behaviors have not yet been fully investigated. The aim of the current paper is, therefore, to carry out a kinetic study of the inhibition of the o-diphenolase and monophenolase activity of mushroom tyrosinase by hexylresorcinol and dodecylresorcinol and to evaluate the kinetic parameters and constants characterizing the system as a model.

2. MATERIALS AND METHODS

2.1. Reagents

Mushroom tyrosinase, hexylresorcinol, and dodecylresorcinol were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Dimethyl sulfoxide (DMSO), L-3,4-dihydroxyphenylalanine (L-DOPA), and L-tyrosine were the products of Aldrich Chemical (St. Louis, MO, USA). All other reagents were homemade analytical grade. The used water was re-distillated and ion-free.

2.2. Enzyme Assays

The diphenolase activity assay was performed as previously reported (Xie et al., 2003). The monophenolase assay was performed with l-tyrosine as substrate. A portion of the enzyme solution (0.1 ml) was added to the assay system (3 ml) containing 50 mM Na2HPO4–NaH2PO4 (pH 6.8), 0.5 mM L-tyrosine and the different concentrations of inhibitor, and the reaction mixture was immediately monitored with optical density at 475 nm for 10 min (after a lag period of 5 s). The molar absorption coefficient is calculated as 3700 M-1 cm-1 (Jiménez et al., 2001) accompanying the oxidation of the substrates (l-tyrosine and l-DOPA). Absorption was recorded using a Beckman (Fullerton, CA, USA) UV-650 spectrophotometer.

2.3. Inhibition of Hexylresorcinol and Dodecylresorcinol on the Enzyme

The inhibitor was first dissolved in DMSO and used for the experiment at 30 times dilution. The final concentration of DMSO in the test solution is 3.3%.

The extent of inhibition by the addition of the sample was expressed as the percentage necessary for 50% inhibition (IC50).

2.4. Determination of the Inhibition Type and the Inhibition Constant of Hexylresorcinol and Dodecylresorcinol on the Diphenolase Activity of Tyrosinase

In the assay system of diphenolase activity, we kept the concentrations of inhibitor (hexylresorcinol or dodecylresorcinol), changed the substrate (L-DOPA) concentrations, and determined the reaction initial rate. The concentrations of L-DOPA ranged from 0.20 to 0.67 mM. The inhibition type of hexylresorcinol or dodecylresorcinol on the enzyme was assayed by the Lineweaver–Burk plot. The inhibition constant was determined by the plot of the apparent Km app versus the concentration of the inhibitor.

3. RESULTS

3.1. Kinetics of Mushroom Tyrosinase

Tyrosinase can catalyze the hydroxylation of monophenols (monophenolase activity) and the oxidation of o-diphenols to o-quinones (diphenolase activity), both depending on molecular oxygen (Jiménez et al., 2001; Robb, 1984). The o-quinones evolve nonenzymatically to yield several unstable intermediates, which then polymerize to render melanins. When the diphenolase activity of tyrosinase was assayed by using L-DOPA as substrate, the reaction course immediately reached a steady-state rate (Fig. 1, curve 1). When the enzymatic reaction was started by the action of tyrosinase on L-tyrosine, a marked lag period characteristic of monophenolase activity was observed simultaneously with the appearance of the first stable product, dopachrome (Fig. 1, curve 3). The system reached a constant rate (the steady-state rate) after the lag period, which was estimated by extrapolation curve to the abscissa. The kinetic behavior of mushroom tyrosinase in the hydroxylation of L-tyrosine and oxidation of L-DOPA has been well studied. Under the conditions employed in the current study, the steady-state rate (vss) for the hydroxylation of L-tyrosine of the enzyme follows Michaelis–Menten kinetics (inset in Fig. 2). Kinetic parameters for the enzyme have been determined, and the results obtained are shown in Fig. 2 as a Lineweaver–Burk plot. The results showed
that \( K_m \) and \( V_m \) values are 0.387 ± 0.025 mM and 54.6 ± 2.0 \( \mu \)M/min, respectively. The oxidation of L-DOPA by the enzyme also follows Michaelis–Menten kinetics, and the \( K_m \) and \( V_m \) values were determined to be 0.493 ± 0.035 mM and 134.3 ± 4.0 \( \mu \)M/min, respectively.

### 3.2. Effects of Hexylresorcinol and Dodecylresorcinol on the Diphenolase Activity of Mushroom Tyrosinase

As the diphenolase activity of tyrosinase was assayed by using L-DOPA as substrate, the reaction course immediately reached a steady-state rate (Fig. 1, curve 1). When 1.2 \( \mu \)M of hexylresorcinol was added into the assay medium, the time course of the enzymatic reaction proceeded as shown in curve 2 in Fig. 1. The slope of the straight line descended, indicating that hexylresorcinol can inhibit the diphenolase activity of the enzyme. In addition, hexylresorcinol also inhibited the monophenolase activity of tyrosinase. As can be seen from curve 4 in Fig. 1, hexylresorcinol extended the lag period and decreased the steady-state rate of tyrosinase when using L-tyrosine as substrate. Taking dodecylresorcinol as inhibitor, the same results were obtained.

### 3.3. Concentration Effects of Hexylresorcinol and Dodecylresorcinol on the Monophenolase Activity of Tyrosinase

The inhibitory effects of the different concentrations of hexylresorcinol and dodecylresorcinol on the oxidation of L-tyrosine by tyrosinase were studied. The kinetics course of the oxidation of the substrate in the presence of different concentrations of hexylresorcinol is shown in Fig. 3. The lag period increased exponentially with increase in the concentration of hexylresorcinol as shown in Fig. 4. The lag period was estimated to be 98 s in the absence of this inhibitor and extended to 260 s in the presence of 2.0 \( \mu \)M of hexylresorcinol. The lag period lengthened 2.6 times. On the other hand, the steady-state rate decreased straight with increasing the concentration of hexylresorcinol as
shown in Fig. 4. When the concentration of this inhibitor reached 2.0 µM, the remaining enzyme activity was determined to be 30%. The inhibitor concentration leading to 50% activity lost (IC50) was estimated to be 1.24 µM. For the same test of dodecylresorcinol as inhibitor, the IC50 was tested to be 1.15 µM. The results obtained are summarized in Table 1. The results indicated that hexylresorcinol and dodecylresorcinol inhibited the hydroxylation of L-tyrosine (monophenolase activity) well.

3.4. Concentration Effects of Hexylresorcinol and Dodecylresorcinol on the Diphenolase Activity of Tyrosinase

The inhibitory effects of the different concentrations of hexylresorcinol and dodecylresorcinol on...
the oxidation of L-DOPA by tyrosinase were studied. The inhibitory course is shown in Fig. 5. With increasing the concentrations of hexylresorcinol and dodecylresorcinol, the diphenolase activity of tyrosinase markedly decreased. Inhibition of the enzyme by hexylresorcinol or dodecylresorcinol was also concentration-dependent. From Fig. 5, the IC50 of hexylresorcinol and dodecylresorcinol for the diphenolase was estimated to be 0.85 µM and 0.80 µM, respectively.

3.5. The Reversible Inhibition of Hexylresorcinol and Dodecylresorcinol on the Diphenolase Activity of Tyrosinase

Taking hexylresorcinol and dodecylresorcinol as effectors, we studied their inhibition mechanism on the enzyme for the oxidation of L-DOPA. The plots of the remaining enzyme activity versus the concentrations of enzyme at different effector concentrations gave a family of straight lines that all passed through the origin (Fig. 6). Increasing the effector concentration resulted in the descending of the slope of the line, indicating that the inhibition of hexylresorcinol and dodecylresorcinol on the enzyme was a reversible reaction course. The presence of these inhibitors did not bring down the amount of the efficient enzyme, but just resulted in the inhibition and the descending of the activity of the enzyme for oxidation of L-DOPA.

3.6. Inhibition of Hexylresorcinol and Dodecylresorcinol on the Diphenolase Activity Following Competitive Mechanism

The kinetic behavior of mushroom tyrosinase during the oxidation of L-DOPA has been studied. Under the conditions employed in the current investigation, the oxidation reaction of L-DOPA by tyrosinase follows Michaelis–Menten kinetics. In the presence of hexylresorcinol, the kinetic studies of the enzyme by the Lineweaver–Burk plot are shown in Fig. 7. The results illustrated in Fig. 7 show that hexylresorcinol was a competitive inhibitor, as increasing the hexylresorcinol concentration resulted in a family of lines with a common intercept on the 1/ν axis but with different slopes. The equilibrium constant for inhibitor binding with free enzyme, $K_I$, was obtained from a plot of the apparent Michaelis–Menten constant ($K_{mapp}$) versus the concentration of hexylresorcinol, which was linear as shown in the inset. The obtained constant is 0.46 µM, summarized in Table 1. Similar results were obtained with dodecylresorcinol. Secondary plot of the apparent Michaelis–Menten constant ($K_{mapp}$) versus the concentration of dodecylresorcinol was also a straight line. The inhibition constant of dodecylresorcinol
obtained from the experimental data is also given in the Table 1.

4. DISCUSSION

Tyrosinase catalyzes a reaction between two substrates, a phenolic compound and oxygen, but the assay was carried out in air-saturated aqueous solutions. Therefore, $K_m$ and $V_m$ values determined in these conditions were only apparent, and the effect of oxygen concentration on these parameters is unknown. As we know, tyrosinase has two distinct kinds of catalysis functions: the hydroxylation of monophenols and the oxidation of $o$-diphenols. In the processing of catalysis, it has three existing forms, $E_{\text{met}}$, $E_{\text{oxy}}$, and $E_{\text{deoxy}}$ (Sanchez-Ferrer et al., 1995). Both the $E_{\text{met}}$ form and the $E_{\text{oxy}}$ form have the activity of oxidation of $o$-diphenols; the $E_{\text{met}}$ form has the activity of hydroxylation of monophenols; and the $E_{\text{deoxy}}$ form could combine with oxygen (Sanchez-Ferrer et al., 1995).

When the inhibitor combines with the $E_{\text{met}}$ form to become $E_{\text{metI}}$, it will decrease the steady-state rate of the diphenolase activity and extend the lag period of the monophenolase activity. When the inhibitor combines with the $E_{\text{oxy}}$ form to become $E_{\text{oxyI}}$, it will decrease the steady-state rate both of the diphenolase activity and the monophenolase activity. In this paper, we took L-tyrosine as substrate for the monophenolase and L-DOPA for the diphenolase activity of the enzyme. The effects of hexylresorcinol on the monophenolase and the diphenolase activity of tyrosinase were studied. The results showed that hexylresorcinol could lengthen the lag period of the monophenolase and decrease the steady-state rate of both monophenolase and diphenolase. The inhibition was displayed as reversible, and the inhibition type was determined to be competitive. It is likely that hexylresorcinol and dodecylresorcinol serve as structural analogues of the substrate ($o$-diphenol) and combine with the free enzyme to form $E_{\text{oxyI}}$ and $E_{\text{metI}}$ complexes. The results indicated that the inhibitory effect depends on the structural part of $m$-diphenol of hexylresorcinol or dodecylresorcinol.

Hexylresorcinol has been used to prevent shrimp and crab melanosis, and it has been recognized as safe for use in the browning control of fresh and hot-air-dried apple slices as well as potatoes, avocados, and grape juices (McEvily et al., 1991). But the depigmenting action mechanism has not yet been fully investigated. Needless to say, the reaction time and amount of oxygen needs to be considered from a practical point of view. Moreover, the results still need to be tested on the biological system. Despite this, it appears that characterization of tyrosinase inhibitors using mushroom tyrosinase is a simple but effective strategy for the initial screening to search for depigmenting agents.

It can be summarized that hexylresorcinol and dodecylresorcinol, which are not oxidized to the corresponding $o$-quinones, act as depigmenting agents. It seems that the nonoxidizable nature of $m$-diphenol can inhibit monophenolase and diphenolase activity of the tyrosinase.

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