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Characterization of a novel recombinant β-glucosidase from *Sphingopyxis alaskensis* that specifically hydrolyzes the outer glucose at the C-3 position in protopanaxadiol-type ginsenosides

Kyung-Chul Shin, Deok-Kun Oh*

Department of Bioscience and Biotechnology, Konkuk University, Seoul 143-701, Republic of Korea

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ABSTRACT

A recombinant β-glucosidase from *Sphingopyxis alaskensis* with a specific activity of 233.3 U mg^{-1} was purified by His-trap chromatography. The native enzyme was a 206 kDa tetramer. The maximum enzyme activity was observed at pH 5.5 and 50 °C. However, above 40 °C, the enzyme stability significantly decreased. The enzyme hydrolyzed only the outer glucose at the C-3 position in protopanaxadiol-type ginsenosides without further hydrolysis. Because of the narrow substrate specificity, the enzyme completely converted ginsenosides Rb_1, Rb_2, Rc, and Rd as substrates to gypenoside XVII, compound O, compound Mc_1, and F_2, respectively, and it converted ginsenoside Rg_3 to Rh_2 with a molar conversion yield of 89%. These results suggest that the recombinant β-glucosidase from *S. alaskensis* is a potential producer of the rare ginsenosides gypenoside XVII, compound O, compound Mc_1, F_2, and Rh_2. Among ginsenoside substrates, Rb_1 was used for the high-level production of the rare ginsenoside gypenoside XVII. The optimum reaction conditions were pH 5.5, 40 °C, 0.5 mg ml^{-1} (116.7 U ml^{-1}) enzyme, and 8.0 g l^{-1} ginsenoside Rb_1. Under these conditions, 6.8 g l^{-1} gypenoside XVII was produced by the enzyme after 1 h with a molar conversion yield of 100% and a productivity of 6.8 g l^{-1} h^{-1}.

1. Introduction

Ginseng (*Panax ginseng* C. A. Meyer) is used as a traditional medicine in Asian countries to strengthen immunity, supply nutrition, and decrease fatigue. These beneficial functions are attributed mainly to the ginsenosides in ginseng. Ginsenosides have various biological and pharmaceutical activities, such as anti-fatigue (Yoshikawa et al., 2003), anti-allergenic (Bae et al., 2002), antioxidant (Cho et al., 2006), anti-inflammatory (Wang et al., 2011a,b), and anti-cancer activities (Lee et al., 2009).

Ginsenosides are classified into three groups based on the type of the aglycone structure: the oleanane, protopanaxadiol (PPD), and protopanaxatriol (PPT) types. The PPD- and PPT-type ginsenosides harbor different sugar moieties linked to C-3 and C-20 in the aglycone PPD (APPD) and to C-6 and C-20 in the aglycone PPT (APPT) by glycosidic bonds, respectively. Major glycosylated ginsenosides (Rb_1, Rb_2, Rc, Rd, Re, and Rg_1) comprise more than 80% of the total ginsenosides in wild ginseng (Noh and Oh, 2009). Deglycosylated ginsenosides are more pharmaceutically active than major glycosylated ginsenosides, because of their smaller size, higher bioavailability, and better permeability across the cell membrane (Xu et al., 2003). Therefore, many studies have focused on the production of deglycosylated ginsenosides by hydrolyzing the sugar moieties linked to the C-3, C-6, and C-20 positions in major glycosylated ginsenosides by heating (Kim et al., 2000), acid treatment (Bae et al., 2004), fermentation (Zhou et al., 2008), and enzymatic conversion methods (Noh et al., 2009). Among these, the enzymatic conversion is the most promising method for the preparation of diverse deglycosylated ginsenosides via the selective hydrolysis of the sugar moieties of ginsenosides because of its high specificity, yield, and productivity.

β-Glucosidases from *Terrabacter ginsenosidimutans* (Jin et al., 2012), *Esteya vermicola* (Hou et al., 2012), and *Spingomonas* sp. 2F2 (Wang et al., 2011a,b) specifically hydrolyze the inner and outer glucose molecules linked to the C-3 position in PPD-type ginsenosides. β-Glucosidases from *Panax ginseng* (Zhang et al., 2001) and *Fusarium proliferatum* (Su et al., 2006) convert ginsenoside Rg_3 to Rh_2 by hydrolyzing only the outer glucose at the C-3 position. However, the substrate specificity of these Rg_3-hydrolyzing enzymes has not been described. Rare ginsenosides have attracted attention as potential materials for screening new biological and pharmaceutical activities. However, the quantitative production of the ginsenosides gypenoside XVII, compound O, compound Mc_1, and F_2 has not been attempted. To produce the rare ginsenosides without byproducts, the discovery of an enzyme that hydrolyzes only the outer glucose at C-3 position in ginsenosides is essential.

* Corresponding author. Tel.: +82 2 454 3118; fax: +82 2 444 5518.
E-mail address: deokkun@konkuk.ac.kr (D.-K. Oh).

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In this study, a ginsenoside-hydrolyzing enzyme from *Sphingopyxis alaskensis* was cloned, purified, and characterized. The substrate specificity of the enzyme indicated it as a β-glucosidase that hydrolyzes only the outer glucose at the C-3 position in PPD-type ginsenosides, and the biotransformation of the major ginsenosides Rb_1, Rb_2, Rc, Rd, and Rg_3, to the rare ginsenosides gypenoside XVII, compound O, compound Mc_1, F_2, and Rh_2, respectively, was also investigated.

2. Materials and methods

2.1. Materials

Ginsenoside standards Rb_1, Rb_2, Rc, Rd, Rg_3, F_2, and Rh_2; and gypenoside XVII, compound O, and compound Mc_1 were purchased from BTGin (Chungnam, Korea) and AMBO institute (Daejeon, Korea), respectively.

2.2. Cloning and gene expression

The genomic DNA from *S. alaskensis* DSM 13593 (DSMZ, Brauschweig, Germany), *Escherichia coli* ER2566 (New England Biolabs, Herfordshire, UK), and pET-22b(+) plasmid (Novagen, Madison, WI) were used as the source of β-glucosidase gene, host cells for cloning and expression, and expression vector, respectively. The gene encoding the β-glucosidase was amplified by PCR using the genomic DNA isolated from *S. alaskensis* as a template. The sequence of the oligonucleotide primers used for gene cloning was based on the DNA sequence of *S. alaskensis* β-glucosidase (GenBank accession number ABF52736). Forward (5′-CATATGATGACGCAGACCATCTTTCCCG-3′) and reverse primers (5′-AAGCTTTCAGCCGAGGTTCCCGCCGTT-3′) were designed to introduce the *Nde*I and *Hind*III restriction sites (underlined), respectively, and the primers were synthesized by Bioneer (Daejeon, Korea). The amplified DNA fragments obtained by PCR were purified and inserted into the pET-22b(+) vector digested with the same restriction enzymes. *E. coli* ER2566 strain was transformed with the ligation mixture using an electroporator (MicroPulser, Bio-Rad, Hercules, CA) and the transformed cells were plated on Luria-Bertani (LB) agar containing 25 μg ml^{-1} ampicillin. An ampicillin-resistant colony was selected, and plasmid DNA from the transformant was isolated using a plasmid purification kit (Promega, Madison, WI). DNA sequencing was carried out on a DNA analyzer (ABI Prism 3730xl, Perkin-Elmer, Waltham, MA). Gene expression was evaluated by both SDS-PAGE and enzyme activity assay.

2.3. Enzyme purification

E. coli cells containing the β-glucosidase/pET-22b(+) gene from *S. alaskensis* were grown in a 2,000 ml flask containing 500 ml of LB medium and 25 μg ml^{-1} kanamycin at 37 °C with shaking at 200 rpm until the optical density of bacteria reached 0.6 at 600 nm. Isopropyl-β-D-thiogalactopyranoside (IPTG) was added to a final concentration of 0.1 mM to induce β-glucosidase expression, and the incubation continued with shaking at 150 rpm at 16 °C for 12 h. The induced cells were harvested and disrupted by sonication on ice for 2 min in 50 mM phosphate buffer (pH 7.0) containing 300 mM NaCl with the addition of 1 mg ml^{-1} lysozyme. The cell debris were removed by centrifugation at 13,000 × *g* for 20 min at 4 °C, and the supernatant obtained was used as the crude extract. The purification was carried out on a fast protein liquid chromatography system (Bio-rad, Hercules, CA) in a cold room at 4 °C. The supernatant was loaded on a His-trap affinity chromatography column (GE Healthcare, Piscataway, NJ) equilibrated with 50 mM phosphate buffer (pH 7.0). The bound protein was then eluted at 4 °C with the same buffer containing 250 mM imidazole at a flow rate of 1 ml min^{-1}. The active fractions were collected and dialyzed at 4 °C for 16 h against 50 mM citrate/phosphate buffer (pH 6.0). The resulting solution was used as the purified enzyme.

2.4. Determination of molecular mass

The subunit molecular mass of β-glucosidase from *S. alaskensis* was examined by SDS-PAGE under denaturing conditions. The pre-stained protein ladder (MBI Fermentas, Hanover, MD, USA) was used as reference. All protein bands were stained with coomassie blue for visualization. The molecular mass of the native enzyme was determined by gel filtration chromatography using a Sephacryl S-300 HR 16/60 preparative-grade column (GE Healthcare, Piscataway, NJ). The enzyme solution was applied to the column and eluted with 50 mM Tris–HCl buffer (pH 7.5) containing 150 mM NaCl at a flow rate of 0.5 ml min^{-1}. The column was calibrated with ferritin (440 kDa), catalase (232 kDa), aldolase (158 kDa), and conalbumin (75 kDa) as reference proteins (GE Healthcare) and the molecular mass of the native enzyme was calculated by comparing it with the migration length of the reference proteins.

2.5. Hydrolytic activity

Unless otherwise stated, the reaction was performed in 50 mM citrate/phosphate buffer (pH 5.5) at 40 °C for 20 min. One unit (U) of enzyme activity used for aryl-glycoside, ginsenoside, or disaccharide was defined as the amount of enzyme required to liberate 1 nmol of nitrophenol (NP), gypenoside XVII from ginsenoside Rb_1 as a substrate, or glucose, per min at 40 °C and pH 5.5, respectively. The aryl-glycoside activity was determined by the increase in the absorbance at 450 nm due to the release of NP. The hydrolytic reactions were performed at 40 °C in 50 mM citrate/phosphate buffer (pH 5.5) containing 1 mM aryl-glycoside and 1.2 U ml^{-1} enzyme, 0.4 mg ml^{-1} ginsenoside and 5.8 U ml^{-1} enzyme, or 1 mM disaccharide and 1.2 U ml^{-1} enzyme. The time course reaction for the conversion of ginsenosides was performed for 7 h.

2.6. Effects of pH and temperature

The effects of pH and temperature on gypenoside XVII production were investigated by varying the pH of 50 mM citrate/phosphate buffer from 4.5 to 7.5 at a temperature of 40 °C, and by varying the temperature from 30 to 60 °C at a constant pH of 5.5. The effect of temperature on enzyme stability was assessed by determining the residual enzyme activity at pH 5.5 and 40 °C after maintaining the solutions of enzymes at five different temperatures (30, 35, 40, 45, and 50 °C) for 30 min.

2.7. Production of gypenoside XVII from ginsenoside Rb_1

The effect of enzyme concentration on gypenoside XVII production was evaluated by varying the concentration of the enzyme from 0.1 (23.3) to 4 mg ml^{-1} (933.2 U ml^{-1}) with 8 mg ml^{-1} ginsenoside Rb_1 for 20 min. The time course reaction of gypenoside XVII from ginsenoside Rb_1 was performed at 40 °C in 50 mM citrate/phosphate buffer (pH 5.5) containing 8 g l^{-1} ginsenoside Rb_1 and 116.7 U ml^{-1} enzyme for 60 min.

2.8. Analytical methods

A reaction solution containing digoxin as an internal standard was extracted with an equal volume of *n*-butanol. The *n*-butanol fraction was then evaporated to dryness, and methanol was added. Ginsenosides were assayed using an HPLC system (Agilent 1100,

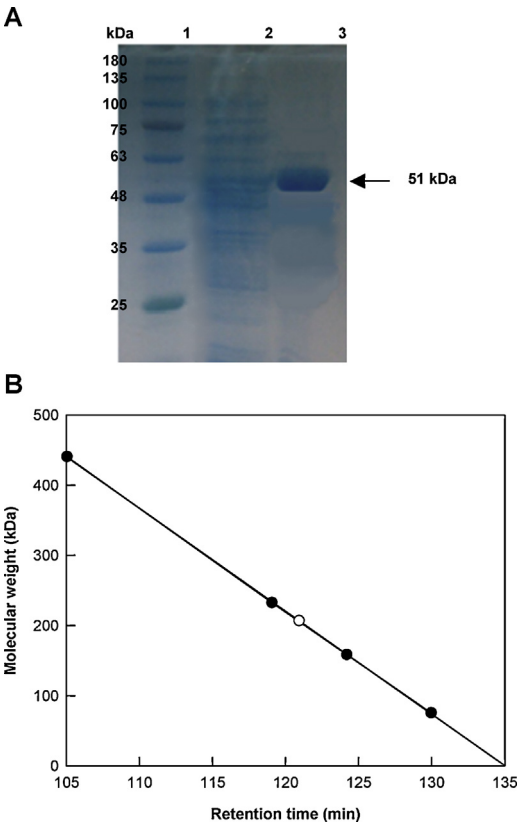

Fig. 1. SDS-PAGE analysis and molecular mass determination of β-glucosidase from *S. alaskensis* (A) SDS-PAGE analysis of β-glucosidase from *S. alaskensis* at each purification step. *Lane 1*, molecular mass markers; *lane 2*, crude extract; *lane 3*, His-Trap HP column product (purified enzyme). (B) Determination of molecular mass of *S. alaskensis* β-glucosidase by gel filtration chromatography. The reference proteins (●) are ferritin (440 kDa), catalase (232 kDa), aldolase (158 kDa), and conalbumin (75 kDa). The purified β-glucosidase (○) eluted at a volume corresponding to 206 kDa.

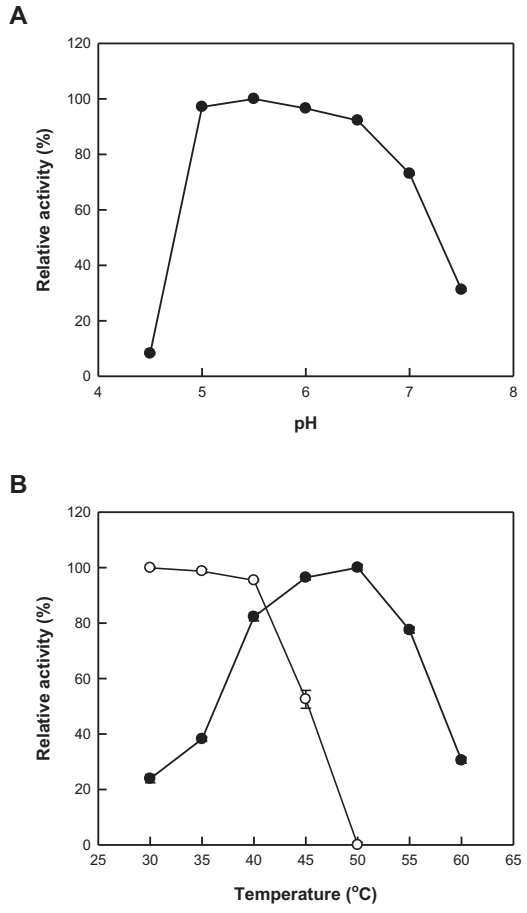

Fig. 2. Effects of pH and temperature on the activity of β-glucosidase from *S.alaskensis*. (a) Effect of pH on the enzyme activity. The reactions were performed with 0.4 mg ml^{-1} ginsenoside Rb_1 and 5.8 U ml^{-1} of enzyme at 40 °C for 20 min. (b) Effect of temperature on the activity (●) and stability (○) of the enzyme. The effect of temperature on the stability for the enzyme was monitored by maintaining the enzyme solutions at each temperature for 30 min. The reactions were performed in 50 mM citrate/phosphate buffer (pH 5.5) containing 0.4 mg ml^{-1} ginsenoside Rb_1 and 5.8 U ml^{-1} of enzyme for 20 min. Data present the means of three experiments and error bars represent standard deviation.

Santa Clara, CA) equipped with a UV detector at a detection wavelength of 203 nm and a C18 column (YMC, Kyoto, Japan). The column was eluted at 37 °C with a linear gradient of acetonitrile/water from 20:80 to 80:20 (v/v) for 80 min at a flow rate of 1 ml min^{-1}. The concentrations of disaccharides were determined using a Bio-LC system (Dionex ICS-3000, Sunnyvale, CA) with an electrochemical detector and a CarboPac PA10 column. The column was eluted at 30 °C with 200 mM sodium hydroxide at a flow rate of 1 ml min^{-1}. The substrates ginsenosides Rb_1, Rb_2, Rc, Rd, and Rg_3 were detected based on retention times of 9.2, 10.6, 9.9, 12.0, and 20.6 min, respectively. The products gypenoside XVII, compound O, compound Mc_1, F_2, and Rh_2 were detected based on the retention times of 13.5, 15.4, 14.4, 17.9, and 28.3 min, respectively (Supplementary Fig. 1). The ginsenosides were identified based on retention times, which were the same as those of the ginsenoside standards.

3. Results and discussion

3.1. Gene cloning, purification, and molecular mass determination of β-glucosidase from S. alaskensis

A gene of 1,344 bp encoding β-glucosidase from *S. alaskensis* was cloned and expressed in *E. coli*. The recombinant enzyme was purified as a soluble protein from crude *E. coli* extracts by His-trap affinity chromatography. β-Glucosidase from *S. alaskensis* was purified with a final purification of 6.3-fold, a yield of 25%, and a specific activity of 233.3 U mg^{-1}. The expressed protein analyzed

Table 1
Substrate specificity of *S. alaskensis* β-glucosidase for aryl-glycosides and disaccharides.

Substrate	Specific activity (U mg^{-1})
*p*NP-β-D-glucopyranoside	309 ± 9.4
*p*NP-α-D-glucopyranoside	0.6 ± 0.01
*o*NP-β-D-glucopyranoside	650 ± 7.8
*p*NP-β-D-galactopyranoside	92 ± 1.5
*p*NP-α-D-galactopyranoside	ND
*p*NP-α-L-arabinopyranose	ND
*p*NP-α-L-arabinofuranose	ND
*p*NP-α-D-mannopyranoside	ND
*p*NP-α-L-rhamnopyranoside	ND
*p*NP-β-D-xylopyranoside	ND
Kojibiose (α-1,2)	5.2 ± 0.1
Maltose (α-1,4)	5.9 ± 0.3
Isomaltose (α-1,6)	5.2 ± 0.1
Sophorose (β-1,2)	230 ± 9.8
Cellobiose (β-1,4)	54 ± 1.3
Gentiobiose (β-1,6)	ND

(): Linkage; ND: not detected.

Table 2
Substrate specificity of *S. alaskensis* β-glucosidase for PPD-type ginsenosides.

Substrate	Product	Specific activity (U mg^{-1})
Rb_1	Gypenoside XVII	233.3 ± 2
Rb_2	Compound O	126.8 ± 1
Rc	Compound Mc_1	113.2 ± 2
Rd	F_2	151.7 ± 4
Rg_3	Rh_2	30.5 ± 1
Gypenoside XVII	–	ND
Compound O	–	ND
Compound Mc_1	–	ND
F_2	–	ND
Rh_2	–	ND

ND: not detected.
Specific activities of other β-glucosidases for the biotransformations of ginsenoside Rb_1, Rb_2, Rc, and Rd to gypenoside XVII, compound O, compound Mc_1, and F_2, respectively, have been not reported yet.

by an SDS-PAGE gel showed a single band with a molecular mass of approximately 51 kDa (Fig. 1a), consistent with the calculated value of 50,903 Da based on the 448 amino acids, which included six histidine residues. The native enzyme was estimated to exist as a tetramer with a molecular mass of 206 kDa, as determined by gel filtration chromatography based on the masses of reference proteins (Fig. 1b).

The amino acid sequence of β-glucosidase from *S. alaskensis* exhibited 73% identity with the characterized ginsenoside-hydrolyzing β-glucosidase from *Sphingomonas* sp. (Wang et al., 2011a,b), which belonged to the glycoside hydrolase (GH) family 1. β-Glucosidase from *Sphingomonas* sp. converted Rb_1, Rb_2, and Rc to gypenoside XVII, compound O, and compound Mc_1, respectively, by hydrolyzing the outer glucose at the C-3 position in PPD-type ginsenosides, which was further converted to F_2 by hydrolyzing the outer glycoside at the C-20 position. Other GH family 1 β-glucosidases from *Pyrococcus furiosus* (Yoo et al., 2011), *Sulfolobus solfataricus* (Noh et al., 2009), and *Sulfolobus acidocaldarius* (Noh and Oh, 2009) reacted with PPD-type ginsenosides by hydrolyzing sugar moieties at the C-3 and C-20 positions linked to APPD.

3.2. Effects of pH and temperature on the activity of β-glucosidase from S. alaskensis

The hydrolytic activity of *S. alaskensis* β-glucosidase for ginsenoside Rb_1 was examined over a pH range from 4.5 to 7.5 at 40 °C (Fig. 2a). Maximum activity was observed at pH 5.5. The effect of temperature on the enzyme activity was investigated at pH 5.5, and maximum activity was recorded at 50 °C (Fig. 2b). At 40 and 55 °C, the activity was approximately 80% of the maximum

Table 3
Specific hydrolysis at the C-3 and C-20 position in PPD-type ginsenosides by ginsenoside-hydrolyzing enzymes.

Cleavage site	Organism	Enzyme	Sugar	Reaction conditions	Substrate	Product	Reference
C-20	*Microbacterium esteraromaticum*	β-Glucosidase	Glucose	pH 7.0, 37 °C	Rb_1	Rd, Rg_3	Quan et al. (2012)
	Paecilomyces bainier	β-Glucosidase	Glucose	pH 3.5, 45 °C	Rb_1	Rd, Rg_3	Yan et al. (2008)
C-3	*Esteya vermicola*	β-Glucosidase	Glucose	pH 5.0, 50 °C	Rb_1 Rd	GypXVII, LXXV F_2, C-K	Hou et al. (2012)
	Terrabacter ginsenosidimutans	Ginsenosidase Type III	Glucose	pH 7.0, 40 °C	Rb_1 Rb_2 Rc Rd Rg_3	GypXVII, LXXV Com-O, Com-Y Com-Mc_1, Com-Mc F_2, C-K Rh_2, APPD	Jin et al. (2012)
C-20 outer	China white jade snail	β-Glucosidase	Glucose	pH 5.6, 50 °C	Rb_1	Rd	Luan et al. (2006)
	Cladosporium fulvum	β-Glucosidase (G I)	Glucose	pH 6.0, 37 °C	Rb_1	Rd	Gao et al. (2010)
	Cladosporium fulvum	β-Glucosidase (G II)	Glucose	pH 5.5, 37 °C	Rb_1	Rd	Zhao et al. (2009)
	Thermus caldophilus	β-Glucosidase	Glucose	pH 5.0, 75 °C	Rb_1	Rd	Son et al. (2008)
	Bifidobacterium breve	α-L-Arabinopyranosidase	Arabinopyranose	pH 5.5, 40 °C	Rb_2	Rd	Shin et al. (2003)
	Bifidobacterium longum	α-L-Arabinopyranosidase	Arabinopyranose	pH 7.0, 37 °C	Rb_2	Rd	Lee et al. (2011)
	Bifidobacterium breve	α-L-Arabinofuranosidase	Arabinofuranose	pH 4.5, 45 °C	Rc	Rd	Shin et al. (2003)
	Bifidobacterium longum	α-L-Arabinofuranosidase	Arabinofuranose	pH 5.5, 37 °C	Rc	Rd	Lee et al. (2011)
	Panax ginseng	α-L-Arabinofuranosidase	Arabinofuranose	pH 5.0, 50 °C	Rc	Rd	Zhang et al. (2002)
	Rhodanobacter ginsenosidimutans	α-L-Arabinofuranosidase	Arabinofuranose	pH 7.5, 37 °C	Rc $C-Mc_1$ C-Mc	Rd F_2 C-K	An et al. (2012)
C-3 outer	*Panax ginseng*	β-Glucosidase	Glucose	pH 5.0, 60 °C	Rg_3	Rh_2	Zhang et al. (2001)
	Fusarium proliferatum	β-Glucosidase	Glucose	pH 5.0, 50 °C	Rg_3	Rh_2	Su et al. (2006)
	Sphingopyxis alaskensis	β-Glucosidase	Glucose	pH 5.5, 40 °C	Rb_1 Rb_2 Rc Rd Rg_3	GypXVII Com-O Com-Mc_1 F_2 Rh_2	This study

Gyp: gypenoside, Com-: compound.

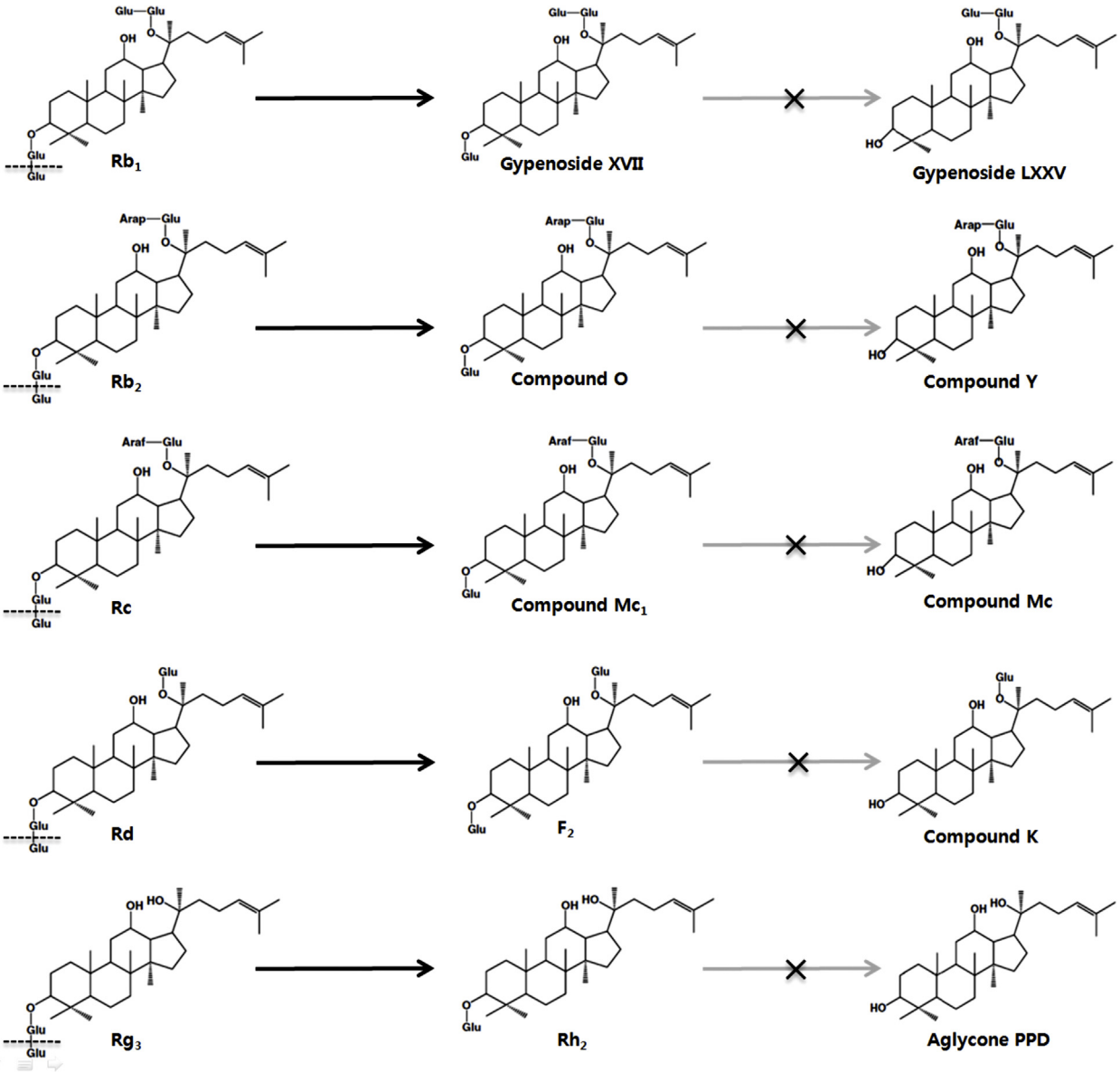

Fig. 3. Schematic representation of the hydrolysis pathway of PPD-type ginsenosides by β-glucosidase from *S. alaskensis*.

activity. The effect of temperature on enzyme stability was investigated by varying the temperature from 30 to 50 °C at a constant pH of 5.5. The enzyme activity was determined after incubating the solution at each temperature for 30 min. The enzyme activity was almost the same within 40 °C. However, above 40 °C, the activity significantly decreased and then was completely lost at 50 °C. Thus, the optimum temperature was found to be 40 °C, and all subsequent experiments were performed at pH 5.5 and 40 °C.

3.3. Substrate specificity of β-glucosidase from S. alaskensis

The substrate specificity of β-glucosidase from *S. alaskensis* was investigated using aryl-glycoside, disaccharides, and ginsenosides. The specific activity for aryl-glycosides and disaccharides as substrates was in the following order: *o*NP-β-D-glucopyranoside > *p*NP-β-D-glucopyranoside > *p*NP-β-D-galactopyranoside > *p*NP-α-D-glucopyranoside and sophorose > cellobiose > maltose > kojibiose > isomaltose, respectively (Table 1). However, no activity was found for *p*NP-α-D-galactopyranoside, *p*NP-α-L-arabinofuranose, *p*NP-α-D-mannopyranoside, *p*NP-α-L-rhamnopyranoside, and *p*NP-β-D-xylopyranoside, and gentiobiose. β-Glucosidase from *S. alaskensis* acted on diverse linkages in glucosides, including β-1,2, β-1,4, α-1,2, α-1,4, α-1,6 linkages. The hydrolytic activities of other β-glucosidases from *Microbacterium esteraromaticum* (Quan et al., 2012), *Paecilomyces bainier* (Yan et al., 2008), *E. vermicola* (Hou et al., 2012), China white jade snail (Luan et al., 2006), *Cladosporium fulvum* (Gao et al., 2010), and *P. ginseng* (Zhang et al., 2001) for aryl-glycosides as substrates were the highest for *p*NP-β-D-glucopyranoside (β-1,4 linkage), whereas that of β-glucosidase from *S. alaskensis* was the highest for *o*NP-β-D-glucopyranoside (β-1,2 linkage). The hydrolytic activity of β-glucosidases from *P. bainier* (Yan et al., 2008), China white jade snail (Luan et al., 2006), and *C. fulvum* (Gao et al., 2010) for disaccharides were the highest for the β-1,6 linked disaccharide gentiobiose. β-Glucosidase from *S. alaskensis* exhibited the highest hydrolytic activity for the β-1,2 linked disaccharide sophorose, but no activity for β-1,6-linked glucosides and other glycosides, including arabinofuranose, arabinopyranose, mannose, rhamnose, and xylose.

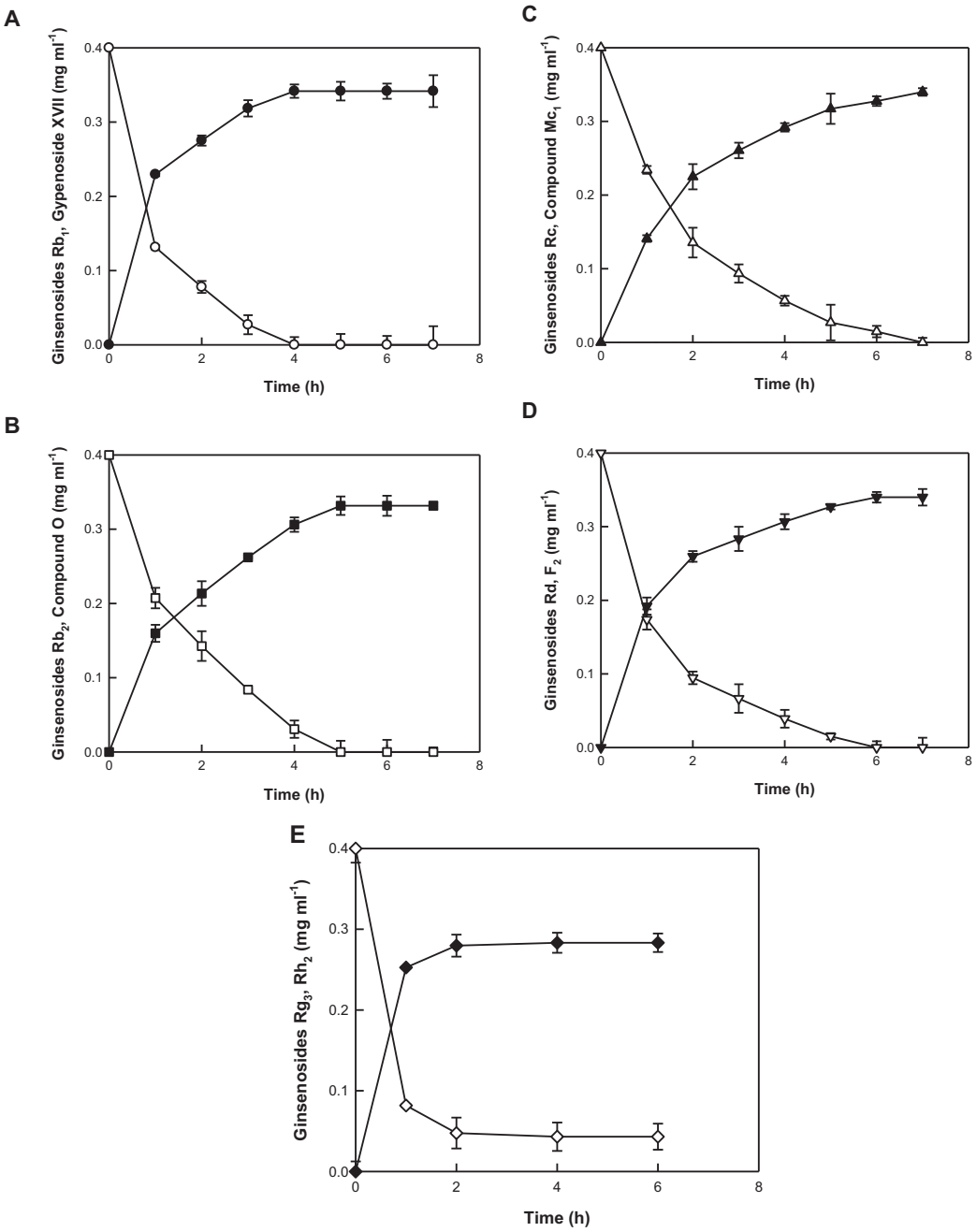

Fig. 4. Biotransformation of major ginsenosides to rare ginsenosides by β-glucosidase from *S. alaskensis*. (A) Biotransformation of ginsenoside Rb_1 (○) to gypenoside XVII (●). (B) Biotransformation of ginsenoside Rb_2 (□) to compound O (■). (C) Biotransformation of ginsenoside Rc (△) to compound Mc_1 (▲). (D) Biotransformation of ginsenoside Rc (▽) to F_2 (▼). (E) Biotransformation of ginsenoside Rg_3 (◊) to Rh_2 (♦). The reactions were performed in 50 mM citrate/phosphate buffer (pH 5.5) containing 0.4 mg ml^{-1} ginsenoside and 5.8 U ml^{-1} of enzyme at 40 °C for 7 h. Data represent the means of three experiments and error bars represent standard deviation.

The specific activity for the PPD-type ginsenosides as substrates was in the order $Rb_1 > Rb_2 > Rc > Rd > Rg_3$ and the products of which were gypenoside XVII, compound O, compound Mc_1, F_2, and Rh_2, respectively (Table 2). The enzyme hydrolyzed only the outer glucose linked to the C-3 position of ginsenosides Rb_1, Rb_2, Rc, Rd, and Rg_3 with a β-1,2 linkage. No activity was observed for gypenoside XVII, compound O, compound Mc_1, F_2, and Rh_2. These results indicate that *S. alaskensis* β-glucosidase hydrolyzes only the outer glucose linked to the C-3 position in PPD-type ginsenosides with a β-1,2 linkage, but the enzyme does not hydrolyze the inner glucose linked to the C-3 position and the outer glycoside (glucose, arabinopyranose, or arabinofuranose) linked to glucose at the C-20 position with a β-1,6 linkage.

The specific hydrolysis at the C-3 and C-20 positions in PPD-type ginsenosides by ginsenoside-hydrolyzing enzymes is summarized in Table 3. β-Glucosidases from *M. esteraromaticum* (Quan et al., 2012) and *Paecilomyces bainer* (Yan et al., 2008) hydrolyzed the inner glucose and the outer glycoside (glucose, arabinopyranose, or arabinofuranose) at the C-20 position in PPD-type ginsenosides. Ginsenosidase Type III from *T. ginsenosidimutans* (Jin et al., 2012) and β-glucosidase from *E. vermicola* (Hou et al., 2012) hydrolyzed the inner and outer glucose at the C-3 position. β-Glucosidases from China white jade snail (Luan et al., 2006), *C. fulvum* (Gao et al., 2010; Zhao et al., 2009), and *Thermus caldophilus* (Son et al., 2008); α-L-arabinopyranosidases from *Bifidobacterium breve* (Shin et al., 2003) and *Bifidobacterium longum* (Lee et al., 2011); and

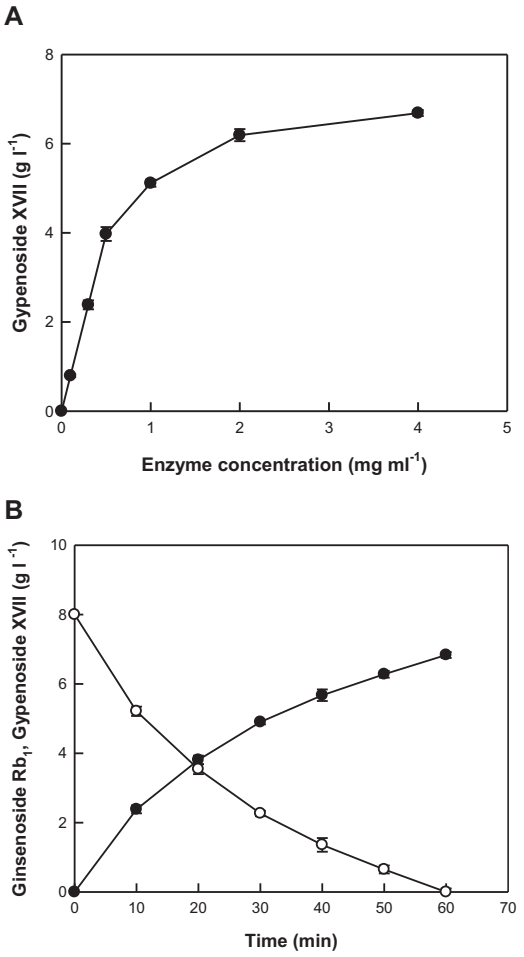

Fig. 5. Production of gypenoside XVII from ginsenoside Rb_1 by β-glucosidase from *S. alaskensis*. (A) Effect of enzyme concentration on gypenoside XVII production from ginsenoside Rb_1. The reactions were performed in 50 mM citrate/phosphate buffer (pH 5.5) containing 8 g l^{-1} ginsenoside Rb_1 at 40 °C for 20 min. (B) Production of gypenoside XVII from ginsenoside Rb_1. The reactions were performed in 50 mM citrate/phosphate buffer (pH 5.5) containing 8 g l^{-1} ginsenoside Rb_1, and 0.5 mg ml^{-1} (116.7 U ml^{-1}) of enzyme at 40 °C for 60 min. Data represent the means of three experiments and error bars represent standard deviation.

α-L-arabinofuranosidases from *B. breve* (Shin et al., 2003), *B. longum* (Lee et al., 2011), *Rhodanobacter ginsenosidimutans* (An et al., 2012), and *P. ginseng* (Zhang et al., 2002) hydrolyzed the outer glucose, arabinopyranose, and arabinofuranose at the C-20 position, respectively. β-Glucosidases from *P. ginseng* (Zhang et al., 2001) and *F. proliferatum* (Su et al., 2006) converted Rg_3 to Rh_2 by hydrolyzing outer glucose at the C-3 position. However, the conversion of these enzymes for other ginsenosides has not been attempted. β-Glucosidase from *S. alaskensis* converted not only Rg_3 to Rh_2 but also Rb_1, Rb_2, Rc, and Rd to gypenoside XVII, compound O, compound Mc_1, and F_2, respectively, by hydrolyzing only the outer glucose at the C-3 position (Fig. 3).

3.4. Biotransformation of major ginsenosides by β-glucosidase from S. alaskensis

The hydrolytic reactions of the major ginsenosides Rb_1, Rb_2, Rc, Rd, and Rg_3 to rare ginsenosides by β-glucosidase from *S. alaskensis* were performed with 0.4 mg ml^{-1} substrate. The enzyme completely converted ginsenosides Rb_1, Rb_2, Rc, and Rd to gypenoside XVII, compound O, compound Mc_1, and F_2, respectively, within 4, 5, 7, and 6 h, respectively (Fig. 4a–d). Ginsenoside Rg_3 was converted to Rh_2 after 2 h, with a molar conversion yield of 89% (Fig. 4e). Subsequently, the production of Rh_2 reached a plateau. The conversion yield of Rg_3 to Rh_2 by this enzyme was 29% higher than those by other β-glucosidases from *P. ginseng* (Zhang et al., 2001) and *F. proliferatum* (Su et al., 2006).

3.5. Production of gypenoside XVII from ginsenoside Rb_1 by β-glucosidase from S. alaskensis

The production of gypenoside XVII from ginsenoside Rb_1 was performed because the specific activity of β-glucosidase from *S. alaskensis* for Rb_1 was the highest among the PPD-type ginsenosides. The effects of enzyme concentration on gypenoside XVII production were investigated with 8 g l^{-1} ginsenoside Rb_1 as the substrate by varying the enzyme concentration from 0.1 (23.3) to 4 mg ml^{-1} (933.2 U ml^{-1}) after 20 min. Gypenoside XVII production increased with increasing the enzyme concentrations. Up to 0.5 mg ml^{-1} (116.7 U ml^{-1}) enzyme, gypenoside XVII production per enzyme concentration used was constant. However, above 0.5 mg ml^{-1}, gypenoside XVII production per enzyme concentration decreased (Fig. 5a), indicating that the optimum concentration of the enzyme was 0.5 mg ml^{-1}. The production of gypenoside XVII from ginsenoside Rb_1 was assessed with 0.5 mg ml^{-1} enzyme for 1 h by varying the concentration of ginsenoside Rb_1 from 0.4 to 8.0 g l^{-1}. Regardless of the substrate concentration used, ginsenoside Rb_1 was completely converted to gypenoside XVII (data not shown). Thus, the time course reaction for the production of gypenoside XVII from ginsenoside Rb_1 by *S. alaskensis* β-glucosidase was performed with 0.5 mg ml^{-1} enzyme and 8.0 g l^{-1} ginsenoside Rb_1 for 1 h (Fig. 5b). Gypenoside XVII has been found as only an intermediate during the production of gypenoside LXXV from ginsenoside Rb_1 by β-glucosidases from *T. ginsenosidimutans* (Jin et al., 2012) and *E. vermicola* (Hou et al., 2012). In the present study, β-glucosidase from *S. alaskensis* produced 6.8 g l^{-1} gypenoside XVII as a final product from 8 g l^{-1} ginsenoside Rb_1 after 1 h, with a molar conversion yield of 100% and a productivity of 6.8 g l^{-1} h^{-1}. To the best of our knowledge, this is the first quantitative production of the rare ginsenosides gypenoside XVII, compound O, compound Mc_1, and F_2.

4. Conclusions

β-Glucosidase from *S. alaskensis* hydrolyzed only the outer glucose at C-3 in PPD-type ginsenosides, but not the inner glucose at the C-3 position and the outer glycoside (glucose, arabinopyranose, or arabinofuranose) linked to glucose at the C-20 position. Because of the narrow substrate specificity, the enzyme completely hydrolyzed ginsenosides Rb_1, Rb_2, Rc, and Rd to gypenoside XVII, compound O, compound Mc_1 and F_2 as final products, respectively. Ginsenoside Rg_3 was converted to Rh_2 as a final product, with a molar conversion yield of 89%. These results suggest that this enzyme is a potential producer of the rare ginsenosides gypenoside XVII, compound O, compound Mc_1, F_2, and Rh_2.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.jbiotec.2013.11.026.

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