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Inhibitory effects of phloridzin dihydrate on the activity of mushroom (*Agaricus bisporus*) tyrosinase

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Abstract—The inhibitory effects of phloridzin dihydrate on the activity of mushroom tyrosinase have been studied. The results show that phloridzin can inhibit the diphenolase activity of the enzyme and the inhibition displays to be reversible. The IC_{50} value was estimated as 110 μM. The kinetic analysis showed that the inhibition of phloridzin on the diphenolase activity of the enzyme is of competitive type, and the inhibition constant (K_I) was determined to be 64.3 μM. The inhibitory effects of the different concentrations of phloridzin on the monophenolase activity were also studied. There were almost no changes in the lag period and the steady-state rate, while the plateaus in the inhibitory curve lowered with increasing the concentration of phloridzin when using tyrosine as a substrate.

1. Introduction

Tyrosinase, also known as polyphenol oxidase (PPO),[1,2] is a copper-containing oxygenase widely distributed in microorganisms, animals, and plants. Three different forms of binuclear coppers in the active site are involved in the reaction.[3] It is responsible not only for melanization in animals but also browning in plants. The latter case is considered to be deleterious to the color quality of plant-derived foods and beverages. This unfavorable darkening from enzymatic oxidation of phenols generally results in a loss of nutritional value and has been of great concern.[4] The inhibitors of this enzyme should be clinically useful for the treatment of some dermatological disorders associated with melanin hyperpigmentation[5] and is also important in cosmetics for whitening and depigmentation after sunburn.[6] In addition, tyrosinase is known to be involved in the molting process of insects[5] and adhesion of marine organisms.[7] Hence, tyrosinase inhibitors should have broad applications.

An important group of browning inhibitors is constituted by compounds structurally analogous to phenolic substrate. These generally show competitive inhibition toward these substrates, although such inhibition may vary depending on the enzyme source and the substrate used.[8,9] Among these groups, L-mimosine,[10] tropolone,[11] kojic acid,[12] and 4-substituted resorcinols[13] have been described as competitive slow-binding inhibitors, according to the classification of reversible enzyme inhibitors established by Morrison.[14] In our continuing investigation of tyrosinase inhibitors, some flavonoids were found to inhibit the *o*-diphenolase activity of mushroom tyrosinase, and almost all of them showed a reversible reaction and served as a competitive inhibitor for the oxidation of L-3,4-dihydroxyphenylalanine.[15] In our efforts to develop new, low-cost, easy-to-prepare, and high inhibitory effect of the enzyme inhibitor, phloridzin dihydrate was found to inhibit the *o*-diphenolase activity of mushroom tyrosinase. The aim of this present experiment was, therefore, to carry out a kinetic study of the inhibition of the diphenolase activity of tyrosinase by phloridzin and to evaluate the kinetic parameters and inhibition constants characterizing the system.

Abbreviations: PPO, polyphenol oxidase; DMSO, Dimethylsulfoxide; Tyr, tyrosine; L-DOPA, L-3,4-dihydroxyphenylalanine; IC_{50}, the inhibitor concentration leading to 50% activity lost.

Keywords: Mushroom tyrosinase; Diphenolase activity; Inhibition; Phloridzin dihydrate.

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2. Results

2.1. Concentration effects of phloridzin on the diphenolase activity of tyrosinase

Diphenolase activity of mushroom tyrosinase was assayed using L-DOPA as substrate. The progress curve of enzyme reaction was a line passing through the origin without lag period. The formation of the product was in proportion to reaction time. The value of the slope of the line indicated the diphenolase activity. Phloridzin dihydrate (see Fig. 1 for the structure) was tested for the effect on the oxidation of L-DOPA by mushroom tyrosinase. The result showed that phloridzin can inhibit the diphenolase activity as shown in Figure 2. With increasing the concentration of phloridzin, the diphenolase activity of mushroom tyrosinase decreased with concentration-dependence. From Figure 2, the inhibitor concentration leading to 50% activity loss (IC_{50}) of phloridzin was estimated to be 110 μM.

2.2. Inhibition of phloridzin on the diphenolase activity of tyrosinase showed to be reversible

Taking phloridzin as an inhibitor, we studied its 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 inhibitor concentrations gave a family of straight lines, which all passed through the origin (Fig. 3). Increasing the inhibitor concentrations resulted in the descent of the slope of the line, indicating that the inhibition of phloridzin on the enzyme undertook a reversible reaction course. The presence of the inhibitor did not bring down the amount of the efficient enzyme, but just resulted in the inhibition and the descent of enzyme activity for the oxidation of L-DOPA.

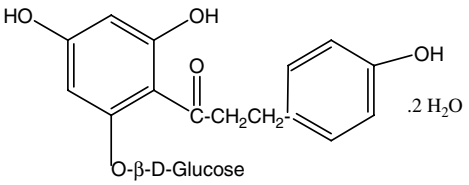

Figure 1. Chemical structure of phloridzin dehydrate.

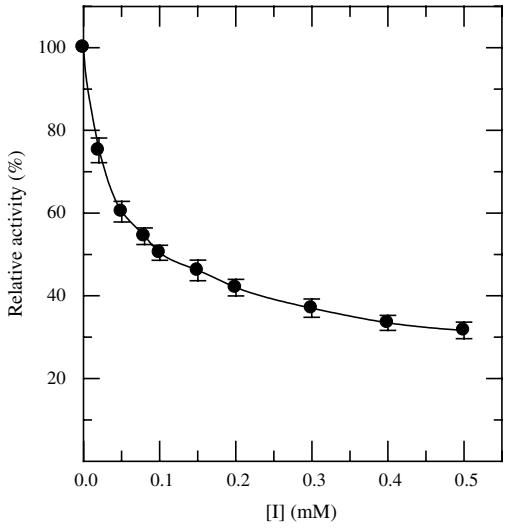

Figure 2. The inhibitions of phloridzin dihydrate on the diphenolase activity of mushroom tyrosinase for the catalysis of DOPA at 30 °C. Assay conditions: 3.0 ml reaction systems contained 50 mM Na_2HPO_4–NaH_2PO_4 buffer (pH 6.8), 0.5 mM DOPA, and 3.3% DMSO. The concentration of mushroom tyrosinase was 6.67 μg/ml.

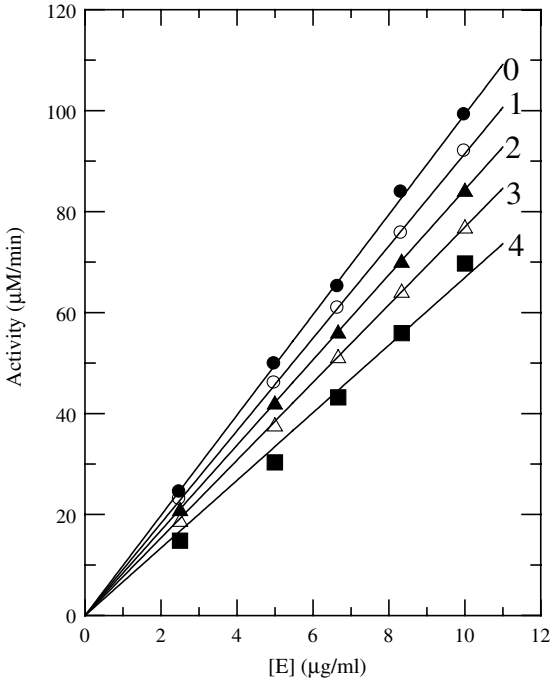

Figure 3. The effect of concentrations of mushroom tyrosinase on its activity for the catalysis of DOPA at different concentrations of phloridzin dihydrate. Concentrations of phloridzin dihydrate for curves 0–6 were 0, 20, 40, 60, and 80 μM, respectively.

2.3. Inhibition type and inhibition constants of phloridzin on the diphenolase activity of mushroom tyrosinase

The kinetic behavior of mushroom tyrosinase during the oxidation of L-DOPA has been studied. Under the conditions employed in the present investigation, the oxidation reaction of L-DOPA by mushroom tyrosinase follows Michaelis–Menten kinetics. In the presence of phloridzin dihydrate, the kinetic studies of the enzyme by the plot of Lineweaver–Burk are shown in Figure 4. The results showed that phloridzin was a competitive inhibitor since increasing the phloridzin concentration resulted in a family of lines with a common intercept on the $1/v$ 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_m) vs the concentration of phloridzin dihydrate, which is a linear as shown in the inset. The obtained constant was 64.3 μM. The results of kinetic constants and inhibition constants are summarized in Table 1.

2.4. Inhibitory effects of phloridzin on the monophenolase activity of mushroom tyrosinase

Tyrosinase can catalyze the hydroxylation of monophenols (monophenolase activity) and the oxidation of

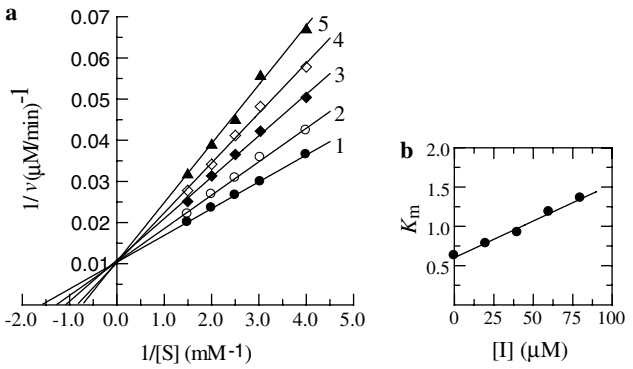

Figure 4. Lineweaver–Burk plots for inhibition of phloridzin dihydrate (a) on the oxidation of DOPA by mushroom tyrosinase. Concentration of phloridzin dihydrate for curves 1–5 was 0, 20, 40, 60, and 80 μM, respectively. (b) The inset represents the plot of K_m versus the concentration of phloridzin dihydrate to determine the inhibition constant. The line is drawn using linear least-squares fit.

Table 1. Kinetic constants and inhibition constants of phloridzin dihydrate on mushroom tyrosinase

Constants	
IC_{50} (μM)	110
K_m (mM)	0.627
V_m (μM/min)	96.33
Inhibition	Reversible
Inhibition type	Competitive
K_I (μM)	64.3

o-diphenols to *o*-quinones (diphenolase activity), both depending on molecular oxygen.[16,17] The *o*-quinones evolve non-enzymatically 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. When the enzymatic oxidation reaction used tyrosine as substrate, a marked lag period, characteristic of monophenolase activity, was observed simultaneously with the appearance of the first stable product, dopachrome. The system reached a constant rate (the steady-state rate) after the lag period, which was estimated by extrapolation curve to the abscissa.[18] The inhibitory effects of the different concentrations of phloridzin on the oxidation of tyrosine by the enzyme were studied. The kinetics course of the oxidation of the substrate in the presence of different concentrations of phloridzin is shown in Figure 5. The lag periods and the steady-state rates almost kept the same with increasing the concentration of phloridzin when using tyrosine as substrate. But, it should be noted that, after several minutes, dopachrome formation reached the plateau as all the available oxygen in the cuvette was consumed.[19] As shown in Figure 5, the plateaus in the inhibitory curve lowered with increasing the concentration of phloridzin and the differences indirectly demonstrate the amount of oxygen in the cuvette used for the oxidation of phloridzin. The results indicated that phloridzin consumed the available oxygen to inhibit the monophenolase activity for the oxidation of tyrosine instead of prolonging the lag periods and decrease the steady-state rates of the enzyme.

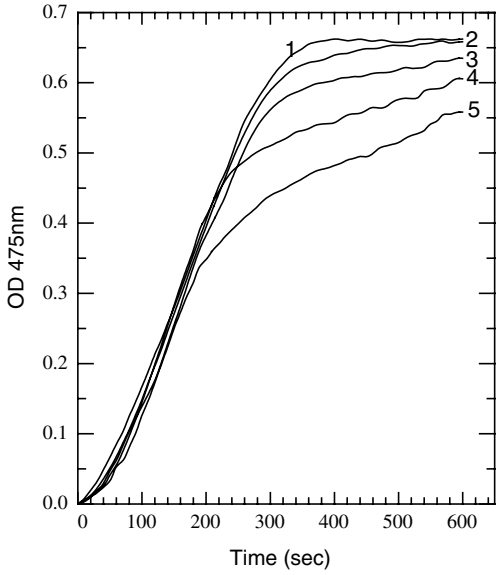

Figure 5. Course of the oxidation of Tyr by mushroom tyrosinase in the presence of different concentrations of phloridzin dihydrate. Assay conditions were 3 ml reaction system containing 50 mM Na_2HPO_4–NaH_2PO_4 buffer (pH 6.8), 0.5 mM L-tyrosine, 33.3 μg/ml of enzyme, and 3.3% DMSO. The concentration of phloridzin dihydrate for curves 1–5 was 0, 20, 40, 60, and 80 μM, respectively.

3. Discussion

Phenolic compounds are efficient antioxidants acting as free radical terminators or metal chelators. Among them, flavonoids, which are derivatives of benzopyrone, are particularly interesting as natural antioxidants for foods and cosmetics. Flavonoids generally occur as glycosylated derivatives in plants where they contribute to the brilliant shades of leaves, flowers, and fruits. Besides their antioxidant activity, some of them have a wide variety of physiological effects in both animals and plants as enzyme activators or inhibitors, transcription regulators, and phytohormones. Phloridzin is a flavonoid glucoside of dihydrochalcone family which is found in *Malus* species (up to 10% of dry weight in young apple leaves and twigs). This flavonoid presents interesting biological activities such as inhibitor of glucose adsorption by cell.[20]

In the paper, we took L-DOPA as substrate for the diphenolase activity and Tyr for the monophenolase activity of the enzyme. The effects of phloridzin on the diphenolase activity and the monophenolase activity of tyrosinase were studied. The results showed that phloridzin could inhibit the diphenolase activity of mushroom tyrosinase. For the diphenolase activity, the inhibition displayed as reversible and the inhibition types were determined to be competitive.

Xie et al.[15] have investigated the inhibition effects of some flavonoids on the activity of mushroom tyrosinase and their results showed that flavonols quercetin, galangin, fisetin, 3,7,4′-trihydroxyflavone, and morin are competitive inhibitors of the enzyme, and their inhibition constants were 29, 58, 75, 154, and 410 μM, respectively. The inhibition constant of phloridzin dehydrate

was 64.3 μM, which is similar to those of galangin and fisetin. Presumably, their inhibition comes from their ability to chelate copper in the active center of the enzyme. The chelation reaction was reversible. The difference in tyrosinase inhibitory activity of flavonoids can be explained by the presence of an intramolecular hydrogen bond between hydroxyl groups which interferes with the chelation of copper in the enzyme involving the hydroxyl and carbonyl groups.

Flavonols exist in many edible plants. Tyrosinase inhibitors isolated from edible plants may be superior to non-natural products. Despite this advantage, the biological significance of flavonols as tyrosinase inhibitors in living systems is still largely speculative. Further work is needed to resolve this issue.

4. Materials and methods

4.1. Chemicals and reagents

Mushroom tyrosinase (EC 1.14.18.1) and phloridzin dihydrate (from apple wood, used to induce experimental glycosuria, dihydrochalcone glycoside found in apple tree) were purchased from Sigma (St. Louis, MO, USA). Dimethylsulfoxide (DMSO), tyrosine (Tyr), and L-3,4-dihydroxyphenylalanine (L-DOPA) were the products of Aldrich (St. Louis, MO, USA). All other reagents were of homemade analytical grade. The used water was re-distillated and made ion-free.

4.2. Assay of the tyrosinase activity

The monophenolase activity and diphenolase activity assay was performed as reported by Chen et al.[3] In this investigation, Tyr was used as the substrate for the monophenolase activity assay, and L-DOPA was used as the substrate for the diphenolase activity assay. The reaction media (3 ml) for activity assay contained 2.0 mM Tyr or 0.5 mM L-DOPA in 50 mM Na_2HPO_4–NaH_2PO_4 buffer (pH 6.8). The final concentration of mushroom tyrosinase was 33.33 μg/ml for the monophenolase activity and 6.67 μg/ml for the *o*-diphenolase activity. The enzyme activity was determined by following the increasing absorbance at 475 nm accompanying the oxidation of the substrates with a molar absorption coefficient of 3700 (M^{-1} cm^{-1})[21] by using a Beckman UV-650 spectrophotometer. Enzymatic activity was defined as 1 μM of dopachrome per minute. The temperature was controlled at 30 °C. Phloridzin dihydrate was dissolved in DMSO and the final concentration of DMSO in the test solution is 3.3%. Controls, without inhibitor but containing 3.3% DMSO, were routinely carried out. The extent of inhibition by the addition of the sample was expressed as the percentage necessary for 50% inhibition (IC_{50}). The inhibition type was assayed by the Lineweaver–Burk plot, and the inhibition constant was determined by the second plots of the apparent K_m/V_m or $1/V_m$ versus the concentration of the inhibitor.

Acknowledgments

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