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Identifying 8-hydroxynaringenin as a suicide substrate of mushroom tyrosinase

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Synopsis

A biotransformed metabolite of naringenin was isolated from the fermentation broth of *Aspergillus oryzae*, fed with naringenin, and identified as 8-hydroxynaringenin based on the mass and ^{1}H- and ^{13}C-NMR spectral data. The compound showed characteristics of both an irreversible inhibitor and a substrate of mushroom tyrosinase in preincubation and HPLC analysis. These results demonstrate that 8-hydroxynaringenin belongs to a suicide substrate of mushroom tyrosinase. The partition ratio between the compound's molecules in the formation of product and in the inactivation of the enzyme was determined to be 283 ± 21. The present study's results, together with our previous findings, which proved that both 8-hydroxydaidzein and 8-hydroxygenistein are suicide substrates of mushroom tyrosinase, show that 7,8,4′-trihydroxyl functional groups on flavonoids' skeletons play important roles in producing suicide substrate properties toward mushroom tyrosinase.

INTRODUCTION

Tyrosinase (EC 1.14.18.1) is a copper-containing monooxygenase widely distributed in nature. This 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 (1). The enzyme also is known as a polyphenol oxidase (PPO) and is responsible for enzymatic browning reactions in damaged fruits during post-harvest handling and processing, which are caused by the oxidation of phenolic compounds in the fruits (2). Both the hyperpigmentation in skin and this enzymatic browning in fruits are undesirable. Hence, inhibiting the tyrosinase activity has been the subject of many studies (3).

Flavonoids are a diverse group of polyphenolic compounds widely distributed in plants with wide-ranging biological properties. Some of them have been demonstrated to be effective inhibitors of mushroom tyrosinase (4–11). In our continuing search for tyrosinase inhibitors from flavonoids, we isolated seven isoflavones from soygerm koji fermented by *Aspergillus oryzae* and demonstrated their potent inhibitory effects on mushroom tyrosinase

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(12,13). Among them, two hydroxylated soy isoflavone derivatives, 8-hydroxydaidzein and 8-hydroxygenistein, biotransformed by *A. oryzae,* possessed a vastly higher inhibitory activity than their precursors, the soy isoflavones daidzein and genistein. Based on our previous findings, we are interested to know whether other kinds of flavonoids could also be biotransformed by *A. oryzae*. If the answer is yes, what are the metabolites' effects on mushroom tyrosinase activity? In the present study, a biotransformed metabolite of naringenin was isolated from the fermentation broth of *Aspergillus oryzae* fed with naringenin and identified as 8-hydroxynaringenin based on the mass and ^{1}H- and ^{13}C-NMR spectral data. The inhibitory property of the metabolite toward mushroom tyrosinase was investigated.

MATERIALS AND METHODS

MICROORGANISMS AND CHEMICALS

Lyophilized culture of *A. oryzae* BCRC 32288 was obtained from the Bioresources Collection and Research Center (BCRC) of the Food Industry Research and Development Institute (FIRDI, Hsinchu, Taiwan, ROC). The stock culture was grown on potato dextrose agar (PDA) and maintained at 25°C. Spore suspension of *A. oryzae* was prepared in sterile water and used for inoculation. Mushroom tyrosinase (5370 U/mg), L-DOPA, dimethyl sulfoxide (DMSO), Sephadex LH-20 gel, and naringenin were purchased from Sigma Chemical (St. Louis, MO). Yeast extract, malt extract, peptone, agar, and potato dextrose agar (PDA) were obtained from Difco Laboratories (Detroit, MI). High-performance liquid chromatography (HPLC)-grade acetonitrile and acetic acid were obtained from J.T. Baker (Phillipsburg, NJ). The other reagents and solvents used are commercially available and were used as received.

FUNGAL CULTIVATION AND METABOLITE PURIFICATION

The cultivation condition and the medium used for *A. oryzae* BCRC32288 were exactly according to the data sheet from the BCRC of the FIRDI. The fermentation experiments were carried out in 250-ml baffled Erlenmeyer flasks containing 20 ml of the medium in the presence of 250 μg naringenin per ml of the medium. Two liters of the cultivations were carried out for metabolite purification. Cultures were incubated for two days with a rotary shaker at a speed of 120 rpm/min and 30°C; then the cultivations were combined and extracted with two liters of ethyl acetate. The ethyl acetate extract was dried under a vacuum. The dry mass (5.8 g) was resuspended with 10 ml of methanol and then fractionated by Sephadex LH-20 gel column chromatography (50 cm × 2.6 i.d.) with methanol as an eluent. Every 50 ml of elution was collected and 20 μl of each fraction was analyzed by HPLC to identify the metabolite's presence. The operational conditions for the HPLC analysis by an analytic C18 reversed-phase column (Spherisorb, 5 μm, 4.6 i.d. × 250 mm, ODS 2, phase separation, Deeside Industrial Park, Clwyd, UK) consisted of an isocratic elution for 15 min with 35% acetonitrile in 1.0% (v/v) acetic acid at a flow rate of 1 ml/min, and detection of absorbance at 280 nm. Fractions 11 to 15 from Sephadex LH-20 gel column chromatography were identified to contain the metabolite and were dried under a vacuum. The dried mass (2.1 g) was then purified by repeated HPLC using a

semi-preparative C18 reversed-phase column (Spherisorb, 5 μm, 10 i.d. × 250 mm, ODS 2, phase separation). The preparative HPLC conditions were the same as those for analytic HPLC, with the exception of the flow rate (3 ml/min) and the injection volume (0.2 ml). The elutions of the metabolite peak were collected and dried under a vacuum. The final purified metabolite (14.2 mg) was analyzed by mass and NMR spectroscopy.

INSTRUMENTAL ANALYSIS OF ISOLATED METABOLITES

^{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 were obtained with a JEOL TMSD-100. The metabolite's ^{1}H- and ^{13}C-NMR spectral data were consistent with those of 8-hydroxynaringenin shown in the literature (14).

IRREVERSIBLE INHIBITORY ACTIVITY ASSAY

In irreversible inhibitory activity assays, 20 units of tyrosinase was preincubated with 100 μM of the tested samples (dissolved in DMSO) in 1 ml of 50 mM phosphate buffer (pH 6.8) at 25°C. At intervals of 0, 0.5, 2.5, and 5 min, 200 μl of the preincubation mixture was mixed with 800 μl of 2.5 mM L-DOPA. The reaction mixture's absorbance at 475 nm was monitored every second with a spectrophotometer. The initial reaction rate was measured from the slope of the linear time-dependent increase in absorbance at 475 nm due to the formation of dopachrome produced from L-DOPA by mushroom tyrosinase. The sample's relative activity was calculated by dividing its initial rate by that of the control reaction at the beginning, in which DMSO replaced the added sample.

DETERMINATION OF THE PARTITION RATIO OF SUICIDE SUBSTRATES OF MUSHROOM TYROSINASE

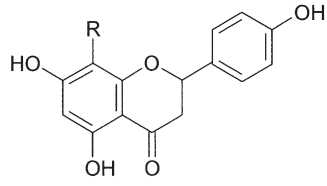
The suicide substrate's partition ratio was determined according to Waley's method by incubating 200 μl of preincubation mixture containing 20 units of tyrosinase (0.03 μM) and 0.9–9.0 μM of 8-hydroxynaringenin at 25°C for 30 min (15). Then 800 μl of 2.5 mM L-DOPA was added to each preincubation mixture, and the reaction mixture's initial rate was determined as described above. Each reaction's relative activity was calculated by dividing the reaction's initial rate with the suicide substrate by that of the reaction without the suicide substrate. The suicide substrate's partition ratio could be determined by plotting the fractional activity remaining against the ratio of the initial concentration of the suicide substrate to that of the enzyme. All enzymatic experiments were repeated at least twice in order to ensure the reproducibility of the results, and the mean values ± SD are reported here.

RESULTS AND DISCUSSION

In an early step of this study, we selected four flavonoids that belong to different classes including apigenin (flavone), quercetin (flavonol), naringein (flavanone), and catechin as the precursors to study the biotransformation of flavonoids by *A. oryzae*. The tested flavonoids were added to the medium and cultivated with the fungus. For different cultivation

periods, the fermentation broth was extracted with methanol and analyzed via HPLC to identify the presence of any metabolite. Among the tested flavonoids, we found that naringenin could be biotransformed by *A. oryzae* during the fermentation process (data not shown). We then scaled up the fermentation quantity and purified the metabolite by ethyl acetate extraction, Sephadex LH-20 gel column chromatography, and semi-preparative HPLC methods. The NMR and mass spectral analysis confirmed the metabolite as 8-hydroxynaringenin (Figure 1) in comparing the spectral data with those of previous reports (14).

Our previous work demonstrated two 8-hydroxyl flavonoid derivatives, 8-hydroxydaidzein and 8-hydroxygenistein, to be potent suicide substrates of mushroom tyrosinase (16). Thus, it is interesting to test the suicide substrate properties of 8-hydroxynaringenin toward mushroom tyrosinase. Because suicide substrates belong to irreversible inhibitors, we then determined the irreversible inhibitory activity of 8-hydroxynaringenin toward mushroom tyrosinase by preincubation experiments. The result is shown in Figure 2. The enzymatic activity remained constant during five minutes in the preincubation mixtures without the tested samples (control) or with 100 μM of naringenin. However, preincubation of tyrosinase with 100 μM of 8-hydroxynaringenin quickly inactivated the enzyme within the first 30 seconds of preincubation and only 1.5% residual activity remained in the preincubation mixture after five minutes of preincubation. These results



Naringenin; R = H

8-Hydroxynaringenin; R = OH

Figure 1. Chemical structures of naringenin and 8-hydroxynaringenin.

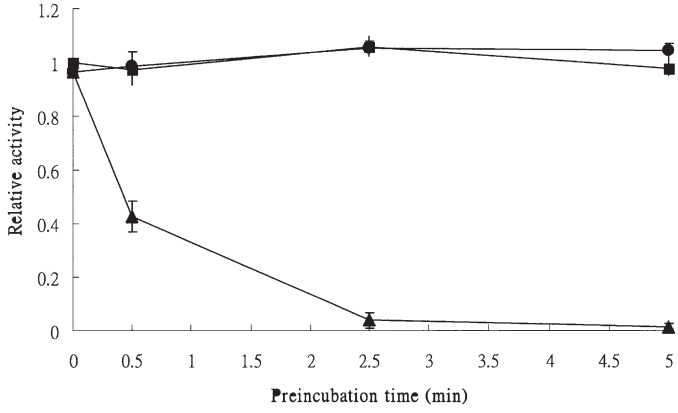


Figure 2. Relative activities of preincubations of mushroom tyrosinase with naringenin (■), 8-hydroxynaringenin (▲), and without a sample (●) for varying durations of preincubation time.

identify 8-hydroxynaringenin as an irreversible inhibitor of mushroom tyrosinase. Besides being an irreversible inhibitor, 8-hydroxynaringenin is also a substrate of mushroom tyrosinase, confirmed by an HPLC method. When incubating with excess native tyrosinase, the amount of 8-hydroxynaringenin in the preincubation mixture quickly decreased to zero within 30 min, but remained constant with heat-denatured tyrosinase (data not shown). The above results show that 8-hydroxynaringenin possesses the characteristics of both a substrate and an irreversible inhibitor of mushroom tyrosinase, and hence was demonstrated to be a new suicide substrate of mushroom tyrosinase.

An important criterion of a suicide substrate is the molar proportion for enzyme inactivation (defined as the partition ratio of a suicide substrate), i.e., the number of molecules of inhibitors required to completely inactivate one molecule of the enzyme. The mechanism of suicide substrates has been studied extensively by Waley, and a suicide substrate's partition ratio may be determined by plotting the fractional activity remaining against the ratio of the initial concentration of the inhibitor to that of the enzyme (15). The result is shown in Figure 3. When mushroom tyrosinase was preincubated with 8-hydroxynaringenin, the fractional enzymatic activity remaining was proportional to the molar ratio of the 8-hydroxynaringenin added to the enzyme. By extrapolation, the new suicide substrate's partition ratio was calculated to be 283 ± 21 from the intercept on the abscissa in Figure 3.

Many flavonoids, including flavones (5,6), flavonols (7,8), catechin (9), and isoflavones (10–12), have been proven to be strong, reversible inhibitors of mushroom tyrosinase. The structure and activity relationships (SAR) between the flavonoids' structures and the reversible inhibitory activity on mushroom tyrosinase have also been investigated in those reports, which found that both the number and position of hydroxyl groups attached to the flavonoids' skeletal structure play important roles in exhibiting their reversible inhibitory activity on mushroom tyrosinase (5,7,8). However, the SAR between the flavonoids' structures and the suicide substrate properties on mushroom tyrosinase has not been well evaluated. Until now, only three flavonoids, 8-hydroxydaidzein, 8-hydroxygenistein, and 8-hydroxynaringenin, have been identified as suicide substrates of tyrosinase. All three of these flavonoids contain 7,8,4′-trihydroxyl functional groups attached to their flavonoid skeletons. The present study's results, together with our previous findings, show that 7,8,4′-trihydroxyl functional groups on flavonoid skeletons play important roles in their exhibiting suicide substrate properties toward mushroom tyrosinase.

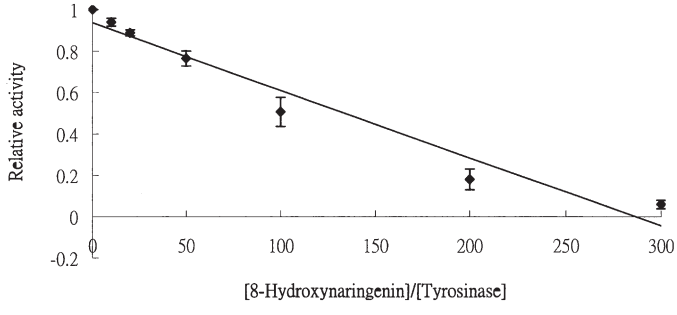


Figure 3. Relative activities of titrations of mushroom tyrosinase with 8-hydroxynaringenin.

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