

One-Pot Synthesis of Ginsenoside Rh2 and Bioactive Unnatural Ginsenoside by Coupling Promiscuous Glycosyltransferase from *Bacillus subtilis* 168 to Sucrose Synthase

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S Supporting Information

ABSTRACT: Ginsenosides, the major effective ingredients of *Panax ginseng*, exhibit various biological properties. UDP-glycosyltransferase (UGT)-mediated glycosylation is the last biosynthetic step of ginsenosides and contributes to their immense structural and functional diversity. In this study, UGT Bs-YjC from *Bacillus subtilis* 168 was demonstrated to transfer a glucosyl moiety to the free C3-OH and C12-OH of protopanaxadiol (PPD) and PPD-type ginsenosides to synthesize natural and unnatural ginsenosides. In vitro assays showed that unnatural ginsenoside F12 (3-O- β -D-glucopyranosyl-12-O- β -D-glucopyranosyl-20(S)-protopanaxadiol) exhibited remarkable activity against diverse human cancer cell lines. A one-pot reaction by coupling Bs-YjC to sucrose synthase (SuSy) was performed to regenerate UDP-glucose from sucrose and UDP. With PPD as the aglycon, an unprecedented high yield of ginsenosides F12 (3.98 g L⁻¹) and Rh2 (0.20 g L⁻¹) was obtained by optimizing the conversion conditions. This study provides an efficient approach for the biosynthesis of ginsenosides using a UGT-SuSy cascade reaction.

KEYWORDS: microbial UDP-glycosyltransferase, glycosylation, protopanaxadiol, unnatural ginsenosides, sucrose synthase, UDP-glucose recycle

INTRODUCTION

Ginseng, the roots and rhizomes of *Panax ginseng* C. A Meyer, is a well-known and highly valuable medicinal and dietary plant.¹ This perennial herb has been used to enhance immunity, reduce fatigue, prevent aging, and provide nutrition in eastern Asia for more than 2000 years.^{2–4} Ginsenosides are the major pharmacologically active components of ginseng.⁵ These natural products form a group of glycosylated triterpene saponins that exhibit various pharmacological effects, such as anticancer, antitumor, antistress, antiaging, anti-inflammatory, and immune-system-enhancing activities.^{6–9}

Protopanaxadiol (PPD)-type ginsenosides represent one of the two major groups of ginsenosides with a structure consisting of a PPD skeleton and one or more sugar moieties attached to the C3-OH and/or C20-OH of PPD by β -glycosidic linkage.¹⁰ Glycosylation mediated by UDP-glycosyltransferase (UGT), the last biosynthetic step of PPD-type ginsenosides, contributes to their immense structural and functional diversity. Most of the key genes involved in the biosynthetic pathway of PPD-type ginsenosides have currently been functionally characterized; an engineered PPD-producing yeast that can accumulate high amounts of PPD has been constructed in our previous study.^{11–13} Diverse natural and unnatural PPD-type ginsenosides, including ginsenosides CK,

Rh2, F2, Rg3, Rd, and 3-O- β -D-glucopyranosyl-12-O- β -D-glucopyranosyl-20(S)-protopanaxadiol, have been biosynthesized through the introduction of an engineered PPD-producing pathway and ginseng or microbial UGTs into yeast cell factories;^{10,14–17} however, the titers of PPD-type ginsenosides (ca. 9–300 mg L⁻¹) produced by yeast cell factories are still considerably low due to the poor catalytic ability of UGTs, the synthetic ability of yeast cells, and the inhibitory effects of the synthesized ginsenosides.

The chemical approach for glycosylation of PPD to synthesize PPD-type ginsenosides is fairly complicated by disadvantages, such as poor stereospecificity, side reactions, low efficiency, and environmental pollution.¹⁸ Although these disadvantages can be alleviated by in vitro enzymatic glycosylation using effective UGTs, UGT-catalyzed glycosylation of PPD requires the costly UDP-glucose (UDPG) as a glucosyl donor, thereby restricting the practical applications of UGTs.¹⁹ Sucrose synthase (SuSy) is a versatile biocatalyst that can efficiently convert cheap sucrose and UDP into UDPG and fructose.^{20,21} Furthermore, the coupling of SuSy and UGT in a

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one-pot reaction can create a UDP recycle, which could instantly regenerate UDPG in the presence of a catalytic amount of UDP, thereby making sucrose the expedient glycosyl donor for the glycosylation of natural products.^{22,23}

Compared with plant UGTs, some naturally occurring microbial UGTs show high aglycon promiscuity, poor regiospecificity, and high catalytic proficiency.^{24–28} Additionally, microbe-derived UGTs are easier to highly express in the engineered bacteria than plant UGTs.²⁹ Thus, microbial UGTs are promising biocatalysts for the in vitro semibiosynthesis of ginsenosides or even novel bioactive ginsenosides. Bs-YjiC from *Bacillus subtilis* 168 is a promiscuous UGT toward a considerable number of structurally diverse compounds.³⁰ Bs-YjiC can also transfer a glucosyl moiety to the free C3-OH, C6-OH, and C12-OH of protopanaxatriol (PPT) to synthesize natural and unnatural PPT-type ginsenosides.³¹ In the present study, the regio- and stereospecificity of Bs-YjiC toward PPD and PPD-type ginsenosides was elucidated by analyzing the glycosylated products using high-performance liquid chromatography–electrospray ionization mass spectrometry (HPLC-ESI-MS) and NMR spectra. Furthermore, a one-pot reaction by coupling Bs-YjiC to SuSy from *Arabidopsis thaliana* (AtSuSy) was established and applied to synthesize PPD-type ginsenosides by using PPD and sucrose as the aglycon and sugar donor, respectively.

MATERIALS AND METHODS

Strains and Chemicals. Authentic PPD and PPD-type ginsenosides were purchased from Chengdu Biopurify Phytochemicals Ltd. (Chengdu, Sichuan, China) and dissolved in dimethyl sulfoxide (DMSO) for use. UDP-glucose (UDPG), UDP-N-acetylglucosamine (UDP-GlcNAc), UDP-galactose (UDP-Gal), and UDP-glucuronic acid (UDP-GlcA) were obtained from Sigma-Aldrich (St. Louis, MO, USA). *Escherichia coli* strains were incubated at 37 °C in Luria–Bertani (LB) medium (5 g L⁻¹ yeast extract, 10 g L⁻¹ NaCl, and 10 g L⁻¹ tryptone) with 100 mg L⁻¹ ampicillin or 50 mg L⁻¹ kanamycin.

Preparation of Recombinant Enzymes. The coding regions of Bs-YjiC (NP_389104) and AtSuSy (NM_001036838) were amplified from the genomic DNA of *B. subtilis* 168 and cDNA of *A. thaliana*, respectively. Bs-YjiC and AtSuSy were inserted into the *Bam*HI and *Sal*I sites of vector pET28a and *Nco*I and *Xho*I sites of vector pET32a to construct the plasmids pET28a-Bs-YjiC and pET32a-AtSuSy, respectively. For the preparation of recombinant proteins, pET28a-Bs-YjiC and pET32a-AtSuSy were transformed into *E. coli* BL21 (DE3). The recombinant *E. coli* BL21 (DE3) strains were cultured at 37 °C and 200 rpm in an LB medium until the absorbance (OD₆₀₀) reached 0.6–0.8. Isopropyl- β -D-thiogalactopyranoside was added to the medium to a final concentration of 0.2 mM, and the recombinant *E. coli* BL21 (DE3) strains were further incubated at 16 °C for approximately 16–18 h.

Recombinant *E. coli* BL21 (DE3) strains were harvested by centrifugation, resuspended in a lysis buffer (25 mM imidazole, 50 mM Tris-HCl, pH 7.5, and 500 mM NaCl), and disrupted using a French press. The cell extracts were centrifuged at 20000g for 1 h. The supernatants containing the target proteins were loaded onto a nickel–nitrilotriacetic acid–agarose column connected to an AKTA Purifier system (GE Healthcare, Piscataway, NJ, USA) and purified using a 25–200 mM imidazole gradient.

Enzyme Activity Assays. Enzymatic assays (0.3 mL) of Bs-YjiC toward PPD or PPD-type ginsenosides were conducted with 1 mM PPD or PPD-type ginsenosides, 5 mM UDPG, UDP-GlcNAc, UDP-Gal, or UDP-GlcA, 50 mM Tris-HCl (pH 8.0), 10 mM MgCl₂, and 5 μ g of purified Bs-YjiC for 0.5 h at 40 °C. The reactions were terminated by adding equal volumes of methanol. The reactants were subsequently centrifuged at 10000g for 5 min, filtered through a 0.22 μ m filter, and directly analyzed by HPLC or HPLC-ESI-MS. A single

unit of enzyme activity was defined as the amount of Bs-YjiC that glycosylated 1 μ mol of PPD per minute.

Enzymatic assays of AtSuSy were conducted in a 0.3 mL volume containing 0.5 mM UDP, 300 mM sucrose, 50 mM Tris-HCl (pH 7.5), and various amounts of purified AtSuSy for 30 min at 40 °C. AtSuSy activity was measured by determining the amount of fructose released from sucrose and analyzed using the bincinchonic acid method.³¹ A single unit of enzymatic activity was defined as the amount of enzyme that released 1 μ mol of fructose from sucrose per min.

Kinetic Analysis of Bs-YjiC. Kinetic studies (300 μ L) of Bs-YjiC toward PPD and ginsenoside Rh2 were conducted with purified Bs-YjiC (2 μ g for PPD and 0.5 μ g for ginsenoside Rh2), 50 mM Tris-HCl (pH 8.0), 10 mM MgCl₂, 10 mM UDPG, and varying concentrations of PPD or ginsenoside Rh2 (50–800 μ M). The reactions were incubated at 40 °C for 20 min and terminated by adding an equal volume of methanol. All the subsequent steps were performed as described above. The kinetic parameters were calculated by nonlinear regression analysis using GraphPad Prism 5.0 software. The k_{cat} values were calculated using the predicted molecular mass of 4.5×10^4 g mol⁻¹ for Bs-YjiC.

Optimizing the Conditions for the Bs-YjiC/AtSuSy Cascade Reaction. Standard Bs-YjiC/AtSuSy cascade reactions containing 1 mM PPD, 0.5 mM UDP, 50 mM Tris-HCl (pH 7.5), 500 mM sucrose, 10% DMSO (v/v), 160 mU mL⁻¹ Bs-YjiC, and 200 mU mL⁻¹ AtSuSy were incubated at 35 °C for 30 min. The optimal pH was determined using 50 mM Na₂HPO₄–NaH₂PO₄ (pH 6.5–7.5) and 50 mM Tris-HCl buffer (pH 7.5–8.5). The optimal temperature was determined ranging from 25 to 45 °C. The optimal concentrations of DMSO, UDP, sucrose, and PPD were individually obtained using different concentrations of DMSO (0–20%), UDP (0.05–0.3 mM), sucrose (0.2–1 M), and PPD (0.5–2 mM) in the standard assay. The optimal ratio of Bs-YjiC and AtSuSy was determined by adding various ratios of Bs-YjiC and AtSuSy in the standard assay.

Fed-Batch Synthesis of Ginsenosides Rh2 and F12 by the Bs-YjiC/AtSuSy Cascade Reaction. The reaction mixtures (10 mL) contained 1 mM PPD, 0.2 mM UDP, 50 mM Tris-HCl (pH 8.0), 0.4 M sucrose, 5% DMSO, 160 mU mL⁻¹ Bs-YjiC, and 200 mU mL⁻¹ AtSuSy. The reaction was performed at 40 °C and 150 rpm. In addition, 50 μ L of reactant was collected and quenched by adding an equal volume of methanol. After centrifugation at 12000g for 10 min, the supernatant was filtered through 0.22 μ m filters and analyzed by HPLC. A 50 μ L amount of PPD (200 mM) was periodically added to the reaction mixtures at 1, 2, 4, 6, and 8 h. Fresh enzymes (160 mU mL⁻¹ Bs-YjiC and 200 mU mL⁻¹ AtSuSy) were added at 6 h.

HPLC and HPLC-ESI-MS Analysis of the Glycosylated Products. HPLC and HPLC-ESI-MS analyses of the reactants were conducted according to our previous study with some minor changes.³² The reactants (20 μ L) were subjected to an XB-C18 column (4.6 $\times$ 250 mm, 5 μ m particles, Welch, Shanghai, China) with a 1 mL/min flow rate and 203 nm UV wavelength. The column was eluted with solvents A (water with 0.1% formic acid) and B (acetonitrile with 0.1% formic acid) using a gradient program of 25–85% B in 0–25 min and 85% B in 25–45 min. The electrospray ionization (ESI) probe was operated in a positive ion mode.

Structural Analysis of the Glycosylated Products. For the structural analysis of ginsenosides Rh2 and F12, the Bs-YjiC/AtSuSy cascade reaction (200 mL) was prepared as described above. The reaction was terminated by adding an equal volume of methanol. The reaction mixtures were subsequently concentrated under reduced pressure distillation and resuspended in chromatographic methanol (15 mL). After centrifugation at 10000g for 10 min and filtration through a 0.22 μ m filter, the glycosylated products were purified using an Agilent 1200 preparative HPLC system coupled with a reverse-phase Ultimate C18 column (21.2 $\times$ 250 mm, 5 μ m particles, Welch, Shanghai, China). The purified products were dissolved in DMSO-*d*₆ or methanol-*d*₄. 1D NMR (¹H NMR and ¹³C NMR) and 2D NMR spectra (heteronuclear multiple-bond correlation spectroscopy [HMBC], heteronuclear singular quantum correlation [HSQC], and

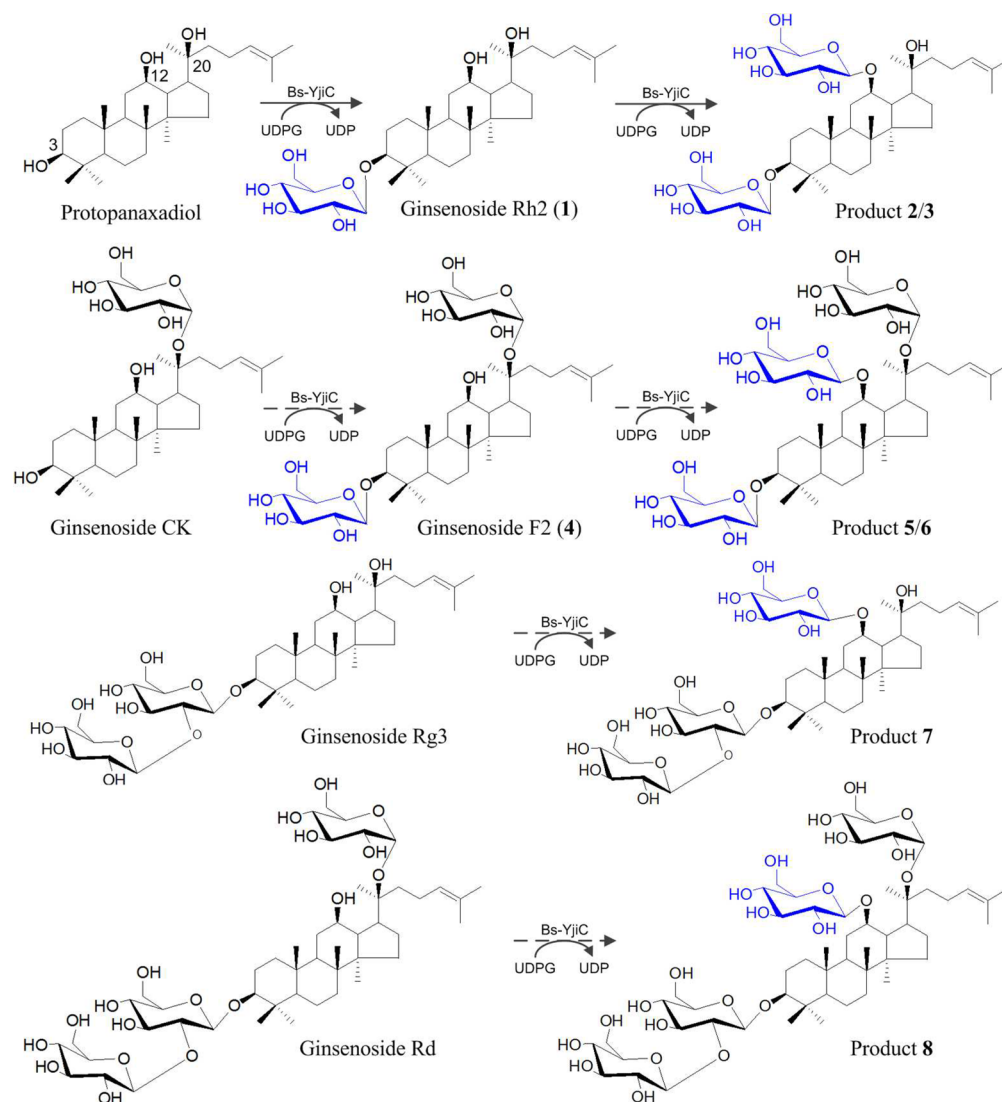


Figure 1. Proposed glycosylation patterns of Bs-YjiC toward PPD and PPD-type ginsenosides. The solid arrows indicated that the synthesized ginsenosides were confirmed by NMR spectra; the dotted arrows signified proposed glycosylation steps.

homonuclear correlation spectroscopy [COSY]) were obtained using a Bruker DMX-600 NMR spectrometer.

Cell Viability Assays. Cell viability assays of unnatural ginsenoside F12 were determined by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) colorimetric assay with ginsenosides Rh2 and Rg3 as the positive controls.³³ Cancer cells were seeded at a density of 1.0×10^4 cells mL^{-1} into a 96-well plate in triplicate. After incubation for 24 h, the cells were treated with different concentrations of ginsenoside Rh2, F12, or Rg3 for 72 h. After incubation, 15 μL of MTT solution (5 mg mL^{-1}) was added to each well, and the cells were incubated for another 4 h. The growth medium was removed, and 150 μL of DMSO was added. The absorbance value of the resulting reaction solution was measured spectrophotometrically at 570 nm using an ELISA reader.

RESULTS AND DISCUSSION

Glycosylation of PPD with Bs-YjiC. Bs-YjiC was heterologously expressed in *E. coli* BL21 (DE3) as an N-terminal His₆-tagged protein. Notably, Bs-YjiC was expressed mainly as a soluble protein within 16–37 °C (Figure S1). The expression level of Bs-YjiC was approximately 600 mg L^{-1} culture medium, which was much higher than those of ginseng

UGTs involved in the biosynthesis of ginsenosides from previous studies.^{15,16}

PPD is the common triterpene skeleton of PPD-type ginsenosides (Figure 1). Therefore, PPD was selected as a probe for the in vitro glycosylation of PPD-type ginsenosides with UDPG as the sugar donor. Two new products (1 and 2) from the Bs-YjiC-catalyzed reaction were identified by HPLC, whereas no new products were detected in the control reaction using the total lysate from induced *E. coli* BL21 (DE3) that expressed an empty vector (Figure 2A). Further mass analysis of product 1 ($[\text{M} + \text{H}]^+ m/z \sim 623.4497^+$, $[\text{M} + \text{H} - \text{H}_2\text{O}]^+ m/z \sim 605.4417^+$, and $[\text{M} + \text{H} - 2\text{H}_2\text{O}]^+ m/z \sim 587.4311^+$) and product 2 ($[\text{M} + \text{H}]^+ m/z \sim 785.5033^+$ and $[\text{M} + \text{H} - \text{H}_2\text{O}]^+ m/z \sim 767.4947^+$) confirmed that these products were monoglucoside and diglucoside derivatives of PPD ($\text{C}_{30}\text{H}_{52}\text{O}_3$, calculated molecular weight, $[\text{M} + \text{H}]^+ m/z \sim 461.3989$) (Figure 2B). Microbial UGTs are usually more flexible than plant-derived UGTs toward both the sugar donors and aglycon acceptors.³⁴ Thus, various reactions by incubating PPD with UDP-Gal, UDP-GlcA, and UDP-GlcNAc were also performed; however, no new products were obtained (data not shown). Thus, Bs-YjiC can distinguish the sugar moiety of UDP-sugars.

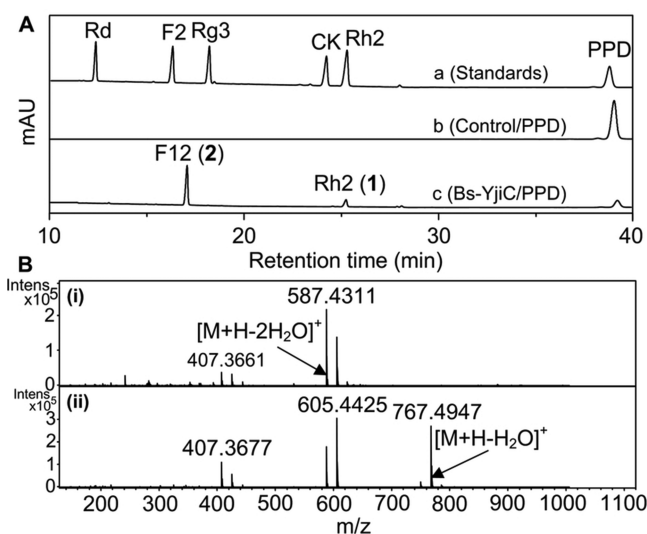


Figure 2. HPLC-ESI-MS analysis of the glycosylated products of PPD. (A) HPLC chromatograms of ginsenoside standards and Bs-YjiC-catalyzed reactant. (B) MS spectra for products 1 (i) and 2 (ii).

Products **1** and **2** were purified using preparative HPLC. Their structures were confirmed by 1D NMR (¹H NMR and ¹³C NMR) and 2D NMR (HMBC, HSQC, and COSY) spectrometry (Supporting Information, Figures S2–S11). The ¹H and ¹³C NMR spectra of product **1** were consistent with those of Rh2 (Table S1). The observation of significant downfield ¹³C shift (~8 ppm, “glycosylation shift”) of the C3 carbon confirmed that a glucosyl moiety was attached to the C3-OH of PPD.^{7,15} Furthermore, detailed HMBC correlations among the C3 of PPD and the anomeric proton of the glucosyl moiety, as well as the large anomeric proton-coupling constant (δ_H 4.14, J = 7.7 Hz), indicated that product **1** was the 3-*O*- β -glucoside, thereby confirming an inverting mechanism of Bs-YjiC. The ¹H and ¹³C NMR spectra of product **2** were highly similar to those of product **1** (Table S2). A notably significant downfield ¹³C-shift (“glycosylation shift”) at δ 90.86 (~12 ppm, C3) and δ 79.66 (~9 ppm, C12) indicated that a glucosyl moiety was attached to the C3-OH and C12-OH of PPD, respectively. In the HMBC, long-range correlations between C3 of the PPD backbone and C1' and C12 of the PPD backbone and C1" further confirmed that product **2** was 3,12-*O*- β -diglucoside of PPD (an unnatural ginsenoside named F12 in this study). The coupling constants (J = 8.0 Hz) of the anomeric protons indicated that both of the glucosyl moieties of ginsenoside F12 adopted the β -configuration. Thus, Bs-YjiC can glycosylate both the C3-OH and C12-OH of PPD and PPD-type ginsenosides with regio- and stereospecificity. Furthermore, the absence of ginsenoside CK or F2 in Bs-YjiC-catalyzed reactants using PPD as an acceptor indicated that Bs-YjiC could not glycosylate the free C20-OH of PPD or ginsenoside Rh2 (Figure 2). Only ginsenosides Rh2 and F12 were detected; however, 12-*O*- β -D-glucopyranosyl-20(*S*)-protopanaxadiol was not observed in the Bs-YjiC-catalyzed reaction mixture (Figure 2). These results indicated that ginsenoside F12 was a C12-glycosylation product of ginsenoside Rh2. UGT109A1 was recently isolated from *B. subtilis* CTCC 63501.¹⁴ The deduced amino acid sequences of UGT109A1 exhibited 94% identity with those of Bs-YjiC; however, UGT109A1 could glycosylate the free C3-OH and C12-OH of PPD to synthesize ginsenoside Rh2, 12-*O*- β -D-glucopyr-

anosyl-20(*S*)-protopanaxadiol, and ginsenoside F12. Thus, further studies should be performed to determine the key amino acids of Bs-YjiC involved in the regiospecific glycosylation of PPD and to the synthesize a specific ginsenoside (e.g., ginsenoside Rh2 or 12-*O*- β -D-glucopyranosyl-20(*S*)-protopanaxadiol) using an engineered Bs-YjiC.

The K_m values of Bs-YjiC for PPD and ginsenoside Rh2 were 163.00 and 139.50 μ M, respectively (Figure S12) (Table 1).

Table 1. Kinetic Parameters of Bs-YjiC toward PPD and Ginsenoside Rh2

substrate	K_m (μ M)	k_{cat} (s^{-1})	k_{cat}/K_m ($s^{-1} M^{-1}$)
PPD	163.00 $\pm$ 16.76	1.31 $\pm$ 0.04	0.34 $\times 10^4$
Rh2	139.50 $\pm$ 21.09	5.68 $\pm$ 0.27	4.07 $\times 10^4$

The K_m values of Bs-YjiC for PPD and ginsenoside Rh2 were compared with those of other UGTs involved in the biosynthesis of ginsenosides.^{10,14,15,17} Nevertheless, the k_{cat} values of Bs-YjiC for PPD (1.31 s^{-1}) and Rh2 (5.68 s^{-1}) were considerably high, thereby resulting in a higher catalytic efficiency (0.34 $\times 10^4 s^{-1} M^{-1}$ for PPD and 4.07 $\times 10^4 s^{-1} M^{-1}$ for ginsenoside Rh2) than those of PgUGT74AE2 from *P. ginseng* and engineered UGT51 from *S. cerevisiae*.^{15,17} The high catalytic efficiencies of Bs-YjiC toward PPD and ginsenoside Rh2 and its remarkable acceptor plasticity were in agreement with the previous notion that naturally occurring UGTs with high catalytic proficiencies generally show broad acceptor tolerance.^{30,35} In addition, the higher catalytic efficiency of Bs-YjiC toward ginsenoside Rh2 than toward PPD was consistent with the results in which ginsenoside F12 was the major product when PPD was used as the substrate, and F12 was synthesized via a continuous two-step glycosylation of PPD.

In Vitro Cytotoxicity of Ginsenoside F12. Glycosylation of C3-OH and/or C20-OH of PPD considerably influences the pharmacological properties of PPD-type ginsenosides.¹⁰ Ginsenoside F12 synthesized by UGT109A1 was recently found to have higher effectiveness against NCI-H460 lung cancer cells than that of ginsenoside Rg3.¹⁴ In the present study, the bioactivity of ginsenoside F12 was further evaluated using Lovo colon cancer cells, DMS53 lung cancer cells, HepG2 liver cancer cells, and SNU719 gastric cancer cells, which were compared with the most active ginsenosides, ginsenosides Rh2 and Rg3. Ginsenoside F12 can inhibit the proliferation of Lovo colon cancer cells, HepG2 liver cancer cells, DMS53 lung cancer cells, and SNU719 gastric cancer cells with IC₅₀ values of 49.2, 58.8, 55.9, and 44.8 μ M, respectively (Table 2). Although both ginsenosides Rg3 and F12 were diglucoside derivatives of PPD, the in vitro cytotoxicity of ginsenoside F12 against cancer cell lines was much higher than that of ginsenoside Rg3. These results indicated that the position of glucosyl moieties attached to PPD remarkably influenced the anticancer activities of PPD-type ginsenosides. Notably, ginsenoside F12 exhibited the highest cytotoxicity against DMS53 lung cancer cells, with an IC₅₀ of 55.9 μ M, whereas the IC₅₀ values of ginsenosides Rh2 and Rg3 against DMS53 lung cancer cells were 107.5 and >400 μ M, respectively. However, ginsenoside Rh2 was still the most active ginsenoside against Lovo colon cancer cells, HepG2 liver cancer cells, and SNU719 gastric cancer cells, with IC₅₀ values of 24.1, 23.3, and 12.0 μ M, respectively. The present study was consistent with previous findings that ginsenosides with a low

Table 2. Cancer Cell Line Cytotoxicity (μM)

compound	Lovo ^a	HepG2 ^b	DMS53 ^c	SNU719 ^d
Rg3	>400	364.1 $\pm$ 20.2	>400	359.4 $\pm$ 30.1
Rh2	24.1 $\pm$ 1.5	23.3 $\pm$ 1.8	107.5 $\pm$ 10.5	12.0 $\pm$ 0.6
F12	49.2 $\pm$ 4.4	58.8 $\pm$ 3.9	55.9 $\pm$ 3.2	44.8 $\pm$ 2.8

^aLovo, colon cancer cells. ^bHepG2, liver cancer cells. ^cDMS53, lung cancer cells. ^dSNU719, gastric carcinoma cells.

degree of glycosylation show stronger inhibitory activity against cancer cells than ginsenosides bearing more sugar moieties.³⁶

Glycosylation of PPD-Type Ginsenosides with Bs-YjiC.

We were interested in the glycosylation of other PPD-type ginsenosides using Bs-YjiC. Thus, the reactions of PPD-type ginsenosides, including ginsenosides Rh2, CK, F2, Rg3, and Rd, were carried out under conditions that were identical to those of PPD (Figure 3). With ginsenoside Rh2, only one diglucoside

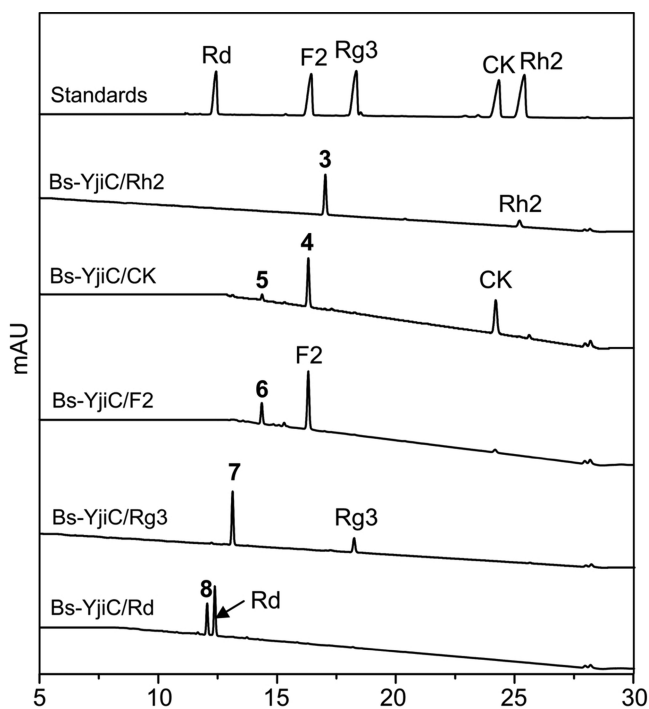


Figure 3. HPLC analysis of the glycosylated products of ginsenosides Rh2, CK, F2, Rg3, and Rd catalyzed by Bs-YjiC.

(product 3, $[M + H]^+ m/z^+ \sim 785.5042$) with the same retention time ($t_R = 17.1$ min) as that of F12 was produced, as confirmed by HPLC-ESI-MS (Figure S13). Product 3 should be ginsenoside F12 because Bs-YjiC catalyzed a continuous two-step glycosylation reaction of PPD (Figure 1). With ginsenoside CK containing the free C3-OH and C12-OH, one diglucoside (product 4, $t_R = 15.9$ min, $[M + H]^+ m/z^+ \sim 785.5017$) and one triglucoside (product 5, $t_R = 13.9$ min, $[M + H]^+ m/z^+ \sim 947.5689$) were confirmed by HPLC-ESI-MS (Figure S14). Considering that Bs-YjiC could only glycosylate the free C3-OH and C12-OH of PPD and the t_R of product 4 was identical with that of ginsenoside F2, we speculated that product 4 should be ginsenoside F2 (Figure 1). For ginsenoside F2, which only contained a free C12-OH, only one triglucoside (product 6, $t_R = 13.9$ min, $[M + H]^+ m/z^+ \sim 947.5655$) was detected (Figure S15). Notably, the t_R values of products 5 and 6 were identical to each other and were not consistent with those of Rd. Thus, we speculated that products 5 and 6 should

be the same derivative of F2 with an additional glucosyl moiety attached to the free C12-OH, namely, 3-*O*- β -D-glucopyranosyl-12-*O*- β -D-glucopyranosyl-20-*O*- β -D-glucopyranosyl-20(*S*)-protopanaxadiol (Figure 1). With ginsenosides Rg3 and Rd, which only contained a free C12-OH, only one new glucoside was detected in each reaction mixtures, as follows: product 7 ($t_R = 12.6$ min, $[M + H]^+ m/z^+ \sim 947.5639$) and product 8 ($t_R = 11.8$ min, $[M + H]^+ m/z^+ \sim 1109.6080$) (Figures S16 and S17). Based on the regiospecificity of Bs-YjiC toward PPD and the speculated glycosylation patterns of Bs-YjiC toward ginsenosides Rh2, CK, and F2, products 7 and 8 should be novel unnatural ginsenosides with an additional glucosyl moiety attached to the free C12-OH of ginsenosides Rg3 and Rd, respectively (Figure 1). Considering that glycosylation is the prominent biological mechanism for the structural and functional diversity of ginsenosides, the unnatural PPD-type ginsenosides 5 or 6, 7, 8, and 9 with a glucosyl moiety attached to the C12-OH might have various novel biological and pharmacological activities that are similar to those of ginsenoside F12 and other PPD-type ginsenosides.³⁶

Optimizing the Parameters for One-Pot Synthesis of Ginsenosides Rh2 and F12. An engineered yeast cell factory that can produce substantial amounts of PPD has been constructed in our previous study, which makes PPD a widely available precursor for the biosynthesis of PPD-type ginsenosides.¹¹ The high expression level and catalytic efficiency of Bs-YjiC toward PPD suggested that Bs-YjiC was a promising biocatalyst for industrial applications. Thus, the AtSuSy gene was amplified from the cDNA of *A. thaliana* and heterologously expressed in *E. coli* BL21 (DE3) as that of Bs-YjiC (Figure S1). A one-pot cascade reaction by coupling Bs-YjiC to AtSuSy was established to create an in situ UDPG regeneration and was applied to synthesize ginsenosides Rh2 and F12 with PPD and sucrose as the aglycon and sugar donor, respectively.

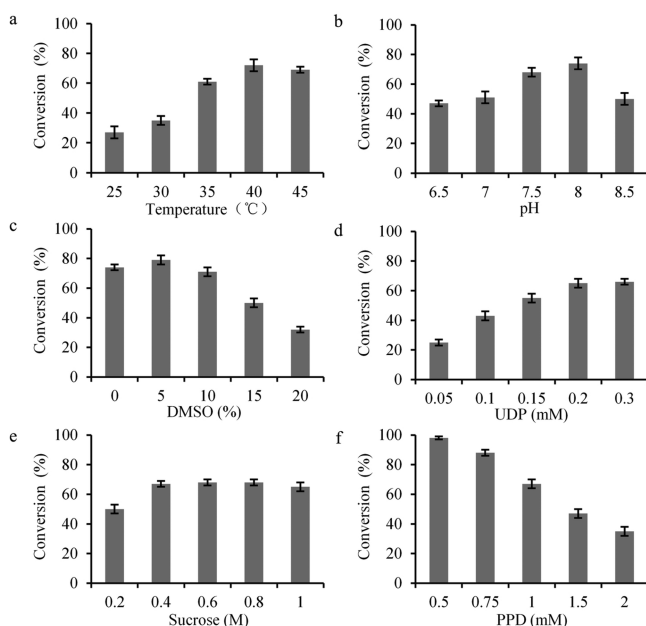
PPD could be transformed into ginsenosides Rh2 and F12 through a Bs-YjiC-AtSuSy cascade reaction as an enzymatic approach using UDPG as the sugar donor (Figure S18). This reaction revealed that AtSuSy could effectively synthesize UDPG. To determine the optimal reaction conditions, the effects of ratios of Bs-YjiC and AtSuSy on the one-pot synthesis of ginsenosides Rh2 and F12 were initially determined (Table 3). Ginsenoside F12 production increased by 237% when increasing the amount of Bs-YjiC from 40 mU mL⁻¹ to 160 mU mL⁻¹; however, only a trace amount of ginsenoside Rh2 (~ 0.04 mM) was detected, which was similar to the Bs-YjiC-catalyzed reaction using UDPG as the sugar donor. When the amount of AtSuSy was increased from 50 mU mL⁻¹ to 200 mU mL⁻¹, ginsenoside F12 production was increased by 373%, whereas ginsenoside Rh2 was decreased from 0.11 mM to 0.05 mM. The molar ratios of ginsenoside Rh2/ginsenoside F12 decreased from 0.58 to 0.08 when the amount of AtSuSy was increased from 50 mU mL⁻¹ to 200 mU mL⁻¹. Considering that AtSuSy was responsible for the formation of UDPG, the present study suggested that ginsenoside Rh2 was favorably produced in the presence of limited UDP-glucose concen-

Table 3. Optimization of the Enzyme Concentrations for the Bs-YjiC/AtSuSy Cascade Reaction

entry	Bs-YjiC (mU/mL)	AtSuSy (mU/mL)	F12 (mM)	Rh2 (mM)
1	40	200	0.27	0.04
2	80	200	0.41	0.04
3	160	200	0.64	0.05
4	320	200	0.72	0.05
5	160	50	0.19	0.11
6	160	100	0.36	0.12
7	160	200	0.63	0.05
8	160	300	0.71	0.04

trations and further reconfirmed that ginsenoside F12 was a C12-glycosylation product of ginsenoside Rh2. Ginsenoside F12 production increased slightly (~13%) when the amount of Bs-YjiC or AtSuSy was doubled. Thus, the optimal ratio of Bs-YjiC and AtSuSy was 160:200 mU mL⁻¹.

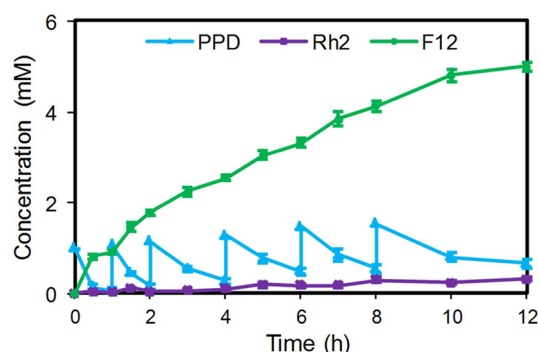
The conversion rates of PPD were determined with different pH values (pH 6.0–8.5), temperatures (30–45 °C), DMSO (0–20%), UDP (0.05–0.3 mM), sucrose (0.2–1 M), and PPD (0.5–2 mM) to further determine the optimal reaction conditions (Figure 4). The highest conversion of PPD was

**Figure 4.** Optimization of the reaction conditions for the Bs-YjiC/AtSuSy cascade reaction.

achieved at 40 °C and pH 8.0 (Figure 4a and b). Although the poor solubility of hydrophobic PPD can remarkably inhibit Bs-YjiC-catalyzed reaction and the addition of DMSO can significantly improve the availability of PPD in the reaction system, the conversions of PPD were slightly increased when DMSO was increased from 0% to 5%. When the concentration of DMSO exceeded 10%, the conversion of PPD rapidly decreased, and only 32% of PPD was glycosylated in the presence of 20% DMSO (Figure 4c). UDP is the most expensive cofactor in the UGT-SuSy reaction mixtures and has intricate effects on the UGT-SuSy reaction due to its importance during the formation of UDPG and inhibiting the activity of UGT. The conversion of PPD increased by 2.3-fold

when the concentration of UDP was increased from 0.05 mM to 0.2 mM and was slightly increased when the concentration of UDP continued to increase (Figure 4d). The highest conversion of PPD was observed in the presence of 0.4 M sucrose and was slightly changed when the concentration of sucrose continued to increase (Figure 4e). The conversion of PPD was decreased from 98% to 32% when the concentration of PPD was increased from 0.5 mM to 2 mM, and maximal ginsenoside F12 production (0.67 mM) was obtained in the presence of 1.0 mM PPD (Figure 4f). In summary, 40 °C, pH 8.0, 5% DMSO, 0.2 mM UDP, 0.4 M sucrose, and 1.0 mM PPD were used for the fed-batch reaction.

One-Pot Synthesis of Ginsenosides Rh2 and F12 by Fed-Batch Reaction. Based on the optimal parameters presented above, a fed-batch reaction by periodic addition of PPD was conducted to avoid the inhibition of a high concentration of PPD. As shown in Figure 5, the glycosylation

**Figure 5.** One-pot synthesis of ginsenosides Rh2 and F12 by fed-batch reaction. One mM PPD was added to the reaction mixtures at 1, 2, 4, 6, and 8 h. Fresh enzymes (160 mU mL⁻¹ Bs-YjiC and 200 mU mL⁻¹ AtSuSy) were added at 6 h.

of PPD was relatively fast, and 1 mM PPD could be completely converted into 0.02 mM ginsenoside Rh2 and 0.92 mM F12 in 1 h (Figure 5). Only a trace amount of ginsenoside Rh2 was detected in the first 3 h, and ginsenoside F12 was the major product, which was consistent with the in vitro enzymatic reaction using UDPG as the glucosyl donor. Nevertheless, the molar ratios of ginsenosides Rh2/F12 increased with the extension of reaction times. After 12 h, 0.32 mM (0.20 g L⁻¹) ginsenoside Rh2 and 5.07 mM ginsenoside F12 (3.98 g L⁻¹) were obtained through the periodic feeding of PPD with the conversion of PPD by 90%. A maximum number of 27.0 was obtained for the UDPG regeneration cycles. To the best of our knowledge, the titer of ginsenoside F12 produced in the present study was much higher than those of other PPD-type ginsenosides synthesized by yeast cell factories.^{14,16,17,37} This is the first report for biosynthesis of triterpene saponins using the UGT-SuSy cascade system. Considering that a PPD-producing yeast cell factory can produce substantial amounts of PPD,¹¹ an in vitro UGT-SuSy cascade reaction could be exploited as a novel promising alternative for semisynthesis of highly valuable PPD-type ginsenosides by using PPD as the precursor.

In summary, Bs-YjiC from *B. subtilis* 168 can regio- and stereospecifically transfer a glucosyl moiety to the free C3-OH and C12-OH of PPD and PPD-type ginsenosides to synthesize natural and unnatural PPD-type ginsenosides. These findings provided insights into the important roles of microbial UGTs for the biosynthesis of ginsenosides. This study suggested that

the in vitro UGT-SuSy cascade reaction could be exploited as an effective approach for biosynthesis of ginsenosides. In vivo and in vitro biological activities of ginsenosides F12 and other unnatural ginsenosides synthesized in this study should be investigated in future studies.

■ ASSOCIATED CONTENT

■ Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.jafc.8b00597.

¹H and ¹³C NMR spectral data for products 1 and 2 (Tables S1 and S2), some experimental results (Figures S1, S2, and S18), and HPLC-Q-TOF/ESI-MS and NMR analysis (Figures S2–S17) (PDF)

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Notes

The authors declare no competing financial interest.

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