Inhibitory Compound of Tyrosinase Activity from the Sprout of *Polygonum hydropiper* L. (Benitade)

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A tyrosinase inhibitor was isolated from the sprout of *Polygonum hydropiper* L. (Benitade) by activity-guided fractionation and identified as (2*R*,3*R*)-(+)-taxifolin (1) by spectroscopic means. Compound 1 inhibited 70% of tyrosinase activity at a concentration of 0.50 mM. ID_{50} (50% inhibition dose) value of compound 1 was 0.24 mM. As compared with tyrosinase inhibitor known cosmetic agent such as arbutin and kojic acid, compound 1 was more inhibited than the former and showed inhibitory effect equal to that of the latter. To study the inhibitory effect of (2*R*,3*R*)-(+)-taxifolin derivatives against tyrosinase activity, 3,7,3′,4′-taxifolin tetraacetate (2) and 5,7,3′,4′-taxifolin teramethyl ether (3) were also assayed together with compound 1.

Key words Benitade; tyrosinase activity; (2*R*,3*R*)-(+)-taxifolin

Melanin biosynthesis inhibitory compounds are useful not only as material used in cosmetics as skin-whitening agents but also as a remedy for disturbances in pigmentation. The color of mammalian skin and hair is determined by a number of factors. The most important factor is the degree and distribution of melanin pigmentation. Tyrosinase (phenol oxidase)[1] is one of the multifunctional enzymes containing copper that is found in fungi, plant materials, and animal tissues[2] and is responsible for melanin biosynthesis.[3,4] Therefore many tyrosinase inhibitors are used in depigmentation drugs and whitening cosmetics,[5,6] whereas compounds that increase melanogenesis may protect human skin from ultraviolet irradiation damage.[7] For example, ascorbic acid,[8] arbutin[9] and kojic acid[10] have been utilized as cosmetic agents. However, a recent study showed that kojic acid has carcinogenic action.[11,12] In the future, the discovery of new compounds to take the place of kojic acid is expected. Tyrosinase is also one of the most important key enzymes in the insect molting process and investigating its inhibitors may be important in finding alternative insect control agents. Furthermore, melanin formation is considered deleterious to the color quality of plant-derived food. This broadens the possible use of tyrosinase inhibitors as food additives, in addition to insect control agents and whitening agents.

Polygonum hydropiper L. (Polygonaceae) is distributed from tropics in the Northern Hemisphere to temperate zone and grows wild in waterside and marsh. It is native to Japan and China. Moreover it has long been used as a hot-tasting spice in Japan, China, and Europe, and also used as a folk medicine against cancer[13] and hemostatic disorders.[14] From the volatile fraction, many drimane-type sesquiterpenes such as polygodial and warburganal were isolated, which possess strong insect antifeedant and antibacterial activities.[15,16] Antioxidant flavonoids were also found from this plant.[17] *P. hydropiper* L. has not been reported to exhibit tyrosinase activity. On the other hand, the sprout is called "Benitade" in Japan and is a cultivar of *P. hydropiper*. It is one of the traditional vegetables in Japan and a well-known garnish mostly for dishes of 'sashimi', sliced raw fish, especially with white fish because of its bright red-purplish appearance and slight pungency. Compounds isolated from Benitade may not be toxic to humans, because safety has been histocically established. These compounds have not been investigated chemically with regard to Benitade and only reported on anthocyanin. There is also no report on inhibitor compounds of tyrosinase activity from Benitade. In this paper, we report the isolation and identification of inhibitor of tyrosinase activity from the sprout of *P. hydropiper* (Benitade).

MATERIALS AND METHODS

Materials Benitade was obtained from Hiroshima Tade Cooperation (Hiroshima, Japan). Tyrosinase was purchased from Funakoshi Co., Ltd. (Tokyo, Japan). L-Tyrosine was purchased from Wako Pure Chemical Ind., Ltd. (Osaka, Japan). Polyoxyethylene nonyl phenyl ether was purchased from Dai-ichi Kogyo Seiyaku Co., Ltd. (Kyoto, Japan).

General Procedure Optical rotation was measured on a Japan Spectroscopic Co. LTDDIP-1000 in acetone. EI-MS spectra were obtained on a JEOL the Tandem MS station JMS-700 (Japan Electron Optics Laboratory Co., Ltd., Tokyo, Japan). Nuclear magnetic resonance (NMR) spectra (δ, J in Hz) were recorded on a JEOL GSX 270 and 500 NMR spectrometers. Tetramethylsilane (TMS) was used as the internal reference (δ 0.00) for ^{1}H-NMR spectra measured in $CDCl_3$ and acetone-d_6. This solvent was also used for ^{13}C-NMR spectra.

Enzymatic Assay of Tyrosinase Tyrosinase activity using L-tyrosine as the substrate was assayed spectrophotometrically. Six-hundred eighty microliters of 0.1 M phosphate buffer (pH 6.8), 80 μl of 20% polyoxyethylene nonyl phenyl ether, 100μl of 0.03% L-tyrosine, and 40μl of test compounds solution (dissolved in DMSO) were added in a test tube and incubated at 37 °C for 10 min. After incubation, 100 μl of tyrosinase solution (528 units/ml, dissolved in 0.1 M phosphate buffer) was added to the test tubes and incubated at 37 °C for 60 min. Enzymatic activity was quantified by measuring the absorbance at 475 nm. Tyrosinase activity was obtained by the following formula.

$$\text{tyrosinase activity (\%)} = [(A-B)/(\text{Cp}-\text{Cn})] \times 100$$

where A is absorbance of test sample (0.1 M phosphate buffer, polyoxyethylene nonyl phenyl ether, L-tyrosine, sample solution, and tyrosinase); B is absorbance of blank (0.1 M phos-

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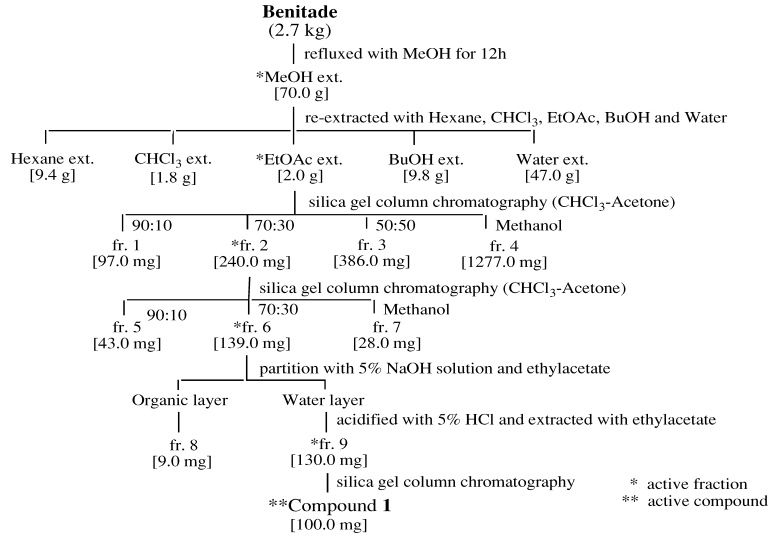

Fig. 1. Isolation Scheme for the Active Compound of Benitade

phate buffer, polyoxyethylene nonyl phenyl ether, distilled water, sample solution, and tyrosinase); Cp is absorbance of positive control (0.1 M phosphate buffer, polyoxyethylene nonyl phenyl ether, L-tyrosine, DMSO, and tyrosinase); and Cn is absorbance of negative control (0.1 M phosphate buffer, polyoxyethylene nonyl phenyl ether, distilled water, DMSO, and tyrosinase). The data are expressed as arithmetic mean±S.D. (n=3) and biological significance $p<0.05$ was determined by Student's t-test.

Fractionation and Isolation of Inhibitory Compound 1 To isolate the inhibitory compound from Benitade, fractionation was carried out as described in Fig. 1 using the tyrosinase inhibitory activity test as a guide. Benitade from Hiroshima (2.7 kg) was refluxed with methanol (3000 ml) for 12 h and evaporated to give a methanol extract (70.0 g). This extract was suspended in water (3000 ml) and re-extracted with hexane, chloroform, ethylacetate, and butanol. Each soluble fraction was evaporated under reduced pressure to give hexane (9.4 g), chloroform (1.8 g), ethylacetate (2.0 g), butanol (9.8 g), and water (47.0 g) fractions. The ethylacetate fraction showed the most potent inhibitory effect (Fig. 2). The ethylacetate fraction was fractionated to fractions 1—4 by silica gel column chromatography with chloroform and acetone as eluents. Fraction 2 showed tyrosinase inhibitory activity, and this fraction was re-fractionated to fractions 5—7 by silica gel column chromatography with chloroform and acetone as eluents. Fraction 6 showed tyrosinase inhibitory activity. Fraction 6 was partitioned with 5% NaOH solution. The water layer was acidified with 5% HCl and then extracted with ethylacetate to yield phenolic fraction 9 (130.0 mg). Fraction 9 was re-chromatographied on silica gel to give 100.0 mg of compound **1**. Compound **1** was identified as (2R,3R)-(+)-taxifolin by EI-MS, IR, and ^{1}H- and ^{13}C-NMR.[18—20]

Structure of Compounds Acetylation and Methylation of Compound **1**: Compound **2** was synthesized by reaction with acetyl chloride and pyridine. Compound **3** was synthesized by reaction with diazomethane. Their structures were identified by EI-MS, IR and ^{1}H- and ^{13}C-NMR.[21—25]

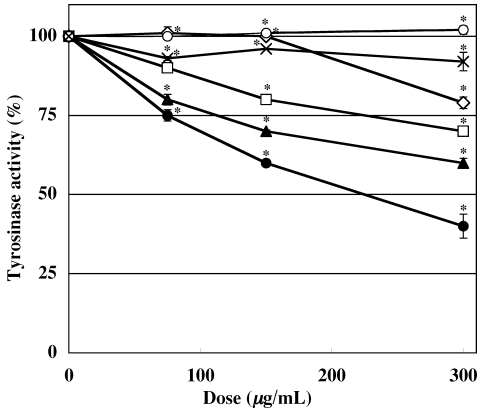

Fig. 2. Inhibitory Effects of Extracts from Benitade against Tyrosinase Activity: (□) Effect of Methanol Extract; (◇) Effect of Hexane Extract; (▲) Effect of Chloroform Extract; (●) Effect of Ethylacetate Extract; (○) Effect of Butanol Extract; (×) Effect of Water Extract

Each value represents the mean±S.D. (n=3). *$p<0.05$ compared with the control (Student's t-test).

RESULTS AND DISCUSSION

Benitade (2.7 kg) was extracted with methanol for 12 h. The methanol extract of Benitade was fractionated to search for inhibitory compound using the tyrosinase activity test as a guide (Fig. 1). To obtain dose–response data, test samples were evaluated at dose levels of 300, 150, and 75 μg/ml. The ethylacetate fraction showed an inhibitory effect on tyrosinase activity (Fig. 2). Fractionation of the ethylacetate extract was carried out as described in Fig. 1. Finally, compound **1** (100.0 mg) was isolated from fraction 9. Compound **1** was identified as (2R,3R)-(+)-taxifolin by EI-MS, IR, and ^{1}H- and ^{13}C-NMR spectroscopy (Fig. 3).

Compounds **1**—**3**, (2R,3R)-(+)-taxifolin (**1**), 3,7,3′,4′-taxifolin tetraacetate (**2**) and 5,7,3′,4′-taxifolin tetramethyl ether (**3**) were investigated for their inhibitory effects against tyrosinase activity together with tyrosinase inhibitor known cosmetic agents such as arbutin and kojic acid (Fig. 4). Compound **1** inhibited 70% of the tyrosinase activity at a concentration of 0.50 mM, and ID_{50} (50% inhibition dose) value was

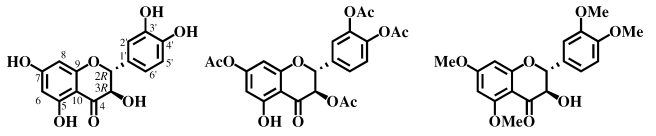

Fig. 3. Chemical Structures of Compounds **1**—**3**

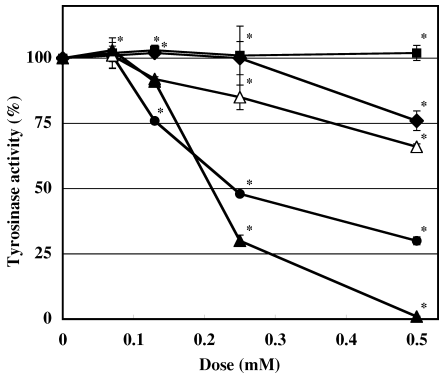

Fig. 4. Inhibitory Effects of Compounds **1**—**3** in Benitade, Arbutin and Kojic Acid against Tyrosinase Activity: (●) Effect of Compound **1**; (◆) Effect of Compound **2**; (■) Effect of Compound **3**; (△) Effect of Arbutin; (▲) Effect of Kojic Acid.

Each value represents the mean±S.D. (n=3). * $p<0.05$ compared with the control (Student's t-test).

0.24 mM. As compared with arbutin and kojic acid, compound **1** more potently inhibited tyrosinase activity than the former and showed inhibitory effect equal to that of the latter. However, compounds **2** and **3** hardly inhibited tyrosinase activity. The difference in structure between compounds **1** and **2** is that compound **1** was acetylated except for a hydroxy group at C-5 position in compound **2**. The difference in structure between compound **1** and **3** is that compound **1** was methylated except for a hydroxy group at the C-3 position in compound **3**. Our results showed that compounds with polarity lower than the mother compound do not show inhibitory effect on tyrosinase activity. Ohad *et al*. reported that a catechol on ring B oxidized to *o*-quinone in the presence of both copper ions and tyrosinase.[26] Tyrosinase inhibitor isolated from black rice bran, protocatechuic acid methyl ester, was assayed together with its derivatives to study the structure–activity relationship.[27] Their results indicated that a hydroxyl group at the C-3 and C-4 position is the most important factor for tyrosinase inhibitory activity. Masamoto *et al*. also reported that hydroxyl groups at the C-6 and C-7 positions of coumarin skeleton play an important role in the expression of tyrosinase inhibitory activity.[28] In consideration of our results, a hydroxyl group at the C-3′ and C-4′ position (catechol subunit on ring B) is the most important factor for inhibition of tyrosinase activity.

We discovered that (2*R*,3*R*)-(+)-taxifolin (**1**) isolated from Benitade may possibly be a of new tyrosinase inhibitor alternative to cosmetic agents such as arbutin and kojic.

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