Syntheses of Arbutin-α-glycosides and a Comparison of Their Inhibitory Effects with Those of α-Arbutin and Arbutin on Human Tyrosinase

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The effects of 4-hydroxyphenyl α-glucopyranoside (α-arbutin) and 4-hydroxyphenyl β-glucopyranoside (arbutin) on the activity of tyrosinase from human malignant melanoma cells were examined. The inhibitory effect of α-arbutin on human tyrosinase was stronger than that of arbutin. The K_i value for α-arbutin was calculated to be 1/20 that for arbutin. We then synthesized arbutin-α-glycosides by the transglycosylation reaction of cyclomaltodextrin glucanotransferase using arbutin and starch, respectively, as acceptor and donor molecules. The structural analyses using ^{13}C- and ^{1}H-NMR proved that the transglycosylated products were 4-hydroxyphenyl β-maltoside (β-Ab-α-G1) and 4-hydroxyphenyl β-maltotrioside (β-Ab-α-G2). These arbutin-α-glycosides exhibited competitive type inhibition on human tyrosinase, and their K_i values were calculated to be 0.7 mM and 0.9 mM, respectively. These arbutin-α-glycosides posessed stronger inhibitory activity than arbutin, but less activity than α-arbutin. These results suggested that the α-glucosidic linkage of hydroquinone-glycosides plays an important role in the inhibitory effect on human tyrosinase.

Key words α-arbutin; arbutin; transglycosylation; human tyrosinase; competitive inhibition

Hyperpigmentation in the epidermis is caused by excessive melanin synthesis. Tyrosinase (EC 1.14.18.1) is one of the keyenzymes involved in melanin synthesis. The enzyme catalyses the first two steps in melanin synthesis: the hydroxylation of tyrosine to 3-(3,4-dihydroxyphenyl)-alanine (DOPA) and the oxidation of DOPA to dopaquinone. Several tyrosinase inhibitors have been used in the cosmetic industry as skin-whitening agents.

4-Hydroxyphenyl β-D-glucopyranoside (arbutin) has been found in the leaves of plants and used as a cosmetic ingredient.[1—4] On the other hand, 4-Hydroxyphenyl α-D-glucopyranoside (α-arbutin) was enzymatically synthesized from hydroquinone and saccharides,[5—8] and its inhibitory effect on melanogenesis was investigated.[6,9] The inhibitory activity of α-arbutin and arbutin against tyrosinases from mushroom and from B16 mouse melanoma cells were previously compared.[6,9] They exhibited different specificity against these tyrosinases.[6,9] However, the inhibitory activity of α-arbutin on human tyrosinase is still unknown.

In this paper we describe the enzymatic synthesis of new arbutin derivatives using cyclomaltodextrin glucanotransferase (CGTase). Comparison of the inhibitory effects of α-arbutin, arbutin and arbutin-α-glycosides against human tyrosinase is also highlighted.

Results and Discussion

We examined the inhibitory effect of α-arbutin and arbutin on the activity of tyrosinase from human malignant melanoma cells, HMV-II,[10] using L-DOPA as the substrate. The inhibition of α-arbutin on human tyrosinase was stronger than that of arbutin. The 50% inhibitory (IC_{50}) value of α-arbutin was 2.1 mM, whereas that of its optical isomer, arbutin, was more 30 mM.

From these results, we were interested in the relationship between the inhibitory effect of hydroquinone glycosides on human tyrosinase activity and the configuration of glucosidic linkage(s) of hydroquinone glucosides. We enzymatically synthesized arbutin-α-glycosides. CGTase from *Bacillus macerans* was successfully used for the transglycosylation reaction to produce arbutin-α-glycosides. Arbutin and soluble starch were used as acceptor and donor molecules, respectively. The synthesis of arbutin-glycosides was confirmed by analysis using high-performance liquid chromatography (HPLC), as shown in Fig. 1. The conversion rate was about 70%, based on the amount of arbutin supplied. When the effect on human tyrosinase was compared, the reaction mixture containing arbutin-glycosides was more inhibitory than arbutin (Fig. 2). The arbutin-transglycosylated products

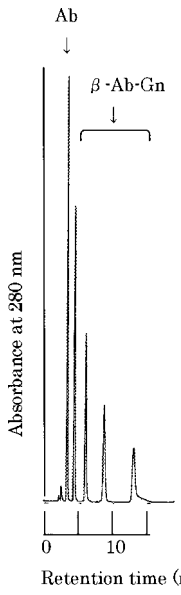

Fig. 1. Synthesis of Arbutin-Glycosides by the Transglycosilation Reaction of CGTase

A mixture (2.5 ml) containing 50 mg of arbutin, 0.5 g of soluble starch, and 37.5 units of CGTase from *Bacillus macerans* in 25 mM sodium phosphate buffer (pH 7.0) was incubated at 40 °C for 16 h. The reaction mixture was analyzed by HPLC. Ab: arbutin, β-Ab-Gn: arbutin mono- and oligo-glucosides.

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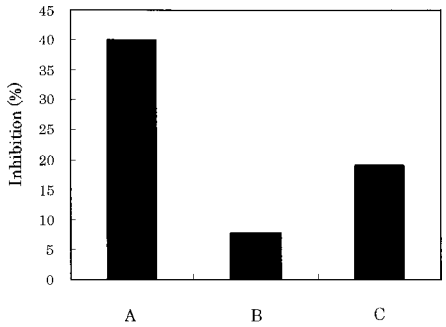

Fig. 2. Inhibitory Effects of α-Arbutin, Arbutin and the Mixture of Arbutin and Arbutin-glycosides on Tyrosinase from Human Malignant Melanoma

One mM α-arbutin or arbutin was used for this experiment. The concentration of the mixture of arbutin and arbutin-glycosides was adjusted to 1 mM based on the amount of arbutin. The other conditions were the same as those for IC_{50} determination. Lanes: A: α-arbutin, B: arbutin, C: the mixture of arbutin and arbutin-glycosides. Results are indicated as the percentage of inhibition with respect to the untreated control.

Table 1. ^{13}C-NMR Data for α-Arbutin, Arbutin and Compounds **1** and **2**

	Compound			
	α-Arbutin[5]	Arbutin	Compound **1**	Compound **2**
C-Aromatic				
1	153.98	154.12	154.14	154.33
2, 6	121.99	121.29	121.24	121.30
3, 5	119.05	119.08	119.03	119.12
4	152.66	153.26	153.16	153.17
C-Glu				
1′	101.12	104.19	103.97	104.04
2′	73.98	75.83	75.64	75.73
3′	75.83	78.43	78.82	78.85
4′	72.20	72.30	79.34	79.59
5′	75.22	78.91	77.48	77.52
6′	63.11	63.40	63.27	63.32
1″			102.43	102.37
2″			74.47	74.34
3″			75.64	76.17
4″			72.12	79.57
5″			75.51	74.07
6″			63.32	63.32
1‴				102.62
2‴				74.60
3‴				75.69
4‴				72.17
5‴				75.57
6‴				63.32

Chemical shifts are given in ppm downfield from TSP.

in the reaction mixture were purified by Sephadex G-15 column chromatography, and two compounds, **1** and **2**, were obtained.

Next, we did the structural analyses of these compounds. Compound **1** was hydrolyzed to glucose and arbutin with a final molar ratio of 1 : 1 by α-glucosidase. The time-of-flight (TOF) MS analysis of compound **1** showed a molecular-related ion peak at *m/z* 457.5 ($C_{18}H_{26}O_{12}Na$). Sixteen signals were observed by ^{13}C-NMR analysis, as shown in Table 1. Four of them (δ 119.03—δ 154.14) were assigned to the aromatic group, and 12 (δ 63.27—δ 103.97) were assigned as maltose. The type of glycosidic linkages was determined to be one of β-configuration and one of α-configuration, based

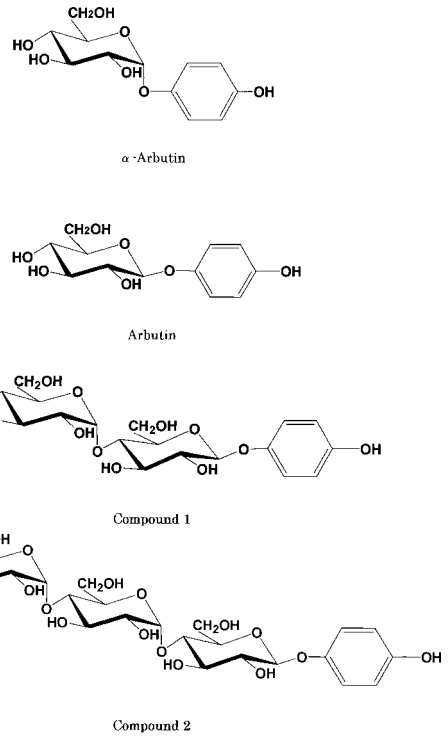

Fig. 3. Structures of α-Arbutin, Arbutin and Compounds **1** and **2**

Table 2. ^{1}H-NMR Chemical Shift Values and Coupling Constants for the Glucosidic Protons

Compound	Glucosidic protons		
α-Arbutin[5]	5.49 (3.7)		
Arbutin	4.99 (7.6)		
Compound **1**	4.99 (7.9)	5.43 (4.0)	
Compound **2**	5.01 (7.9)	5.41 (4.0)	5.44 (4.0)

Chemical shifts were given in ppm downfield from TSP. Coupling constants (Hz) are shown in parentheses.

on the values of the coupling constants (J=7.9, J=4.0) of the anomeric protons, as shown in Table 2. From these results, we concluded that compound **1** was 4-hydroxyphenyl β-maltoside (β-Ab-α-G1) (Fig. 3).

Similarly, compound **2** was hydrolyzed to glucose and arbutin with the final molar ratio of 2 : 1 by α-glucosidase treatment. The TOF MS analysis of compound **2** showed a molecular-related ion peak at *m/z* 619.7 ($C_{24}H_{36}O_{17}Na$). Twenty-two signals were observed by ^{13}C-NMR analysis (Table 1). Four of them (δ 119.12—δ 154.33) were assigned to the aromatic group, and 18 (δ 63.32—δ 104.04) as maltotriose. The type of glycosidic linkage was determined to be one of β-configuration and two of α-configurations, based on the values of the coupling constants (J=7.9, J=4.0, J=4.0) of the anomeric protons, as shown in Table 2. Therefore, we concluded that compound **2** was 4-hydroxyphenyl β-maltotrioside (β-Ab-α-G2) (Fig. 3).

To investigate the inhibitory activity of these purified arbutin-glycosides, the IC_{50} values against human tyrosinase were measured. When the IC_{50} values of β-Ab-α-G1 and β-Ab-α-G2 were compared with those of α-arbutin and arbutin, β-Ab-α-G1 and β-Ab-α-G2 exhibited stronger inhibitory activity against the human tyrosinase than arbutin,

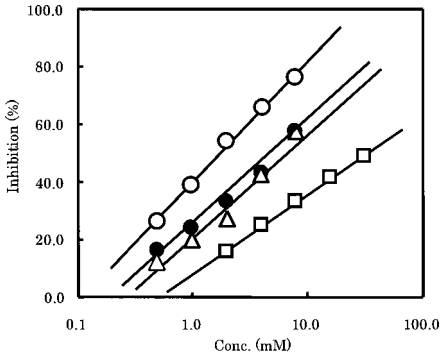

Fig. 4. Inhibitory Effects of α-Arbutin, Arbutin, β-Ab-α-G1 and β-Ab-α-G2 on Tyrosinase from Human Malignant Melanoma

Tyrosinase activity was measured using 3.3 mM L-DOPA as the substrate. Results are expressed as the percentage of inhibition by α-arbutin (○), arbutin (□), β-Ab-α-G1 (●) or β-Ab-α-G2 (△) with respect to the untreated control.

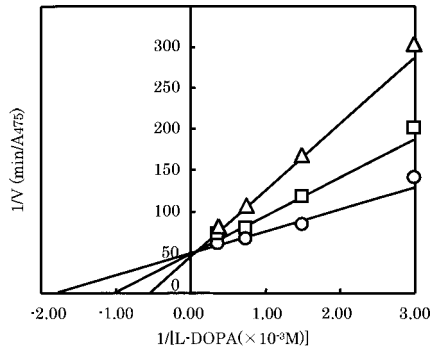

Fig. 5. Lineweaver–Burk Plots Showing the Reciprocal of the Velocity (1/V) of the Human-Tyrosinase Reaction *vs.* the Reciprocal of the Substrate Concentration (1/S) in the Presence and Absence of the Inhibitor

The rate of the tyrosinase reaction in the presence of 0.3 mM (□) or 0.5 mM (△) of α-arbutin and in the absence of inhibitor (○). A_{475} represents an increase in the absorbance at 475 nm.

but weaker than α-arbutin (Fig. 4). The IC_{50} values of β-Ab-α-G1 and β-Ab-α-G2 were 5.7 mM and 6.1 mM, respectively (Table 3). According to Lineweaver-Burk plots of the tyrosinase activity, the inhibition of α-arbutin against human tyrosinase was indicated to be competitive, as shown in Fig. 5. Based on these kinetic data, the K_i value for α-arbutin was calculated as 0.2 mM (Table 3). Similar kinetic analyses indicated that the inhibitions of β-Ab-α-G1 and β-Ab-α-G2 were also competitive. The K_i values for β-Ab-α-G1 and β-Ab-α-G2 and arbutin were 0.7 mM, 0.9 mM and 4.2 mM, respectively (Table 3). The K_i values for β-Ab-α-G1 and β-Ab-α-G2 were about 6-fold lower than that for arbutin. Jergil *et al.*[11] reported that the K_M value for human tyrosinase was 0.5 mM when L-DOPA was used as the substrate, but 3 mM when D-DOPA was used as the substrate. It would be interesting to examine whether the difference in inhibitory activity of α-arbutin and arbutin is correlated with the high stereo specificity of human tyrosinase. We are now in the process of synthesizing α-arbutin-α-glycosides and analyzing their effect on human tyrosinase activity.

The inhibitory effects of α-arbutin and arbutin have been previously compared.[6,9] Nishimura *et al.*[9] reported that α-arbutin, as well as arbutin, inhibited mushroom tyrosinase. Funayama *et al.*[6] reported that α-arbutin did not inhibit mushroom tyrosinase, while arbutin did. These works suggested that α-arbutin was not a better skin-whitening agent than arbutin. On the other hand, Funayama *et al.*[6] reported that α-arbutin strongly inhibited the tyrosinase from B16 mouse melanoma cells, while arbutin did not. According to our study described here, α-arbutin inhibited human tyrosinase much more strongly than arbutin. These results obtained by using the tyrosinase from mammalian cells indicated that α-arbutin is a more effective skin-whitening agent than arbutin. Since mushroom tyrosinase is commercially available, it might have been thought that the enzyme was useful for the first screening of a tyrosinase inhibitor. However, the amino acid sequence identity between human tyrosinase[12] and mushroom tyrosinase (Gene bank accession No., O42713) is only 23%. On the other hand, human tyrosinase and murine tyrosinase[13] are highly homologous (82% sequence identity). Therefore, it is definitely important to use human tyrosinase for screening skin-whitening agents.

α-Arbutin is the strongest inhibitor of human tyrosinase among the hydroquinone-glycosides which we studied so far. Further investigation of the inhibitory effect of α-arbutin on melanogenesis is now in progress.

Table 3. Effects of α-Arbutin, Arbutin, β-Ab-α-G1 and β-Ab-α-G2 on Human Tyrosinase

Compound	IC_{50} (mM)	K_i (mM)
α-Arbutin	2.1	0.2
Arbutin	>30.0	4.2
β-Ab-α-G1 (**1**)	5.7	0.7
β-Ab-α-G2 (**2**)	6.1	0.9

Experimental

General Experimental Procedures Mass spectra were recorded on a KRATOS Compact-Maldiseq instrument. ^{1}H-NMR (500 MHz) and ^{13}C-NMR (125 MHz) spectra were obtained using a JEOL JNM-A500 spectrometer in D_2O containing [2,2,3,3-D4] sodium 3-3-(trimethylsilyl) propanoate as an internal standard.

Chemicals Arbutin and L-DOPA were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). CGTase from *Bacillus macerance* was purchased from Amano Pharmaceutical Co., Ltd. (Nagoya, Japan). Other chemicals were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan).

Preparation of α-Arbutin α-Arbutin was prepared by the method described by Nishimura *et al.*[5] α-Amylase from *Bacillus* sp. strain X-23 was added to 100 ml of 50 mM sodium acetate buffer solution (pH 5.0) containing 5% hydroquinone and 20% maltopentaose. After incubation at 40 °C for 16 h, glucoamylase from *Aspergillus niger* was added, and the mixture was incubated at 40 °C for 4 h. α-Arbutin was purified from the reaction mixture by extraction with ethylacetate and charcoal column chromatography.

Preparation of Tyrosinase from Human Malignant Melanoma Human malignant melanoma cells, HMV-II,[10] were provided by the Cell Resource Center for Biomedical Research, Tohoku University. The cells were cultured in F12/DMEM supplemented with 10% fetal bovine serum and 100 μM L-DOPA at 37 °C in a humidified atmosphere of 5% CO_2 in air. On the 10th day, the cells were scraped out from the tissue culture plate with Mg^{2+}-, Ca^{2+}-free phosphate-buffered saline [PBS(−)], and were homogenized in PBS(−) with a glass homogenizer at 4 °C. And the homogenate was fractionated by differential centrifugation based on the method of Claude.[14,15] The homogenate was centrifuged at 1000×$\boldsymbol{g}$ for 10 min. The precipitate was sonicated in PBS(−) on ice, and the mixture was centrifuged at 10000×$\boldsymbol{g}$ for 30 min. The supernatant containing tyrosinase was used for the measurement of the inhibitory effects.

Assay of Tyrosinase Activity Tyrosinase activity was assayed according to the method of Funayama *et al.*[16] with slight modification. The reaction mixture (90 μl) contained 3.3 mM L-DOPA in 0.33 M phosphate buffer (pH 7.0) and the enzyme in the presence or absence of inhibitors. Thirteen units

or 17 units of tyrosinase was used to determine the IC_{50} value or the K_i value, respectively. The reaction mixtures in 96-well plates were incubated at 37 °C for 10 min, and the absorbance was measured at 475 nm in a Benchmark microplate reader (Bio-Rad Laboratories). One unit of enzyme was defined as the amount of enzyme which increased the absorbance value by 0.001 at 475 nm per minute under the condition described above.

HPLC Analyses of Arbutin and Arbutin-α-glycosides In order to elucidate the transglycosylation products by the CGTase, the reaction mixtures were analyzed by HPLC. HPLC was carried out under the following conditions: column, LiChrosorb NH_2 (4.0×250 mm, Merck Co., Frankfurt, Germany); solvent, acetonitrile–water (3 : 1, v/v); flow rate, 1.0 ml/min; pump, Shimadzu LC-6AD; detector, Shimadzu SPD-6A (at 280 nm).

Preparation and Purification of Arbutin-α-glycosides The mixture of arbutin-α-glycosides was obtained by incubating the reaction mixture (2.5 ml) containing 50 mg of arbutin, 0.5 g of soluble starch, and 37.5 units of CGTase from *Bacillus macerans* in 25 mM sodium phosphate buffer (pH 7.0) at 40 °C for 16 h. The reaction was terminated by the addition of nine volumes of acetone. Then the mixture was centrifuged at 3000×***g*** for 10 min. The supernatant was dried by evaporation, then dissolved in 0.6 ml of distilled water. Subsequent purification of arbutin-α-glycosides was performed by a column (1.8×40 cm) of Sephadex G-15. The fractions containing arbutin-glycosides were concentrated and applied again to the same column that had been equilibrated with distilled water. The fractions containing compound **1** were collected and freeze-dried, and 20 mg of compound **1** was obtained.

Five mg of compound **2** was obtained by the same procedure.

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