Production of the Rare Ginsenosides Compound K, Compound Y, and Compound Mc by a Thermostable β-Glycosidase from *Sulfolobus acidocaldarius*

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The rare ginsenosides compound K, compound Y, and compound Mc were produced from the major ginsenosides Rb_1, Rb_2, Rc, and Rd by a thermostable β-glycosidase from *Sulfolobus acidocaldarius via* three pathways: Rb_1→Rd→compound K, Rb_2→compound Y→compound K, and Rc→compound Mc. Each of the ginsenosides was identified by high-performance liquid chromatography using standards and liquid chromatography-mass spectrometry based on their molecular weights. The catalytic efficiency of the enzyme for ginsenosides followed the order Rb_1 (4.8)>Rc (4.5)>Rd (1.0)>Rb_2 (0.77 mM^{-1} min^{-1}). The enzyme converted 1 mg/ml reagent-grade Rb_1, Rb_2, and Rc to 0.53 mg/ml compound K, 0.56 mg/ml compound Y, and 0.70 mg/ml compound Mc, respectively, at pH 5.5 and 85 °C after 180 min, corresponding to mole conversion yields of 94, 80, and 100% (mol/mol), respectively. The enzyme converted the major ginsenosides Rb_1, Rb_2, Rc, and Rd in 10% (w/v) ginseng root extract to the rare ginsenosides with a mole yield of 99% after 24 h. These results suggest that β-glycosidase from *S. acidocaldarius* can be used to produce compound K, compound Y, and compound Mc.

Key words ginsenoside; biotransformation; β-glycosidase; compound K; compound Y; compound Mc

Panax ginseng has been used in traditional medicine to strengthen immunity, provide nutrition, and reduce fatigue. The anticancer, anti-inflammatory, and anti-proliferative effects of *P. ginseng* are attributed to ginsenosides found in the plant.[1—3] Pharmaceutically active ginsenosides present at low concentrations or absent in ginseng root are referred to as rare ginsenosides. These rare ginsenosides, including compound K, compound Y, and compound Mc can be produced by the hydrolysis of sugar moieties from the major ginsenosides Rb_1, Rb_2, Rc, and Rd, which account for more than 50% of the total ginsenosides.[4] Compound K (20-*O*-β-D-glucopyranosyl-20(*S*)-protopanaxadiol) and compound Mc (20-*O*-[α-L-arabinofuranosyl-(1→6)-β-D-glucopyranosyl]-20(*S*)-protopanaxadiol) reportedly have antitumor, anti-inflammatory, antiallergic, and hepatoprotective effects,[5—8] whereas the functionality of compound Y (20-*O*-[α-L-arabinopyranosyl-(1→6)-β-D-glucopyranosyl]-20(*S*)-protopanaxadiol) has yet to be reported.

Compound K production has been achieved from ginseng root extract by enzymatic methods using Pectinex, β-galactosidase from *Aspergillus oryzae*, and β-glycosidase from *Sulfolobus solfataricus*.[9—11] Compound K, compound Y, and compound Mc were produced using a crude extract from *A. niger*, while compound K and compound Mc were produced by fermentation from *Fusarium sacchari*.[12,13] The fungi used in the production of the rare ginsenosides exhibited low productivity, and the enzymes involved were unknown. β-Glycosidase from *S. solfataricus* produced mainly compound K but not compound Y due to its high level of hydrolytic activity toward the sugar groups in the ginsenosides. To produce the rare ginsenosides compound K, compound Y, and compound Mc more efficiently, the discovery of a new enzyme with different hydrolytic activity toward these sugar groups is necessary.

In this study, the ginsenosides produced from the major ginsenosides Rb_1, Rb_2, Rc, and Rd by a thermostable β-glycosidase from *S. acidocaldarius* were identified and their production was attempted using ginseng root extract and reagent-grade Rb_1, Rb_2, and Rc.

MATERIALS AND METHODS

Materials Regent-grade Rb_1, Rb_2, Rc, and Rd were purchased from BTGin (Chungnam, Korea). Compound K was kindly provided by Professor Dong-Hyun Kim of Kyunghee University. Ginseng root extract was prepared by the previously reported method.[4]

Bacterial Strains, Plasmid, and Culture Conditions The genomic DNA from *S. acidocaldarius* DSMZ 639 (DSMZ, Brauschweig, Germany), *Escherichia coli* ER2566 (New Englands Biolabs, Herfordshire, U.K.), and pTrc99a plasmid (Pharmacia, Piscataway, NJ, U.S.A.) were used as a source of β-glycosidase gene, a host, and a vector for expression, respectively. *S. acidocaldarius* was grown anaerobically at 70 °C on *Sulfolobus* medium (DSM media formulation No. 88) for 5 d. The recombinant *E. coli* for protein expression was cultivated in a 2-l flask containing 500 ml of Luria–Bertani (LB) medium and 50 μg/ml of ampicillin at 37 °C with agitation at 200 rpm until the optical density of bacteria reached 0.8 at 600 nm. Isopropyl-β-D-thiogalactopyranoside (IPTG) was added to the culture medium at a final concentration of 0.1 mM, and the enzyme was induced and expressed at 16 °C for 12 h.

Cloning of β-Glycosidase Gene from *S. acidocaldarius* The genomic DNA from *S. acidocaldarius* was extracted using the genomic DNA extraction kit (Qiagen, Hilden, Germany). The gene encoding β-galactosidase was amplified from the genomic DNA as a template by polymerase chain reaction (PCR) using *Pfu* DNA polymerase (Solgent, Daejeon, Korea). The sequence of the oligonucleotide primers used for gene cloning was based on the DNA sequence of *S. acidocaldarius* β-glycosidase (GenBank accession number, YP 256448). Forward (5′-GAATTCATGTTATCATTCCCAAAGGGTTTC-3′) and reverse primers (5′-CTGCAGT-

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TAATGTCTCAAAGGTTTTATTGGTGG-3′) were designed as primers to introduce the *Eco*RI and *Pst*I restriction sites (underline), respectively and were synthesized by Bioneer (Daejon, Korea). The amplified DNA fragment obtained by PCR was purified and digested with both *Eco*RI and *Pst*I endonucleases (New England Biolabs, Hertfordshire, U.K.). The digested DNA fragment was extracted, and then inserted into the pTrc99a vector digested with the same restriction enzymes. The plasmid was transformed into *E. coli* ER2566 strain with the ligation mixture and grown on LB medium containing 20 μg/ml of ampicillin. An ampicilin-resistant colony was selected, and plasmid DNA from the transformant was isolated using a plasmid purification kit (Promega, Madison, WI, U.S.A.). DNA sequencing was performed at the facility of Solgent (Daejeon, Korea). The expression of the gene was determined by both SDS-PAGE and used on the assay of enzyme activity.

Preparation of β-Glycosidase from *S. acidocaldarius* *E. coli* ER2566 cells containing β-glycosidase/pTrc99a gene from *S. acidocaldarius* were grown in a Luria–Bertani (LB) medium containing 20 μg/ml of ampicillin at 37 °C with agitation at 200 rpm until an optical density (OD) at 600 nm of 1.0 was reached. Isopropyl-β-D-thiogalactopyranoside (IPTG) was added to the culture medium at a final concentration of 0.1 mM, and incubation continued with agitation at 150 rpm at 16 °C for 16 h. The induced cells were harvested and disrupted by sonication in 50 mM citrate buffer (pH 5.5). The unbroken cells and cell debris were removed by centrifugation at 13000×***g*** for 20 min, and the supernatant was treated at 90 °C for 10 min to remove proteins derived from *E. coli*. After heat treatment, the suspension was centrifuged at 13000×***g*** for 20 min to remove insoluble denatured proteins. The supernatant was used as the soluble enzyme.

Hydrolytic Activity of β-Glycosidase from *S. acidocaldarius* The hydrolytic activity of β-glycosidase was determined using various aryl-glycosides including *p*-nitrophenyl(*p*NP)-β-D-glucopyranoside, *p*NP-β-D-galactopyranoside, *p*NP-β-L-arabinopyranoside, *p*NP-α-L-arabinopyranoside, *p*NP-α-D-glucopranoside, *p*NP-α-D-galactopyranoside, *p*NP-β-L-arabinofuranoside, and *p*NP-α-L-arabinofuranoside (Sigma, St. Louis, MO, U.S.A.). The hydrolytic reaction was performed with 1 mM aryl-glycoside in 50 mM citrate buffer at 85 °C for 5 min and then quenched by adding 100 μl of 2 M Na_2CO_3. The increase in the absorbance at 420 nm due to the release of *p*-nitrophenol was measured. Protein concentrations of the enzyme preparations were measured by the Bradford method with bovine serum albumin as the standard. One unit (U) of enzyme activity was defined as the amount of enzyme required to liberate the equivalent of 1 μmol of *p*-nitrophenol per min under the assay conditions.

Determination of Kinetic Parameters for Ginsenosides Various concentrations of regent-grade ginsenosides Rb_1 (from 2.1 to 11 mM), Rb_2 (from 1.1 to 10 mM), Rc (from 0.76 to 4.2 mM), and Rd (from 1.1 to 10 mM) were used to determine kinetic parameters of the enzyme. The reactions were performed in citrate buffer (pH 5.5) at 85 °C for 5 min. The enzyme kinetic parameters, K_m (mm) and k_{cat} (min^{-1}) values were determined by fitting to the Michaelis–Menten equation.

Analytical Methods Digoxin of 10 μl as an internal standard was added to 40 μl sample and then extracted with 50 μl *n*-butanol for 2 min using a vortex. After centrifugation at 13000×***g*** for 5 min, the upper organic phase as *n*-butanol fraction was transferred into clean tube and evaporated to dryness in the centrifugal evaporator (Eyela CVE-3100, Tokyo, Japan). The residue was then reconstituted by methanol for analysis.[14]

Ginsenosides were assayed by an HPLC system (Agilent 1100, Santa Clara, CA, U.S.A.) equipped with a UV detector at 203 nm using a C_{18} column (150×3.0 mm, YMC, Kyoto, Japan). The column was eluted initially with the mixture of acetonitrile and water of 20 : 80 (v/v) as the mobile phase, gradient to 40 : 60 in 40 min, and then gradient to 80 : 20 in 80 min. The flow rate was 1.0 ml/min and column temperature was 37 °C. The ginsenosides were separated on a C_{18} column (100×2.0 mm, Varian, Palo Alto, CA, U.S.A.) with a gradient of 0.1% formic acid (buffer A) and acetonitrile containg 0.1% formic acid (buffer B). LC/MS for ginsenosides was analyzed by Varian 500-MS with negative polarity and an ion trap analyzer. Ion spray was operated under condition of 5 l/min N_2, 3.5 kV, 25 psi, and 300 °C.

Time Course of Rare Ginsenosides Production The production of compound K, compound Y, and compound Mc was followed at 85 °C for 180 min in 50 mm citrate buffer (pH 5.5) with 33, 187, and 110 U/ml enzyme using approximately 1 mg/ml reagent-grade Rb_1, Rb_2, and Rc, respectively, and for 24 h with 30 U/ml enzyme using 10% (w/v) ginseng root extract.

RESULTS

HPLC Analysis of the Ginsenosides in Ginseng Root Extract in the Presence and Absence of β-Glycosidase from *S. acidocaldarius* Because rare ginsenosides can be produced by the hydrolysis of sugar moieties from major ginsenosides using ginsenoside-hydrolyzing β-glycosidases,[15—18] the above reaction was performed using a thermostable β-glycosidase from the hyperthermophilic bacterium *S. acidocaldarius* and an extract prepared from ginseng root at pH 5.5 and 85 °C for 24 h. The ginsenosides in the ginseng root extract were analyzed by HPLC using a C_{18} column. Four typical major ginsenosides were detected with the same retention times as Rb_1, Rb_2, Rc, and Rd (Fig. 1A). The ginsenosides converted by the β-glycosidase were also analyzed, and three peaks were observed (Fig. 1B). The transformation of Rb_1, Rb_2, Rc, and Rd in various microorganisms has been characterized as follows: Rb_1, Rb_2, or Rc→Rd→F_2→compound K, Rb_1→gypenoside XVII→gypenoside LXXV→compound K, Rb_2→compound O→compound Y→compound K, and Rc→compound Mc→compound K.[19,20] Thus, the peaks could be identified as three among F_2, gypenoside XVII, gypenoside LXXV, compound O, compound Y, compound Mc, and compound K.

Confirmation of the Rare Ginsenosides by LC/MS The above biotransformation was achieved using reagent-grade Rb_1, Rb_2, and Rc and β-glycosidase from *S. acidocaldarius*. The samples obtained after biotransformation were subjected to LC/MS to determine their molecular weights. Compound K obtained from Rb_1 was identified by HPLC using a standard and confirmed by LC/MS based on its molecular weight of 623 (Fig. 1C). The molecular weights of the

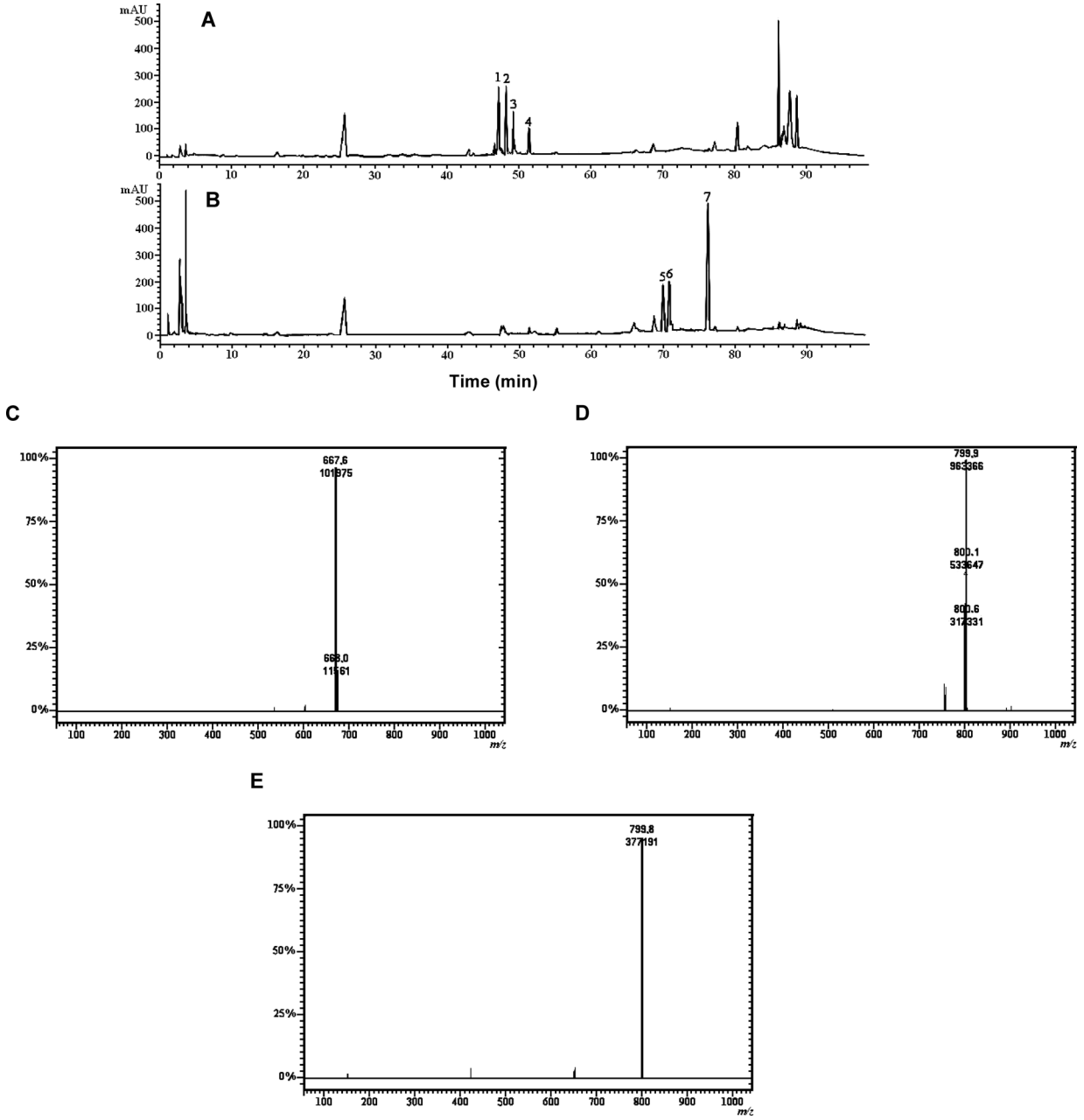

Fig. 1. HPLC Chromatographies (A, B) and Mass Spectra (C—E) of Ginsenosides after the Reaction of *S. acidocaldarius* β-Glycosidase

(A) HPLC of ginsenosides in ginseng root extract. 1. Rb_1, 2. Rc, 3. Rb_2, 4. Rd. (B) HPLC of ginsenosides produced from ginseng root extract by *S. acidocaldarius* β-glycosidase after 24 h. 5. Compound Mc, 6. Compound Y, 7. Compound K. The reactions were performed using 10% (w/v) ginseng root extract in 50 mm citrate buffer (pH 5.5) at 85 °C with 30 U/ml enzyme for 24 h. (C) Mass spectrum of compound K, m/z=668=[MW+formic acid+H], MW=623. (D) Mass spectrum of compound Y, m/z=800=[MW+formic acid+H], MW=755. (E) Mass spectrum of compound Mc, m/z=800=[MW+formic acid+H], MW=755. The samples of compound K, compound Y, and compound Mc were obtained after the reactions for 180 min in 50 mm citrate buffer (pH 5.5) at 85 °C with 33, 187, and 110 U/ml enzyme for reagent-grade Rb_1, Rb_2, and Rc, respectively.

ginsenosides obtained from Rb_2 and Rc were both 755 (Figs. 1D, E), indicating that they were compound Y and compound Mc, respectively, based on the reported transformation pathways.[20]

Hydrolytic Activity of β-Glycosidase from *S. acidocaldarius* The hydrolytic activity of β-glycosidase from *S. acidocaldarius* was investigated with aryl-glucosides, aryl-galactosides, and aryl-arabinosides (Table 1). The hydrolytic activity for the *p*NP substrates followed the order *p*NP-β-D-glucopyranoside>*p*NP-β-D-galactopyranoside>*p*NP-α-L-arabinopyranoside>*p*NP-β-L-arabinopyranoside. However, the enzyme exhibited no activity for *p*NP-α-D-glucopyranoside, *p*NP-α-D-galactopyranoside, *p*NP-β-L-arabinofuranoside, and *p*NP-α-L-arabinofuranoside.

The Michaelis–Menten constants (K_m), turnover numbers (k_{cat}), and catalytic efficiencies (k_{cat}/K_m) for reagent-grade Rb_1, Rb_2, Rc, and Rd are presented in Table 2. The catalytic efficiencies decreased as Rb_1 (4.8), Rc (4.5), Rd (1.0), and Rb_2 (0.77 $mM^{-1}\,min^{-1}$).

Biotransformation of Reagent-Grade Ginsenosides to Rare Ginsenosides Since the optimum conditions for producing the rare ginsenosides were pH 5.5 and 85 °C (data not shown), the reactions were allowed to run for 180 min with 33 U/ml β-glycosidase for reagent-grade Rb_1, 187 U/ml for Rb_2, and 110 U/ml for Rc. Rb_1 and Rb_2 were converted to Rd and compound Y as intermediates, respectively. The used

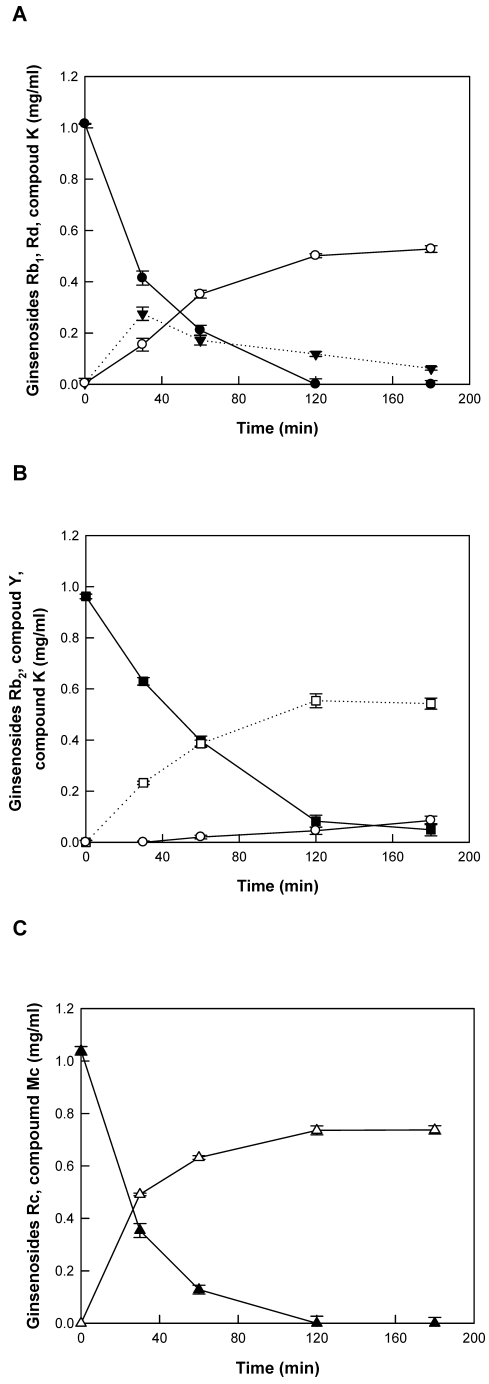

Fig. 2. Production of Compound K, Compound Y, and Compound Mc from Reagent-Grade Rb_1, Rb_2, and Rc by β-Glycosidase from *S. acidocaldarius*

(A) Rb_1 (●)→Rd (▼)→compound K (○). (B) Rb_2 (■)→compound Y (□)→compound K (○). (C) Rc (▲)→compound Mc (△). The reactions were performed for 180 min in 50 mM citrate buffer (pH 5.5) at 85 °C with 33, 187, and 110 U/ml enzyme for approximately 1 mg/ml reagent-grade Rb_1, Rb_2, and Rc, respectively. Data represent the means of three experiments and error bars represent standard deviation.

amount of the enzyme for the reactions followed the order Rb_2>Rc>Rb_1 because the hydrolytic activity followed the order Rb_1>Rc>Rb_2 (Table 2). The intermediates were then converted to compound K (Figs. 2A, B). After the reactions, compound K and compound Y were produced mainly from Rb_1 and Rb_2, respectively. Rc was converted to compound Mc, but not to compound K (Fig. 2C). The enzyme converted 1 mg/ml reagent-grade (0.90 mM) Rb_1, (0.93 mM) Rb_2, and (0.93 mM) Rc to 0.53 mg/ml (0.85 mM) compound K, 0.56 mg/ml (0.74 mM) compound Y, and 0.70 mg/ml (0.93 mM) compound Mc, respectively, corresponding to a mole conversion yield of 94, 80, and 100%, respectively. The transformation of reagent-grade Rb_1, Rb_2, and Rc by β-glycosidase from *S. acidocaldarius* suggests the following three transformation pathways: Rb_1→Rd→compound K, Rb_2→compound Y→compound K, and Rc→compound Mc (Fig. 3).

Table 1. Hydrolytic Activity of *S. acidocadarius* β-Glycosidase

Substrate	Relative activity (%)
p-Nitrophenyl-β-D-glucopyranoside	100±0.45
p-Nitrophenyl-β-D-galactopyranoside	28±0.22
p-Nitrophenyl-α-L-arabinopyranoside	17±0.05
p-Nitrophenyl-β-L-arabinopyranoside	8±0.03
p-Nitrophenyl-α-D-glucopyranoside	ND
p-Nitrophenyl-α-D-galactopyranoside	ND
p-Nitrophenyl-β-L-arabinofuranoside	ND
p-Nitrophenyl-α-L-arabinofuranoside	ND

ND: activity is not detected by the analytical method used in this study. Data represent the means of three separate experiments.

Table 2. Kinetic Parameters of *S. acidocaldarius* β-Glycosidase for Ginsenosides

Ginsenoside	K_m (mM)	k_{cat} (min^{-1})	k_{cat}/K_m ($mM^{-1}\,min^{-1}$)
Rb_1	3.0±0.04	14±0.29	4.8±0.02
Rb_2	8.6±0.04	6.6±0.12	0.77±0.01
Rc	1.2±0.04	5.4±0.13	4.5±0.04
Rd	7.7±0.03	8.0±0.04	1.0±0.01

Data represent the means of three separate experiments.

Biotransformation of the Major Ginsenosides in Ginseng Root Extract to Rare Ginsenosides The rare ginsenosides compound K, compound Y, and compound Mc were produced from an extract prepared from ginseng root at pH 5.5 and 85 °C for 24 h with β-glycosidase from *S. acidocaldarius*. The enzyme converted 1.70 mg/ml (1.53 mM) Rb_1, 0.53 mg/ml (0.49 mM) Rb_2, 0.96 mg/ml (0.89 mM) Rc, and 0.27 mg/ml (0.29 mM) Rd to 1.20 mg/ml (1.93 mM) compound K, 0.26 mg/ml (0.35 mM) compound Y, and 0.67 mg/ml (0.89 mM) compound Mc over a 24-h period (Fig. 4). The initial molarity of the major ginsenosides was 3.20 mM while the final molarity of the rare ginsenosides was 3.17 mM, corresponding to a mole yield of 99%. This suggests that β-glycosidase from *S. acidocaldarius* can be used to produce compound K, compound Y, and compound Mc from a ginseng root extract containing Rb_1, Rb_2, Rc, and Rd. The mole yield from Rc to compound Mc was 100%, indicating the complete conversion of Rc into compound Mc. Starting with ginseng root extract, the reaction time required to produce the rare ginsenosides was significantly longer than that using a single reagent-grade ginsenoside. This retardation was previously reported to be due to the inhibition of enzymatic activity by the ginsenosides.[4)]

DISCUSSION

The specific activity of β-glycosidase from *S. acidocalda-*

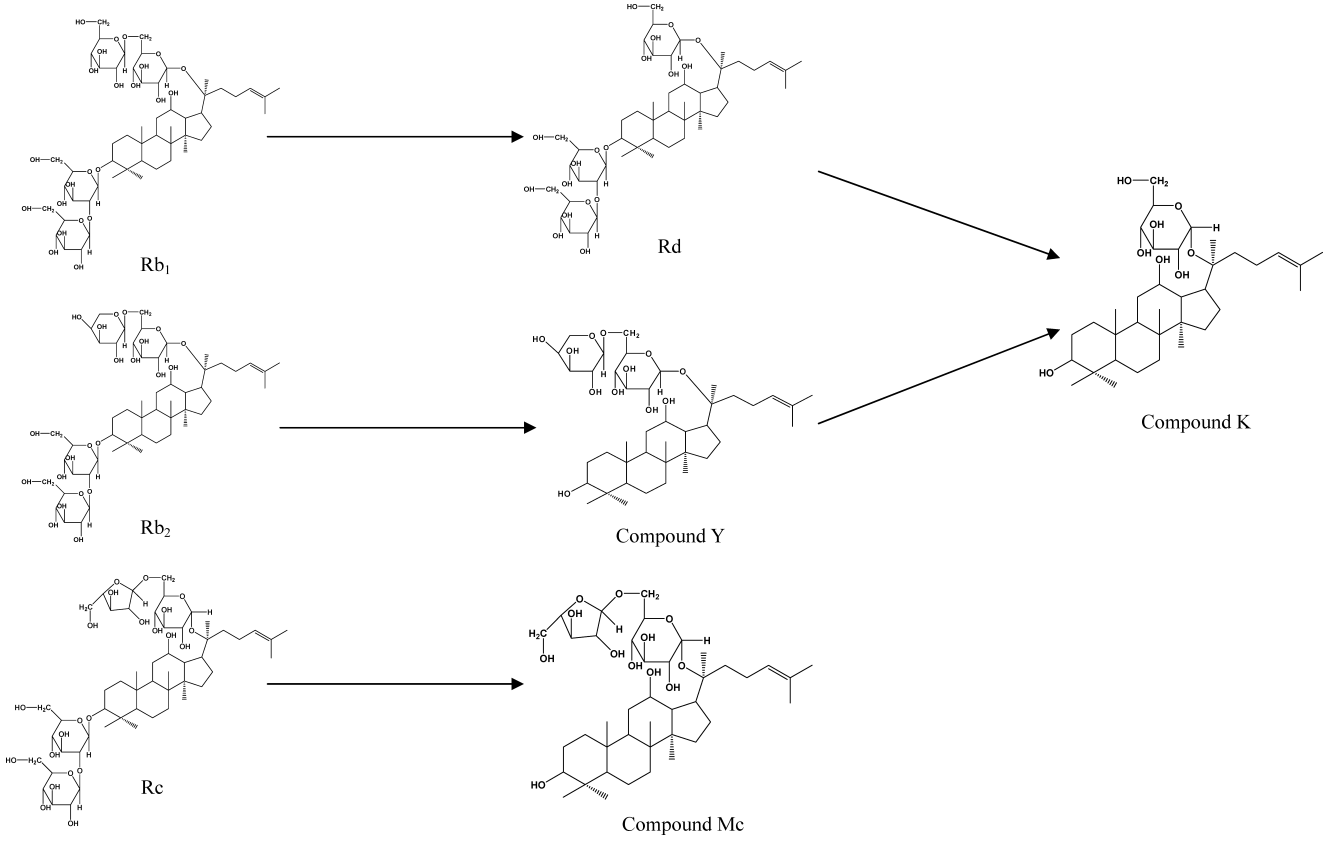

Fig. 3. Transformation Pathways from Ginsenoside Rb_1, Rb_2, and Rc to Compound K, Compound Y, and Compound Mc by β-Glycosidase from *Sulfolobus acidocaldarius*, Respectively

The three transformation pathways were Rb_1→Rd→compound K, Rb_2→compound Y→compound K, and Rc→compound Mc.

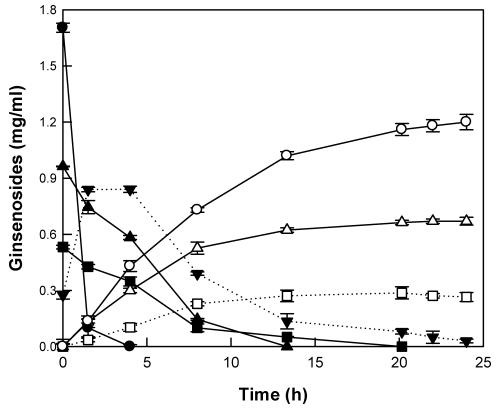

Fig. 4. Production of Compound K (○), Compound Y (□), and Compound Mc (△) from Rb_1 (●), Rb_2 (■), Rc (▲), and Rd (▼) in Ginseng Root Extract by β-Glycosidase from *Sulfolobus acidocaldarius*

The reactions were performed using 10% (w/v) ginseng root extract in 50 mM citrate buffer (pH 5.5) at 85 °C with 30 U/ml enzyme for 24 h. Data represent the means of three experiments and error bars represent standard deviation.

rius for *p*NP-β-D-glucopyranoside was higher than that for *p*NP-α-L-arabinopyranoside. However, the enzyme exhibited no activity for *p*NP-α-L-arabinofuranoside (Table 1). Similar results were also observed in the hydrolysis of ginsenosides. The enzyme showed greater hydrolytic activity toward the 3-*O*-β-D-glucopyranosyl-(1→2)-β-D-glucopyranosyl linkage in panaxadiol (Rd→compound K, Rb_2→compound Y, and Rc→compound Mc) and β-D-glucopyranose linkage of 20-*O*-β-D-glucospyranosyl-(1→6)-β-D-glucopyranose (Rb_1→Rd) than toward the α-L-arabinopyranose linkage in 20-*O*-α-L-arabinopyranosyl-(1→6)-β-D-glucopyranose (compound Y→compound K). The enzyme did not convert compound Mc to compound K due to no activity for the α-L-arabinofuranose linkage in 20-*O*-α-L-arabinofuranosyl-(1→6)-β-D-glucopyranose. Thus, β-glycosidase from *S. acidocaldarius* produced compound K from Rb_1 and Rd, compound Y from Rb_2, and compound Mc from Rc (Fig. 3), suggesting that the enzyme is a potential producer of the rare ginsenosides compound K, compound Y, and compound Mc from the major ginsenosides Rb_1, Rb_2, Rc, and Rd.

In contrast, β-glycosidase from *S. solfataricus* had broader substrate specificity and greater hydrolytic activity toward the α-L-arabinopyranose group in 20-*O*-β-(1→6)-glucosyl-α-L-arabinopyranose compared to β-glycosidase from *S. acidocaldarius*. Moreover, the enzyme converted compound Mc to compound K because it had activity for the α-L-arabinofuranose linkage in 20-*O*-α-L-arabinofuranosyl-(1→6)-β-D-glucopyranose. As a result, β-glycosidase from *S. solfataricus* produced more compound K and utilized different pathways for Rb_1, Rb_2, and Rc (*i.e.*, *via* Rb_1 or Rb_2→Rd→F_2→compound K and Rc→compound Mc→compound K)[10] comparing to β-glycosidase from *S. acidocaldarius*. A cell extract of *A. niger* converted the reagent-grade ginsenosides Rb_2 and Rc to compound K, compound Y, and compound Mc after 48 h.[12] The fugus *F. sachari* converted Rb_1, Rb_2, Rb_3, and Rc to compound K, compound Mx, and compound Mc after 6 d of fermentation.[13]

β-Glycosidase from *S. acidocaldarius* utilized three path-

ways for the transformation of the major ginsenosides: Rb_1→Rd→compound K, Rb_2→compound Y→compound K, and Rc→compound Mc. Although the cell extract from *A. niger* also produced compound K, compound Y, and compound Mc, its transformation pathways for Rb_1, Rb_2, and Rc were Rb_1→Rd→F_2→compound K, Rb_2→compound O→compound Y→compound K, and Rc→compound Mc→compound K.[20] The detection of F_2 and compound O may have occurred because the reaction was slower than the enzymatic reaction. The *A. niger* extract had hydrolytic activity toward the α-L-arabinofuranose linkage in compound Mc, whereas the β-glycosidase from *S. acidocaldarius* had no activity toward the α-L-arabinofuranose linkage.

Comparing to previous reports, the production of rare ginsenosides by β-glycosidase from *S. acidocaldarius* in this study was improved in several respects: (i) The rare ginsenosides have been produced using not enzymes but cells, and the enzymes involved in the cells were unknown and their productivities were very low.[8,12,13,20] On the other hand, the study using the enzyme β-glycosidase from *S. acidocaldarius* demonstrated clearly and quantitatively the hydrolytic process of ginsenoside with a high productivity. (ii) The production of compound K, compound Y, and compound Mc in this study relied on a recombinant enzyme. Recombinant enzymes have several advantages over native enzymes, including ease of purification, large-scale production, and genetic improvement through directed evolution.[10] (iii) Rare ginsenosides were produced using a thermostable β-glycosidase from *S. acidocaldarius*. Thermostable enzymes have several advantages, including an increased reaction velocity, increased solubility of substrate, and reduced risk of contamination. The reaction temperature of β-glycosidase from *S. acidocaldarius* for the production of the rare ginsenosides was 85 °C, whereas those of the fungal cells were in the range of 30—37 °C.[8,12,13,20] (iv) The specific conversions of compound K from Rb_1 *via* Rd, compound Y from Rb_2, and compound Mc from Rc using one enzyme have not been reported previously. The enzyme exhibited novel features based on its broad substrate specificity. Moreover, no reports have described the production of compound Y using enzyme. (v) β-Glycosidase from *S. solfataricus* in this study converted the major ginsenosides Rb_1, Rb_2, Rc, and Rd to the rare ginsenosides compound K, compound Y, and compound Mc with a mole yield of 99% after 24 h, indicating that the enzyme is a potential producer of the rare ginsenosides.

In conclusion, the hydrolytic activity of β-glycosidase from *S. acidocaldarius* for the glucopyranose linkage in panaxadiol was higher than that for the α-L-arabinopyranose linkage. However, the enzyme had no activity for the α-L-arabinofuranose linkage. As a result, the enzyme converted Rb_1, Rb_2, Rc, and Rd to compound K, compound Y, and compound Mc with a high conversion yield *via* three transformation pathways: Rb_1→Rd→compound K, Rb_2→compound Y→compound K, and Rc→compound Mc. Thus, β-glycosidase from *S. acidocaldarius* can be used to produce the rare ginsenosides compound K, compound Y, and compound Mc.

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