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Oxidative coupling reaction of arbutin and gentisate catalyzed by horseradish peroxidase

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Abstract

Horseradish peroxidase catalyzed an oxidative coupling reaction of 4′-hydroxyphenyl β-glucoside (arbutin) and 2,5-dihydroxybenzoic acid sodium salt (gentisate) using H_2O_2 as an electron acceptor to yield a precipitating yellow compound. An approximate arbutin/gentisate ratio of 1:2 was effective for the synthesis. The addition of 100–300 mM H_2O_2 to the mixtures of 100 mM arbutin and 200 mM gentisate attained optimized yields of 50–60% in the initial arbutin. Mass spectrometry, infrared spectroscopy, and nuclear magnetic resonance data revealed that the precipitated compound was a novel glycoside bound between the C3′-position of the hydroxyphenyl moiety of arbutin and the C6-position of gentisate, followed by the intramolecular esterification between the phenolic hydroxyl group of arbutin moiety and the carboxyl group of gentisate moiety. The product exerted anti-oxidation and competitive inhibition against mushroom tyrosinase higher than arbutin, while it inhibited mouse melanoma tyrosinase lower than arbutin.

Keywords: Peroxidase; Arbutin; Gentisate; Coupling; Glycoside

1. Introduction

Peroxidase (donor: hydrogen peroxide oxidoreductase, EC 1.11.1.7) is a redox enzyme that is important in the colorimetric determination of various biological substances. The colored compounds are formed by oxidative coupling of aromatic compounds, such as 4-aminoantipyrine and phenol depending on the hydrogen peroxide (H_2O_2) generated by appropriate oxidases [1,2]. The enzyme has also been investigated as a catalyst that polymerizes a number of aromatic compounds [3–9]. The products are often a new class of polymers that are difficult to synthesize by conventional chemical methods. Horseradish peroxidase (HRP), for instance, polymerizes arbutin (4′-hydroxyphenyl β-D-glucoside, Arb) to produce a polymer, poly(Arb), which are subsequently deglycosylated to poly(hydroquinone), a redox active polymer, constructed regularly from a 1,4-dihydroxy-2,6-phenylene unit [7–9].

In this study, the HRP-catalyzed coupling reaction was applied to the synthesis of a new phenolic glucoside from Arb and 2,5-dihydroxybenzoic acid (sodium salt, gentisate, GA). The reaction is regarded as a potential method of enabling the specific and efficient modification of aglycone structures of phenolic glycosides without protection and de-protection steps.

2. Materials and methods

2.1. Materials

Arb, GA (sodium salt), catechol (Cat, 1,2-dihydroxybenzene), resorcinol (Res, 1,3-dihydroxybenzene), 3-(3,4-dihydroxyphenyl)-L-alanine (L-DOPA), 1,1-diphenyl-2-pyridilhydradine (DPPH), and H_2O_2 were purchased from Nakalai Tesque (Tokyo). α-Arbutin (4′-hydroxyphenyl α-D-glucopyranoside, α-Arb) [10–12] was donated by Ezaki Glico (Osaka). The other aromatic compounds were purchased from Nakalai Tesque and Sigma–Aldrich (St. Louis, MO, USA).

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2.2. Enzymes

HRP (138 units/mg) was a commercial product (Toyobo, Kyoto). Tyrosinase from mouse melanoma B16 was supplied by Oppen Cosmetics (Shiga).

2.3. Thin layer chromatography

Thin layer chromatography (TLC) was performed by the ascending method using Silica gel 60F (0.5 mm thick, containing fluorescent indicator; Merck, Darmstadt, Germany) and a solvent of ethyl acetate–acetic acid–water (3:1:1, v/v/v). Spots were detected by spraying sulfuric acid in methanol (1:1, v/v) followed by heating at 130 °C.

2.4. High performance liquid chromatography

High performance liquid chromatography (HPLC) was performed under the following conditions using a Shimadzu LC-10AD (Shimadzu, Kyoto) equipped with RID-10A and SPD-10AV UV–vis detectors: Column, Cosmosil Sugar-D (Nakalai Tesque); temperature, 40 °C; solvent, 70% (v/v) CH_3CN; flow rate, 1.0 ml/min.

2.5. Spectrometries and other analytical methods

^{1}H nuclear magnetic resonance (300 MHz, NMR) and ^{13}C NMR (75 MHz) spectra were taken with a JEOL AL-300 spectrometer (Tokyo). Acetone (δ 2.10) or 3-(trimethylsily)-propanesulfonic acid, sodium salt (DSS, δ 0) were used as the internal standards for ^{1}H NMR. 1,4-Dioxane (δ 66.5) was used as the standard for ^{13}C NMR. ^{1}H and ^{13}C NMR signals were assigned by means of one-dimension (1D) proton decoupling technique, distortionless enhancement by polarization transfer (DEPT), 2D HH-correlation spectroscopy (COSY), and CH-COSY. Fourier transform infrared spectroscopy (FT-IR) spectra were taken with a Shimadzu FT-IR 8200. Electrospray ionization (ESI) mass spectra were taken with a Finnigan LCQ^{DECA}. Matrix-assisted laser desorption ionization (MALDI)-time-of-flight mass spectrometry (TOF-MS) was performed using a Voyager^{TM} (PerSeptive Biosystems, Framingham, MA, USA). α-Cyano-4-hydroxy-cinnamic acid was used as a matrix. Ultraviolet–visible spectroscopy (UV–vis) spectra were taken with a JASCO UV/vis spectrophotometer V-530 (Tokyo). Melting points (m.p.) were measured with a Yanaco micro-melting point apparatus (Kyoto).

2.6. Synthesis of hydroxyphenyl glucosides

2.6.1. Catechol α-glucoside (2′-hydroxyphenyl α-D-glucopyranoside, Cat-Glc)

A reaction mixture (100 ml) containing 2.0% (w/v) Cat, 5.0% (w/v) α-cyclodextrin (Ensuiko Sugar Refining, Yokohama), and cyclodextrin glucanotransferase (14 units/ml, from *Bacillus stearothermophilus*, Hayashibara Biochemical Laboratories, Okayama) in 50 mM acetate buffer (pH 5.5) were incubated at 40 °C for 18 h. The mixture was boiled for 20 min to stop the reaction. The maltooligosylated Cat(s) were digested to produce Cat-Glc with glucoamylase (2.0 units/ml, from *Rhizopus niveus*, Seikagaku Kogyo, Tokyo) at 40 °C for 16 h. After evaporation, the sample (20 ml) was loaded onto a column of Bio-Gel P-2 (5 cm × 93 cm, Bio-Rad Laboratories. Hercules, CA, USA) equilibrated with 5.0% (v/v) ethanol. The elution was performed at 20 ml/h, and 5-ml fractions were collected. The components were monitored by the phenol–sulfuric acid method, adsorption at 260 nm, and TLC. The fractions containing Cat-Glc were collected and evaporated (isolated yield, 1170 mg): white powder; m.p. 76–78 °C; ^{1}H NMR (300 MHz, D_2O) δ 7.14 (d, 1H, ^3J = 8.1 Hz, H6′), 6.95–6.80 (m, 3H, H3′-5′), 5.50 (d, 1H, $^3J_{H1,H2}$ = 3.9 Hz, H1), 3.87 (t, 1H, $^3J_{H2,H3} = {}^3J_{H3,H4}$ = 9.5 Hz, H3), 3.73 (m, 1H, H5), 3.67 (m, 2H, H6), 3.64 (dd, 1H, $^3J_{H1,H2}$ = 3.9 Hz, $^3J_{H2,H3}$ = 9.5 Hz, H2), 3.42 (t, 1H, $^3J_{H3,H4} = {}^3J_{H4,H5}$ = 9.5 Hz, H4); ^{13}C NMR (75 MHz, D_2O) δ 145.7 (C1′), 144.2 (C2′), 123.8 (C4′), 120.9 (C5′), 117.2 (C6′), 116.6 (C3′), 98.1 (C1), 72.8 (C3), 72.5 (C5), 71.3 (C2), 69.2 (C4), 60.2 (C6); FT-IR (cm^{-1}, KBr disk) 3385 (O–H), 1597 (C=C), 1021 (ethers); ESI-MS *m/z* 271 (M–H)$^-$ (negative mode); $[\alpha]_D$ 177° (*c* 0.5, H_2O).

2.6.2. Resorcinol α-glucoside (3′-hydroxyphenyl α-D-glucopyranoside, Res-Glc)

Res-Glc was synthesized from Res and α-cyclodextrin and isolated in a similar manner (isolated yield, 1380 mg): white powder; m.p. 84–86 °C; ^{1}H NMR (300 MHz, D_2O) δ 7.13 (t, 1H, $^3J_{H4',H5'} = {}^3J_{H5',H6'}$ = 8.3 Hz, H5′), 6.63 (dd, 1H, $^3J_{H4',H5'}$ = 8.3 Hz, $^4J_{H2',H4'}$ = 2.3 Hz, H4′), 6.57 (t, 1H, $^4J_{H2',H4'} = {}^4J_{H2',H6'}$ = 2.3 Hz, H2′), 6.51 (dd, 1H, $^3J_{H5',H6'}$ = 8.3 Hz, $^4J_{H2',H6'}$ = 2.3 Hz, H6′), 5.50 (d, 1H, $^3J_{H1,H2}$ = 3.6 Hz, H1), 3.80 (t, 1H, $^3J_{H2,H3} = {}^3J_{H3,H4}$ = 9.6 Hz, H3), 3.64 (m, 3H, H5, H6), 3.59 (dd, 1H, $^3J_{H1,H2}$ = 3.6 Hz, $^3J_{H2,H3}$ = 9.6 Hz, H2), 3.42 (m, 1H, $^3J_{H3,H4} = {}^3J_{H4,H5}$ = 9.6 Hz, H4); ^{13}C NMR (75 MHz, D_2O) δ 157.4 (C3′), 156.8 (C1′), 130.5 (C5′), 109.9 (C6′), 108.8 (C4′), 104.4 (C2′), 97.1 (C1), 73.0 (C3), 72.4 (C5), 70.7 (C2), 69.2 (C4), 60.2 (C6); FT-IR (cm^{-1}, KBr disk) 3389 (O–H), 1580 (C=C), 1022 (ethers); ESI-MS *m/z* 271 (M–H)$^-$ (negative mode); $[\alpha]_D$ 162° (*c* 0.5, H_2O).

2.7. Reaction of Arb and various aromatic compounds

Benzoic acid derivatives were neutralized with 2.0 M NaOH prior to the reactions. To the mixtures (185 μl) consisted of Arb (final concentration, 100 mM), aromatic compounds (100 mM), phosphate buffer (50 mM, pH 7.0), and HRP (50 μg), portions of H_2O_2 solution (2.36 M, 2.5 μl) were added six times at 5-min intervals to avoid the enzyme inactivation (final concentration of H_2O_2, 177 mM). The reaction was carried out at 40 °C. After 30 min of the final addition of H_2O_2, the products were analyzed by TLC.

2.8. Reaction of various phenolic glucosides and GA

Reaction mixtures (200 μl) consisted of 100 mM glucosides (Arb, α-Arb, Res-Glc, and Cat-Glc), 200 mM GA, 50 mM phosphate buffer (pH 7.0), 177 mM H_2O_2, and HRP (50 μg) in final

concentrations. H_2O_2 was also added stepwise as described above. After incubation at 40 °C for 30 min, the products were analyzed by TLC.

2.9. Production of the coupling product at different concentrations of GA

Reaction mixtures (2.5 ml, in centrifuge tubes) consisted of 100 mM Arb, 0–500 mM GA, 189 mM H_2O_2, 100 mM phosphate buffer (pH 7.0), and HRP (100 μg/ml) in final concentrations. Portions (25 μl) of H_2O_2 solution (2.36 M, 200 μl) were added stepwise at 5-min intervals. The reaction was carried out at 25 °C. (1) Arb was measured by HPLC under the conditions described above. (2) Poly(Arb) was determined as follows: before the precipitation of the coupling product (30–60 min after the addition of H_2O_2), a portion of the reaction mixtures (100 μl) was mixed with 400 μl of ethanol. The precipitates were collected by centrifugation at 12,000 rpm for 10 min, washed with 80% (v/v) ethanol, dissolved in 1.0 ml water, and determined by the phenol–sulfuric acid method using Glc as a standard. (3) The coupling product was determined as follows: acetic acid (1.0 M, 0.5 ml) was added to the reaction mixture for stopping the reaction and for promoting the precipitation. After 7 days at 5 °C, precipitates were collected by centrifugation at 10,000 rpm for 15 min, washed twice with water, dried *in vacuo*, dissolved in dimethyl formamide (DMF), and determined by the phenol–sulfuric acid method.

2.10. Production of the coupling product with different amount of H_2O_2

Reaction mixtures (2.5 ml, in centrifuge tubes) consisted of 100 mM Arb, 200 mM GA, 100 mM phosphate buffer (pH 7.0), and HRP (100 μg/ml) in final concentrations. Small portions (25 μl) of H_2O_2 solution (2.36 M, 100–500 μl) were added stepwise at 5-min intervals. The reaction was carried out at 25 °C. Arb, poly(Arb), and the coupling products were measured in the same manner as described above.

2.11. Preparation of the coupling product

Reaction mixtures (50 ml) consisted of 100 mM Arb, 200 mM GA, 189 mM H_2O_2, 100 mM phosphate buffer (pH 7.0), and HRP (100 μg/ml) at final concentrations. H_2O_2 solution (2.36 M, 4.0 ml) was added dropwise. The reaction was carried out at 25 °C. After 10 days of precipitation of the coupling product at 5 °C, the yellowish precipitates were collected by centrifugation at 10,000 rpm for 15 min, washed well with water, and dried *in vacuo*.

2.12. Determination of anti-oxidizing activity

DPPH was used as a model radical compound [13]. The aqueous solutions of Arb-GA were prepared by dissolving in 1.0 M NaOH followed by neutralizing with 1.0 M HCl. DPPH solution (0.01%, w/v, 100 μl, in ethanol) and the Arb-GA solution (100 μl) were mixed, incubated at 20 °C for 10 min, and the absorbance at 540 nm was measured. Arb, GA, and ascorbic acid (AsA) dissolved in water were used as reference compounds.

2.13. Determination of inhibitory activity against tyrosinases

2.13.1. Mushroom tyrosinase

The coupling product was dissolved in a minimum volume of 1.0 M NaOH, neutralized with 1.0 M HCl prior to the reactions. L-DOPA (5.0 mM) in 50 mM phosphate buffer (pH 7.0, 1.0 ml) and the sample (the coupling product, Arb, and GA) solutions (1.0 ml) were mixed in a 1-cm cuevet, placed in a Shimadzu UV-1600 spectrophotomer equipped with cell temperature controller. After preliminary incubation for 5 min at 40 °C, the reaction was started by the addition of 20 μl of a tyrosinase solution (4000 units/ml). Dopaquinone was measured at 475 nm.

2.13.2. Mouse melanoma tyrosinase

The coupling product was dissolved in a minimum volume of DMSO and filled up to different concentrations with water. The sample solutions of Arb and GA were also prepared in the same manner. L-DOPA (3.3 mM) in 50 mM phosphate buffer (pH 7.0, 500 μl), the sample solutions (500 μl), and the tyrosinase solution (20 μl, 4000 units/ml) were mixed in a 1-cm cuevet and reacted at 37 °C for 10 min to determine dopaquinone at 475 nm.

3. Results and discussion

3.1. Coupling reaction of Arb and GA

HRP polymerized Arb using H_2O_2 as an electron acceptor [7–9]. The polymerized product, poly(Arb), was detected at the origin in TLC analysis (Fig. 1). The enzyme also oxidized GA, which was recognizable from the reaction mixture developing a

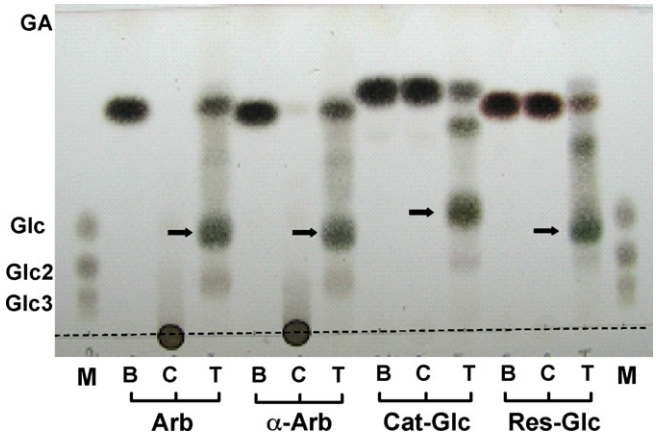

Fig. 1. TLC analysis of products from hydroxyphenyl glucosides and GA. A, Arb; B, α-Arb; C, Cat-Glc; D, Res-Glc; M, markers; Glc2, maltose; Glc3, maltotriose; B, blank (without HRP); C, control (reaction without GA); T, test (reaction in the coexistence of the glucoside and GA). The reactions were performed as described in Section 2.8. Spots were visualized by spraying H_2SO_4/methanol followed by heating. Note that GA gave no clear spots with H_2SO_4. The arrows show major reaction products from the glucosides and GA.

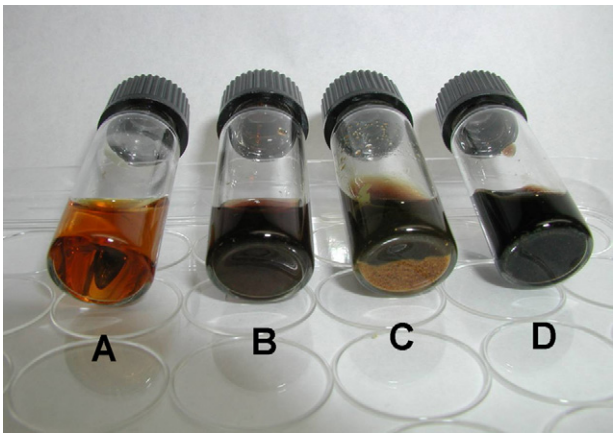

Fig. 2. Appearance of the reaction solutions of Arb and/or GA. The reactions conditions were same as those for Fig. 1. (A) The reaction solution for Arb (in the absence of GA) after keeping for 4 days at 20 °C; (B) the reaction solution of Arb and GA that was incubated for 30 min at 40 °C after completion in the addition of H_2O_2; (C) the reaction solution B after keeping for 4 days at 20 °C; (D) the reaction solution for GA (in the absence of Arb) after keeping for 4 days at 20 °C.

brown color (Fig. 2). In the coexistence of Arb and GA, however, unknown compounds were produced instead of poly(Arb) as shown in Fig. 1. The products, one major and a few minors, were positive not only for sugars that turned brown with H_2SO_4 (Fig. 1), but also for aromatic compounds that gave fluorescent spots under UV light. Such water-soluble products were formed soon after the addition of H_2O_2. On standing the mixture for several days, yellow precipitates appeared gradually (Fig. 2) as the main spot on TLC disappeared. The individual reactions of Arb and GA afforded no such precipitations (Fig. 2). Peroxidases catalyze radical coupling of various aromatic compounds [1–9]. We revealed that the unknown and precipitating compound was a coupling product of the two phenolic substrates. In addition to HRP, peroxidases from soybean and *Arthromyces* also synthesized the same coupling product (data not shown).

3.2. Reaction products from Arb and several aromatic compounds

HRP was reacted with Arb in the presence of several aromatic compounds to analyze the products by TLC as described in Section 2.7. Among benzoate derivatives, a faint spot of a similar mobility to that of the product from GA was detected in the reaction with 3,4-dihydroxybenzoate (protocatechuate). The similar mobility suggested that the product was structurally relevant to the coupling product from GA, although the yield was too low to identify. No particular products except poly(Arb) were detected in the reactions of Arb with the following aromatic compounds: (1) phenol, benzoate, and benzyl alcohol; (2) 1,2-dihydroxybenzene, 1,3-dihydroxybenzene, and 1,4-dihydroxybenzene; (3) 2-nitrophenol, 3-nitrophenol, and 4-nitrophenol; (4) 2-hydroxybenzoate, 3-hydroxybenzoate, 4-hydroxybenzoate, and 4-hydroxybenzyl alcohol; (5) 2,3-dihydroxybenzoate, 2,4-dihydroxybenzoate, and 2,6-dihydroxybenzoate; (6) 3,4,5-trihydroxybenzoate.

3.3. Reaction products from a few phenolic glucosides and GA

HRP was reacted with a few phenolic glycosides in the presence of GA. As shown in Fig. 1, sugar spots of similar chromatographic behaviors to that of the coupling product from Arb and GA were detected in the reaction of 4′-hydroxyphenyl α-D-glucopyranoside with another anomeric linkage, which afforded a polymer in the absence of GA, as well as in the reactions of 2′-hydroxyphenyl α-D-glucopyranoside and 3′-hydroxyphenyl α-D-glucopyranoside with different anomeric and aglycone structures, which afforded no polymers. Yellow precipitates appeared in the reaction of Cat-Glc in several days, while α-Arb and Res-Glc did not. The products were expected to be structurally relevant to that of Arb and GA, considering the fact that they were particularly synthesized in the coexistence of the hydroxyphenyl glucosides and GA, although their identification and characterization were remained to be investigated.

4-*O*-α-glucosyl Arb [9,12], which possessed elongated glycosyl moiety, also gave a soluble relating product (data not shown). The following glycosides neither gave such particular products, nor polymers in the presence of GA: phenyl α-glucoside, 2′-hydroxymethyl β-glucoside, 2′-nitrophenyl β-galactoside, 3′-nitrophenyl β-galactoside, 4′-nitrophenyl β-galactoside, 4′-nitrophenyl β-glucoside, and 4′-nitrophenyl α-mannoside.

3.4. Synthesis and some properties of Arb-GA

The following study focused on the coupling of Arb and GA because of the high yield, and the facile isolation of the product by precipitation. The insoluble product was abbreviated as Arb-GA.

First, the synthesis was performed at different ratios of the phenolic substrates to optimize the yield of Arb-GA (Fig. 3): Arb-GA was synthesized from 100 mM Arb, 189 mM H_2O_2, and different concentrations of GA. A concentrated solution of H_2O_2 was added in small divided portions to the mixtures

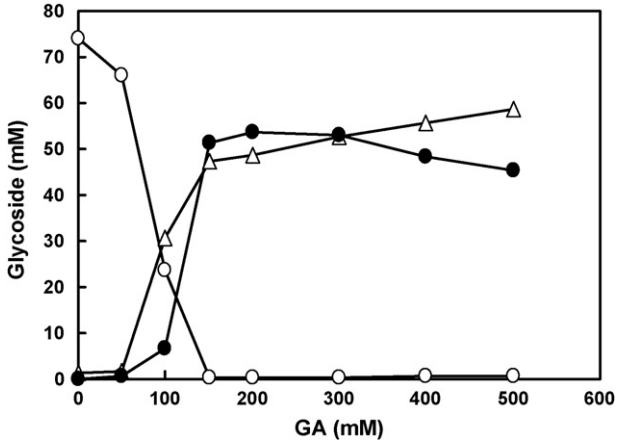

Fig. 3. Production of Arb-GA at different concentrations of GA. The reaction conditions were described in Section 2.9. Open triangle, Arb (mM); open circle, poly(Arb) (mM as Glc); closed circle, Arb-GA (mM as Glc).

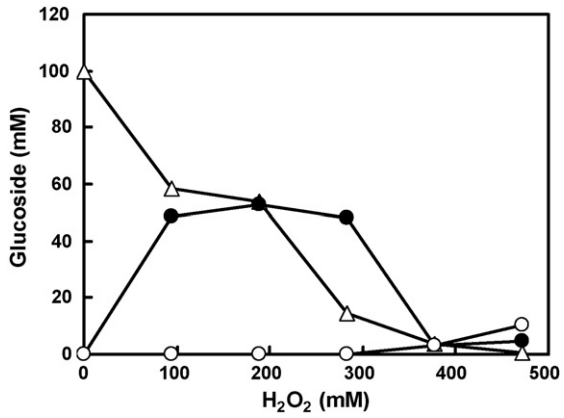

Fig. 4. Production of Arb-GA with different amounts of H_2O_2. The reaction conditions were described in Section 2.10. Open triangle, Arb (mM); open circle, poly(Arb) (mM as Glc); closed circle, Arb-GA (mM as Glc).

of the phenolic substrates and HRP to suppress the enzyme inactivation. As shown in Fig. 3, Arb was polymerized predominantly to poly(Arb) at low GA concentrations (0–100 mM). Even in the presence of sufficient GA (more than 150 mM), the yield of Arb-GA flattened at approximately 50% and more than half of Arb remained. H_2O_2 may be consumed dominantly for the oxidation of GA under such conditions. We employed, therefore, an Arb/GA ratio of 1:2 for efficient synthesis. Fig. 4 shows the effects of H_2O_2 added to 100 mM Arb and 200 mM GA on the synthesis. Arb-GA was synthesized in approximate yields of 50–60% of Arb by the addition of 100–300 mM H_2O_2. More excessive H_2O_2 resulted in the reduction of Arb-GA and poly(Arb). Unknown radicals or oligomeric products seemed to be formed according to the complex chromatogram in TLC analysis. Consequently, Arb-GA was synthesized in a mixture of 100 mM Arb, 200 mM GA, and 189 mM H_2O_2 at final concentrations.

Arb-GA was precipitated at 5 °C, collected by centrifugation, washed with water, and lyophilized to obtain pale yellow powder. Irrespective of such simple isolation procedures, the purity was judged to be high enough to give NMR spectra without any extra peaks as shown in Fig. 5. The compound was soluble in DMF and dimethyl sulfoxide (DMSO), but scarcely soluble in water (solubility, less than about 0.1 mg/ml at 20 °C), methanol, ethanol, acetone, and chloroform. Arb-GA was

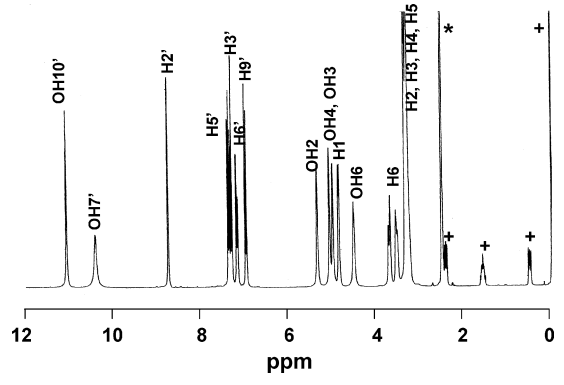

Fig. 5. ^{1}H NMR spectrum of Arb-GA. (*) Peak of solvent and (+) peaks of DSS.

Table 1
^{13}C NMR chemical shifts for Arb-GA

Carbon number	Chemical shift
1	102.7
2	74.8
3	78.6
4	71.0
5	78.3
6	62.0
1′	155.6
2′	119.0
3′	120.5[a]
4′	145.9
5′	118.8
6′	117.1
7′	149.0
8′	127.8
9′	118.9
10′	156.2
11′	107.1
12′	120.3[a]
13′	166.8

The carbon numbers were shown in Fig. 6. The chemical shifts were given in ppm.
[a] Signals may be interchangeable.

soluble in alkaline water, although the solution turned brown in a few days. The aqueous solution (0.05 mg/ml, not in alkaline solution) showed absorption maxima at 373, 235, and 213 nm. A DMSO solution showed strong absorption at 377 nm in addition to the medium ones at 273 and 280 nm. The melting point was 218–219 °C.

3.5. Structure of Arb-GA

Arb-GA exclusively gave a molecular-related ion peak $[C_{19}H_{18}O_{10}+H]^+$ at *m/z* 407 in MALDI-TOF-MS, indicating that the product was composed of Arb (molecular mass, 272) and gentisic acid (154) with the reduction of the mass corresponding to two hydrogen atoms (2) and H_2O (18). In the IR spectrum, the stretching vibration peak of the carbonyl group (C=O) was observed at 1655 cm^{-1}. The following typical absorptions were also observed; at 3396 cm^{-1} due to the O–H linkage, at 1599 cm^{-1} due to the C=C linkage in the aromatic ring, and at 1190 and 1076 cm^{-1} due to ether (C–O–C) linkage. Table 1 shows the results of ^{13}C NMR analysis in DMSO-d_6. The compound gave 19 carbon signals. Six signals were assigned to the carbons of the glucosyl moiety, which were similar to those of Arb. The carbonyl carbon signal was detected at δ 166.8. In ^{1}H NMR analysis (Fig. 5), the proton signals were assigned by H–H-COSY and C–H-COSY. COLOC was also applied for the measurement of long range coupling for the assignment of hydroxyl protons and carbons without protons. Glucosyl moiety gave a ^{1}H NMR spectrum similar to that of Arb and signals were observed at δ 3.6–5.4: 5.41, 5.13, 5.05, 4.57 (1H, s, OH of glucosyl group); 4.91 (1H, d, $^2J_{H1,H2}$ = 6.42 Hz, H1); 3–4 (6H, m, H1–6). Only two signals of phenolic protons appeared at δ 11.2 (1H, s) and δ 10.5 (1H, s), which were assigned to those of H10′ and H7′, respectively. In addition, the signals were

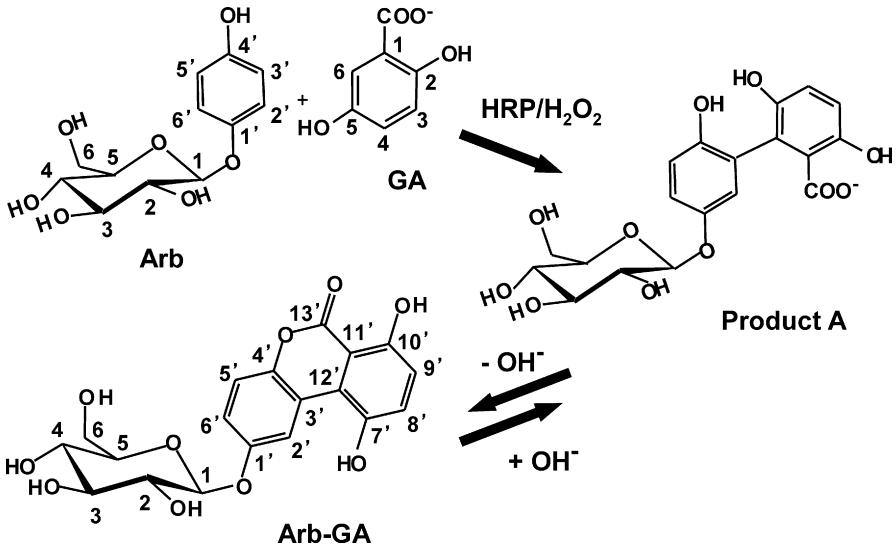

Fig. 6. Scheme for the coupling reaction of Arb and GA. The carbon numbers appeared in the text and Table 1 are shown in this figure. Product A, suspected intermediate product.

reasonably assigned to the protons of the aglycone moiety. Consequently, Arb-GA was elucidated to be a β-glucoside with a novel aglycone structure shown in Fig. 6. The reaction scheme was reasonably deduced from the structure of Arb-GA together with the known action of HRP that catalyzes radical coupling of aromatic compounds: C3′ of Arb and C6 of GA were linked to give a suspected intermediate compound termed "Product A", followed by intramolecular ester formation to produce Arb-GA. Conversion of Product A to Arb-GA seemed to be a slow step as described in Section 3.1, suggesting the difficulty of the ester formation between the carboxylate group and the OH groups. So far, however, Product A has not been identified because of the instability and the difficulty in isolation.

Although the formation of Product A is in line with the known oxidation action of peroxidase, the following mechanism may also be possible: the radicals initially formed from the substrates give rise to quinine compounds by disproportionation and then quinone/phenol interaction ultimately occur. This mechanism may explain the observed regioselectivity successfully. At this time, however, the detailed mechanism for the formation of Product A as well as other postulated radical and quinone compounds remained to be established.

3.6. Anti-oxidizing activity and inhibition activity against tyrosinases

The anti-oxidizing activity of Arb-GA was evaluated by the neutralizing of DPPH, a model radical compound [13]. The concentrations required for reducing DPPH to 50% (IC_{50}) were as follows: GA, 50 μM; AsA, 93 μM; Arb-GA, 143 μM; Arb, 800 μM. Arb-GA exhibited slightly lower activity than GA and AsA, which was nevertheless much stronger than Arb as an anti-oxidizing compound.

Arb inhibits melanogenesis and is used in topical skin-lightening cosmetics [11,12]. Tyrosinase is a key enzyme that plays a regulatory role in the melanin synthesis. Fig. 7 shows the inhibition of Arb-GA against tyrosinases from mushroom

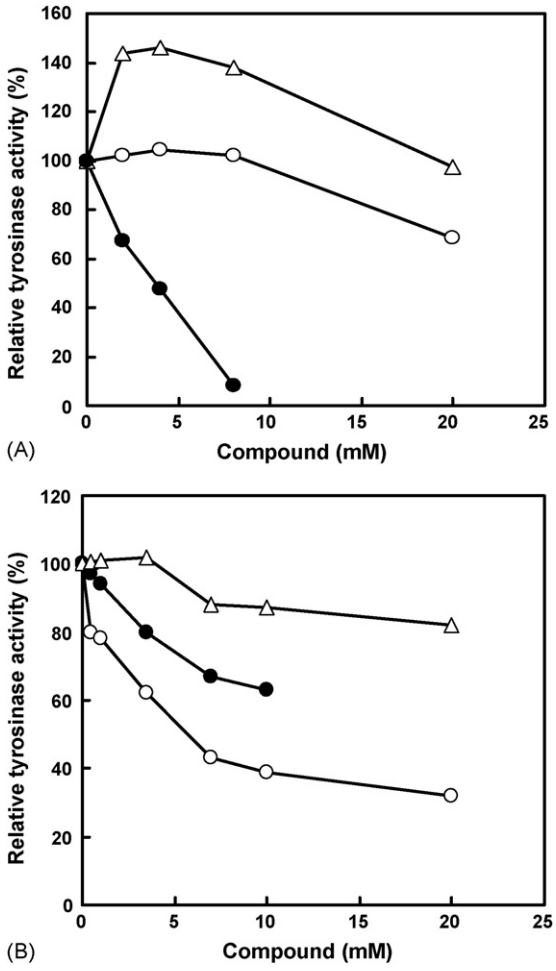

Fig. 7. Inhibition of Arb-GA against tyrosinases from mushroom and mouse melanoma cells. A, inhibition against the mushroom tyrosinase; B, inhibition against the melanoma tyrosinase; closed circle, Arb-GA; open circle, Arb; open triangle, GA. The reaction conditions were described in Section 2.13. Note that the mushroom enzyme formed significantly colored substance from GA and DOPA especially at low GA concentrations, and eventually the enzyme activity was apparently promoted.

and mouse melanoma cells. Arb-GA did not act as a substrate of the tyrosinases. For the mushroom enzyme (Fig. 7A), Arb and GA exhibited a weak inhibition or even an apparent promotion especially at low concentrations. On the contrary, Arb-GA inhibited the mushroom enzyme more strongly than each starting constituent. A Lineweaver–Burk plot made from the reactions at different concentrations of L-DOPA (0.08–0.2 mM) and Arb-GA (0–0.8 mM) suggests that the inhibition was competitive type with a K_i value of 1.1 mM (data not shown). The considerable inhibition of Arb-GA was attributed to a high affinity with the enzyme, which was comparable to that of L-DOPA (K_m, 1.6 mM). On the contrary, Arb-GA exhibited a lower inhibition against the mammalian tyrosinase than Arb (Fig. 7B). Some phenolic glycosides exert such different behaviors for the tyrosinases from mushroom and mammalian cells [14,15]. For instance, glucosylated catechin inhibits the mushroom enzyme much more significantly than the melanoma enzyme [14], but Arb and α-Arb inhibits the enzymes in the opposite manner [11,12,15]. A lower inhibition against the mammalian tyrosinase is not necessarily favorable to its application to cosmetics, even though it may be worth evaluating further with respect to, for example, the toxicity and the suppression of melanin synthesis in the cells.

4. Conclusions

We revealed the formation of Arb-GA, a glucoside with a novel aglycone structure, through HRP-catalyzed coupling of Arb and GA followed by spontaneous intramolecular esterification. Arb-GA was isolated efficiently as precipitates from the reaction mixtures. The glycoside exhibited an enhanced inhibition against the fungal tyrosinase compared to Arb in spite of a lower inhibition against the mammalian tyrosinase. In general, phenolic glycosides have potential applications, for example, as building blocks of new materials for cosmetics and pharmaceuticals. Therefore, the enzymatic coupling described here may provide a possible modification method for the aglycone structures of phenolic glycosides.

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