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ARTICLE

Enzymatic Synthesis and Characterization of Hydroquinone Galactoside Using *Kluyveromyces lactis* Lactase

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Hydroquinone galactoside (HQ-Gal) as a potential skin whitening agent was synthesized by the reaction of lactase (β-galactosidase) from *Kluyveromyces lactis*, *Aspergillus oryzae*, *Bacillus circulans*, and *Thermus* sp. with lactose as a donor and HQ as an acceptor. Among these lactases, the acceptor reaction involving HQ and lactose with *K. lactis* lactase showed a higher conversion ratio to HQ-Gal (60.27%). HQ-Gal was purified using butanol partitioning and silica gel column chromatography. The structure of the purified HQ-Gal was determined by nuclear magnetic resonance, and the ionic product was observed at *m*/*z* 295 $(C_{12}H_{16}O_7Na)^+$ using matrix assisted laser desorption ionization time-of-flight mass spectrometry. HQ-Gal was identified as 4-hydroxyphenyl-β-D-galactopyranoside. The optimum conditions for HQ-Gal synthesis by *K. lactis* determined using response surface methodology were 50 mM HQ, 60 mM lactose, and 20 U mL^{-1} lactase. These conditions produced a yield of 2.01 g L^{-1} HQ-Gal. The half maximal inhibitory concentration (IC_{50}) of diphenylpicrylhydrazyl scavenging activity was 3.31 mM, indicating a similar antioxidant activity compared to β-arbutin (IC_{50} = 3.95 mM). The K_i value of HQ-Gal (0.75 mM) against tyrosinase was smaller than that of β-arbutin (K_i = 1.97 mM), indicating its superiority as an inhibitor. HQ-Gal inhibited (23%) melanin synthesis without being significantly toxic to the cells, while β-arbutin exhibited only 8% reduction of melanin synthesis in B16 melanoma cells compared with the control. These results indicate that HQ-Gal may be a suitable functional component in the cosmetics industry.

KEYWORDS: *Kluyveromyces lactis*; lactase; hydroquinone; acceptor reaction; galactosylation; DPPH assay; Tyrosinase inhibition; MTT assay

INTRODUCTION

Hydroquinone (HQ) is a constituent of β-arbutin. The compound is a good general-purpose inhibitor of tyrosinase (one of the key enzymes in melanin synthesis), a stabilizer against light or oxidation and an antioxidant agent (*1*). HQ can function as a skin-whitening agent, but it has the potential to cause skin irritation and contact dermatitis. The United States Food and Drug Administration recently issued a proposal to ban HQ-containing over-the-counter and prescription products. The prospect of the curtailed availability of HQ has spurred intense interest in the cosmetics industry in alternatives, including naturally occurring compounds, for the inhibition of melanin synthesis (*2*).

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Transglycosylation catalyzed by enzymes from various bacteria has been used to improve physiochemical properties such as water solubility and oxidative stability of various compounds. Glucansucrase-mediated glycosylation of HQ potently obviates lipid oxidation and produces synergistic effects superior to β-arbutin, a cosmetic ingredient isolated from the leaves of various plant species (*3*, *4*). Glucansucrase-mediated glycosylation of epigallocatechin gallate (EGCG) and quercetins increases the water solubility of these compounds, compared to their nonglycoslyated counterparts (*5*, *6*). Enzymatic transgalactosylation has also been used to modify bioactive substances with the aim of improving their functionality. For example, the synthesis of myricitrin glycosides using β-galactosidase (lactase) from *Bacillus circulans* with lactose as a substrate produced a galactosylated myricitrin that was 480 times more soluble than myricitrin (*7*). Lactase also catalyzes the transfer of the galactosyl residue from

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lactose to various acceptor molecules. When the acceptor is water, the reaction is termed hydrolysis (*8*). When the acceptors are carbohydrates or hydroxyl-containing nucleophiles, lactase transfers the lactose galactosyl moiety to a variety of acceptor compounds possessing hydroxyl groups (*8*−*10*), which has been exploited to produce a hypocholesterolemic agent (*11*) and an antitumor agent (*12*). The potential of this enzymatic approach in the cosmetic, food, and pharmaceutical industries has been recognized (*3*−*5*).

In this study, the novel synthesis of the HQ galactoside 4-hydroxyphenyl-β-D-galactopyranoside via acceptor reactions of lactase with HQ and lactose is described. The transgalactosylated HQ was purified, and the structure was analyzed by thin-layer chromatography (TLC), high-performance liquid chromatography (HPLC), nuclear magnetic resonance (NMR), and matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS). The compound's antioxidant activities, tyrosinase inhibition activities, cell viability, and reduction of melanin synthesis in B16 melanoma cells were also examined and are described.

MATERIALS AND METHODS

Materials. HQ, lactose, diphenylpicrylhydrazyl (DPPH), 2,5-dihydroxybenzoic acid (DHB), and 3-(4,5-dimethylthiazol-2yl)-2,5-diphenyl-2*H*-tetrazolium bromide (MTT) were purchased from Sigma-Aldrich (St. Louis, MO). Fetal bovine serum, penicillin, streptomycin, and Dulbecco's modified Eagle media (DMEM) were purchased from GIBCO BRL (Grand Island, NY). Lactase was provided by GenoFocus Co. (Daejeon, Korea). Silica-gel (40−60 μm) was purchased from Acros Organics (Geel, Belgium). B16 melanoma cell was provided by AMOREPACIFIC Co. (Yongin, Korea). Other chemical reagents were commercially available and of chemically pure grade.

Galactosylation of Hydroquinone. The reaction mixture consisting of 100 mM HQ and 100 mM lactose with lactase from *Kluyveromyces lactis*, *Aspergillus oryzae*, *Bacillus circulans*, or *Thermus* sp. was incubated at each optimum condition for 6 h. The first acceptor reaction mixture used *K. lactis* lactase in sodium (Na) phosphate buffer (pH 7.0) at 28 °C. The second acceptor reaction mixture used *A. oryzae* lactase in Na acetate buffer (pH 4.3) at 50 °C. The third acceptor reaction mixture used *B. circulans* lactase in Na phosphate buffer (pH 6.0) at 60 °C. The fourth acceptor reaction mixture used *Thermus* sp. lactase in Na phosphate buffer (pH 7.0) at 70 °C. Each enzyme reaction was halted by boiling for 10 min. Galactosylation of HQ was confirmed using TLC.

Analysis of Acceptor Reaction Product by TLC. TLC was conducted at room temperature, using a silica gel 60 F_{254} TLC plate (Merck, Darmstadt, Germany). A 0.9 L volume of each reaction digest was spotted onto the silica gel plate, and the plate was developed using a solvent mixture of acetonitrile−water (85:15, v/v). The compounds were developed on the plate by rapidly dipping the plate into a solution containing 3 g of *N*-(1-naphthyl)ethylenediamine and 50 mL of concentrated H_2SO_4 in 1 L of methanol. The plate was dried and then placed in an oven for 10 min at 120 °C (*5*).

Purification of HQ-Gal. The 0.9 L reaction digest in 20 mM Na acetate (pH 5.2) consisting of 100 mM HQ, 100 mM lactose, and lactase from *K. lactis* (10 U mL^{-1}) was concentrated under vacuum to 90 mL using a rotary evaporator (EYELA, Tokyo, Japan) at 65 °C. Five milliliters of the concentrate was applied to a 3 cm × 62 cm silica gel column, and each HQ acceptor reaction product was purified with 90% (v/v) acetonitrile in water. The column was washed with distilled water (total 3 L, flow rate 1 mL min^{-1}) in order to remove the remaining sugars (polymers, lactose, galactose, and glucose). The HQ-Gal was confirmed for purification using HPLC (LC-10AD; Shimadzu, Kyoto, Japan) under the following conditions: reverse column, μ-Bondapak C_{18} (19 mm × 300 mm); mobile phase, methanol/water = 4:6 (v/v); flow rate, 3.0 mL min^{-1}; room temperature; detection, RID-10A model RI detector (Shimadzu). The eluants were concentrated at 65 °C with a rotary evaporator (EYELA) (*3*).

Matrix-Assisted Laser Desorption/Ionization Mass Spectrometry (MALDI-TOF MS) Analysis. Purified HQ-Gal was diluted with deionized water and then mixed with a matrix, 2,5-dihydroxybenzoic acid (1 mg mL^{-1}) dissolved in water in a 1:1 v/v ratio. The mixed solution (1 μL) was then spotted onto a stainless steel plate and slowly dried at room temperature. The water vaporized, leaving only the recrystallized DHB but now with HQ-Gal spread throughout the DHB crystals. The DHB and HQ-Gal are said to be cocrystallized in a MALDI spot. The mass spectrum was acquired using a Voyager DE-STR MALDI-TOF mass spectrometer (Applied Biosystems, Foster City, CA). The laser is fired at the crystals in the MALDI spot. The mass spectra were obtained in the positive linear mode with delayed extraction (average of 75 laser shots) with an accelerating voltage of 65 kV (*5*).

Nuclear Magnetic Resonance (NMR) Analysis. Approximately 5 mg of the purified HQ-Gal was dissolved in 250 μL of deuterated water and dispensed into 3 mm NMR tubes. NMR spectra were obtained on a VNMRS spectrometer (Varian, Palo Alto, CA) equipped with a carbon-enhanced cryogenic probe operating at 600 MHz for ^{1}H and 150 MHz for ^{13}C at 25 °C. Linkages between HQ and galactose were evaluated using the spectra of homonuclear correlation spectroscopy (COSY), heteronuclear single quantum coherence (HSQC), and heteronuclear multiple bond correlation (HMBC) (*5*).

Experimental Design for Optimization of Acceptor Reaction. The experimental response surface method (RSM) data were fitted via the response surface regression procedure using the following second-order polynomial equation (*13*):

$$Y_i = \beta_0 + \sum_i \beta_i x_i + \sum_{ii} \beta_{ii} x_i^2 + \sum_{ij} \beta_{ij} x_i x_j$$

where Y_i is the predicted response, $x_i x_j$ is the independent variables, β_0 is the offset term, β_i is the ith linear coefficient, β_{ii} is the ith quadratic coefficient, and β_{ij} is the ijth interaction coefficient. In this study, however, the independent variables were coded as X_1, X_2, and X_3. Thus, the second-order polynomial equation can be presented as follows: $Y = \beta_0 + \beta_1 X_1 + \beta_2 X_2 + \beta_3 X_3 + \beta_{11} X_1^2 + \beta_{22} X_2^2 + \beta_{33} X_3^2 + \beta_{12} X_1 X_2 + \beta_{13} X_1 X_3 + \beta_{23} X_2 X_3$. Design-Expert 6.0.11 CCD RSM software (State-Ease, Minneapolis, MN) was also used for regression analysis and graphical analysis of the data obtained during whole experiments. Analysis of variance (ANOVA) was used to estimate the statistical parameters. The second-order polynomial equation was employed to fit the experimental data. The significance of the model equation and model terms was evaluated by Fisher's test. The quality of fit for the polynomial model equation was expressed by the coefficient of determination (R^2) and adjusted R^2. The fitted polynomial equation was expressed as three-dimensional surface plots to show the relationship between the responses and the experimental levels of each variables used in the design. The combination of different parameters producing maximum response was determined to verify the model. From a preliminary experiment, three factors (lactase unit, lactose, and hydroquinone concentrations) were selected to optimize the synthesis of HQ-Gal: lactase from *K. lactis*, 5−50 U mL^{-1}; lactose concentration, 20−200 mM; HQ concentration, 10−250 mM.

Antioxidant Activity. The antioxidant activities of HQ, HQ-Gal, and β-arbutin were assessed using diphenylpicrylhydrazyl (DPPH). DPPH is a stable free radical that accepts an electron or hydrogen radical to become a stable diamagnetic molecule. DPPH reacts with an antioxidant compound that can donate hydrogen and becomes reduced. The change in color (from deep violet to light yellow) that occurs upon the reduction was measured. The intensity of the yellow color depends on the amount and nature of radical scavenger present in the sample or standard compounds. Each sample (0.01, 0.05, 0.1, 0.5, 1, 2, and 5 mM) was dissolved in 30 μL of ethanol and mixed thoroughly with 100 μM DPPH in 270 μL of ethanol. After 1 h at room temperature in total darkness, the absorbance of each mixture was measured at 517 nm on a SmartSpec 3000 spectrophotometer (Bio-Rad, Hercules, CA). The DPPH radical-scavenging activity was determined according to the decrease in absorbance of the DPPH radical in the sample and compared to that observed with an ethanol blank (*5*, *14*). The half maximal inhibitory concentration (IC_{50}) designates the concentration of a sample at which the DPPH radicals were decreased by 50%.

Tyrosinase-Inhibitory Effect. A 90 μL reaction mixture contained 3-(3,4-dihydroxyphenyl)-L-alanine, 3,4-dihydroxyphenylalanine (DOPA; 0.33, 1, and 3 mM in 100 mM sodium phosphate buffer, pH 7.0), and enzyme in the presence or absence of inhibitors. An amount of 10 units of tyrosinase was used to determine the K_i value. To determine which type of

inhibition occurred, a Dixon plot of the relationship between the reciprocal of the velocity and the HQ-Gal concentration (0, 0.1, 0.5, and 2 mM) was made using three substrate concentrations (0.33, 1, and 3 mM DOPA). The absorbance at 475 nm of the reaction mixtures in wells of a 96-well plate was measured using a microplate reader (Molecular Devices, Sunnyvale, CA) to determine the tyrosinase inhibition (*3*, *15*).

Cell Culture. B16 melanoma cells were cultured in DMEM, supplemented with 10% fetal bovine serum, 10 μg mL^{-1} penicillin, and 10 μg mL^{-1} streptomycin at 37 °C in a humidified CO_2 incubator.

Cell Proliferation Assay. The study of cell proliferation was carried out using the MTT-based colorimetric assay (*16*). Aliquots (100 μL) containing 1×10^4 B16 cells were inoculated into 96-well microplates. Each compound was added to three 100 μL replicates of cells in wells of a 96-well microplate, and the plates were incubated at 37 °C in an incubator with 5% CO_2 for 24 h. The final concentration of each compound in the well was 10 or 50 μg mL^{-1}. At the end of incubation, MTT solution (20 μL, 5 mg mL^{-1} in PBS) was added to the wells. After 4 h of incubation, the medium was removed and 100 μL of dimethyl sulfoxide was added to dissolve the formazan produced in the cells. The absorbance of each well was then read at 570 nm. The optical density of formazan formed by control cells was assumed to be 100%.

Melanin Content Assay. The melanin content of B16 cells was measured according to a modification of a previously described method (*17*). B16 cells were plated in wells of a 24-well plate at a density of 1×10^5 cells mL^{-1}. Concentrations (50 μg mL^{-1}) of samples (control, β-arbutin, HQ, and HQ-Gal) were prepared by dissolution in culture medium. After addition of the samples, the plates were incubated at 37 °C in a CO_2 incubator for 72 h. After incubation, the medium was removed and the cells were washed with sterile PBS (pH 7.4) and pellets were solubilized in 400 μL of 1 M NaOH at 60 °C for 1 h. Optical densities were determined at a wavelength of 405 nm.

RESULTS

Synthesis and Purification of HQ-Gal. After the acceptor reaction involving HQ and lactose was performed using lactase from the four selected bacterial species, a common reaction product determined to be HQ-Gal was identified by TLC (**Figure 1**). As shown in **Table 1**, *K. lactis* lactase synthesized 1.79 mM HQ-Gal, representing a yield of 60.27% based (here and hereafter) on the lactose amount used from 100 mM lactose. *A. oryzae* lactase synthesized 0.30 mM HQ-Gal (10.95% yield), *B. circulans* lactase synthesized 2.29 mM HQ-Gal (18.72% yield), and *Thermus* sp. lactase synthesized 0.55 mM HQ-Gal (11% yield). The acceptor reaction mixtures were purified using silica gel column chromatography producing HQ-Gal with a purification yield of 89.1% using HPLC (data not shown).

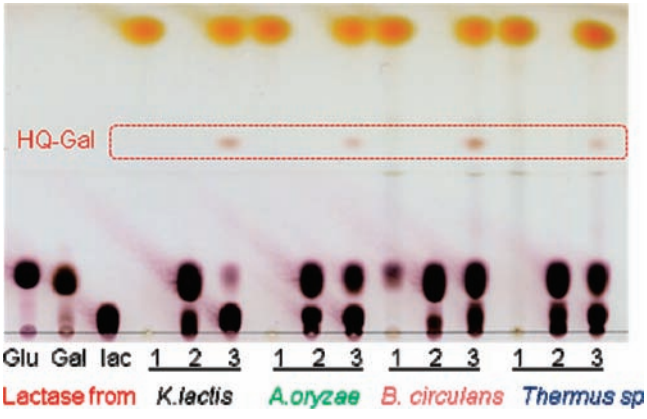

Figure 1. TLC analyses of the lactase acceptor reaction products: lane 1, lactase reaction digest with HQ; lane 2, lactase reaction digest with lactose; lane 3;, acceptor reaction digest of lactose, HQ, and lactase. TLC solvent system is acetonitrile/water = 85:15, v/v.

Structural Determination of HQ-Gal. The number of galactose units attached to purified HQ-Gal was determined using MALDI-TOF MS. HQ-Gal molecular ion was observed at *m*/*z* 295 $(C_{12}H_{16}O_7Na)^+$, indicating a single galactose attachment to HQ. The glycosidic linkage for HQ-Gal was determined using ^{1}H, ^{13}C, ^{1}H−COSY, HSQC, and HMBC analyses (**Figure 2**). ^{1}H NMR (D_2O, 600 MHz): δ 6.90 (2H, d, J = 9.0, H-2, H-6), 6.70 (2H, d, J = 8.4, H-3, H-5), 4.76 (1H, d, J = 7.8, H-1′), 3.60 (1H, m, H-2′), 3.62 (1H, m, H-3′), 3.83 (1H, d, J = 3.0, H-5′), 3.65 (1H, s, H-4′), 3.49 (1H, dd, J = 4.2,4.2, H-6′a), 3.40 (2H, q, J = 6.0, H-6′b). ^{13}C NMR (D_2O, 150 MHz): δ 151.03 (C-1), 118.25 (C-2, C-6), 116.10 (C-3, C-5), 150.47 (C-4), 101.79 (C-1′), 70.51 (C-2′), 72.49 (C-3′), 75.24 (C-4′), 68.40 (C-5′), 60.66 (C-6′). A doublet signal at 4.76 ppm (J = 7.8 Hz) could be assigned to the anomeric proton, showing that only one galactosyl residue is β-linked to HQ. Ten signals were observed by ^{13}C NMR. Four of the signals (151.03−116.10 ppm) were assigned to carbons of HQ, and six signals (101.79−60.66 ppm) were assigned to carbons of galactose. In our HMBC data, the H-1′ of the galactosyl residue was observed at 4.76 ppm, with the couplings occurring at the C-4 of the HQ ring. This correlation allowed for the precise assignment of the signals of the linkage between HQ and galactose. The structure of HQ-Gal produced by the *K. lactis* lactase acceptor reaction appears to be commensurate with 4-hydroxyphenyl-β-D-galactopyranoside (**Figure 2**).

Determination of Polynomial Equation Coefficients for Optimum HQ-Gal Synthesis. The design matrix and the corresponding results of RSM experiments are shown in **Table 2** along with the mean predicted values. The selected reaction conditions including lactose concentration unit (X_1), HQ concentration (X_2), and lactase unit (X_3) were investigated. RSM was used to study the interaction of these variables within a range of −1.682 to +1.682 in relation to HQ-Gal synthesis (**Table 2**). A total of 20 experiments were performed with different combinations of these factors (**Table 3**). HQ-Gal synthesis varied with changes of lactase units, lactose concentration, and HQ concentration. As

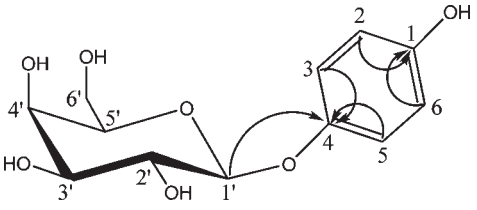

Figure 2. Structures and HMBC correlations of HQ-Gal. Approximately 5 mg of purified HQ-Gal was dissolved in 250 μL of deuterated water and placed into 3 mm NMR tubes. NMR spectra were acquired on a VNMRS spectrometer operating at 600 MHz for ^{1}H and 150 MHz for ^{13}C at 25 °C.

Table 1. Reaction Conditions for Galactosylation Using Four Kinds of Lactase

lactase	optimum pH	optimum temp (°C)	product concn (mM)[a]	concn of lactose used (mM)	conversion ratio (%)[b]
1. *Kluyveromyces lactis*	7.0	28	1.79	2.97	60.27
2. *Aspergillus oryzae*	4.3	50	0.30	2.74	10.95
3. *Bacillus circulans*	6.0	60	2.29	12.23	18.72
4. *Thermus* sp.	7.0	70	0.55	5.00	11.00

[a] 100 mM lactose, 100 mM hydroquinone, 10 U mL^{-1} lactase. [b] Conversion ratio (%): [product concentration (mM)/concentration of lactose used (mM)] × 100.

Table 2. Experimental Codes, Ranges, and Levels of the Independent Variab1es for RSM Experiment[a]

			level				
variable	unit	symbol code	−1.682[b]	−1	0	+1	+1.682[b]
lactose	mM	X_1	0	10	60	110	144.08
hydroquinone	mM	X_2	0	10	50	90	117.27
enzyme	U/mL	X_3	0	5	20	35	45.22

[a] $Y = \beta_0 + \beta_1X_1 + \beta_2X_2 + \beta_3X_3 + \beta_{11}X_1^2 + \beta_{22}X_2^2 + \beta_{33}X_3^2 + \beta_{12}X_1X_2 + \beta_{13}X_1X_3 + \beta_{23}X_2X_3$. [b] Based on program design value.

Table 3. Central Composite Design Matrix for the Experimental Design and Predicted Responses for HQ-Gal Synthesis[a]

	coded level			HQ-Gal synthesis (mM)	
run no.	X_1	X_2	X_3	actual	predicted
1	10	10	35	1.00	−0.17
2	110	10	35	4.51	5.11
3	60	50	20	7.08	6.91
4	60	50	20	7.97	6.91
5	60	50	0	0	1.75
6	60	117.27	20	9.42	9.42
7	60	50	20	7.18	6.91
8	10	90	35	3.52	3.60
9	60	50	45.22	5.00	4.85
10	60	50	20	7.88	6.91
11	10	10	5	1.00	−0.08
12	60	0	20	0	1.70
13	110	90	5	10.43	10.91
14	60	50	20	7.40	6.91
15	144.08	50	20	15.94	15.48
16	10	90	5	0.95	−0.33
17	110	90	35	17.06	17.46
18	110	10	5	3.32	2.56
19	0	50	20	0	2.79
20	60	50	20	6.91	6.91

[a] $Y = -1.6441 - 0.01625X_1 + 0.03483X_2 + 0.27256X_3 + 0.00023X_1^2 - 0.00057X_2^2 - 0.00752X_3^2 + 0.001074X_1X_2 + 0.000876X_1X_3 + 0.00167X_2X_3$.

ascertained from the central points of the corresponding contour plots, the three variables were 20 U mL^{-1} lactase activity, 60 mM lactose, and 50 mM HQ. From ANOVA analysis, values of "Prob > F" < 0.05 indicated that the model terms were significant. X_2, X_3, X_1^2, X_2^2, X_3^2, X_1X_2, and X_1X_3 were significant model terms (**Table 4**). The model F value was 22.54, implying the significance of the model. The regression equation coefficient was calculated, and the data were fitted to a second-order polynomial equation. The response, HQ-Gal synthesis by lactase acceptor reaction, was expressed in terms of the following regression equation: $Y = -1.6441 - 0.01625X_1 + 0.03483X_2 + 0.27256X_3 + 0.00023X_1^2 - 0.00057X_2^2 - 0.00752X_3^2 + 0.001074X_1X_2 + 0.000876X_1X_3 + 0.00167X_2X_3$ where X_1 is lactose concentration (mM), X_2 is HQ concentration (mM), and X_3 is lactase unit (U mL^{-1}). The regression equation obtained from ANOVA indicated a R^2 (multiple correlation coefficient) value of 0.9530 (a value of >0.75 indicates fitness of the model). This was an estimate of the fraction of overall variation in the data accounted by the model, and the model was capable of explaining 95.30% of the variation in response. The "adequate precision value" of the present model was 17.025, suggesting that the model can be used to navigate the design space. The "adequate precision value" is an index of the signal-to-noise ratio, and values exceeding 4 are desirable prerequisites for a model to be a good fit. On the basis of the model, the predicted response for HQ-Gal synthesis was 6.91 mM and the observed experimental value was 7.40 ± 0.44 mM using 20 U mL^{-1} lactase, 60 mM lactose, and 50 mM HQ, representing near-identical results from the predicted and actual HQ-Gal synthesis.

Table 4. ANOVA for the RSM Parameters Fitted to Second-Order Polynomial Equation[a]

source	sum of squares	degree of freedom	mean square	F	$P > F$
model	442.99	9	49.22	22.54	<0.0001
residual	21.83	10	2.18		
lack of fit	20.88	5	4.18	21.95	0.0021
pure error	0.95	5	0.19		
cor total	464.82	19			

[a] Standard deviation = 1.48, R^2 = 0.9530, CV = 25.35, Adj R^2 = 0.9108.

Antioxidant Activity. HQ-Gal displayed antioxidatant activity similar to that of β-arbutin. The IC_{50} value of HQ-Gal based on DPPH radical scavenging activity was 3.31 mM, which was lower than that of HQ (IC_{50} = 0.21 mM) but marginally greater than that of β-arbutin (3.95 mM). This result was consistent with the suggestion that the attachment of galactose on HQ decreases its antioxidant activity in vitro.

Tyrosinase-Inhibitory Effect. The tyrosinase-inhibitory effect by HQ-Gal was compared with β-arbutin. The inhibition for HQ-Gal was identified as a competitive type; the K_i value of HQ-Gal was 0.75 mM, which represented a higher tyrosinase-inhibitory effect compared with β-arbutin (1.97 mM, 2.6 times better as an inhibitor).

Cell Viability and Inhibition of Melanin Synthesis. The viability of B16 cells after treatment was examined using the MTT assay. HQ-Gal was not significantly cytotoxic to B16 cells when treated at 10 or 50 μg mL^{-1}, although HQ showed significant cytotoxicity in a dose-dependent manner (**Figure 3A**). To investigate the effect of HQ-Gal on melanogenesis, B16 cells were cultured in the presence of 50 μg mL^{-1} of each sample. β-Arbutin exhibited an 8% reduction of melanin synthesis and HQ-Gal exhibited a 23% inhibition of melanin synthesis compared with the control (**Figure 3B**).

DISCUSSION

HQ-Gal is synthesized via the acceptor reaction of lactases of different origins with HQ and lactose. There have been many efforts to synthesize the analogue or derivatives of β-arbutin by glycosylation with enzymes including glucansucrase and glycosidase (*3*, *18*−*21*). But to our knowledge, galactosylation of HQ with lactase has not been described. HQ was a suitable acceptor for the transgalactosylation of lactase. The modified HQ was purified by column chromatography and identified as 4-hydroxyphenyl-β-D-galactopyranoside by ^{13}C NMR, ^{1}H NMR, and MALDI-TOF-MS analyses. By use of the optimized conditions (50 mM HQ, 60 mM lactose, and 20 U mL^{-1} *K. latics* lactase), HQ-Gal could be produced at 2.01 g L^{-1}.

HQ derivatives displayed differing antioxidant activities depending on their structural configurations (α-linkage or β-linkage). We previously reported that 4-hydroxyphenyl-α-glucopyranoside (HQ-Glu) shows lower antioxidant activity compared with that of HQ but displays a higher antioxidant activity compared to β-arbutin (*4*). 4-Hydroxyphenyl-β-fructofuranoside (HQ-Fru) also displays similar or a slightly higher antioxidant activity compared to β-arbutin (*3*). The α-glucosidic linkage of HQ conveys a higher antioxidant activity than a β-glucosidic linkage while maintaining its potential as an antioxidant ingredient in cosmetics (*18*). HQ-Gal also showed 1.19 times higher antioxidant activity than β-arbutin. Tyrosinase inhibition is important in the cosmetics industry as an essential component of skin-whitening agents. In previous studies, HQ-Fru showed a

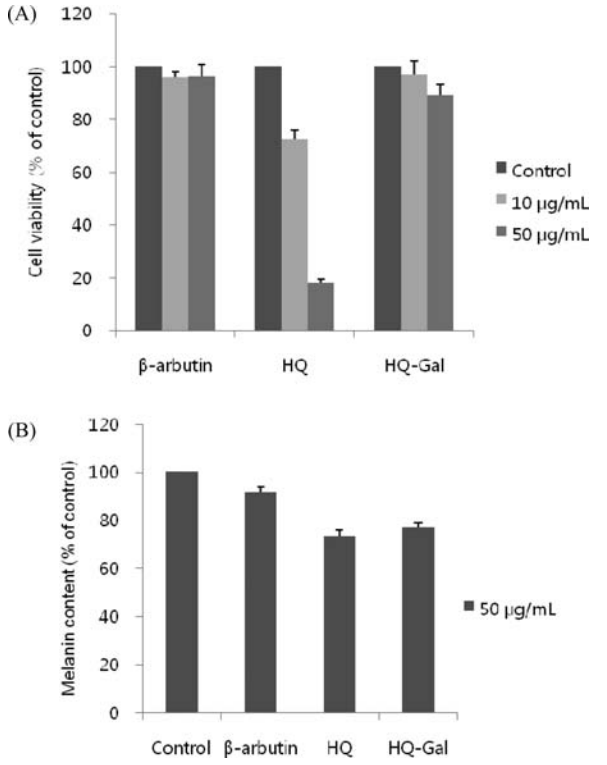

Figure 3. Cell viability (A) and melanin content (B) of B16 melanoma cells treated with the indicated concentrations. Cells in wells of a 96-well plate (104 cells per well) and 24-well plate (105 cells per well) were incubated overnight, then treated with the each sample and incubated further for 72 h. The data are reported as the mean ± SEM of five independent experiments.

higher tyrosinase-inhibitory effect compared to β-arbutin; the determined K_i value of HQ-Fru showed it was 1.8 times better as an inhibitor (*3*). 4-Hydroxyphenyl-β-xylopyranoside (HQ-Xyl) has a higher tyrosinase-inhibitory effect than β-arbutin; the IC_{50} value of HQ-Xyl was determined to be 1.7 times better as an inhibitor than β-arbutin (*20*). HQ-Xyl produces an increasing inhibitory activity with increasing molecular weight compared to 4-hydroxyphenyl β-xylopyranoside. In the case of HQ-Glu, the K_i value previously calculated indicated a 20 times stronger inhibitory effect against human tyrosinase than β-arbutin (*22*). 4-Hydroxyphenyl β-maltoside and 4-hydroxyphenyl β-maltotrioside have exhibited stronger inhibitory activities than β-arbutin against human tyrosinase in several studies (*18*, *19*, *22*). Inhibition can be competitive (*3*, *20*, *22*, *23*) or noncompetitive (*15*). β-D-Glucopyranosyl-(1→3)-arbutin has also been synthesized and shown to possess more effective depigmentation activity. It is less cytotoxic than β-arbutin (*21*). HQ-Gal exhibited 2.8 times more inhibition activity for melanin synthesis, without being significantly toxic to the cells, compared to β-arbutin, which is a current commercial active whiting ingredient of cosmetics. HQ-Gal inhibition was identified as competitive.

DPPH radical scavenging activity, tyrosinase-inhibitory effect, and reduction of melanin synthesis in B16 melanoma cells indicate that HQ-Gal may be a suitable functional component in the cosmetics industry. The determination of the relationship between the inhibitory effects and physicochemical properties of inhibitors is an important approach to identify the structural environments of the active center of enzymes for further study.

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