Biochemistry (Moscow), Vol. 68, No. 4, 2003, pp. 487-491. Translated from Biokhimiya, Vol. 68, No. 4, 2003, pp. 598-602.

Inhibitory Effects of Some Flavonoids on the Activity of Mushroom Tyrosinase

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Received June 18, 2002
Revision received August 21, 2002

Abstract—Mushroom tyrosinase (EC 1.14.18.1) is a copper containing oxidase that catalyzes both the hydroxylation of tyrosine into *o*-diphenols and the oxidation of *o*-diphenols into *o*-quinones, and then forms brown or black pigments. In the present study, the effects of some flavonoids on the oxidation of L-3,4-dihydroxyphenylalanine (L-DOPA) have been studied. The results show that flavonoids can lead to reversible inhibition of the enzyme. A kinetic analysis showed that the flavonols are competitive inhibitors, whereas luteolin is an uncompetitive inhibitor. The rank order of inhibition was: quercetin > galangin > morin; fisetin > 3,7,4′-trihydroxyflavone; luteolin > apigenin > chrysin.

Key words: mushroom tyrosinase, flavonoids, inhibitory mechanism

Tyrosinase (EC 1.14.18.1) is a copper containing mixed-function oxidase. It is widely distributed in microorganisms, animals, and plants. This enzyme catalyzes both the hydroxylation of monophenols and the oxidation of *o*-diphenols into *o*-quinones, which then polymerize to form brown or black pigments [1-5]. In some vegetables and fruits, tyrosinase is responsible for browning and is considered to be deleterious to the color quality of plant-derived foods and beverages. This unfavorable darkening from enzymatic oxidation generally results in a loss of nutritional and market values. In animals, tyrosinase is responsible for melanization. Tyrosinase has three forms: *met*, *deoxy*, and *oxy* [6]. Structural models for the active site of these three enzyme forms have been proposed [7]. Mushroom tyrosinase, with a molecular mass of 120 kD, is composed of two H subunits (43 kD) and two L subunits (13 kD) and contains two active sites [8].

Tyrosinase inhibitors have attracted concern recently due to hyperpigmentation [9] resulting from the increased use of tyrosinases in medicinal [10] and cosmetic products [11]. Hence there is interest in tyrosinase inhibitors, which should have broad applications. It is well known that mushroom tyrosinase can be inhibited by aromatic aldehydes and acids [12], tropolone, and kojic acid [13]. Recently, copper chelators have been targeted for inhibition of the enzyme. The characterization of quercetin was reported as a principal tyrosinase inhibitor [14]. This common flavonoid was also noted to inhibit mushroom tyrosinase in the oxidation of L-3,4-dihydroxyphenylalanine (L-DOPA) [15]. In the present investigation, a number of flavonoids were compared for their effects on mushroom tyrosinase: their inhibitory kinetics and the mechanism involved were investigated.

MATERIALS AND METHODS

Chemicals. 3,5,7,3′,4′-Pentahydroxyflavone (quercetin, **a**), 3,5,7-trihydroxyflavone (galangin, **b**), 3,7,3′,4′-quatrihydroxyflavone (fisetin, **c**), 3,7,4′-trihydroxyflavone (**d**), 3,5,7,4′,6′-pentahydroxyflavone (morin, **e**), 5,7,3′,4′-quatrihydroxyflavone (luteolin, **f**), 5,7,4′-trihydroxyflavone (apigenin, **g**), 5,7-dihydroxyflavone (chrysin, **h**), quercetin-3-rutinoside (**i**), and dimethyl sulfoxide (DMSO) were purchased from Sigma (USA). Tyrosinase (EC 1.14.18.1) from mushroom was also purchased from Sigma. The specific activity of the enzyme was 6680 U/mg. L-3,4-Dihydroxyphenylalanine (L-DOPA) was from Aldrich (USA). All other reagents were of analytical grade. Double-distilled, ion-free water was used throughout.

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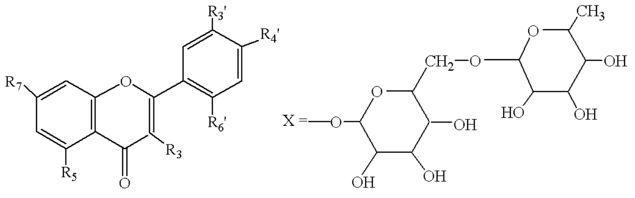
Enzyme assay. Tyrosinase catalyzes the reaction between two substrates, a phenolic compound and oxygen. The assay was carried out in air-saturated solutions. The enzyme activity was monitored by dopachrome formation at 475 nm ($\varepsilon = 3700\ M^{-1}\cdot cm^{-1}$) [16] accompanying the oxidation of the substrate (L-DOPA). The assay was performed as previously described [17] with slight modifications. The reaction media (3 ml) for *o*-diphenolase activity contained 0.5 mM L-DOPA in 50 mM sodium phosphate buffer (pH 6.8), the indicated concentration of inhibitor, and 3.3% DMSO. The final concentration of mushroom tyrosinase was 6.67 µg/ml. In this method, 0.1 ml of different concentrations of effector dissolved in DMSO solution was added into the test tube. Then, 2.8 ml substrate solution in sodium phosphate buffer preincubated at 30°C was mixed and 0.1 ml of the aqueous solution of mushroom tyrosinase (20 µg) added. This solution was immediately monitored for 1 min (after a lag period of 5 sec) for the formation of dopachrome by measuring the linear increase in absorbance at 475 nm. The reaction was carried out at constant temperature of 30°C. Absorption was recorded using a Beckmann UV-600 spectrophotometer (Germany). The kinetic and inhibition constants were obtained by the method previously described [18-21].

RESULTS

Effects of the flavonoids on the activity of mushroom tyrosinase. The flavonoids quercetin (**a**), galangin (**b**), fisetin (**c**), 3,7,4′-trihydroxyflavone (**d**), morin (**e**), luteolin (**f**), apigenin (**g**), chrysin (**h**), and quercetin-3-rutinoside (**i**) (see Fig. 1 for structures) were tested for their effects on the oxidation of L-DOPA by mushroom tyrosinase. Compounds **a**, **b**, **c**, **d**, **e**, **f**, and **g** showed inhibitory effects on the enzyme activity (Fig. 2). Among these flavonoids, quercetin was the most potent inhibitor, galangin the second and fisetin the third most potent. The IC_{50} values were estimated as 50 (**a**), 101 (**b**), 130 (**c**), 240 (**f**), 270 (**d**), and 720 µM (**e**). In comparison, apigenin (**g**) exhibited only a 20% inhibition at 150 µM, and the degree of inhibition remained unchanged at higher concentration. It was noted that compound **g** was only partially soluble in the assay medium. The assay medium was turbid in the presence of 200 µM (**g**) and above, which influenced the accuracy of the enzyme assay.

Inhibitory mechanism of mushroom tyrosinase by flavones. Using quercetin (**a**), galangin (**b**), fisetin (**c**), 3,7,4′-trihydroxyflavone (**d**), morin (**e**), and luteolin (**f**) as the effectors, we studied their mechanism of inhibition of oxidation of L-DOPA. Plots of the remaining enzyme activity versus enzyme concentration at different effector concentrations gave a family of straight lines which passed through the origin (Fig. 3), indicating that the inhibition by these flavonoids was reversible. Other effectors exhibited similar behavior. They were all reversible inhibitors of the oxidation of L-DOPA by mushroom tyrosinase.

Inhibition of the enzyme activity by quercetin (a), galangin (b), fisetin (c), 3,7,4′-trihydroxyflavone (d), and morin (e) is competitive. The kinetic behavior of mushroom tyrosinase during the oxidation of L-DOPA was studied. Under the conditions employed in the present



a: $R_3 = R_5 = R_7 = R_3' = R_4' = OH;\ R_6' = H$
b: $R_3 = R_5 = R_7 = OH;\ R_3' = R_4' = R_6' = H$
c: $R_3 = R_7 = R_3' = R_4' = OH;\ R_5 = R_6' = H$
d: $R_3 = R_7 = R_4' = OH;\ R_5 = R_3' = R_6' = H$
e: $R_3 = R_5 = R_7 = R_4' = R_6' = OH;\ R_3' = H$
f: $R_5 = R_7 = R_3' = R_4' = OH;\ R_3 = R_6' = H$
g: $R_5 = R_7 = R_4' = OH;\ R_3 = R_3' = R_6' = H$
h: $R_5 = R_7 = OH;\ R_3 = R_3' = R_4' = R_6' = H$
i: $R_5 = R_7 = R_3' = R_4' = OH;\ R_6' = H;\ R_3 = OX$

Fig. 1. Chemical structure of some flavonoids: quercetin (**a**), galangin (**b**), fisetin (**c**), 3,7,4′-trihydroxyflavone (**d**), morin (**e**), luteolin (**f**), apigenin (**g**), chrysin (**h**), and quercetin-3-rutinoside (**i**).

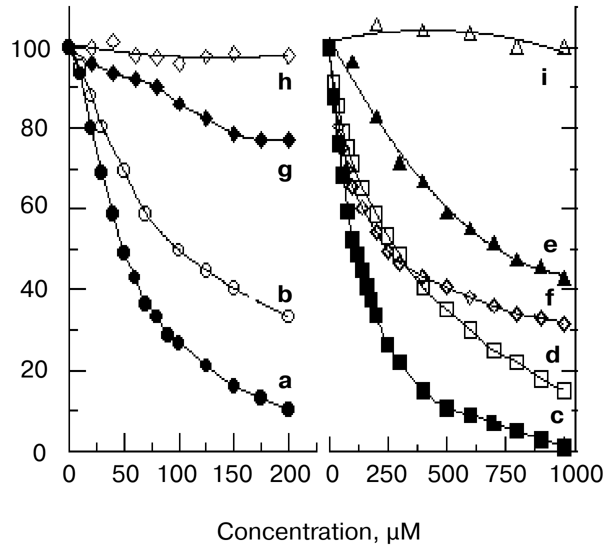


Fig. 2. Inhibition of mushroom tyrosinase by flavonoids (letter designations are the same as in Fig. 1 legend).

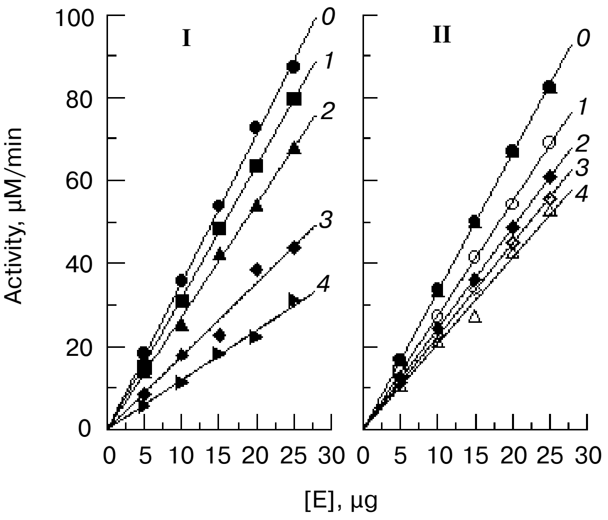


Fig. 3. Catalytic activity of mushroom tyrosinase at different enzyme concentrations and different concentrations of quercetin (I) and luteolin (II). Concentrations of quercetin for curves *0-4* were 0, 10, 20, 30, and 40 μM, respectively. Concentrations of luteolin for curves *0-4* were 0, 25, 50, 75, and 100 μM, respectively.

investigation, the oxidation of L-DOPA by mushroom tyrosinase followed Michaelis–Menten kinetics. The results illustrated in Fig. 4 showed that quercetin was a competitive inhibitor. The equilibrium constant for inhibitor binding with free enzyme, K_I, was obtained from a plot of the apparent Michaelis–Menten constant (K_m^{app}) versus the concentration of quercetin, which was linear as shown in the inset. Similar results were obtained with compounds **b**, **c**, **d**, and **e**.

Inhibition of the enzyme by luteolin (f) is uncompetitive. Inhibition of mushroom tyrosinase by luteolin (**f**) is illustrated in Fig. 5. Double-reciprocal plots yield a family of parallel straight lines with the same slopes, which indicated that luteolin is an uncompetitive inhibitor of the enzyme. This behavior is observed when the inhibitor binds at a site distinct from the substrate site and combines with the enzyme–substrate complex (ES) but not with the free enzyme (E). The equilibrium constant for inhibitor binding with the enzyme–substrate complex (ES), K_{IS} was obtained from a plot of the vertical intercept ($1/V_{max}^{app}$) versus the concentration of luteolin (see Fig. 5 inset).

DISCUSSION

Tyrosinase has two distinct catalytic functions: the hydroxylation of monophenol and the oxidation of *o*-diphenol. This paper reports on the effects of some flavonoids on the oxidation of L-DOPA by mushroom tyrosinase. The results show that flavonols quercetin (**a**), galangin (**b**), fisetin (**c**), 3,7,4′-trihydroxyflavone (**d**), and morin (**e**) are competitive inhibitors of the enzyme.

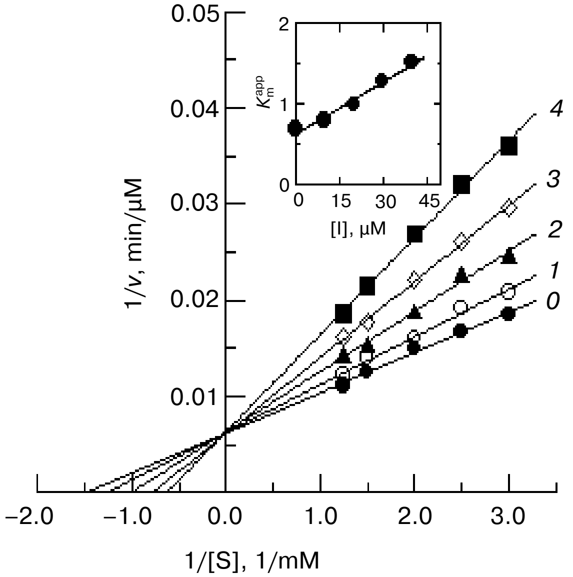


Fig. 4. Lineweaver–Burk plots for the catalysis of L-DOPA by mushroom tyrosinase at 30°C, pH 6.8. Concentration of quercetin for plots *0-4* was 0, 10, 20, 30, and 40 μM, respectively; the enzyme concentration 6.67 μg/ml. The inset represents the plot of apparent Michaelis constant (K_m^{app}) versus the concentration of quercetin for determining the inhibition constant K_I. The line is drawn using a linear least squares fit.

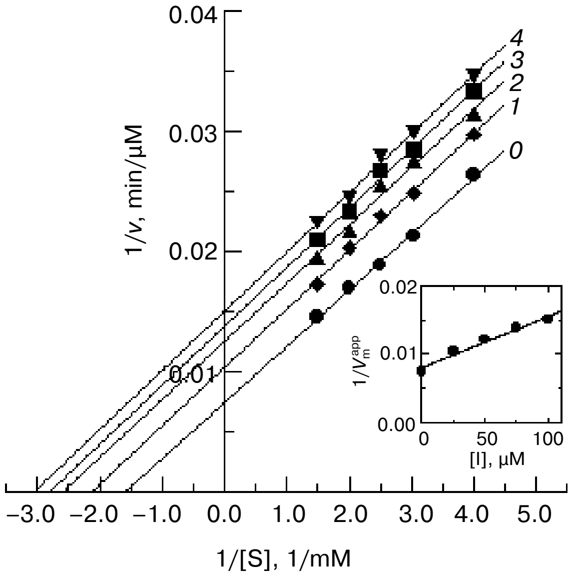


Fig. 5. Lineweaver–Burk plots for uncompetitive inhibition of mushroom tyrosinase by luteolin for the catalysis of L-DOPA at 30°C, pH 6.8. Concentration of luteolin for plots *0-4* was 0, 25, 50, 75 and 100 μM, respectively. The experimental conditions were as for Fig. 4. The inset represents the plot of $1/V_{max}$ versus the concentration of luteolin for determining the ES complex inhibition constant K_{IS}. The line is drawn using a linear least squares fit.

Inhibition of mushroom tyrosinase by flavonols

Compound	Type of inhibition	Inhibition constants, µM	
		K_I	K_{IS}
Quercetin (**a**)	competitive	29	/
Galangin (**b**)	competitive	58	/
Fisetin (**c**)	competitive	75	/
3,7,4′-Trihydroxyflavone (**d**)	competitive	154	/
Morin (**e**)	competitive	410	/
Luteolin (**f**)	uncompetitive	/	103
Apigenin (**g**)	*	*	*
Chrysin (**h**)	no effect		
Quercetin-3-rutinoside (**i**)	no effect		

* Not tested.

Presumably, their inhibition comes from their ability to chelate copper in the active center of the enzyme (Fig. 6). In contrast, luteolin (**f**), apigenin (**g**), and chrysin (**h**) do not chelate copper in the enzyme. Moreover, when the 3-hydroxyl group was protected by a large group such as rutinoside as in quercetin-3-rutinoside (**i**), no inhibition was observed. The chelation mechanism seems to be specific to flavonols as long as the 3-hydroxyl group is free. The chelation reaction was reversible and the substrate exerted a very strong protection. It can also be seen from the table that the ratio of inhibition constant, K_I, of morin with quercetin was 14.3, indicating that the inhibition by quercetin (**a**) of mushroom tyrosinase is about 14-fold more potent than that by morin (**e**). The difference in tyrosinase inhibitory activity of quercetin (**a**) and morin (**e**) likely derives from their different substituents in the B-ring. This difference in inhibitory activity can be explained by the presence of an intramolecular hydrogen bond between the 3- and 2′-hydroxyl groups which interferes with chelation of copper in the enzyme involving the 3-hydroxyl and 4-carbonyl groups.

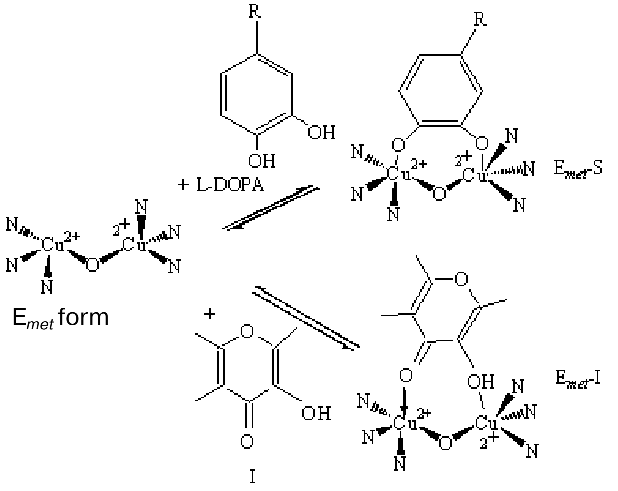


Fig. 6. Inhibition by flavonols of mushroom tyrosinase showing the 3-hydroxy-4-keto moiety chelating copper in the active center. The inhibitor competes with the substrate (L-DOPA) for binding to the enzyme.

Luteolin (**f**) can also inhibit the enzyme; apigenin (**g**) inhibits the enzyme slightly, and chrysin (**h**) had no effects on the enzyme. These three flavones differ in the hydroxyl substituent in the B-ring. It would appear that the 3′,4′-dihydroxyl groups substituent is a more potent inhibitor than the 4′-hydroxyl group substituent, i.e., **f** > **g**. The B-ring with the hydroxyl group substituent is a more potent inhibitor than that without the hydroxyl group (**g** > **h**). Similar conclusions can be drawn from the results with other flavonols, where the inhibitory intensity order is: **a** > **b** and **c** > **d**.

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.

The present investigation was supported in part by grant B0110002 of the Natural Science Foundation of Fujian Province for Q. X. Chen, grant 2001AA621140 from the National High Technology Research and Development of China (863 Program), and the Fundamental Research Foundation of Tsinghua University). We thank Dr. Bruce Livett, University of Melbourne, Australia, for comments on the manuscript.

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