Research article

Rare ginsenoside Ia synthesized from F1 by cloning and overexpression of the UDP-glycosyltransferase gene from Bacillus subtilis: synthesis, characterization, and in vitro melanogenesis inhibition activity in BL6B16 cells

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Background: Ginsenoside F1 has been described to possess skin-whitening effects on humans. We aimed to synthesize a new ginsenoside derivative from F1 and investigate its cytotoxicity and melanogenesis inhibitory activity in B16BL6 cells using recombinant glycosyltransferase enzyme. Glycosylation has the advantage of synthesizing rare chemical compounds from common compounds with great ease.

Methods: UDP-glycosyltransferase (BSGT1) gene from Bacillus subtilis was selected for cloning. The recombinant glycosyltransferase enzyme was purified, characterized, and utilized to enzymatically transform F1 into its derivative. The new product was characterized by NMR techniques and evaluated by MTT, melanin count, and tyrosinase inhibition assay.

Results: The new derivative was identified as (20S)-3β,6α,12β,20-tetrahydroxydammar-24-ene-20-O-D-glucopyranosyl-3-O-b-D-glucopyranoside (ginsenoside Ia), which possesses an additional glucose linked into the C-3 position of substrate F1. Ia had been previously reported; however, no in vitro biological activity was further examined. This study focused on the mass production of arduous ginsenoside Ia from accessible F1 and its inhibitory effect of melanogenesis in B16BL6 cells. Ia showed greater inhibition of melanin and tyrosinase at 100 μmol/L than F1 and arbutin. These results suggested that Ia decreased cellular melanin synthesis in B16BL6 cells through downregulation of tyrosinase activity.

Conclusion: To our knowledge, this is the first study to report on the mass production of rare ginsenoside Ia from F1 using recombinant UDP-glycosyltransferase isolated from B. subtilis and its superior melanogenesis inhibitory activity in B16BL6 cells as compared to its precursor. In brief, ginsenoside Ia can be applied for further study in cosmetics.

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1. Introduction

Glycosyltransferases (GTs; EC 2.4.x.y) consist of a ubiquitous family of enzymes that can catalyze glycosylation on a wide range of acceptor molecules [1,2], which is paramount for the biosynthesis of oligosaccharides, polysaccharides, and glycoconjugates [3]. GTs are important in the development of complex natural glycosylated products. Based on their sequence, signature motif, stereochemistry of the glycoside linkage, and known target specificity, GTs are classified into 78 families [4]. In addition, GT Family 1 contains UDP-glucosyltransferases (UGTs), which have been found in bacteria, plants, and animals. UGTs can use UDP-activated sugar moieties such as UDP-glucose as a donor and some small molecules, such as flavonoids, antibiotics, and plant hormones, as sugar acceptors [5]. The Bacillus cereus genome was completely sequenced and found to contain four UDP-glycosyltransferases [6]. One of these, BcGT-1, was reported to carry out reactions on flavonoids [5]. However, there are few reports
on the glycosylation of ginsenoside by UDP-glycosyltransferases [7]. Glycosylation plays a key biological role in many synthesis processes of natural saponins [5]. GTs are the most common enzymes used in glycosylation, and UDP-glucose is the necessary and specific sugar for the conversion of saponins.

Ginseng, the root of Panax ginseng Meyer, is a well-known traditional medicine widely used in China, Korea, Japan, and other Asian countries [8]. Among many other report pharmacological efficacies, ginseng has been used for more than 2,000 years to strengthen immunity [9,10] and reduce fatigue. Ginsenosides, the principal components in ginseng, have been documented to possess various pharmacological activities, such as immune-modulatory [11], anti-inflammatory [12], antitumor [13], anti-diabetic [14], and anti-aging effects [15]. More than 128 ginsenosides have been isolated from ginseng roots and are classified into three groups, namely protopanaxadiol (PPD), and protopanaxatriol, and oleanane [16]. Amidst the numerous ginsenosides, ginsenoside F1 is a minor ginsenoside with glucose in the 20-C position of protopanaxatriol aglycone. It is present naturally at low concentrations as a minor ginsenoside with glucose in the 20-C position of protopanaxatriol aglycone. It is present naturally at low concentrations and is a minor ginsenoside with glucose in the 20-C position of protopanaxatriol aglycone. It is present naturally at low concentrations and is a minor ginsenoside with glucose in the 20-C position of protopanaxatriol aglycone.

Glycosylation ability was assayed with overexpressed BSGT1 with UDP-glucose; and (3) control 3 (C3) consisted of BSGT1 with UDP-glucose; and (3) control 3 (C3) consisted of BSGT1 with UDP-glucose; and (3) control 3 (C3) consisted of BSGT1 with UDP-glucose; and (3) control 3 (C3) consisted of BSGT1 with UDP-glucose.

2.2. Molecular cloning, expression, and purification of recombinant UDP-glycosyltransferase (BSGT1) gene from B. subtilis

Genomic DNA from B. subtilis was isolated using a genomic DNA extraction kit (GeneAll, Korea). The UDP-glycosyltransferase gene was amplified from genomic DNA using polymerase chain reaction (PCR) with Pfu DNA polymerase (GeneAll, Korea). The BSGT1 gene (GenBank accession number KU 500621) was amplified using the following primers (with NdeI and EcoRI restriction sites in boldface): BSGT1 F (5′-CCATATGGATTTACATGGTTGGTGAAA-3′) and BSGT1 R (5′-CCGATTAAGAAAGCCTGATAATACG-3′).

The amplified fragment was digested with NdeI and EcoRI and then inserted into a pMAL-c5X vector to generate a maltose-binding protein (MBP)-BSGT1 gene fusion using the EzFusion Cloning Kit (Enzymonics, Korea). The amplified gene was sequenced and confirmed at the Genotech facility (Daejeon, Korea). Escherichia coli BL21 (DE3) transformed with recombinant pMAL-BSGT1 were grown in Luria–Bertani medium with ampicillin at 37 °C to an optical density of 0.4 at 600 nm, and protein expression was subsequently induced through the addition of 0.3mM isopropyl-β-D-thio-galactopyranoside (IPTG). The bacteria were incubated for additional 9 h at 20 °C and harvested by centrifuging at 5,000g for 20 min at 4 °C. The cells were washed twice with 20mM sodium phosphate buffer (pH 7.0, 1mM EDTA, and 1mM NaCl) and then resuspended in 20mM sodium phosphate buffer (pH 7.0). The cells were sonicated on ice, and debris was removed by centrifugation at 12,000g at 4 °C for 20 min. The MBP-tagged fusion protein was purified in an amylose resin column. The supernatant was collected, and the homogeneity of the protein was assessed using 12% SDS-PAGE and Coomassie Blue staining. Initial biosynthesis experiments using ginsenoside F1 as the substrate revealed that the presence of MBP fused to BSGT1 did not affect the enzyme activity. Therefore, the MBP fusion protein was used to determine the specificity and selectivity of BSGT1 for the biosynthesis using UDPG as well as SDS-PAGE. The protein concentration was determined using bovine serum albumin as the standard according to the Bradford method.

2.3. Enzyme characterization

The Michaelis–Menten constant, Km (mol/L), and the kinetic parameters, Vmax (μmol/L·min), were measured by Michaelis–Menten plot using different substrate concentrations ranging from 0.5mM to 5mM UDP-glucose [29]. The values of Km and Vmax were calculated by the Lineweaver–Burk plot.

2.4. Biosynthesis of metabolites

Glycosylation ability was assayed with overexpressed BSGT1 enzyme and F1. The reaction mixtures contained 100 μL of 0.5mM F1 and 100 μL of 2.5mM UDP-glucose and 800 μL of purified enzyme (final concentration at 0.1 mg/mL) (pH 7.0). The mixtures were incubated at 30 °C for 24 h. Moreover, three groups of controls were incubated under the same conditions: (1) control 1 (C1) consisted of ginsenoside F1 with BSGT1; (2) control 2 (C2) consisted of BSGT1 with UDP-glucose; and (3) control 3 (C3) consisted of ginsenoside F1 with UDP-glucose.

2.5. Analysis of optimal conditions for purified enzyme activity according to pH, temperature, and metal ion concentrations

Initial biotransformation experiments were determined in 1 mL of reaction mixture using 0.5mM ginsenoside F1 as a substrate. The
activity of BSGT1 was assessed in the presence of 10mM of Co$^{2+}$, Mg$^{2+}$, Fe$^{3+}$, Na$^+$, Cu$^{2+}$, NH$_4^+$, K$^+$, Ca$^{2+}$, and Zn$^{2+}$ for 30 min at 30°C and subsequently compared with a control without metal ions. The effect of pH of enzyme activity was determined at 30°C using 0.5mM ginsenoside F1 as the substrate in 20mM glycine-HCl buffer (pH 3.0), citric acid-sodium citrate buffer (pH 4.0–5.0), sodium phosphate buffer (pH 6.0–7.0), Tris HCl buffer (pH 8.0–9.0), and glycine-NaOH buffer (pH 10.0) at optimum metal ion concentration. The effect of temperature on enzyme activity was determined at 20°C, 30°C, 37°C, 50°C, and 60°C at optimum pH and metal ion conditions for 24 h in the presence of 0.5mM ginsenoside F1. Samples were analyzed at regular intervals. An equal volume of water-saturated n-butanol was added to each sample to stop the reaction. The supernatant containing ginsenoside F1 and bio-transformed products was analyzed using TLC, HPLC, and HR/MS.

2.6. TLC analysis of metabolites

The extract was collected and evaporated to dryness at room temperature. TLC analysis was performed using Silica gel 60 plates with the developing solvent CHCl$_3$:CH$_3$OH:H$_2$O (65:35:10, v/v/v, temperature. TLC analysis was performed using Silica gel 60 plates.

2.7. HPLC, NMR, and MS analysis of metabolite 1

The reaction mixture was extracted in n-butanol saturated with H$_2$O and evaporated in a vacuum. HPLC was performed using an Agilent 1260 system (Agilent). HPLC-grade acetonitrile and water were purchased from SK Chemicals (Ulsan, Korea). Separation was performed on the C$_{18}$ column (250 × 4.6 mm, ID 2.6 μm) using acetonitrile (solvent A) and distilled water (solvent B) mobile phases at 85% B for 5 min, 79% B for 20 min, 42% B for 55 min, 10% B for 12 min, and 85% B for 18 min at a flow rate of 1.6 mL/min. The sample was detected using UV (203 nm) absorbance [30]. The structure was identified using $^1$H-NMR and $^{13}$C-NMR. $^1$H-NMR and $^{13}$C-NMR spectra were obtained by a Bruker Av 600NMR spectrometer at 100 MHz with CD$_3$OD as the solvent. MS analysis was performed on a Finnigan LCQ-Advantage mass spectrometer.

2.8. In vitro application in B16BL6 cell lines

2.8.1. Cell culture

B16BL6 cells were cultured in Dulbecco’s modified Eagles medium supplemented with 10% fetal bovine serum and 1% penicillin-streptomycin at 37°C in a humidified 95% air/5% CO$_2$ atmosphere, as described previously [31].

2.8.2. Cell viability assay

Cell viability was determined for ginsenoside F1 and metabolite 1 using MTT (Life Technologies) conversion to formazan [32]. Cells were seeded at a density of 1 × 10$^5$ cells/well in a 96-well plate (Corning Costar), cultured for 24 h, and treated with various concentrations from 1μM to 200μM of F1 and metabolite 1 for 5 d [19]. Finally, 10 μL of MTT [5 mg/mL in phosphate-buffered saline (PBS)] was added to each well. Cells were incubated at 37°C for 3 h, and then dimethyl sulfoxide (DMSO, 100 μL) was added to dissolve the formazan crystals. The absorbance was measured at 570 nm with the reference wavelength of 630 nm using an ELISA reader (Bio-Tek Instruments, USA).

2.8.3. Melanin content measurement

The melanin content of the cultured B16BL6 cells was measured, as described previously with slight modifications [33,34]. The cells were seeded at a density of 2 × 10$^5$ cells/well with α-MSH (1μM) in six-well plates. After 24 h, the cells were treated with 100μM F1, metabolite 1, or arbutin for 5 d. After the cells were washed with PBS, they were harvested by trypsinization. The cell pellet was solubilized in 500 μL of 1M NaOH containing 10% DMSO at 80°C for 1 h. The relative melanin content was determined by measuring the absorbance at 475 nm using an ELISA reader (Bio-Tek Instrument, USA). A standard curve for synthetic melanin (0–500 μg/mL) was prepared for each experiment. Melanin production was expressed as a percentage of untreated controls.

2.8.4. Tyrosinase activity assay

Tyrosinase activity was assayed indirectly via dopa oxidase activity [35,36]. The cells were cultured in six-well plates at a density of 4 × 10$^5$ cells/well. After 24 h, the cells were treated with 100μM of F1, metabolite 1, or arbutin for 5 d. Next, the medium was removed, the cells were washed with ice-cold PBS, and subsequently lysed with phosphate buffer (pH 6.9) containing 1% Triton X-100. The mixture was freeze-thawed by incubating at –80°C for 15 min and then room temperature for 10 min. The samples were clarified by centrifugation at 12,000g for 15 min. After quantification of protein levels using the Bradford method and adjusting concentrations with lysis buffer, 10 μL of prewarmed freshly prepared substrate (15mM L-DOPA in 48mM sodium phosphate buffer pH 7.1) was added to 50 μL of supernatant and incubated at 37°C for 1 h. The absorbance was then read at 475 nm using an ELISA reader (Bio-Tek Instrument, USA).

2.8.5. Statistical analysis

All data are presented as mean ± standard deviation (SD), and all experiments were independently performed in triplicate. The mean values of the treatment groups were compared with those of the untreated groups using Student t test. A p value of 0.05 or less was considered statistically significant.

3. Results and discussion

3.1. Heterologous expression of BSGT1 gene in E. coli BL21

The BSGT1 gene was successfully cloned from the B. subtilis genome and consisted of 1,179 bp encoding 393 amino acid
residues and was homologous to the protein domain of the GT family 1. The BSGT1 gene was inserted into a pMAL-C5X vector and then expressed in E. coli BL21 (DE3). Different conditions for protein induction were determined to maximize the yield of the fusion protein. Induction with 0.3mM IPTG at 20°C for 9 h produced the maximum level of soluble active fusion enzyme. The MBP-BSGT1 fusion protein was purified in MBP amylose resin, and then supernatant from cell lysates as well as purified protein was subjected to SDS-PAGE analysis. The molecular weight of the recombinant MBP-BSGT1 calculated via an amino acid sequence was 86.12 kDa, which was similar to the mass of 86.1 kDa detected by SDS-PAGE (Fig. 1).

3.2. Enzyme kinetic properties

The kinetics of biosynthesis of metabolite 1 by BSGT1 were determined using the Michaelis–Menten model. The purified enzyme was incubated in the presence of a nonradiolabeled substrate UDP-glucose at concentrations varying from 0.5mM to 5mM. To measure the kinetic values, the product was detected by HPLC. The efficiency of BSGT1 is shown in Fig. 3A. Km and Vmax values were determined from the Lineweaver–Burk plot and were estimated to be 0.015 mol/L and 0.073 µmol/L*min, respectively.

3.3. Biosynthesis of metabolites

Transglycosylation is the formation of a glycosidic bond by transferring a glycosyl molecule from the donor to the acceptor. In this study, we aimed to synthesize a new transglycosylation metabolite from UDP-glucose as a donor and ginsenoside F1 as an acceptor using recombinant UDP-glycosyltransferase BSGT1. The mixtures were extracted by n-butanol, and a series of new spots were detected in TLC (Fig. 2). The new spots were not present in the control mixtures, which included ginsenoside F1 and enzyme without UDP-glucose, UDP-glucose and enzyme without ginsenoside F1, and UDP-glucose and ginsenoside F1 without enzyme. Two new spots appeared in TLC; metabolites 1 and 2 were detected by HPLC analysis (Fig. 3B). However, due to the low concentration of metabolite 2, we focused on the study of metabolite 1.

3.4. Optimal conditions of BSGT1 enzymes for metabolite production

Based on the metal ion effect, the enzyme activity appeared to be significantly stimulated by Co²⁺, Mg²⁺, Fe³⁺, Na⁺, Cu²⁺, NH₄⁺, K⁺, Ca²⁺, and Zn²⁺. Furthermore, the optimum metal ions with regard to enzyme activity were strongly affected by Co²⁺ (Fig. 4A). The enzyme showed optimal activity at pH 7 in 20mM sodium phosphate buffer and had more potent activity than those at other pH values (Fig. 4B). The optimum temperature range of BSGT1 was 20–37°C; however, the recombinant BSGT1 had the highest activity at 30°C (Fig. 4C).

3.5. Biotransformation pathway of ginsenoside F1 by BSGT1

For large-scale production, final concentrations of 0.5mM ginsenoside F1, 2.5mM UDP-glucose, and 10mM Co²⁺ with BSGT1 enzyme (pH 7.0) were combined in 25 mL and reacted for 24 h at 30°C in a shaking incubator. The resulting metabolite 1 was purified by open silica column chromatography with a solvent system of CHCl₃:CH₃OH:H₂O (65:35:10, v/v/v, lower phase); further purification was performed by HPLC analysis.
separation and purification were achieved by repeated prep HPLC using a Nova-pak C18 column for further characterization [37]. The yield of metabolite 1 was 40.6%. Metabolite 1, a white amorphous powder, had the molecular formula C_{42}H_{72}O_{14} as deduced by a molecular ion peak at m/z 987.5532 ([M-H]^-, calculated as C_{42}H_{71}O_{14} 799.4844) (Fig. 5) in the negative HRFABMS and ^{13}C-NMR (DEPT) spectra. The IR spectrum of metabolite 1 showed absorption bands at 3401 cm\(^{-1}\) and 1649 cm\(^{-1}\) due to an OH group and double bond, respectively. The ^{1}H-NMR and ^{13}C-NMR data were similar to those of ginsenoside F1 with the exception of an additional hexose sugar. The ^{13}C-NMR signals of the sugar were observed as a hemiacetal (\(\delta_{C} 107.1\)), four oxygenated methines (\(\delta_{C} 78.2, 78.1, 76.4, 74.0\)), and an oxygenated methylene (\(\delta_{C} 62.9\)), indicating the sugar to be \(\beta\)-glucopyranose. The \(\beta\)-configuration of the anomer hydroxyl group was confirmed from the coupling constant of the anomer proton signal (\(\delta_{H} 4.31, d, J = 7.8 \text{ Hz}\)). The sugar was linked to the C-3 hydroxyl group as observed from the chemical shift of C-3 (\(\delta_{C} 90.8\)) downfield by 11.3 ppm compared to that of ginsenoside F1 owing to the glycosylation. Also, the positions of the two sugars were confirmed to be C-3 and C-20 from the HMBC spectrum. Two anomer proton signals H-1' and H-1" (\(\delta_{H} 4.61, d, J = 7.8 \text{ Hz}\)) showed cross peaks with the oxygenated methine carbon signal (\(\delta_{C} 90.8, \text{C-3}\)) and the oxygenated quaternary carbon signal (\(\delta_{C} 85.0, \text{C-20}\)), respectively. Taken together, these results indicate that metabolite 1 is (20\(S\))-3\(\beta\),6\(\alpha\),12\(\beta\),20-tetrahydroxydammar-24-ene-20-O-\(\beta\)-D-glucopyranosyl-3-O-\(\beta\)-D-glucopyranoside (Fig. 6), which is ginsenoside Ia [Supplementary Table 1] [28].

Previously, ginsenoside Ia had been reported to be isolated from the leaves of \(P. \) ginseng Meyer [28]. In this work, we were successful to bioconvert ginsenoside F1 to rare ginsenoside Ia by means of recombinant UDP-glycosyltransferase isolated from \(B. \) subtillis.

Fig. 4. (A) Effect of metal ions on recombinant BSGT1 activity in the synthesis of ginsenoside Ia. (B) Effect of pH on recombinant BSGT1 activity in the synthesis of ginsenoside Ia. (C) Effect of temperature on the recombinant BSGT1 activity in the synthesis of ginsenoside Ia.

Fig. 5. Mass spectrum of ginsenoside Ia after transformation by recombinant BSGT1.
clustering of B16 cells and the intracellular signaling cascade [19]. However, considering the poor solubility of ginsenoside and the absence of pharmacological activity of ginsenoside Ia in literatures, the glycosylation product Ia was expected to be applied as a novel skin cancer drug. Additionally, we aimed to conduct further in vitro study of ginsenoside Ia on B16BL6 skin cell line.

3.6. In vitro cytotoxicity assay of ginsenoside Ia on B16BL6 skin cells

To evaluate the cytotoxic effects of ginsenoside Ia on melanoma cells, we performed the assay for over 5 d. The cell viability assay results are shown in Fig. 7A; the cell viabilities were greater than 90% even at the highest concentration of ginsenoside Ia (200μM). We compared these results to the MTT results for ginsenoside F1, which showed 68% cell viability at 200μM.

3.7. Inhibition effect of ginsenoside Ia on melanin and tyrosinase activity on B16BL6 skin cells

Melanin is chiefly responsible for the dark color of skin by UV irradiation [38]. Furthermore, the tyrosinase is the key enzyme for the formation of melanin. Thus, the inhibition of content of melanin and tyrosinase is critical for the reduction of skin darkness [39]. Previously Yoo et al [19] reported the whitening effect of
ginsenoside F1 on B16 cells [19]. Based on the chemical similarity between ginsenoside F1 and ginsenoside Ia, we assay the effect of ginsenoside Ia on melanin synthesis and tyrosinase regulation in B16BL6 cells after stimulation with α-MSH. Fig. 7B shows that the treatment with α-MSH increases the melanin production in B16 cells, and this induction could be inhibited by ginsenoside Ia. Cellular melanin content was significantly decreased to 56 ± 0.02% at 100 μM after treatment with ginsenoside Ia. However, cells treated with equal concentrations of arbutin and ginsenoside F1 showed that the melanin content decreased to 96 ± 0.02% and 65 ± 0.02%, respectively. Ginsenoside Ia decreased cellular tyrosinase activity in B16 cells (Fig. 7C); this result is in agreement with the decrease of melanin content. Tyrosinase enzyme is a key regulator in melanin production in cells. Ginsenoside Ia resulted in a greater decrease in the cellular tyrosinase activity in α-MSH-stimulated B16BL6 cells than F1, similar to that observed with an equal concentration of arbutin.

Overall, ginsenoside Ia showed better efficacy than F1 and arbutin in preventing pigmentation, and it can be applied as a newer and more potent skin-whitening agent in cosmetics.

4. Conclusion

In this study, the glycosyltransferase BSGT1 gene from B. subtilis was cloned and overexpressed in E. coli BL 21 (DE3). The recombinant enzyme was purified and characterized. The molecular weight of the purified MBP-BSGT1 was 86.1 kDa as determined by SDS-PAGE. Ginsenoside F1 was transformed into (20R)-3β,6α,12β,20-tetrahydroxydammar-24-ene-20-O-β-D-glucopyranosyl-3-O-β-D-glucopyranoside (ginsenoside Ia) by GT with high efficiency and specificity to the C-3 position of F1. Therefore, the enzyme is considered potentially useful for mass production of arduous ginsenoside Ia from F1.

Skin-fin compounds suppress melanogenesis in B16BL6 melanoma cells through cytotoxic effects on melanocytes, direct tyrosinase inhibition, and melanin biosynthesis inhibition. In the present study, we investigated the inhibitory effect of enzymatically-synthesized ginsenoside Ia on melanogenesis in B16BL6 cells. Ginsenoside Ia had no significant cytotoxicity at 0–200 μM. Furthermore, ginsenoside Ia significantly suppressed melanin synthesis and tyrosinase activity at 100 μM, which demonstrated greater efficacy than ginsenoside F1 and arbutin at the same concentration. These results suggest that ginsenoside Ia decreases cellular melanin synthesis in B16BL6 cells through downregulation of tyrosinase activity.

In conclusion, we have identified a potential antimelanogenic ginsenoside Ia which decreases melanin production without reducing B16BL6 melanin cell viability. However, further study is needed to determine the molecular mechanism of melanin content reduction by ginsenoside Ia in B16BL6 cells.

Conflicts of interest

The authors have no conflicts of interest to report.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.jgr.2016.12.009.

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