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Two Potent Suicide Substrates of Mushroom Tyrosinase: 7,8,4′-Trihydroxyisoflavone and 5,7,8,4′-Tetrahydroxyisoflavone

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The inhibitory characteristics of two isoflavone metabolites, 7,8,4′-trihydroxyisoflavone and 5,7,8,4′-tetrahydroxyisoflavone, on mushroom tyrosinase were investigated. The two isoflavones were isolated from soygerm koji and inhibited both monophenolase and diphenolase activities of tyrosinase. Their inhibition type was demonstrated to be irreversible inhibition by preincubation and recovery experiments. By using HPLC analysis, it was found that mushroom tyrosinase could catalyze the two isoflavones. These results revealed that the two isoflavones belonged to suicide substrates of mushroom tyrosinase. The partition ratios between molecules of suicide substrate in the formation of product and in the inactivation of enzyme were determined to be 81.7 ± 5.9 and 35.5 ± 3.8 for 7,8,4′-trihydroxyisoflavone and 5,7,8,4′-tetrahydroxyisoflavone, respectively. From kinetic studies, maximal inactivation rate constants and Michaelis constants were 0.79 ± 0.08 and 1.01 ± 0.04 min^{-1} and 18.7 ± 2.31 and 7.81 ± 0.05 μM for 7,8,4′-trihydroxyisoflavone and 5,7,8,4′-tetrahydroxyisoflavone, respectively, when L-DOPA was used as the enzyme substrate. Structure analysis comparing the inactivating activity between the two isoflavones and their structure analogues showed that not only the 7,8-dihydroxyl groups but also the isoflavone skeleton of the two isoflavones played an important role in inactivating tyrosinase activity. The present study demonstrated that 7,8,4′-trihydroxyisoflavone and 5,7,8,4′-tetrahydroxyisoflavone are potent suicide substrates of mushroom tyrosinase.

KEYWORDS: Inhibitor; irreversible; isoflavone; suicide substrate; tyrosinase

INTRODUCTION

Tyrosinase (EC 1.14.18.1) is a copper-containing monooxygenase widely distributed in nature. The structures of model tyrosinases have been elucidated (*1*, *2*). The enzyme catalyzes the first two reactions of melanin synthesis, the hydroxylation of L-tyrosine to 3,4-dihydroxyphenylalanine, L-DOPA, and the oxidation of L-DOPA to dopaquinone. This *o*-quinone is a highly reactive compound and can polymerize spontaneously to form melanin (*3*). The enzyme is also known as a polyphenol oxidase (PPO) (*4*) and is responsible for enzymatic browning reactions in damaged fruits during postharvest handling and processing, which is caused by the oxidation of phenolic compounds in the fruits (*5*). Both the hyperpigmentation in skin and the enzymatic browning in fruits are not desirable, and inhibiting the tyrosinase activity has been the subject of many studies (*6*−*9*). There is a concerted effort to search for naturally occurring tyrosinase inhibitors from plants, because plants constitute a rich source of bioactive chemicals and many of them are largely free from harmful adverse effects (*10*).

In our continuing search for tyrosinase inhibitors from known compounds, 6,7,4′-trihydroxyisoflavone (**Figure 1**) was first identified as a potent tyrosinase inhibitor in our laboratory (*11*). Because several isoflavone derivatives, including 6,7,4′-trihydroxyisoflavone, have been isolated from some fermented soybean foods (*12*−*14*), we decided to study the tyrosinase inhibitors from these soy products. We recently isolated seven isoflavone derivatives from soygerm koji as tyrosinase inhibitors. These tyrosinase inhibitors could be divided into two groups on the basis of their inhibitory characterizations. One group could only reversibly inhibit monophenolase activity of tyrosinase and belonged to competitive inhibitors on the L-tyrosine binding site of mushroom tyrosinase. This group included 6,7,4′-trihydroxyisoflavone, daidzein, glycitein, daidzin, and genistin. The other group contained 7,8,4′-trihydroxyisoflavone and 5,7,8,4′-tetrahydroxyisoflavone (**Figure 1**), which could inhibit both monophenolase and diphenolase activities of tyrosinase. In this study, the two isoflavone metabolites were demonstrated to be suicide substrates of mushroom tyrosinase. The partition ratios, kinetics parameters, and structure comparisons of the newly found tyrosinase inhibitors were also studied.

MATERIALS AND METHODS

Materials. Mushroom tyrosinase (2870 units/mg), L-tyrosine, L-DOPA, dimethyl sulfoxide (DMSO), and 5,7,4′-trihydroxyisoflavone (genistein) were purchased from Sigma Chemical Co. (St. Louis, MO). One unit of mushroom tyrosinase is defined as the amount of the enzyme that could induce 0.001 ΔA_{280} per min at pH 6.5 at 25 °C in

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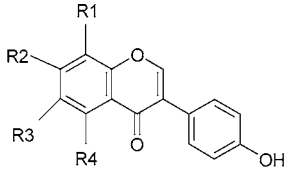


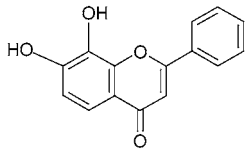
7,8,4'-Trihydroxyisoflavone; R1 = R2 = OH, R3 = R4 = H

5,7,8,4'-Tetrahydroxyisoflavone; R1 = R2 = R4 = OH, R3 = H

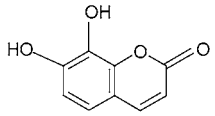
5,7,4'-Trihydroxyisoflavone; R1 = R3 = H, R2 = R4 = OH

6,7,4'-Trihydroxyisoflavone; R1 = R4 = H, R2 = R3 = OH

7,4'-Dihydroxyisoflavone-8-glucoside; R1 = Glc, R2= OH, R3 = R4 = H



7,8-Dihydroxyflavone



7,8-Dihydroxycoumarin

Figure 1. Chemical structures of investigated compounds.

3 mL of reaction mixture containing l-tyrosine. 7,4′-Dihydroxyisoflavone-8-glucoside (puerarin) was obtained from Fluka Chemical Co. (Buchs, Switzerland). 7,8-Dihydroxycoumarin and 7,8-dihydroxyflavone were from Tokyo Chemical Industry Co. (Tokyo, Japan). High-performance liquid chromatography (HPLC) grade acetonitrile and acetic acid were from J. T. Baker (Phillipsburg, NJ). Other reagents and solvents used were of analytical grade and were used as received.

Isolation of 6,7,4′-Trihydroxyisoflavone, 7,8,4′-Trihydroxyisoflavone, and 5,7,8,4′-Tetrahydroxyisoflavone from Soygerm Koji. The purification process of 6,7,4′-trihydroxyisoflavone, 7,8,4′-trihydroxyisoflavone, and 5,7,8,4′-tetrahydroxyisoflavone in soygrem koji was carried out by using the anti-tyrosinase activity assay as a guide. Soygerm koji (500 g) was refluxed with 5 L of methanol for 3 h to give a methanol extract (102 g). The extract was suspended in water (0.1 L) and re-extracted with hexane and ethyl acetate. Each solute fraction was concentrated under vacuum to give hexane (54 g), ethyl acetate (5.43 g), and water (37 g) fractions. The ethyl acetate fraction (100 mg/mL in DMSO) showed the highest anti-tyrosinase activity (IC_{50} = 0.19 mg/mL). The ethyl acetate extract was then fractionated by silica gel column chromatography (50 × 2.6 cm i.d.) with 0.5 L each of hexane/ethyl acetate (3:1), hexane/ethyl acetate (1:1), ethyl acetate, ethyl acetate/methanl (1:1), and methanol as eluents. The ethyl acetate fraction showed strongest anti-tyrosinase activity and was purified by repeated HPLC using a 250 × 10 mm i.d., ODS 2 Spherisorb semipreparative C18 reversed-phase column (Phase Separation Ltd., Deeside Industrial Park, Clwyd, U.K.). The gradient elution using water (A) containing 0.1% (v/v) acetic acid and acetonitrile (B) consisted of an isocratic elution for 10 min with 14% B and a linear gradient for 50 min with 20% to 40% B at a flow rate of 3 mL/min. The elution of the peaks was collected, dried, and assayed for anti-tyrosinase activity. The chemical structures of purified 6,7,4′-trihydroxyisoflavone, 7,8,4′-trihydroxyisoflavone, and 5,7,8,4′-tetrahydroxyisoflavone were identified by mass and NMR spectrometry.

Instrumental Analyses of 6,7,4′-Trihydroxyisoflavone, 7,8,4′-Trihydroxyisoflavone, and 5,7,8,4′-Tetrahydroxyisoflavone. ^{1}H NMR spectra were recorded with a Varian Gemini NMR spectrometer at 400 MHz and ^{13}C NMR spectra with a Varian Gemini NMR spectrometer at 100 MHz in DMSO. FAB MS spectra were obtained with a JEOL TMSD-100. The physicochemical properties of 6,7,4′-trihydroxyisoflavone, 7,8,4′-trihydroxyisoflavone, and 5,7,8,4′-tetrahydroxyisoflavone are given next.

6,7,4′-Trihydroxyisoflavone: ^{1}H NMR (DMSO-d_6), δ 6.78 (2H, d, J = 8.8 Hz, H-3′, 5′), 6.84 (1H, s, H-8), 7.34 (2H, d, J = 8.8 Hz, H-2′, 6′), 7.36 (1H, s, H-5), 8.21 (1H, s, H-2), 9.57 (3H, br s, OH-6,7,4′); ^{13}C NMR (DMSO-d_6), δ 174.8 (C-4), 157.3 (C-4′), 152.8 (C-7), 152.6 (C-2), 151.2 (C-9), 145.0 (C-6), 130.4 (C-2′, 6′), 123.2 (C-1′), 123.1 (C-3), 116.9 (C-10), 115.3 (C-3′, 5′), 108.4 (C-5), 103.0 (C-8). FAB MS, m/z 271 $[M + H]^+$.

7,8,4′-Trihydroxyisoflavone: ^{1}H NMR (DMSO-d_6), δ 6.79 (2H, d, J = 8.3 Hz, H-3′, 5′), 6.94 (1H, d, J = 8.7 Hz, H-6), 7.37 (2H, d, J = 8.3 Hz, H-2′, 6′), 7.45 (1H, d, J = 8.7 Hz, H-5), 8.30 (1H, s, H-2), 9.46 (1H, br s, OH-7), 9.58 (1H, br s, OH-4′), 10.37 (1H, br s, OH-8); ^{13}C NMR (DMSO-d_6), δ 175.6 (C-4), 157.4 (C-4′), 153.0 (C-2), 150.2 (C-7), 147.0 (C-9), 133.2 (C-8), 130.4 (C-2′, 6′), 123.2 (C-1′), 123.0 (C-3), 117.7 (C-10), 116.0 (C-5), 115.3 (C-3′, 5′), 114.5 (C-6); FAB MS, m/z 271 $[M + H]^+$.

5,7,8,4′-Tetrahydroxyisoflavone: ^{1}H NMR (DMSO-d_6), δ 6.29 (1H, s, H-6), 6.81 (2H, d, J = 9.0 Hz, H-3′, 5′), 7.36 (2H, d, J = 9.0 Hz, H-2′, 6′), 8.31 (1H, s, H-2), 8.86 (1H, br s, OH-7), 9.70 (1H, br s, OH-4′), 10.71 (1H, br s, OH-8); ^{13}C NMR (DMSO-d_6), δ 180.5 (C-4), 157.2 (C-4′), 153.8 (C-2), 153.3 (C-5), 153.0 (C-7), 145.7 (C-9), 130.1 (C-2′, 6′), 124.8 (C-8), 121.7 (C-1′), 121.3 (C-3), 115.0 (C-3′, 5′), 103.9 (C-10), 98.6 (C-6); FAB MS, m/z 287 $[M + H]^+$.

Enzymatic Assay of Tyrosinase. Ten microliters of the test sample (dissolved in DMSO) was mixed with 970 μL of 0.112 mM substrate (L-tyrosine or L-DOPA dissolved in 50 mM phosphate buffer, pH 6.8) at 25 °C for 2 min. Then, 20 μL of tyrosinase (1000 units/mL in phosphate buffer) was added to initiate the reaction. The increase in absorbance at 475 nm due to the formation of dopachrome was monitored with a spectrophotometer.

In irreversible inhibitory activity assays, 20 units of tyrosinase was preincubated with a 3 or 10 μM concentration of the tested isoflavone (dissolved in DMSO) in 1 mL of 50 mM phosphate buffer (pH 6.8) at 25 °C. At intervals of 0, 2, 7, 12, and 30 min, 200 μL of the preincubation mixture was mixed with 800 μL of 2.5 mM L-DOPA and incubated at 25 °C for 10 min. The formation of dopachrome in each reaction was monitored with a spectrophotometer. The relative activity was calculated by dividing the absorbance at 475 nm of each reaction by that of the control reaction, in which DMSO replaced the added isoflavone. For recovery experiments, the preincubation mixture incubated for 30 min was either dialyzed twice against 200 mL of phosphate buffer at 4 °C for 1 h with stirring or centrifuged through a Sephadex G-25 spin column (Sigma). Then, the residual tyrosinase activities of the mixtures from the two treatments were assayed as described above.

The partition ratio of the suicide substrate was determined according to the method of Waley (*15*) by incubating 500 μL of preincubation mixture containing 0.1 μM tyrosinase and 0.55−7.7 μM 7,8,4′-trihydroxyisoflavone or 0.1−3.5 μM 5,7,8,4′-tetrahydroxyisoflavone at 25 °C for 30 min. Then, 200 μL of preincubation mixture was mixed with 800 μL of 2.5 mM L-DOPA. The absorbance of the reaction mixture at 475 nm was monitored every 1 s with a spectrophotometer. The initial reaction velocities were measured from the slope at the first 2 min of the time course of the reaction curve. The relative activity of each reaction was calculated by dividing the initial velocity of the reaction with suicide substrate by that of the reaction without suicide substrate. The partition ratio of suicide substrate could be determined by plotting the fractional activity remaining against the ratio of the initial concentration of the suicide substrate to that of enzyme.

The Michaelis constants (K_I) and maximal inactivation rate constants (k_{i-max}) of suicide substrates were determined according to the method of Frere et al. (*16*). The inactivation reactions were carried out in the presence of 0.03 μM mushroom tyrosinase, 2.5 mM L-DOPA, and the suicide substrate at concentrations ranging from 50 to 300 μM, and the formation of dopachrome was monitored every second for 2 min with a spectrophotometer. Under these conditions, the rate of oxidation of L-DOPA progressively decreased and the apparent first-order rate constant (k_{obs}) for the inactivation was computed from the plots of ln(v_t/v_0) against t, where v_0 and v_t are the rates of increase of absorbance at 475 nm at zero time and at time t, respectively. Both K_I and k_{i-max}

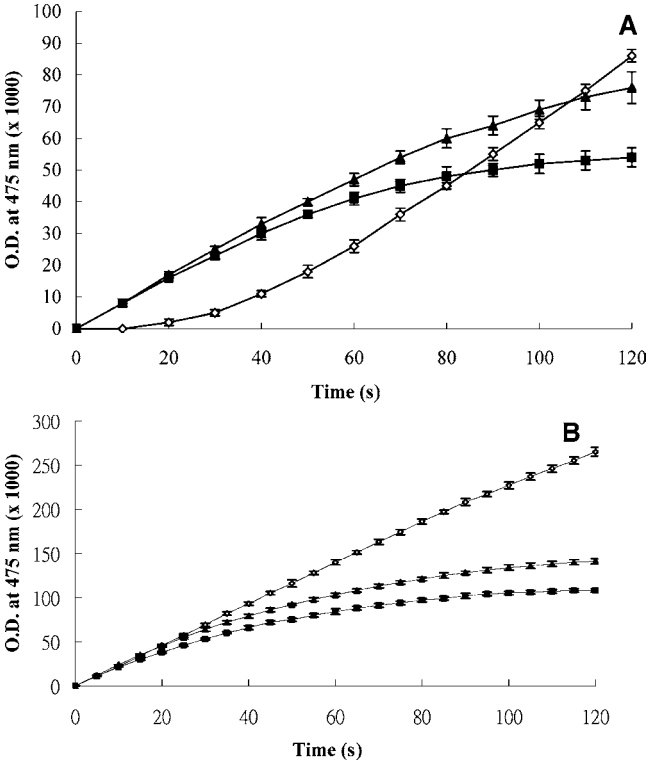


Figure 2. Time course of tyrosinase reaction (◇) inhibited by 0.05 mM 7,8,4′-trihydroxyisoflavone (▲) or 5,7,8,4′-tetrahydroxyisoflavone (■) with mushroom tyrosinase (100 units/mL). L-Tyrosine (**A**) or L-DOPA (**B**) at 0.1 mM was used as the substrate.

could thus be calculated, assuming a competition between the added isoflavone and L-DOPA.

For structure analysis of the two isoflavones on the inhibitory effects of mushroom tyrosinase, 20 units of tyrosinase was preincubated with the tested compound (10 μM for 7,8,4′-trihydroxyisoflavone and 5,7,8,4′-tetrahydroxyisoflavone; 100 μM for others) in 200 μL of 50 mM phosphate buffer (pH 6.8) at 25 °C for 30 min. Then, 800 μL of 2.5 mM L-DOPA was added, and the reaction mixture was incubated at 25 °C for 10 min. For comparison, another set of experiments was conducted by mixing immediately the tested compound, tyrosinase, and L-DOPA in 1 mL of phosphate buffer and incubated at 25 °C for 10 min. The formation of dopachrome in each reaction was monitored with a spectrophotometer. The relative activity was calculated by dividing the absorbance at 475 nm of each reaction mixture by that of the control reaction, in which DMSO replaced the tested compound. All enzymatic reactions described above were carried out at least three times independently, and the average values are presented.

HPLC Analysis. HPLC analysis was performed on a Hitachi D-7000 HPLC (Hitachi, Ltd., Tokyo, Japan) system equipped with an L-7400 UV detector and a 250 × 4.6 mm i.d., ODS 2 Spherisorb C18 reversed-phase column (Phase Separation Ltd.). The operating conditions were as follows: solvent, 30% acetonitrile/water containing 1% acetic acid; flow rate, 0.8 mL/min; detection, 262 nm; injected volume, 20 μL from a 1 mL assay system containing 100 μM isoflavone and 1000 units of mushroom tyrosinase in 50 mM phosphate buffer (pH 6.8).

RESULTS AND DISCUSSION

Identification of 7,8,4′-Trihydroxyisoflavone and 5,7,8,4′-Tetrahydroxyisoflavone as Suicide Substrates of Mushroom Tyrosinase. To study the tyrosinase inhibition by 7,8,4′-trihydroxyisoflavone and 5,7,8,4′-tetrahydroxyisoflavone, the inhibitory effects of the two isoflavones on both monophenolase and diphenolase activities of mushroom tyrosinase were examined. The results are shown in **Figure 2**. When the enzymatic reaction was carried out with L-tyrosine as a substrate, a marked

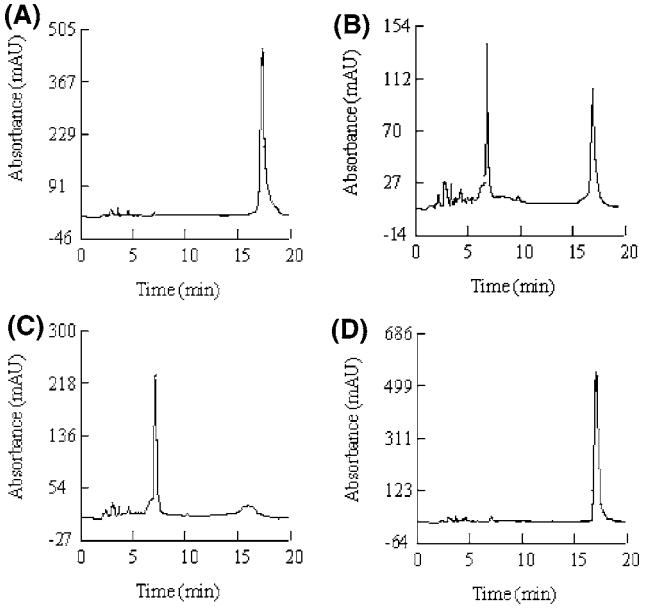


Figure 3. HPLC chromatograms of the reaction mixture containing 100 μM 5,7,8,4′-tetrahydroxyisoflavone and tyrosinase (1000 units/mL, **A**–**C**) or heat-denatured tyrosinase (1000 units/mL, **D**) in 1 mL of 50 mM phosphate buffer (pH 6.8). Samples were collected at 0 sec (**A**), 10 sec (**B**), 30 sec (**C**), and 10 sec (**D**).

lag time, characteristic of monophenolase activity (*17*), was observed, simultaneously with the appearance of dopachrome (**Figure 2A**). The lag time is the time required to reach the steady-state concentration of *o*-diphenol. The length of the lag time can be shortened or abolished by the presence of reducing agents or *o*-diphenol substrates, such as L-DOPA (*18*). As can be seen from **Figure 2A**, L-tyrosine was oxidized by the enzyme without the lag time in the presence of each of the two isoflavones. Because the two compounds contain an *o*-diphenol structure, the result implied that the two isoflavones might act as substrates of mushroom tyrosinase. In this situation, the *met* form of the enzyme, which is the major form in the enzyme resting state, was quickly reduced to its *deoxy* form by catalyzing the *o*-diphenol substrates. Then the *deoxy* form of tyrosinase spontaneously is converted to its *oxy* form, which is the only form that could bind with L-tyrosine. On the other hand, the two isoflavones slowed and stopped the formation of dopachrome when L-tyrosine was used as a substrate, behaving, therefore, as an inhibitor of the monophenolase activity of mushroom tyrosinase. Furthermore, when the diphenolase activity of tyrosinase was examined by using L-DOPA as a substrate, the reaction immediately reached a steady state (**Figure 2B**). The presence of each of the two isoflavones in the assay medium resulted in reduction in the diphenolase activity (**Figure 2B**). The above results revealed that the two isoflavones inhibited both monophenolase and diphenolase activities of mushroom tyrosinase.

To ascertain whether the two isoflavones behaved as the substrates of mushroom tyrosinase, the enzymatic reactions of tyrosinase with 5,7,8,4′-tetrahydroxyisoflavone and 7,8,4′-trihydroxyisoflavone were studied by mixing the isoflavone and tyrosinase in phosphate buffer at pH 6.8. The reaction mixture was analyzed by HPLC, and the results are shown in **Figure 3**. The isoflavone (t_R = 17.3 min) started to decrease and a new peak (t_R = 7.1 min) gradually appeared during the catalytic reaction with active tyrosinase. In contrast, the isoflavone remained constant during the catalytic reaction with the heat-denatured tyrosinase. A similar result was also obtained with

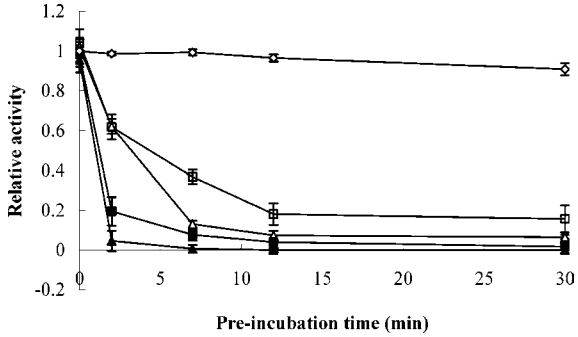


Figure 4. Inhibitory effects of 7,8,4′-trihydroxyisoflavone (3 μM, △; 10 μM, ▲) and 5,7,8,4′-tetrahydroxyisoflavone (3 μM, □; 10 μM, ■) on mushroom tyrosinase activity (◇) with various durations of preincubation.

7,8,4′-trihydroxyisoflavone as the substrate (data not shown). The results revealed that the two isoflavones acted as the substrates of mushroom tyrosinase. However, determination of the structure of the enzymatic oxidation products of the two isoflavones (**Figure 3**) seemed to be difficult because sufficient quantities of the purified products were not available. Coexistence of several byproducts along with the lower concentrations of the products resulting from the use of lower amounts of the original substrate, such as 5,7,8,4′-tetrahydroxyisoflavone, rendered the purification difficult. Despite the lack of detailed information about the chemical structure of the oxidized isoflavone, the two isoflavones in the present study might be catalyzed by tyrosinase to their corresponding *o*-quinones first. The *o*-quinones are usually unstable and might change to other more stable intermediates. Kubo et al. recently reported the study of the oxidation products of a commercial flavonol, quercetin, catalyzed by mushroom tyrosinase (*19*). They found that quercetin was oxidized to the corresponding *o*-quinone and subsequent isomerization to *p*-quinone methide type intermediate followed by the addition of water on C-2, yielding a relatively stable intermediate. Although the oxidation process of quercetin by tyrosinase gives some clues, the oxidation process of the two isoflavones in the present study by mushroom tyrosinase needs further studies.

The above results showed that the two compounds possess the characteristics of both a substrate and an inhibitor for mushroom tyrosinase. It is known that tyrosinase could be irreversibly inhibited by its *o*-diphenol substrates, such as L-DOPA and catechol (*20*). These substrates were also named as suicide substrates or mechanism-based inhibitors (*15*). We therefore investigated further whether 7,8,4′-trihydroxyisoflavone and 5,7,8,4′-tetrahydroxyisoflavone could irreversibly inhibit tyrosinase. The results are shown in **Figure 4**. The enzyme activity in the preincubation mixture without the addition of the two isoflavones remained constant during 30 min of reaction. However, preincubation of tyrosinase with each of the two isoflavones quickly inactivated the enzyme within the first 2 min of preincubation. With the addition of 10 μM isoflavone in the preincubation mixture, the enzyme was totally inactivated after 7 min of preincubation. Moreover, the enzyme activity in the preincubation mixture was not restored by using dialysis or molecular exclusion chromatography to remove compounds of low molecular weight such as the two isoflavones (data not shown). From these results, 7,8,4′-trihydroxyisoflavone and 5,7,8,4′-tetrahydroxyisoflavone were identified as irreversible inhibitors and they belonged to suicide substrates or mechanism-based inhibitors for mushroom tyrosinase.

Determination of Partition Ratios of the Two Suicide Substrates. An initial step, which is of prime importance in every quantitative work with suicide substrates, is to determine the molar proportion for inactivation, that is, the number of molecules of inhibitors required to completely inactivate one molecule of the enzyme. The mechanism of suicide substrate has been extensively studied by Waley (*15*), who proposed a simple branched reaction pathway as follows, in which an intermediate Y may give either active enzyme and product or inactive enzyme.

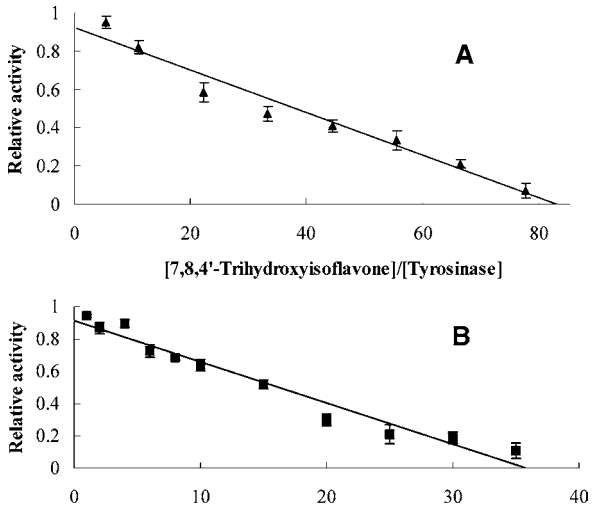
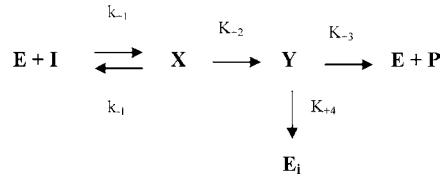


Figure 5. Titration of mushroom tyrosinase with either 7,8,4′-trihydroxyisoflavone (**A**) or 5,7,8,4′-tetrahydroxyisoflavone (**B**). The enzyme (0.1 μM) and the isoflavone (0.55–7.7 μM in panel **A** and 0.1–3.5 μM in panel **B**) were preincubated in 1 mL of 50 mM phosphate buffer (pH 6.8) at 25 °C for 30 min.



In the above scheme, E and E_i are enzyme and inactivated enzyme, respectively; P is product; X is the first intermediate and Y is another intermediate. The intermediate Y has a choice of reaction, governed by the partition ratio r, where $r = k_{+3}/k_{+4}$. The molar proportion for inactivation, as defined above, may be determined by plotting the fractional activity remaining against the ratio of the initial concentration of inhibitor to that of enzyme. The intercept on the abscissa is $1 + r$ in the plot, when $r > 1$ (*21*). The result is shown in **Figure 5**. When tyrosinase was preincubated with each of varied amounts of 7,8,4′-trihydroxyisoflavone or 5,7,8,4′-tetrahydroxyisoflavone, the fractional activity remaining was proportional to the molar ratio of the added isoflavone to enzyme. By extrapolation, 82.7 $\pm$ 5.9 and 36.5 $\pm$ 3.8 molecules of 7,8,4′-trihydroxyisoflavone and 5,7,8,4′-tetrahydroxyisoflavone, respectively, were required to inactivate 1 molecule of the enzyme. When less than 83 and 37 molar proportions of 7,8,4′-trihydroxyisoflavone and 5,7,8,4′-tetrahydroxyisoflavone, respectively, were used, the suicide reaction ceased because all of the suicide substrates had been consumed, and the fractional activity remaining was not appreciably different when the duration of incubation was varied from a few minutes to a few hours. Therefore, the partition ratios of the two suicide substrates were calculated to be 81.7 $\pm$ 5.9 and 35.5 $\pm$ 3.8 for 7,8,4′-trihydroxyisoflavone and 5,7,8,4′-

tetrahydroxyisoflavone, respectively, from the intercept on the abscissa in **Figure 5**, which is $1 + r$ (*15*).

Determination of Michaelis Constant and Maximal Inactivation Rate Constant of the Two Suicide Substrates. The kinetics of inhibition of 7,8,4′-trihydroxyisoflavone and 5,7,8,4′-tetrahydroxyisoflavone were studied by using the method of Frere et al. (*16*) and by measuring the oxidation of L-DOPA by mushroom tyrosinase in the presence of each of the two suicide substrates. During the assay, the concentration of the tested suicide substrate was much higher than that of the enzyme. When the progress of the inactivation of the enzyme was monitored at high suicide substrate/enzyme ratios (*21*), the concentration of suicide substrate ([*I*]) could be considered as constant throughout the process and a pseudo-first-order rate constant (k_i) could be determined by using eq 1, where k_{i-max} and K_I represent the maximal inactivation velocity and the Michaelis constant of the inactivator.

$$k_i = \frac{k_{i-max}[I]}{[I] + K_I} \quad (1)$$

In the presence of L-DOPA, the exponential decrease in the rate of oxidation of L-DOPA gave an apparent first-order rate constant k_{obs}, which was computed from plots of $\ln(v_t/v_0)$ against t, where v_0 and v_t are the rates of increase of absorbance at 475 nm, at zero time and at time t, respectively. Assuming a competitive interaction between the added isoflavone and L-DOPA with the enzyme, the variation of k_{obs} with the concentrations of the isoflavone ([I]) and L-DOPA ([S]) are given by eq 2

$$k_{obs} = \frac{k_{i-max}[I]}{[I] + K_I(1 + [S]/K_S)} \quad (2)$$

where K_S and K_I are the Michaelis constants for L-DOPA and the isoflavone, respectively. When eq 2 is written as

$$[I]/k_{obs} = [I]/k_{i-max} + K_I/k_{i-max}\,(1 + [S]/K_S) \quad (3)$$

it is clear that a plot of $[I]/k_{obs}$ against [I] will be linear and that k_{i-max} and K_I can be found from the intercept and slope. The result is shown in **Figure 6**. Under the same experimental conditions, K_S was determined to be 0.25 ± 0.01 mM. Hence, the values of k_{i-max} and K_I were calculated to be 0.79 ± 0.08 and 1.01 ± 0.04 $min^{-1}$and 18.70 ± 2.31 and 7.81 ± 0.05 μM for 7,8,4′-trihydroxyisoflavone and 5,7,8,4′-tetrahydroxyisoflavone, respectively, when L-DOPA was used as the enzyme substrate. The high k_{i-max} and low K_I values of the two suicide substrates meant the second-order rate constants (k_{i-max}/K_I) were large. Thus, the two isoflavones are "high-reactivity, high-affinity" suicide substrates of mushroom tyrosinase.

Structure Analysis on the Potency of Suicide Substrate. To verify the relationship between the chemical structure and the potency of suicide substrate of mushroom tyrosinase, we used irreversible inhibitory ability as a primary guide. The tested structural analogues of 7,8,4′-trihydroxyisoflavone included 5,7,4′-trihydroxyisoflavone, 6,7,4′-trihydroxyisoflavone, 7,4′-dihydroxyisoflavone-8-glucoside, 7,8-dihydroxycoumarin, and 7,8-dihydroxyflavone (**Figure 1**). The irreversible inhibitory assay was conducted by the reaction of preincubation of the compound with mushroom tyrosinase. As a consequence, we found that none of the tested analogous compounds irreversibly inhibited diphenolase activity of mushroom tyrosinase. Hence, it was clear that when the hydroxyl group at the C8 position of the A-ring in 7,8,4′-trihydroxyisoflavone was exchanged with

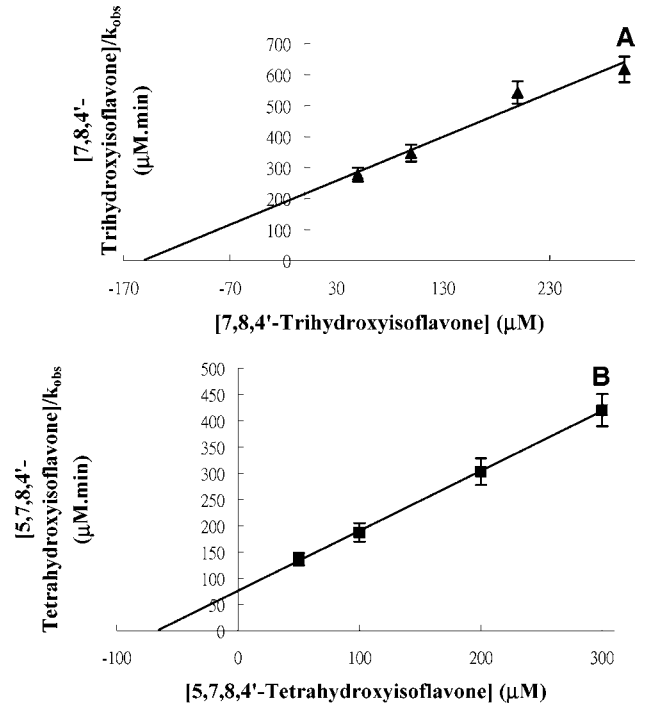


Figure 6. Determination of Michaelis constants and maximal inactivation rate constants of 7,8,4′-trihydroxyisoflavone (**A**) or 5,7,8,4′-tetrahydroxyisoflavone (**B**).

that at the C5 (5,7,4′-trihydroxyisoflavone) or C6 (6,7,4′-trihydroxyisoflavone) position or exchanged with the glucoside (7,4′-dihydroxyisoflavone-8-glucoside), the potency of the irreversible inhibitory activity totally disappeared. This indicated the 7,8-dihydroxyl groups in both 7,8,4′-trihydroxyisoflavone and 5,7,8,4′-tetrahydroxyisoflavone played an important role in the suicide nature of the substrate for mushroom tyrosinase. On the other hand, when the isoflavone skeleton was replaced with that of flavone (7,8-dihydroxyflavone) or coumarin (7,8-dihydroxycoumarin), the irreversible inhibitory activity also disappeared, even when the two hydroxyl groups at the C7 and C8 positions in the A-ring were maintained. From the above results, it was thus concluded that not only the 7,8-dihydroxyl groups but also the isoflavone skeleton in both 7,8,4′-trihydroxyisoflavone and 5,7,8,4′-tetrahydroxyisoflavone were absolutely necessary for the compounds to function as potent suicide substrates of mushroom tyrosinase. The results also revealed that the two suicide substrates are unique.

Suicide inactivation of tyrosinase has been reported in early studies (*22*, *23*) and, since then, many researchers have investigated this phenomenon for tyrosinases from different organisms (*20*, *24*−*27*), as well as for the catalase activity of tyrosinase (*28*). Suicide substrates of tyrosinase will be useful as skin-depigmenting and food-antibrowning agents, and their presence must be prevented in biosensors (*29*) and phenol removal applications (*30*) of tyrosinase. Potent suicide substrates have rarely been discovered. However, the two isoflavones 7,8,4′-trihydroxyisoflavone and 5,7,8,4′-tetrahydroxyisoflavone, in the present study, were proven to be potent and unique suicide substrates of mushroom tyrosinase with low partition ratios, low Michaelis constants, and high maximal inactivation rate constants. It is worthwhile to further investigate the applications of these two suicide substrates.

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