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Food Chemistry 105 (2007) 1430–1438
Food Chemistry
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Mushroom tyrosinase inhibitory effects of isoflavones isolated from soygerm koji fermented with *Aspergillus oryzae* BCRC 32288

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Received 25 October 2006; received in revised form 27 February 2007; accepted 9 May 2007

Abstract

The inhibition of mushroom tyrosinase in soygerm koji, fermented with *Aspergillus oryzae* BCRC 32288, was investigated. A methanol extract of the soygerm koji was partitioned into hexane, ethyl acetate and water. The ethyl acetate extract showed potent anti-tyrosinase activity with an IC_{50} value of 0.19 mg/ml. The active compounds were isolated by activity-guided silica gel column chromatography and high-performance liquid chromatography (HPLC) methods. Seven tyrosinase inhibitors were purified and identified as 6,7,4′-trihydroxyisoflavone, 7,8,4′-trihydroxyisoflavone, 5,7,8,4′-tetrahydroxyisoflavone, 7,4′-dihydroxyisoflavone (daidzein), 6-methoxy-7,4′-dihydroxyisoflavone (glycitein), 4′-hydroxyisoflavone-7-*O*-glucoside (daidzin), and 5,4′-dihydroxyisoflavone-7-*O*-glucoside (genistin) by comparing their mass, ^{1}H NMR, and ^{13}C NMR spectral data with those in the literature. The purified seven isoflavones from fermented soygerm koji were divided into two groups, based on their inhibitory effects on mushroom tyrosinase. Five isolated isoflavones showed inhibitory activity against monophenolase activity of mushroom tyrosinase only, with IC_{50} values of 0.009 ± 0.001 (6,7,4′-trihydroxyisoflavone), 0.203 ± 0.018 (daidzein), 0.218 ± 0.007 (glycitein), 0.267 ± 0.008 (daidzin), and 0.343 ± 0.013 (genistin) mM. The kinetic study indicated that the five inhibitors significantly lengthened the lag time of the monophenolase activity of tyrosinase and acted competitively for the L-tyrosine binding site of the enzyme. So, the five isoflavones were competitive inhibitors for the monophenolase activity of tyrosinase. The other two isoflavones, 7,8,4′-trihydroxyisoflavone and 5,7,8,4′-tetrahydroxyisoflavone, inhibited both monophenolase and diphenolase activities of tyrosinase. Moreover, pre-incubation of each of the two isoflavones with tyrosinase resulted in total irreversible inhibition of the enzyme activity, even at concentrations as low as of 10 μM. Hence, 7,8,4′-trihydroxyisoflavone and 5,7,8,4′-tetrahydroxyisoflavone were irreversible inhibitors of mushroom tyrosinase.

Keywords: Inhibitor; Isoflavone; Irreversible; Soygerm; Tyrosinase

1. Introduction

Tyrosinase (EC 1.14.18.1) is a copper-containing monooxygenase, widely distributed in nature. 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 (Seo, Sharma, & Sharma, 2003). Although the pigment melanin in human skin is a major defence mechanism against the ultraviolet light of the sun, the production of abnormal pigmentation, such as melasma, freckles, age-spots, liver spots, and other forms of melanin hyperpigmentation can be a serious aesthetic problem (Briganti, Camera, & Picardo, 2003). Hence, inhibiting the tyrosinase activity (and preventing the abnormal pigmentations) has been the subject of many studies (Kim et al., 2002).

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0308-8146/$ - see front matter © 2007 Elsevier Ltd. All rights reserved.
doi:10.1016/j.foodchem.2007.05.019

In the food industry, tyrosinase, which is also known as a polyphenol oxidase (PPO) (Mayer, 1987), is responsible for enzymatic browning reactions in damaged fruits during post-harvest handling and processing. Browning is caused by the oxidation of phenolic compounds in fruits (Labuza, Lilemo, & Taoukis, 1992). This reaction could produce undesirable changes in colour, flavour, and nutritive value for some products. Control of enzymatic browning during processing is important in fruit pulp manufacturing. Therefore, there is also 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 of harmful adverse effects (Lee & Lee, 1997).

Isoflavones are naturally occurring dietary phytoestrogens in different plants, and specifically in legumes, such as soybeans (Franke, Custer, Cerna, & Narala, 1994). In recent years, the isoflavones have been under intensive investigation due to their possible role in preventing certain hormone-dependent and other diseases, including breast and prostate cancers, osteoporosis, and cardiovascular diseases (Messina, 2000). Our recent study on the screening of new tyrosinase inhibitors resulted in the identification of a known compound, 6,7,4′-trihydroxyisoflavone, as a potent tyrosinase inhibitor (Chang, Ding, & Lin, 2005). This compound exists in several fermented soybean foods, including tempe (Gyorgy, Murata, & Ikkhata, 1964), miso (Hirota et al., 2004) and sake (Esaki, Kawakishik, Morimitsu, & Osawa, 1999). In fact, in addition to 6,7,4′-trihydroxyisoflavone, there are many isoflavone analogues produced from the preparation processes of those fermented soybean foods. So, we were interested in finding out if any other isoflavone metabolites in the fermented soybean products showed anti-tyrosinase activity. In this study, we investigated the natural compounds found in soygerm koji with anti-tyrosinase activity. The inhibition mode and kinetic study of the newly found tyrosinase inhibitors were also studied.

2. Materials and methods

2.1. Materials

Rice and soybean were purchased from the local market. Soygerm was obtained from Hai-Yin Oil Corporation (Tainan, Taiwan, ROC). Lyophilized culture of *Aspergillus oryzae* BCRC 32288 was obtained from the Bioresources Collection and Research Center (BCRC, Food Industry Research and Development Institute, 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 (2870 U/mg), L-tyrosine, L-DOPA, dimethyl sulfoxide (DMSO), Sephadex G-25 spin column, and silica gel (70–230 mesh) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Kojic acid was obtained from Aldrich Chemical Co. (Milwaukee, WI, USA). HPLC-grade acetonitrile and acetic acid were supplied by J.T. Baker (Phillipsburg, NJ, USA). Other reagents and solvents were commercially available and used as received.

2.2. Preparation of fermented koji

Each substrate for preparing koji (rice, soybean, or soygerm) was steeped in water for 1 h and steamed at 121 °C for 15 min for sterilization. After cooling to room temperature, a suspension of *A. oryzae* spore was sprayed onto the surface of the steamed substrate (inoculation of 10%, v/w) and incubated at 25 °C without shaking for one week.

2.3. Isolation and identification of tyrosinase inhibitors from soygerm koji

The purification process of the tyrosinase inhibitors in goygrem koji was carried out as shown in Fig. 1, 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 cm × 2.6 i.d.) with 0.5 l each of hexane/ethyl acetate (3:1), hexane/ethyl acetate (1:1), ethyl acetate, ethyl acetate/methanol (1:1) and methanol as eluents. Both ethyl acetate and ethyl acetate/methanol (1:1) fractions showed anti-tyrosinase activity and were purified by repeated HPLC using a semi-preparative C18 reversed-phase column (Spherisorb, 5 μM, 10 i.d. × 250 mm, ODS 2, Phase Separation Ltd., Deeside Industrial Park, Clwyd, UK). The gradient elution using water (A), containing 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 ethyl acetate fraction (1.56 g) and ethyl acetate/methanol fraction (0.862 g) were suspended in 15 ml and 8 ml DMSO, respectively, and repeatedly injected into the HPLC column with an injection volume of 250 μl. The eluted peaks were collected, dried, and assayed for anti-tyrosinase activity. Five peaks from the ethyl acetate fraction were identified with anti-tyrosinase activity. Among them, one compound (7,8,4′-trihydroxyisoflavone) was of high purity judged by the single peak pattern in HPLC chromatography. Two peaks (6,7,4′-trihydroxyisoflavone and 5,7,8,4′-tetrahydroxyisoflavone) interfered with each other and were repetitively separated by HPLC under the same conditions as described above until the purities of the two compounds were qualified by single peak patterns in the HPLC chromatography. The other two peaks (daidzein and glycitein) also interfered with each other and were

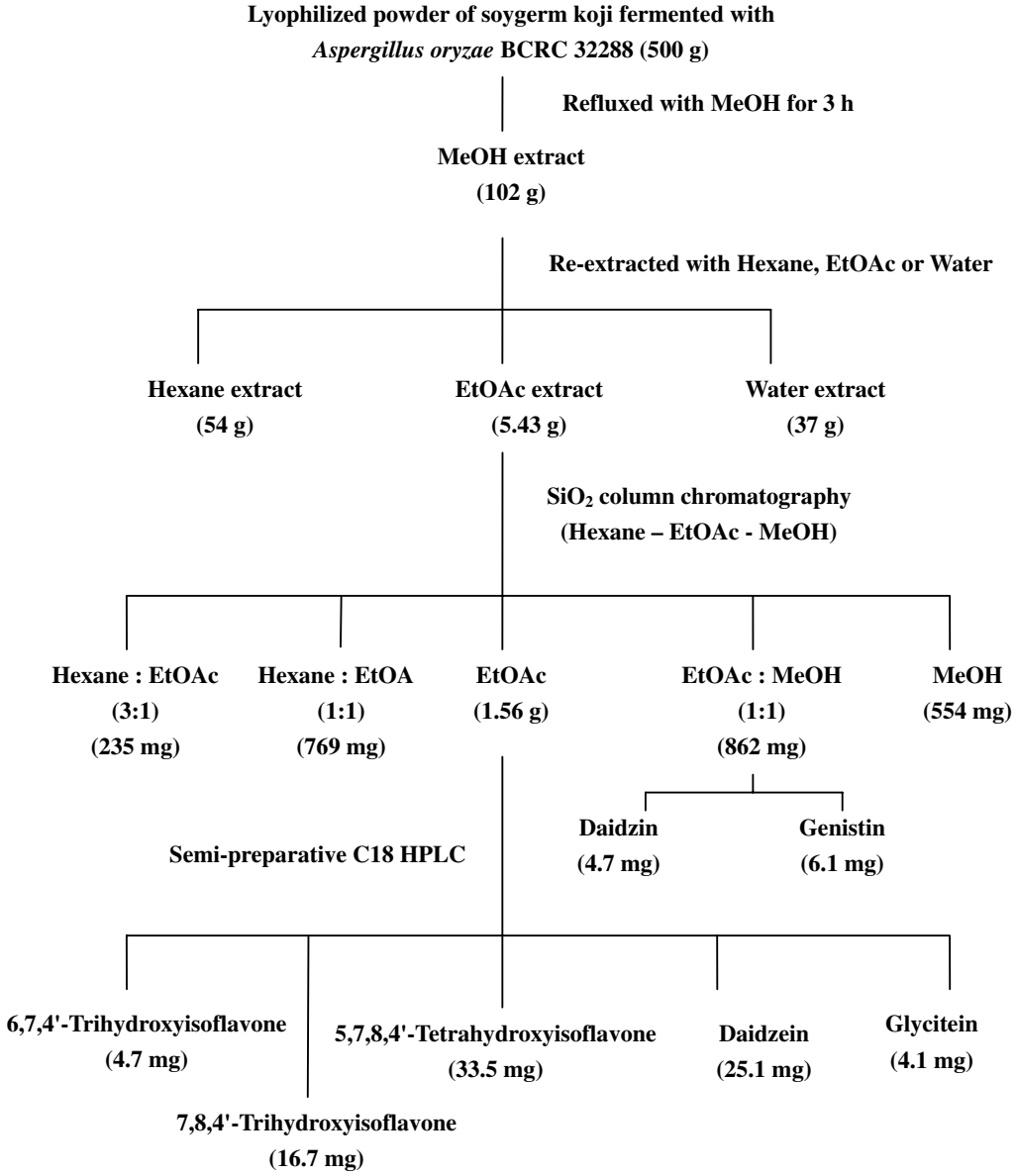


Fig. 1. Isolation scheme for tyrosinase inhibitors from soygerm koji.

isolated in advance in the same way. Two peaks (daidzin and genistin) from the ethyl acetate/methanol fraction were identified with anti-tyrosinase activity and both of them were of high purity also, judged by the single peak pattern in the HPLC chromatography. The final seven isolated compounds were then subjected to mass spectral and NMR analyses.

The chemical structures of the seven isolated compounds were determined by mass and NMR spectrometry. FAB MS were obtained with a JEOL TMSD-100 mass spectrometer performing in positive ionization mode. The voltage used was 10.0 kV. The NMR spectra were measured in DMSO-d_6 with a Varian Gemini NMR spectrometer operating at 301 K and 400 MHz for ^{1}H NMR and 100 MHz for ^{13}C NMR, with complete proton decoupling. The sweep width, pulse angle, repetition delay and acquisition time for ^{1}H NMR were 7000.0 Hz, 4.2–4.5 μs, 1 and 1.7–2.3 s, respectively, and for ^{13}C NMR were 30,000.0 Hz, 4.5–4.9 μs, 2.0 and 0.5–1 s, respectively. The chemical shifts are reported in parts per million (ppm) from tetramethylsilane. The chemical shifts were defined by assigning the deuterated DMSO-d_6 resonance peak to 3.305 ppm for ^{1}H NMR and 49.15 ppm for ^{13}C NMR. The resulting spectral data and identifications of the compounds are given below.

6,7,4′-Trihydroxyisoflavone (Esaki et al., 1999). The molecular formula of the active compound was determined to be $C_{15}H_{10}O_5$ from the mass spectral data; FAB $MS_{m/z}$: 271 $[M + H]^+$. The NMR spectral data of the active compound were: ^{1}H NMR (DMSO-d_6) δ: 6.78 (2 H, 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).

7,8,4′-Trihydroxyisoflavone (Esaki, Onozakik, Morimitsu, Kawakishi, & Osawa, 1998). The molecular formula of the active compound was determined to be $C_{15}H_{10}O_5$ from the mass spectral data; FAB $MS_{m/z}$: 271 $[M+H]^+$. The NMR spectral data of the active compound were: ^{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 (1 H, 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).

5,7,8,4′-Tetrahydroxyisoflavone (Esaki et al., 1998). The molecular formula of the active compound was determined to be $C_{15}H_{10}O_6$ from the mass spectral data; FAB $MS_{m/z}$: 287 $[M+H]^+$. The NMR spectral data of the active compound were: ^{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).

Daidzein (Kinjo et al., 1987). The molecular formula of the active compound was determined to be $C_{15}H_{10}O_4$ from the mass spectral data; FAB $MS_{m/z}$: 255 $[M+H]^+$. The NMR spectral data of the active compound were: ^{1}H NMR (DMSO-d_6) δ: 6.77 (2H, d, $J=8.4$ Hz, H-3′, 5′), 6.83 (1H, d, $J=2.1$ Hz, H-8), 6.90 (1H, dd, $J=8.7$, 2.1 Hz, H-6), 7.36 (2H, d, $J=8.4$ Hz, H-2′, 6′), 7.94 (1H, d, $J=8.7$ Hz, H-5), 8.25 (1H, s, H-2). ^{13}C NMR (DMSO-d_6) δ: 174.7 (C-4), 162.5 (C-7), 157.4 (C-9), 157.2 (C-4′), 152.7 (C-2), 130.1 (C-2′, 6′), 127.2 (C-5), 123.6 (C-3), 122.8 (C-1′), 116.7 (C-10), 115.1 (C-6), 115.0 (C-3′, 5′), 102.3 (C-8).

Glycitein (Park et al., 1999). The molecular formula of the active compound was determined to be $C_{16}H_{12}O_5$ from the mass spectral data; FAB $MS_{m/z}$: 285 $[M+H]^+$. The NMR spectral data of the active compound were: ^{1}H NMR (DMSO-d_6) δ: 3.85 (3H, s, OCH_3), 6.78 (2H, d, $J=8.6$ Hz, H-3′, 5′), 6.92 (1H, s, H-8), 7.36 (2H, d, $J=8.6$ Hz, H-2′, 6′), 7.41 (1H, s, H-5), 8.23 (1H, s, H-2). ^{13}C NMR (DMSO-d_6) δ: 174.4 (C-4), 157.1 (C-4′), 152.7 (C-2), 152.5 (C-7), 151.7 (C-9), 146.8 (H-6), 130.0 (C-2′, 6′), 123.1 (C-3), 122.6 (C-1′), 116.2 (C-10), 115.1 (C-3′, 5′), 104.8 (C-5), 102.8 (C-8), 55.8 (OCH_3).

Daidzin (Hirakura et al., 1997). The molecular formula of the active compound was determined to be $C_{21}H_{20}O_9$ from the mass spectral data; FAB $MS_{m/z}$: 417 $[M+H]^+$. The NMR spectral data of the active compound were: ^{1}H NMR (DMSO-d_6) δ: 3.23 (1H, m, H-4″), 3.25 (1H, m, H-3″), 3.31 (1H, m, H-2″), 3.53 (1H, m, H-6″a), 3.57 (1H, m, H-5), 3.69 (1H, d, $J=10.7$ Hz, H-6″b), 5.10 (1H, d, $J=7.2$ Hz, H-1″), 6.82 (2H, d, $J=8.3$ Hz, H-3′, 5′), 7.15 (1H, d, $J=8.8$ Hz, H-6), 7.41 (2 H, d, $J=8.3$ Hz, H-2′, 6′), 8.06 (1H, d, $J=8.8$ Hz, H-5), 8.37 (1H, s, H-2), 9.58 (1H, br. s, OH-4′). ^{13}C NMR (DMSO-d_6) δ: 175.1 (C-4), 161.5 (C-7), 157.3 (C-9), 157.2 (C-4′), 153.3 (C-2), 130.2 (C-2′, 6′), 127.1 (C-5), 123.9 (C-3), 122.5 (C-1′), 118.6 (C-10), 115.8 (C-6), 115.2 (C-3′, 5′), 103.6 (C-8), 100.3 (C-1″), 77.5 (C-5″), 76.6 (C-3″), 73.4 (C-2″), 69.8 (C-4″), 60.7 (C-6″).

Genistin (Wang, Nair, Strasburg, Booren, & Gray, 1999). The molecular formula of the active compound was determined to be $C_{21}H_{20}O_{10}$ from the mass spectral data FAB $MS_{m/z}$: 433 $[M+H]^+$. The NMR spectral data of the active compound were: ^{1}H NMR (DMSO-d_6) δ: 3.17 (1H, t, $J=8.6$ Hz, H-4″), 3.27 (1H, t, $J=8.6$ Hz, H-2″), 3.29 (1H, t, $J=8.6$ Hz, H-3″), 3.42 (1H, m, H-5), 3.49 (1H, dd, $J=10.1$, 4.7 Hz, H-6″a), 3.70 (1H, d, $J=10.1$ Hz, H-6″b), 5.02 (1H, d, $J=7.3$ Hz, H-1″), 6.46 (1H, s, H-6), 6.69 (1H, s, H-8), 6.81 (2H, d, $J=8.5$ Hz, H-3′, 5′), 7.37 (2H, d, $J=8.5$ Hz, H-2′, 6′), 8.35 (1H, s, H-2), 9.62 (1H, br. s, OH-4′), 12.91 (1H, br. s, OH-5). ^{13}C NMR (DMSO-d_6) δ: 181.0 (C-4), 163.5 (C-7), 162.2 (C-5), 161.9 (C-9), 157.8 (C-4′), 154.9 (C-2), 130.7 (C-2′, 6′), 123.2 (C-3), 121.6 (C-1′), 115.5 (C-3′, 5′), 106.6 (C-10), 100.4 (C-6), 100.0 (C-1″), 95.1 (C-8), 77.6 (C-5″), 76.7 (C-3″), 73.5 (C-2″), 70.0 (C-4″), 61.1 (C-6″).

2.4. Enzymatic assay of tyrosinase

Monophenolase and diphenolase activities of mushroom tyrosinase were determined according to the methods described in our previous report (Chang et al., 2005) with some modifications. 880 μl of 2 mM substrate (L-tyrosine or L-DOPA dissolved in 50 mM phosphate buffer, pH 6.8) were mixed with 100 μl of the tested sample (dissolved in DMSO) at 25 °C for 2 min. Then, 20 μl of tyrosinase (1000 U/ml in phosphate buffer) were added to initiate the reaction. The assay mixture was incubated at 25 °C for 10 min. The increase in absorbance at 475 nm, due to the formation of dopachrome, was monitored with a spectrophotometer. The percentage inhibition of tyrosinase activity was calculated as follows: % Inhibition $=[(A-B)/A]\times 100$, where *A* is the absorbance at 475 nm with DMSO instead of the tested sample and *B* is the absorbance at 475 nm with the sample. The concentration of a compound at which 50% of the enzyme activity was inhibited (the IC_{50} value) was obtained by linear curve fitting. For the crude samples obtained from the purification process and different koji extracts, only the monophenolase activity was assayed. The inhibition kinetics of the isoflavones were analyzed by the Lineweaver–Burk method. Experiments were carried out using the same protocol described above, except for the concentration of L-tyrosine (0.1–0.5 mM).

For irreversible inhibitory activity assay, 20 units of tyrosinase was pre-incubated with the tested isoflavone (dissolved in DMSO) in 0.2 ml of 50 mM of phosphate buffer (pH 6.8) at 25 °C for 30 min. Then, 0.8 ml of 2.5 mM

L-DOPA or L-tyrosine were added and the reaction mixture was incubated at 25 °C for 10 min. For comparison, further experiments were conducted by immediately mixing the tested compound, tyrosinase, and L-DOPA in 1 ml of phosphate buffer and incubating at 25 °C for 10 min. The formation of dopachrome product 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 pre-incubation mixture, incubated for 30 min, was first 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. Then, the residual tyrosinase activities of the mixtures from the two treatments were assayed as described above. All experiments were repeated at least twice in order to ensure the reproducibility of the results, and the mean values are reported here.

3. Results and discussion

3.1. Isolation, purification, and identification of tyrosinase inhibitors from soygerm koji fermented with A. oryzae BCRC 32288

In our preliminary study, 6,7,4′-trihydroxyisoflavone was demonstrated to be a potent tyrosinase inhibitor (Chang et al., 2005). Because many of the isoflavone analogues are produced during the fermentation of soy koji, we were interested to know whether these isoflavone metabolites possessed tyrosinase inhibitory activity. Koji was prepared by fermenting rice, soybean and soygerm using *A. oryzae* BCRC 32288. After fermentation, the methanol extracts of the three koji products and their corresponding unfermented substrate were assayed for tyrosinase inhibitory activity. The results are shown in Fig. 2. The extract of soygerm koji showed maximal anti-tyrosinase activity with an IC_{50} value of 0.96 mg/ml. Neither the methanol extract of rice nor that of rice koji showed any anti-tyrosinase activity, even at the high concentration of 5 mg/ml. In contrast, all the methanol extract of soybean, soygerm and their corresponding koji products showed tyrosinase inhibitory activity. Comparisons between anti-tyrosinase activities of the extracts from the fermented and unfermented materials implied that both the isoflavone content and the fermentation process could improve anti-tyrosinase activity in the extract. Based on the above results, we selected the fermented soygerm koji as the material for isolating tyrosinase inhibitors.

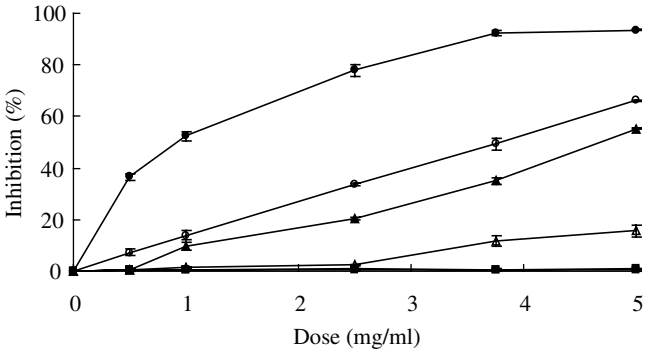
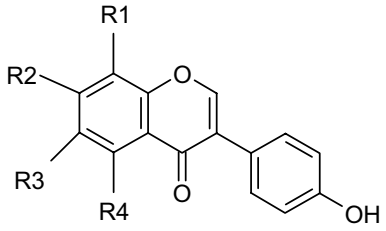


Fig. 2. Inhibitory effects of methanol extracts from rice (□), rice koji (■), soybean (△), soybean koji (▲), soygerm (○), and soygerm koji (●) on tyrosinase activity with L-tyrosine as a substrate.

Bioassay-guided purification of the methanol extract of the fermented soygerm koji was carried out as shown in Fig. 1. The methanol extract was partitioned with hexane, ethyl acetate, and water in succession. The active fraction, the ethyl acetate layer, was further separated by silica gel column chromatography and repeated semi-preparative HPLC with the bioactivity as the guide. Finally, seven active compounds were isolated. The chemical structures of the isolated compounds were determined by mass, ^{1}H NMR, and ^{13}C NMR analyses and the resulting spectral data were shown in Section 2. Due to large amounts of various compounds isolated and identified in various fermented or unfermented soybean foods, it is straightforward to confirm the chemical structures of the isolated compounds in the present study by comparing their mass, ^{13}C NMR, and ^{1}H NMR spectral data with those in the literature. As a result, the spectral characteristics of all the seven isolated compounds were individually found to be identical to those reported in the literature as described in Section 2 and the compounds were identified as 6,7,4′-trihydroxyisoflavone, 7,8,4′-trihydroxyisoflavone, 5,7,8,4′-tetrahydroxyisoflavone, daidzein, glycitein, daidzin and genistin, respectively. The structures of the isolated compounds are shown in Fig. 3.

3.2. Characterization of tyrosinase inhibitors from soygerm koji

The inhibitory potencies of the seven isoflavones toward both monophenolase and diphenolase activities of mushroom tyrosinase were investigated. The results are shown in Table 1. All inhibitory activities were dose-dependent. Among them, five isoflavones, including 6,7,4′-trihydroxyisoflavone, daidzein, glycitein, daidzin and genistin, showed potent monophenolase inhibitory activity, but poor diphenolase inhibitory activity. The inhibitory effects of these five isoflavones on diphenolase activity, with L-DOPA as the substrate, were over 100 times lower than that on monophenolase activity with L-tyrosine as the substrate. For example, even at the high concentration of 0.5 mM of 6,7,4′-trihydroxyisoflavone, the diphenolase activity of tyrosinase was not inhibited, while 30.4% of the monophenolase activity of tyrosinase was inhibited in the presence of only 0.005 mM of the same compound. This phenomenon is similar to the report of Fu, Li, Wang, Lee, and Cui (2005), who identified three chalcones, including licuraside, isoliquiritin and licochalcone A, as being potent monophenolase inhibitors but poor diphenolase inhibitors of mushroom tyrosinase. The differences between the two



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

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

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

7,4'-Dihydroxyisoflavone (Daidzein); R1 = R3 = R4 = H, R2 = OH

6-Methoxy,7,4'-dihydroxyisoflavone (Glycitein); R1 = R4 = H, R2 = OH, R3 = OCH_3

4'-Hydroxyisoflavone-7-*O*-glucoside (Daidzin); R1 = R3 = R4 = H, R2 = OGlc

5,4'-Dihydroxyisoflavone-7-*O*-glucoside (Genistin); R1 = R3 = H, R2 = OGlc, R4 = OH

Fig. 3. Chemical structures of compounds investigated in this study.

Table 1
Inhibitory effects of investigated compounds on both monophenolase and diphenolase activities of mushroom tyrosinase

Test sample	IC_{50} (mM) against monophenolase activity	IC_{50} (mM) against diphenolase activity	Inhibition mode	Michaelis constants K_I (μM)
6,7,4′-Trihydroxyisoflavone	0.009 ± 0.001	–[a]	Reversibly competitive[b]	1.93 ± 0.77
7,8,4′-Trihydroxyisoflavone	0.191 ± 0.011	0.184 ± 0.023	Irreversible[c]	–[a]
5,7,8,4′-Tetrahydroxyisoflavone	0.181 ± 0.009	0.212 ± 0.015	Irreversible[c]	–[a]
Daidzein	0.203 ± 0.018	–[a]	Reversibly competitive[b]	19.4 ± 0.4
Glycitein	0.218 ± 0.007	–[a]	Reversibly competitive[b]	50.6 ± 8.76
Daidzin	0.267 ± 0.008	–[a]	Reversibly competitive[b]	15.1 ± 1.02
Genistin	0.343 ± 0.013	–[a]	Reversibly competitive[b]	17.6 ± 0.21
Kojic acid	0.054 ± 0.001	0.058 ± 0.003	Mixed[c,d]	

[a] Unable to establish.
[b] With respect to L-tyrosine.
[c] With respect to both L-tyrosine and L-DOPA.
[d] Chen et al., 1991.

inhibitory activities of the three tyrosinase inhibitors are all over 10-fold. In addition, Nerya et al. (2003) also identified three compounds, including glabridin, isoliquiritigenin and glabrene with potent monophenolase inhibitory activity but poor diphenolase inhibitory activity. The IC_{50} values of the three compounds for anti-diphenolase activity were 15–1000 times higher than those for anti-monophenolase activity. The reasons why these tyrosinase inhibitors had much higher inhibitory activity on monophenolase activity than on diphenolase activity of mushroom tyrosinase are still unknown.

Tyrosinase naturally exists in three forms (*met*-, *oxy*-, and *deoxytyrosinase*) with different binuclear copper structures of the active site (Rescigno, Sollai, Pisu, Rinaldi, & Sanjust, 2002). The *met* form of tyrosinase, which is unable to act on monophenols, oxidizes *o*-diphenols to the corresponding *o*-quinones with concomitant reduction to the *deoxy* form, which then reacts with molecular oxygen and converts to the *oxy* form. The converted *oxy* form turns to the *met* form by either catalyzing monophenols to *o*-diphenols or catalyzing *o*-diphenols to *o*-quinones and continuing the catalytic action. Due to the fact that the *met* form is the main one in the enzyme resting state, the monophenolase activity is time-dependent with a characteristic lag time at the initial stage, when the monophenols are used as the enzyme substrates. The small amount of the *oxy* form generally present in the resting enzyme preparations could catalyze the monophenols to *o*-diphenols, which are needed to reduce the *met* form to the *deoxy* one and then to the *oxy* one. Hence, the lag time is the time required to reach the steady-state concentration of the cumulated amount of *o*-diphenols. Obviously, the length

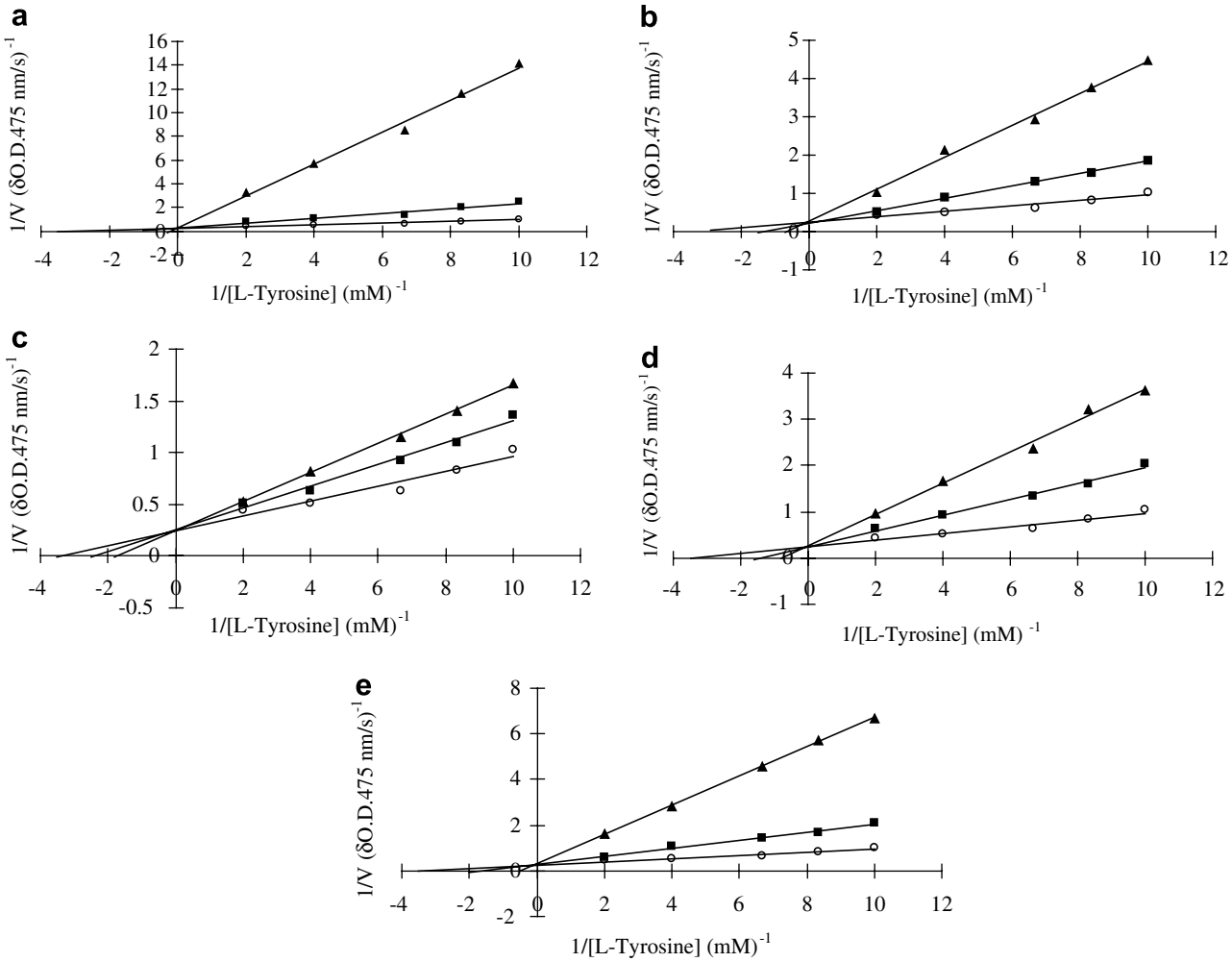


Fig. 4. Lineweaver–Burk plots of mushroom tyrosinase and L-tyrosine without (○) and with 1.85 μM (■) and 4.625 μM (▲) of 6,7,4′-trihydroxyisoflavone (a); with 29.25 μM (■) and 81.9 μM (▲) of daidzein (b); with 29.25 μM (■) and 81.9 μM (▲) of glycitein (c); with 18 μM (■) and 50.4 μM (▲) of daidzin (d); with 17.25 μM (■) and 48.3 μM (▲) of genistin (e).

of the lag time can be shortened or eliminated by the presence of small amounts of *o*-diphenols or reducing agents. In contrast, it has been known that inhibitors of monophenolase activity, such as tropolone, would extend the lag time (Kahn & Andrawis, 1985). In the present study, all the five compounds described above significantly lengthened the lag time (data not shown). This result again revealed that the five isoflavones are monophenolase inhibitors. In addition, the inhibition kinetics of the five isoflavones were analyzed by the Lineweaver–Burk method. The Lineweaver–Burk plots of $1/V$ versus $1/[S]$ of all the five isoflavones resulted in straight lines passing through the same point on the vertical axis (Fig. 4). The results indicated that they were all competitive inhibitors.

In contrast to the above five isoflavones, which were competitive inhibitors of the monophenolase activity of mushroom tyrosinase, 7,8,4′-trihydroxyisoflavone and 5,7,8, 4′-tetrahydroxyisoflavone showed both monophenolase and diphenolase inhibitory activities (Table 1). This indicated that the two isoflavones inhibited tyrosinase differently, based on the inhibitory effect on diphenolase activity of mushroom tyrosinase. In order to ascertain the inhibition mode of the two isoflavones in advance, we used a pre-incubation assay to conduct the inhibitory activity of the two isoflavones on mushroom tyrosinase. The results are shown in Fig. 5. Pre-incubation of tyrosinase with 7,8,4′-trihydroxyisoflavone or 5,7,8,4′-tetrahydroxyisoflavone for 30 min totally inactivated the enzyme activity at concentrations as low as 10 μM. Furthermore, after pre-incubation of tyrosinase with either of the two compounds, using dialysis or molecular exclusion chromatography (Sephadex G-25) to remove compounds of low molecular weight, did not restore tyrosinase activity. In contrast, the other five isoflavones described above did not possess the property. From the results obtained above, 7,8,4′-trihydroxyisoflavone and 5,7,8,4′-tetrahydroxyisoflavone were identified as irreversible inhibitors of mushroom tyrosinase. The detailed properties of these two newly identified irreversible inhibitors were described in a separated paper (Chang, 2007).

The present study isolated seven soy isoflavone derivatives from soygerm koji as tyrosinase inhibitors. The tyrosinase inhibitors could be divided into two groups based on

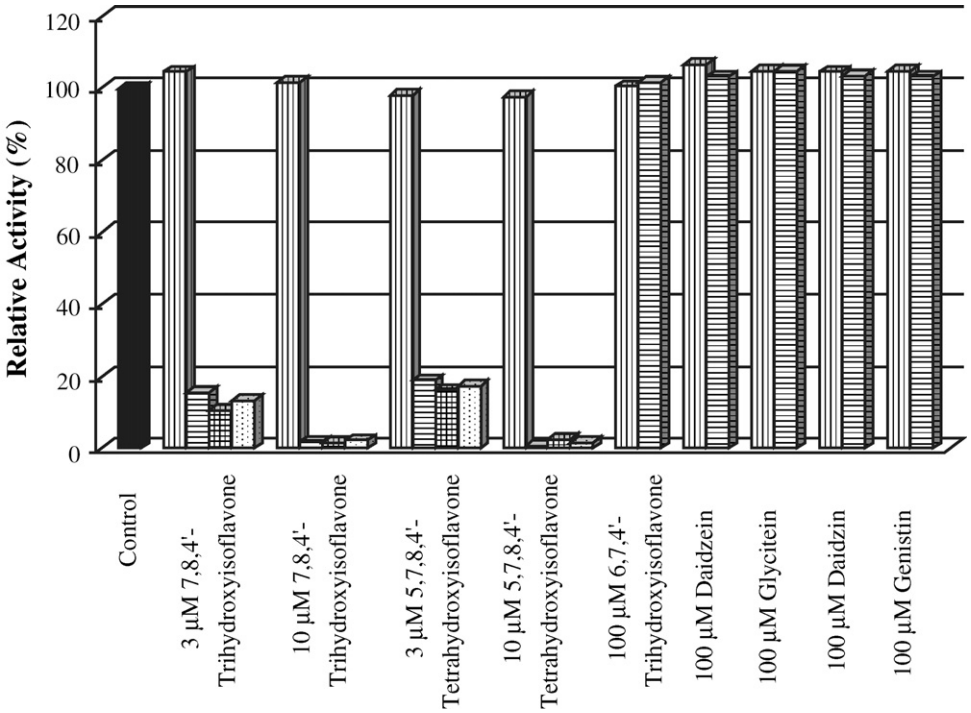


Fig. 5. Inhibitory effects of isoflavone derivatives on tyrosinase activity with L-DOPA as a substrate. The columns represent the corresponding activities of the reactions from immediate addition of inhibitor, enzyme, and substrate (vertical lines), pre-incubation of inhibitor with the enzyme (horizontal lines), dialysis of the pre-incubation mixture (cubic), and gel-filtration of the pre-incubation mixture (dotted). The detailed procedure is given in Section 2.

their inhibitory characterizations (Table 1). One group could only reversibly inhibit monophenolase activity of tyrosinase and constituted competitive inhibitors of 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, which could irreversibly inhibit both monophenolase and diphenolase activities of tyrosinase.

Besides, it is interesting to note that the position and number of the hydroxyl group in the A-ring of the isoflavone structure could strongly affect both the inhibitory strength and the inhibitory mode of the isoflavones on mushroom tyrosinase. As examples, the isoflavone with hydroxyl groups at both C6 and C7 positions in the A-ring (6,7,4′-trihydroxyisoflavone) could increase (over 10 times) both inhibitory activity (judged by IC_{50} values) and affinity for the enzyme (judged by Michaelis constants) to more than that of isoflavones with either only one hydroxyl group at the C7 position (daidzein and glycitein) or without any hydroxyl group in the A-ring (daidzin and genistin) (Table 1). Alternatively, the hydroxyl groups at both C7 and C8 positions (7,8,4′-trihydroxyisoflavone and 5,7,8,4′-tetrahydroxyisoflavone) could completely change the inhibitory mode of the isoflavones from reversible to irreversible form. However, the hydroxyl group at the C7 position alone does not significantly affect the inhibitory potency, as shown by comparing the inhibitory activities between daidzein and glycitein (with hydroxyl group at C7 position) and daidzin and genistin (with *O*-glucoside at C7 position). The elucidation of a detailed mechanism of the effects of the hydroxyl groups in the isoflavones on their inhibitory activities toward tyrosinase needs further studies.

Being safe is an important demand for tyrosinase inhibitors used in foods and cosmetic products. Soy isoflavones and their derivatives occur in many fermented soybean foods in amounts of several mg/g, which is much higher than the concentration needed to protect against enzymatic browning. Besides, many investigations have demonstrated that the soy isoflavones and their metabolites contain cancer chemoprotective (Lamartiniere, Wang, Smith-Johnson, & Eltoum, 2002), antioxidant (Esaki et al., 1998), estrogenic (Santell, Chang, Nair, & Helferich, 1997), antifungal and antibacterial (Chang, Nair, & Nitiss, 1995) activities. In addition to the potent anti-tyrosinase activity, safety, and health benefits for human consumption, the soy isoflavones and their metabolites exist in large amounts in soybean and are available on an industrial scale. On the basis of our data and some earlier findings, the isoflavones from soygerm koji evidently have potential to be further developed as effective anti-browning agents for foodstuffs or as skin-whitening agents in cosmetics.

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