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Mushroom tyrosinase inhibitory effects of isoflavones isolated from soygerm koji fermented with *Aspergillus oryzae* BCRC 32288

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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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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