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ORIGINAL ARTICLE

Inhibition of tyrosinase activity and melanine pigmentation by 2-hydroxytyrosol

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KEY WORDS

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B16 melanoma cells

Abstract 2-Hydroxytyrosol (2-HT), originally reported as a synthetic compound, was isolated for the first time as a fungal metabolite. 2-HT was found to inhibit mushroom tyrosinase with an IC_{50} value of 13.0 μmol/L. Furthermore, 2-HT dose-dependently inhibited tyrosinase activity (IC_{50}, 32.5 μmol/L) in the cell-free extract of B16 melanoma cells and α-melanocyte stimulating hormone (α-MSH)-stimulated melanin formation in intact B16 melanoma cells.

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1. Introduction

Melanin is essential for protecting human skin against radiation, but the accumulation of abnormal melanin induces pigmentation disorders, such as melasma, freckles, ephelides, and senile lentigines[1]. Melanogenesis is conducted in melanocytes, located in the basal layer of the epidermis and controlled by tyrosinase[2].

Tyrosinase (EC 1.14.18.1), also known as polyphenol oxidase (PPO), is a copper-containing monooxygenase enzyme involved in melanogenesis[3]. The enzyme is widely distributed in fungi, higher plants and animals[4], and is involved in the first two steps of the melanin biosynthesis, in which L-tyrosine is hydroxylated to 3,4-dihydroxyphenylalanine (L-DOPA, monophenolase activity) and the latter is subsequently oxidated to dopaquinone (diphenolase activity)[2]. A large number of moderate to potent tyrosinase inhibitors from natural and synthetic resources have been reported during the last decade[5–9]. Tyrosinase inhibitors such as arbutin, kojic acid and hydroquinones have been used as whitening or antihyperpigment agents because of their ability to suppress dermal-melanin production[10,11]. However, arbutin and kojic acid hardly showed inhibitory activity against pigmentation in intact melanocytes or in a clinical trial[12], and hydroquinones are considered to be cytotoxic to melanocytes and potentially mutagenic to mammalian cells[11]. Therefore, it remains necessary to search for new tyrosinase inhibitors without side effects.

During our course of screening for mushroom tyrosinase inhibitors of microbial origin, 2-hydroxytyrosol (2-HT, Fig. 1) was isolated from the fungal culture broth of *Metarhizium* sp. OB-0098. 2-HT was originally reported to be a synthetic compound[13], but its biological activity has not been reported. In this study, tyrosinase inhibitory activities and melanin formation in mouse B16 melanoma cells of 2-HT were described.

2. Results

2.1. Inhibition of mushroom tyrosinase activity by 2-hydroxytyrosol

In this assay, the conversion of L-DOPA to dopaquinone by mushroom tyrosinase was observed at 450 nm. As shown in Fig. 2, 2-HT dose-dependently inhibited mushroom tyrosinase activity with an IC_{50} value of 13.0 μmol/L. Under the same conditions, kojic acid also inhibited the activity with IC_{50} of 14.8 μmol/L.

2.2. Inhibition of melanin pigmentation in B16 melanoma cells by 2-hydroxytyrosol

To investigate whether 2-HT inhibited melanogenesis, the effect of 2-HT on melanin pigmentation in intact B16 melanoma cells was studied. α-MSH was added to this assay system, because melanin production was markedly enhanced. 2-HT was found to inhibit the melanin pigmentation of B16 melanoma cells in a dose-dependent manner with IC_{50} of 571 μmol/L (Fig. 3). Under the same conditions, arbutin inhibited the melanin pigmentation with IC_{50} of 1130 μmol/L, and kojic acid inhibited it by 45.7% at 735 μmol/L. Furthermore, the cytotoxic effects of these inhibitors on B16 melanoma cells were investigated by the MTT assay. The IC_{50} values of 2-HT, kojic acid and arbutin were 1.3, 3.0 and 1.8 mmol/L, respectively.

2.3. Inhibition of B16 cells tyrosinase activity by 2-hydroxytyrosol

To confirm the inhibition of melanin pigmentation in intact B16 melanoma cells by 2-HT, the effect of 2-HT on tyrosinase activity

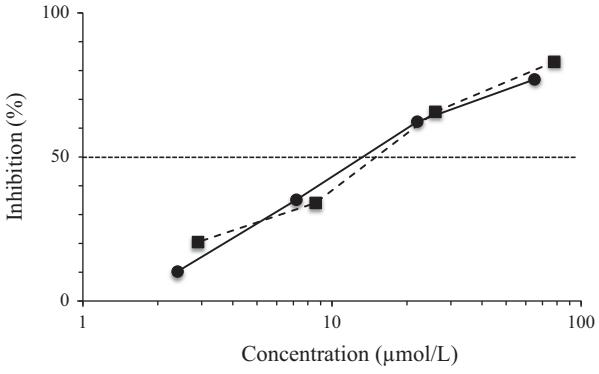

Figure 2 Inhibitory effects of 2-HT(●) and kojic acid (■) against mushroom tyrosinase.

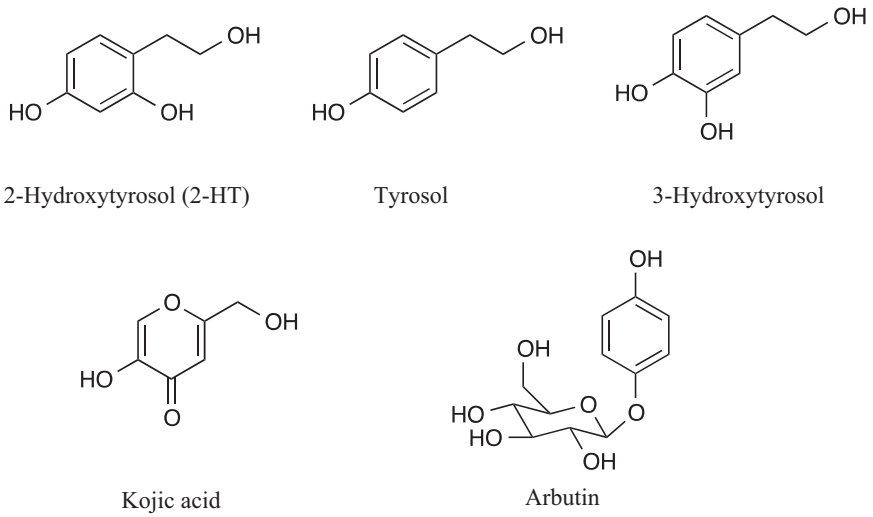

Figure 1 Structures of 2-hydroxytyrosol (2-HT) and tyrosinase inhibitors.

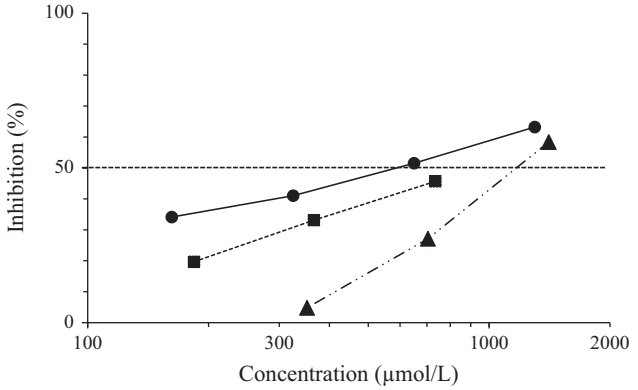

Figure 3 Inhibitory effects of 2-HT, kojic acid and arbutin on α-MSH-induced melanin production in B16 melanoma cells. The pellets were resuspended in 2 mol/L NaOH solution and absorbance of supernatants was measured at 450 nm using a microplate reader. (●) 2-HT, (■) kojic acid and (▲) arbutin.

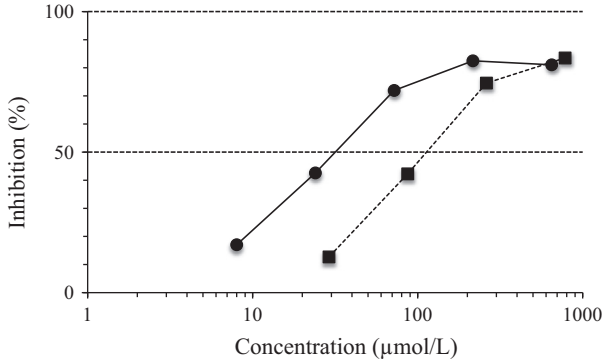

Figure 4 Inhibitory effects of 2-HT (●) and kojic acid (■) against tyrosinase in B16 melanoma cell crude lysates.

in B16 cell lysate was examined. As shown in Fig. 4, 2-HT showed inhibitory activity with IC_{50} of 32.5 μmol/L. Under the same conditions, the IC_{50} of kojic acid was calculated to be 113 μmol/L.

3. Discussion

In our screening for tyrosinase inhibitors of microbial origin using mushroom tyrosinase, 2-HT was isolated and identified from the fungal culture broth. Although 2-HT was originally reported as a synthetic compound[13], we showed for the first time that 2-HT is also a fungal metabolite. Furthermore, 2-HT was found to inhibit tyrosinase in this study. Structurally related tyrosol and 3-hydroxytyrosol were isolated from olive oil as antioxidants (Fig. 1)[14].

Kojic acid is a well-known tyrosinase inhibitor. In this study, we showed that the inhibitory activity of 2-HT (IC_{50}, 13 μmol/L) against mushroom tyrosinase is as potent as that of kojic acid (IC_{50}, 14.8 μmol/L), and even more potent against B16 melanoma tyrosinase than kojic acid. Furthermore, we demonstrated that 2-HT suppressed melanin production on α-MSH-treated B16 melanoma cells. 2-HT is a phenolic compound having three hydroxyl groups in the structure, which appears structurally related to L-DOPA, the substrate of tyrosinase. Therefore, it is plausible that 2-HT works competitively with respect to the substrate. These findings suggested that 2-HT is a promising lead compound for the treatment of skin pigmentation disorders.

4. Materials and methods

4.1. General experimental procedure

Various NMR spectra were obtained using an Agilent Technologies XL-400 (400 MHz) spectrometer (Agilent Technologies, Santa Clara, CA, USA). Electronionization mass spectrometry (EI-MS) was conducted on a JEOL JMS-T100LP spectrometer (JEOL, Tokyo, Japan).

4.2. Materials

3,4-Dihydroxy-L-phenylalanine (L-DOPA), kojic acid and α-arbutin were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Lyophilized mushroom tyrosinase and α-melanocyte stimulating hormone (α-MSH) were purchased from Sigma-Aldrich Co. (St. Louis, MO, USA). The B16 melanoma cell line, JCRB0202, was obtained from the Health Science Research Resources Bank (Tokyo, Japan).

4.3. Identification of producing fungus OB-0098

Strain fungus OB-0098 was isolated from an unidentified univalve shell collected on Okinawa main island, Okinawa, Japan. The genetic sequence information of the rDNA ITS (including 5.8S rDNA) gene[15] was designated as a strain belonging to the genus *Metarhizium*[16].

4.4. Fermentation of Metarhizium sp. OB-0098

A stock culture of strain *Metarhizium* sp. OB-0098 was grown and maintained on 2.4% potato dextrose agar (Becton, Dickinson and Company, NJ, USA) medium (non-adjusted pH). For the production of 4-(2-hydroxyethyl)-1,3-benzenediol, the seed medium used contained 2.4% potato dextrose broth (PDB) medium (non-adjusted pH). The production medium was composed of 50 g Vialonenano rice (Masi, VR, Italy) and 25 mL of 2.4% PDB (non-adjusted pH).

A loopful of spores of *Metarhizium* sp. OB-0098 was inoculated into a 500 mL Erlenmeyer flask with 100 mL seed medium and incubated on a rotary shaker at 27 °C for 3 days. The production culture was initiated by transferring 3 mL seed culture into each of fifty 500 mL culture bottles (As one, Osaka, Japan) containing production medium, and the fermentation was carried out at 27 °C for 14 days under stationary conditions.

4.5. Isolation procedure of 2-hydroxytyrosol

The culture (2.5 g) was treated with EtOH (5.0 L) for 2 h, and EtOH extracts were filtered to remove the mycelium and fermentation media. After concentration of the extracts to remove EtOH, the aqueous solution (0.33 L) was extracted with $CHCl_3$. Further, the aqueous layer was adjusted to pH 3.0 and extracted with EtOAc (0.33 L). The organic layer was dried over Na_2SO_4 and concentrated under reduced pressure to give brown material (0.6 g). The material (75 mg) containing 2-HT was dissolved in a small amount of MeOH and purified by HPLC using a reverse-phase C30 column under the

following conditions: column, Develosil C30 (250 mm × 10 mm), Nomura Scientific Co., Ltd., (Aichi, Japan); column temperature, 40 °C; mobile phase, 5% CH_3CN in 0.05% TFA.; flow rate, 3 mL/min; detection, UV 210 nm. 2-HT was eluted as a peak with a retention time of 16 min. The fraction of the peak was collected and concentrated to dryness to give pure 2-HT (2.73 mg).

4.6. Structure determination of 2-hydroxytyrosol

From the spectral data including ^{1}H NMR, ^{13}C NMR, and MS, and the search results of SciFinder Scholar, 2-HT was identified to be the same as the known synthetic compound 4-(2-hydroxyethyl)-1,3-benzenediol (Fig. 1)[13]. In this study, 2-HT was named as 2-hydroxytyrosol.

2-hydroxytyrosol: ^{1}H NMR (400 MHz, CD_3OD): δ 6.86 (1H, d, J=8 Hz, H-8), δ 6.27 (1H, d, J=2 Hz, H-5), δ 6.21 (1H, dd, J=2, 8 Hz, H-7), δ 3.68 (2H, t, J=7.5 Hz, H-1), δ 2.72 (2H, t, J=7.5 Hz, H-2). ^{13}C NMR (100 MHz, CD_3OD): δ 157.9 (s, C-6), δ 157.4(s, C-4), δ 132.2 (d, C-8), δ 117.8 (s, C-3), δ 107.4 (d, C-7), δ 103.6 (d, C-5), δ 63.6 (t, C-1), δ 34.5 (t, C-2). LR-EI-MS *m/z*: 154 $[M]^+$ HR-EI-MS *m/z*: $[M]^+$ calcd. for $C_8H_{10}O_3$, 154.0630; found, 154.0622.

4.7. Assay for mushroom tyrosinase activity

Tyrosinase inhibitory activity was measured spectrophotometrically according to the method of Masamoto et al.[17] with some modifications. First, 10 µL solution of 2-HT (2.4–65 µmol/L) in DMSO was added to a 96-well microplate and mixed with 60 µL 50 mmol/L phosphate buffer (pH 6.8) on ice. Then, 20 µL 0.9 mg/mL L-DOPA in phosphate buffer was added. Finally, 10 µL mushroom tyrosinase (500 U/mL in phosphate buffer) was added and the assay mixture was then incubated at 27 °C for 10 min. Following incubation, the amount of dopachrome production in the reaction mixture was determined spectrophotometrically at 450 nm (OD450) in a microplate reader. Kojic acid (2.9–77 µmol/L) dissolved in 50 mmol/L phosphate buffer was used as a positive control. The concentration for 50% inhibition (IC_{50}) was determined. Each measurement was performed at least in duplicate.

4.8. Cell culture

The murine melanoma B16 cell line, JCRB0202[18] (obtained from the National Institute of Biomedical Innovation) was maintained in Minimum Essential Medium (MEM) supplemented with 10% heat-inactivated fetal bovine serum (FBS), penicillin (100 units/mL)/streptomycin (100 mg/mL) and cultured at 37 °C in a humidified atmosphere with 5% CO_2. All experiments were performed in triplicate and were repeated 3 times to ensure reproducibility.

4.9. Cell proliferation

The cell viability assay of B16 cells was performed using the 3-(4,5-dimethyl-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay[19]. The cells were grown in 96-well plates at a density of 7.5×10^3 cells/well. After 22 h, the cells were then incubated in the presence of 100 nmol/L α-MSH and treated with various concentrations of sample. After 48 h of incubation, the cells were rewashed and 10 µL MTT solution (5 µg/mL) was added and incubated for approximately 3 h. After the cells were lysed with 40% *N*,*N*-dimethylformamide, 20% sodium dodecyl sulfate (SDS), 2.0% acetic acid, and 0.03% hydrochloric acid, the amount of formazan salt was quantified by measuring the absorbance at 570 nm with a microplate reader.

4.10. Measurement of melanin pigmentation on B16 melanoma cells

The melanin content was determined in accordance with the procedure described by Komiyama et al.[20] with some slight modifications. The B16 melanoma cells were seeded at a density of 2.5×10^3 cells/well in 6-well culture plates and then incubated for 5 days. The cells were washed twice in phosphate-buffered saline and treated with various concentrations of sample in the presence of 100 nmol/L α-MSH. After incubation for 3 days, the cells were washed in phosphate-buffered saline and was removed from the bottom of the well with 0.25% trypsin-EDTA solution (1 mL/well), and the solution containing the cells was transferred to a 1.5 mL microtube. After centrifugation for 10 min at 10,000×*g*, the pellets were dissolved in 2 mol/L NaOH (50 µL) for 15 min at 60 °C. The absorbance of the sample at 450 nm was measured with a microplate reader.

4.11. Assay for tyrosinase activity in B16 melanoma cell crude lysates

Crude tyrosinase was prepared by the method of Ohguchi et al.[21] with some slight modifications. Aliquots of B16 melanoma cells (5.0×10^6 cells/mL) in 100 mmol/L phosphate buffer (pH 6.8) containing 0.1% Triton X-100 (13.4 mL) were homogenized using a sonicator (Bioruptor; Cosmo Bio Co., Ltd., Tokyo, Japan). After centrifugation (11,000×*g*, 30 min, 4 °C), the supernatant was used as a crude tyrosinase enzyme solution. Protein in this crude enzyme solution was measured by the Lowry method, using bovine serum albumin as a standard.

Tyrosinase activity in B16 cell crude lysates was estimated using a modified mushroom tyrosinase assay. First, 50 µL 50 mmol/L phosphate buffer (pH 6.8), 20 µL L-DOPA (0.9 mg/mL, dissolved in 50 mmol/L phosphate buffer, pH 6.8) and 10 µL 2-HT or kojic acid were mixed. Then 20 µL crude lysates (2500 µg/mL) was added, and the amount of dopachrome in the reaction mixture was determined by optical density at 450 nm after 5 h at 37 °C.

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