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Highly selective biotransformation of arbutin to arbutin-α-glucoside using amylosucrase from *Deinococcus geothermalis* DSM 11300

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ABSTRACT

Arbutin (Ab, 4-hydroxyphenyl β-glucopyranoside) is a glycosylated hydroquinone known to prevent the formation of melanin by inhibiting tyrosinase. An arbutin-α-glucoside was synthesized by the transglycosylation reaction of amylosucrase (AS) of *Deinococcus geothermalis* (DGAS) using arbutin and sucrose as an acceptor and a donor, respectively. The maximum yield of the arbutin transglycosylation product was determined to be over 98% with a 1:0.5 molar ratio of donor and acceptor molecules (sucrose and arbutin), in 50 mM sodium citrate buffer pH 7 at 35 °C. TLC and HPLC analyses revealed that only one transglycosylation product was observed, supporting the result that the transglycosylation reaction of DGAS was very specific. The arbutin transglycosylation product was isolated by preparative recycling HPLC. The structural analyses using ^{13}C and ^{1}H NMR proved that the transglycosylated product was 4-hydroxyphenyl β-maltoside (Ab-α-glucoside), in which a glucose molecule was linked to arbutin via an α-(1 → 4)-glycosidic linkage.

1. Introduction

Melanin is a class of compounds which function primarily as pigments in living organisms. The photochemical properties of melanin make it an excellent photoprotectant. It absorbs destructive UV-radiation and transforms the energy into harmless amounts of heat through an ultrafast internal conversion process [1]. In humans, however, excess melanin production or abnormal distribution can cause anomalous hyperpigmentation of the skin [2,3]. One of the key enzymes in mammalian melanine biosynthesis is tyrosinase [4,5]. Tyrosinase (monophenol monooxygenase; EC 1.14.18.1), a membrane-bound copper-containing glycoprotein [6], is an enzyme catalyzing the first two steps in melanin biosynthesis. Therefore, inhibition of tyrosinase activity has typically been targeted to improve or prevent hyperpigmentary disorders, such as melasma and age spots [7]. Many natural or synthetic tyrosinase inhibitors have been described, including arbutin, catechols, hinokitol, kojic acid, linoleic acid, and naturally occurring hydroquinones, but arbutin is the most popular and effective skin-whitening or depigmenting agent used because of its very low cytotoxicity [4,8,9].

Arbutin (Ab; hydroquinone-*O*-D-glucopyranoside) is a glycosylated benzoquinone extracted from bearberry plants in the genus *Arctostaphylos*. It is an extremely effective natural skin lightener because it inhibits the oxidation of L-tyrosine catalyzed by mushroom tyrosinase by competing for active binding sites in tyrosinase without being oxidized [10–12]. There are two anomeric (α- and β-) forms of Ab. Ab is naturally occurred anomer that inhibited both tyrosinase activities from mushroom and mouse melanoma, whereas α-Ab inhibited only the tyrosinase from mouse melanoma. However, the inhibitory effect of α-Ab on the activity of tyrosinase from human malignant melanoma cells was estimated to be ten times stronger than that of Ab [13]. Recently, a number of Ab-glucoside derivatives have been synthesized by enzymatic biotransformation and examined for their inhibitory effects on mushroom or human tyrosinase [14–16]. Various enzymes having transglycosylation activity have been employed to modify Ab. Cyclomaltodextrin glucanotransferase (CGTase) from *Bacillus macerans* was successfully employed to produce α-Ab-α-glucosides using α-Ab and soluble starch as an acceptor and a donor molecule, respectively [15]. Three Ab-β-glucosides were synthesized by β-glucosidase from hyperthermophilic *Thermotoga neapolitana* using Ab and cellobiose as an acceptor and a donor molecule, respectively [16]. α-Glucosidase from baker's yeast was used to synthesize α-Ab from hydroquinone and maltose as glucosyl donor by transglucosylation in a water system [17].

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Amylosucrase (EC 2.4.1.4; AS) is a member of family 13 of the glucoside hydrolases (the α-amylase), although its biological function is the synthesis of amylose-like polymers from sucrose [18]. Among the glucansucrases, AS is structurally unique because it does not have a circularly permuted $(\beta/\alpha)_8$-barrel assembly [19,20]. In addition, it synthesizes only α-1-4-glycosidic linkages to make a glucan polymer, whereas other glucansucrases make heterogeneous glycosidic bonds such as α-1-2-, α-1-3-, and α-1-6- in the final glucan polymer products [21,22]. Recently, the presence of AS-homologous genes in various microorganisms has been inferred by microbial whole genome sequencing projects, although ASs from only two genera, *Neisseria* and *Deinococcus*, have been reported [23–25]. When sucrose is used as a sole substrate, AS performs three different catalytic reactions: (1) a sucrose hydrolysis reaction to produce glucose and sucrose, (2) sucrose isomerization to form sucrose isomers (turanose and trehalulose), and (3) polymer synthesis to create a soluble maltooligosaccharide and insoluble glucan [25,26]. The polymerization activity of recombinant AS from *Neisseria polysaccharea* was used to glucosylate glycogen particles and thereby to synthesize carbohydrate-based dendritic nanoparticles [27].

Except for various starches and glycogen, other molecules are not examined as acceptors for the transglycosylation of AS. In this work, we have employed the transglycosylation activity of recombinant AS from *Deinococcus geothermalis* (DGAS) to synthesize Ab-glucoside. Unexpectedly, only a single product was formed with exceptionally high yield by DGAS. The structure of the resulting product was determined by nuclear magnetic resonance (NMR) and mass spectrometry (MS) analyses and its melanogenesis inhibitory activity was discussed.

2. Experimentals

2.1. Chemicals and enzymes

All chemicals including Ab used in this study were purchased from Sigma Chemical Co. and were of reagent grade. DNA-modifying enzymes such as restriction endonucleases, T4 DNA ligase and *Pfu* DNA polymerase were purchased from New England Biolabs (Beverly, MA, USA), Solgent (Seoul, Korea) or Promega (Madison, WI, USA). A Glutathione-Sepharose™ High Performance affinity column was obtained from Amersham Biosciences and used for the purification of glutathione S-transferase (GST)-fused DGAS.

2.2. Bacterial strains

The PCR method has been used to clone the gene corresponding to DGAS from the genomic DNA of *D. geothermalis* DSM 11300. The detailed cloning procedure for DGAS was described previously [23–25]. *Escherichia coli* DH10B [F^- *ara*D139 Δ(*ara leu*)7697 Δ*lac*X74 *gal*U *gal*K *rps*L *deo*R Φ80*lac*ZΔM15 *end*A1 *nup*G *rec*A1 *mcr*A Δ(*mrr hsdRMS mcrBC*)] was used for general recombinant DNA procedures while *E. coli* BL21 [F^-, *omp*T, *hsd*S_B(r_B-, m_B-), *dcm*, *gal*, λ(DE3)] was employed as a host for recombinant *dgas* expression studies. In general, recombinant *E. coli* cells were grown in Luria-Bertani (LB) medium containing 1% (w/v) bactotryptone, 0.5% (w/v) yeast extract, and 0.5% (w/v) NaCl, supplemented with ampicillin (100 μg/mL).

2.3. Preparation of recombinant DGAS

Recombinant *E. coli* BL21 cells harboring pGEX-DGAS, a plasmid for DGAS expression, were grown in 1 L of LB medium supplemented with 0.1 mg/mL ampicillin at 37 °C with vigorous agitation. When the optical density of the cells reached around 0.6, 1 mM (final concentration) of isopropyl-β-D-thiogalactopyranoside (IPTG) was added into the culture to induce *dgas* gene expression. After a 3 h induction, the cells were harvested and resuspended in phosphate-buffered saline (PBS, 5 mL/g (wet weight) of cells) buffer composed of 140 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, and 1.8 mM KH_2PO_4 (pH 7.3). The crude enzyme solution was prepared by sonication (Sonifier 450, Branson, Danbury, CT, USA; output 4, 6 times for 10 s, constant duty), followed by centrifugation at 12,000 rpm for 10 min at 4 °C. The purified recombinant DGAS was obtained by affinity chromatography using a Glutathione-Sepharose™ High Performance affinity column as suggested by the manufacturer. The removal of fused GST protein from the purified recombinant DGAS was performed with thrombin treatment. The apparent homogeneity of recombinant DGAS was confirmed by SDS–PAGE analysis as previously mentioned [25] and protein concentration was determined with the Bradford reagents kit (Bio-Rad, Hercules, CA, USA) with bovine serum albumin as a standard. The specific activity of purified enzymes was 69 U/mg.

2.4. Determination of enzyme activity

In general, the activity assay of DGAS was performed with 40 mg/mL sucrose at 45 °C in 50 mM Tris–HCl (pH 8.0). One unit of DGAS activity was defined as the amount of enzyme that produces 1 μmol of fructose per minute in the reaction conditions [25]. Fructose concentration was determined by the dinitrosalicylic acid method using fructose as standard.

2.5. Transglycosylation reaction and purification of Ab-glucoside

An Ab transglycosylation product was obtained by incubating the reaction mixture (5 mL) containing 200 mg of sucrose, 250 mg of Ab, and 50 U of DGAS in 50 mM Tris–HCl buffer (pH 8) at 30 °C for 12 h. The reaction was stopped by boiling for 10 min, and freeze-dried for further use. The freeze-dried sample was dissolved in 2.5 mL of distilled water and purified by the preparative recycling HPLC system (LC-9104, JAI, Tokyo, Japan) equipped with refractive index detection. The transglycosylation reaction mixture (2.5 mL) was loaded onto a JAIGEL-W252 column (2 cm × 50 cm; Japan Analytical Industry Co., Ltd., Tokyo, Japan), a gel permeation chromatography column for water soluble and low molecular weight samples, which was connected to two JAIGEL-W251 columns (2 cm × 50 cm) and an RI detector RI-50 (JAI, Tokyo, Japan). Deionized water was used for the elution of the sample at a flow rate of 3 mL/min. The purification of the Ab transglycosylation product was analyzed by TLC. The purified products were lyophilized and used for NMR analysis.

2.6. Thin layer chromatography (TLC) and high performance liquid chromatography (HPLC) analyses

TLC and HPLC analyses were carried out to detect Ab and the Ab transglycosylation product. A TLC silica gel plate (Whatman K5F, Whatman, Kent, UK) was activated at 110 °C for 30 min. The samples on the TLC plate were separated by a solvent of *n*-butanol:acetic acid: water (3:1:1, v/v/v). After allowing the solvent to ascend twice, the TLC plate was dried in a hood and then developed by being soaked in 0.3% (w/v) N-(1-naphthyl)-ethylenediamine and 5% (v/v) H_2SO_4 in methanol. Finally, the spots in the TLC plate were visualized by placing the plate in a 110 °C oven for 10 min. The yield of the Ab transglycosylation product was calculated from the reduction of the area of the Ab peak in HPLC analysis. For the HPLC analysis, the reaction mixtures were filtered through a 0.45 μm filter and analyzed using a SUPELCOSIL™ LC-NH_2 column (5 μm, 25 cm × 4.6 mm;