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Production of Ginsenoside Rd from Ginsenoside Rc by α-L-Arabinofuranosidase from *Caldicellulosiruptor saccharolyticus*

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Ginsenoside Rd was produced from ginsenoside Rc using a thermostable recombinant α-L-arabinofuranosidase from *Caldicellulosiruptor saccharolyticus*. The optimal reaction conditions for the production of ginsenoside Rd from Rc were pH 5.5, 80°C, 227 U enzyme/ml, and 8.0 g/l ginsenoside Rc in the presence of 30% (v/v) *n*-hexane. Under these conditions, the enzyme produced 7.0 g/l ginsenoside Rd after 30 min, with a molar yield of 100% and a productivity of 14 g l^{-1} h^{-1}. The conversion yield and productivity of ginsenoside Rd are the highest reported thus far among enzymatic transformations.

Key words: Ginsenoside Rd, ginsenoside Rc, biotransformation, α-L-arabinofuranosidase, *Caldicellulosiruptor saccharolyticus*

Ginseng ginsenosides are used as traditional medicine in Asian countries to reinforce diverse pharmacological activities, such as antifatigue [13], antiallergenic [2], and antioxidant [3] activities. Ginsenoside Rd is produced by the hydrolysis of sugar moieties from the major ginsenosides (Rb_1, Rb_2, and Rc) and exhibits neuroprotective [14], anti-inflammatory [12], and anticancer activities [6].

Ginsenoside Rd-producing activity from ginsenoside Rc by α-L-arabinofuranosidase has been reported in *Bifidobacterium longum* [5], *Bifidobacterium breve* [9], and *Rhodanobacter ginsenosidimutans* [1]. Additionally, ginsenoside Rd-producing activity from ginsenoside Rb_2 by α-L-arabinopyranosidase has also been demonstrated in *B. longum* and *B. breve* [9]. However, the quantitative production of ginsenoside Rd from ginsenoside Rc or Rb_2 has not yet been reported. The production of ginsenoside Rd from Rb_1 has been performed by β-glucosidases from *Cladosporium fulvum* [15] and *Thermus caldophilus* [11]. However, the conversion yield and productivity of these enzymes are low for the effective production of ginsenoside Rd.

In this study, the production of ginsenoside Rd with a high conversion yield and productivity was attempted by the hydrolysis of the α-L-arabinofuranose residue linked at the 20-C in ginsenoside Rc using the recombinant α-L-arabinofuranosidase from *Caldicellulosiruptor saccharolyticus*, which exhibited hydrolytic activity for the arabinofuranose, but not for the arabinopyranose moiety in ginsenosides.

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Materials and Methods

Preparation of Ginsenosides

Reagent-grade ginsenosides Rb_1, Rb_2, and Rc were purchased from Ambo Institute (Daejeon, Korea). Ginsenoside compound Mc was prepared from the conversion of reagent-grade ginsenoside Rc by β-glucosidase from *Sulfolobus acidocaldarius* [8]. The reaction solution was eluted with a mixture of acetonitrile and water [3:7 (v/v)] using an ODS column (YMC, Kyoto, Japan). Compound Mc was purified from the eluted fraction and then used as a substrate.

Bacterial Strains, Plasmid, and Culture Conditions

Genomic DNA from *C. saccharolyticus* DSM 8903, *Escherichia coli* ER2566, and plasmid pTrc99A were used as the source of the α-L-arabinofuranosidase gene, host cells, and expression vector, respectively. *C. saccharolyticus* was cultivated in "*Caldicellulosiruptor*" medium (DSM Media Formulation No. 640) and grown at 70°C under anaerobic conditions with 100% N_2 gas in a 3-L anaerobic jar (Difco, Sparks, MD, USA) for 5 days. The recombinant *E. coli* for protein expression was cultivated with shaking at 200 rpm in a 2,000 ml flask containing 500 ml of Luria–Bertani (LB) medium at 37°C with 20 μg/ml of kanamycin until the OD_{600} reached 0.8. Isopropyl-β-D-thiogalactopyranoside (IPTG) was added to a final concentration of 0.1 mM to induce enzyme expression. The culture was grown at 16°C with shaking at 150 rpm for 16 h.

Gene Cloning and Expression, and Enzyme Purification

The methods of gene cloning and expression, and enzyme purification were performed as described previously [7].

Hydrolytic Activity

One unit (U) of enzyme activity was defined as the amount of enzyme required to liberate 1 nmol of ginsenoside Rd from ginsenoside Rc as a substrate per minute at 85°C and pH 5.5. One unit corresponded to 0.057 μg of protein. The hydrolytic reactions of α-L-arabinofuranosidase from *C. saccharolyticus* were performed at 85°C in the presence of 30% *n*-hexane for 10 min in 50 mM citrate/phosphate buffer (pH 5.5) containing 0.4 mg/ml ginsenoside (Rb_1, Rb_2, Rc, or compound Mc) and 15 U/ml enzyme, or containing 1 mM aryl-glycoside and 3.8 U/l enzyme. The nitrophenyl-glycoside activity was determined by the increase in absorbance at 420 nm due to the release of nitrophenol, and the ginsenoside activity was measured by the increase of the ginsenoside product.

Effects of pH, Temperature, and Solvent

To evaluate the effects of pH and temperature on α-L-arabinofuranosidase activity, the pH values were varied from 4.5 to 7.0 at 85°C, and the temperatures were varied from 70°C to 95°C at pH 5.5 in 50 mM citrate/phosphate buffer containing 0.4 mg ml^{-1} ginsenoside Rc and 15 U/ml enzyme. The effect of temperature on enzyme stability was monitored as a function of incubation time by applying the enzyme solution at four different temperatures (70°C, 75°C, 80°C, and 85°C) in 50 mM citrate/phosphate buffer (pH 5.5). Samples were withdrawn at time intervals and then assayed in 50 mM citrate/phosphate buffer (pH 5.5) containing 0.4 mg/ml ginsenoside Rc and 15 U/ml enzyme at 85°C for 10 min. The half-life of the enzyme was calculated using Sigma Plot 9.0 software (Systat Software, San Jose, CA, USA). The effect of solvent on the activity of α-L-arabinofuranosidase from *C. saccharolyticus* was evaluated using DMSO, ethanol, methanol, acetone, 3-methyl-1-butanol, *n*-hexane, and toluene. The reactions were performed at 85°C for 10 min in 50 mM citrate/phosphate buffer (pH 5.5) containing 0.4 mg/ml ginsenoside Rc, 15 U/ml enzyme, and 5% (v/v) or 10% (v/v) solvent. The effect of *n*-hexane concentration on ginsenoside Rd acid production was investigated by varying its concentration from 0% to 50% (v/v). The reactions were performed at 85°C for 10 min in 50 mM citrate/phosphate buffer (pH 5.5) containing 0.4 mg/ml ginsenoside Rc, 15 U/ml enzyme, and *n*-hexane.

Production of Ginsenoside Rd from Ginsenoside Rc by α-L-Arabinofuranosidase from *C. saccharolyticus*

To evaluate the effect of the amount of enzyme on the production of ginsenoside Rd, the reactions were performed in 50 mM citrate/phosphate buffer (pH 5.5) containing 4 g/l ginsenoside Rc and 30% (v/v) *n*-hexane at 80°C for 10 min by varying the enzyme amount from 76 to 379 U/ml.

The time courses for the production of ginsenoside Rd from ginsenoside Rc by α-L-arabinofuranosidase from *C. saccharolyticus* were investigated. The reactions were performed at 80°C in 50 mM citrate/phosphate buffer (pH 5.5) containing 8 g/l ginsenoside Rc, 227 U/ml enzyme, and 30% (v/v) *n*-hexane for 30 min.

Analytical Methods

Digoxin as an internal standard was added to the samples and then extracted with an identical volume of *n*-butanol for 1 min *via* vortexing. After centrifugation at 13,000 ×*g* for 5 min, the upper organic phase as a *n*-butanol fraction was transferred into a clean tube and evaporated to dryness in the centrifugal evaporator (Eyela CVE-3100; Tokyo, Japan). The residue was then reconstituted by methanol for HPLC analysis [4]. Ginsenosides were assayed using an HPLC system (Agilent 1100; Santa Clara, CA, USA) equipped with a UV detector at 203 nm using a C18 column (250×4.6 mm; YMC, Kyoto, Japan). The column was initially eluted with a mixture of acetonitrile and water of 20:80 (v/v) as the mobile phase, a gradient of 40:60 for 40 min, and then a gradient of 80:20 for 80 min. The flow rate was 1.0 ml/min and the column temperature was 37°C.

Results and Discussion

Effects of pH and Temperature on the Activity of *C. saccharolyticus* α-L-Arabinofuranosidase for Ginsenoside Rd Production

A gene encoding a putative α-L-arabinofuranosidase from *C. saccharolyticus*, with the same sequence as the gene reported in GenBank (Accession No. CP000679), was cloned and expressed in *E. coli.* The enzyme was purified from crude extract obtained from harvested cells as a soluble protein by heat treatment and Hi-trap Q HP anion-exchange chromatography with a purification of 25.6-fold, a yield of 11%, and a specific activity of 75.7 U/mg for ginsenoside Rc. The molecular mass of the expressed protein analyzed by SDS-PAGE was approximately 58 kDa, consistent with the calculated value of 57,882 Da based on the 505 amino acid residues [7]. The native enzyme existed as an octamer with a molecular mass of 460 kDa, as determined by Sephacryl S-300 gel filtration chromatography (data not shown).

No ginsenosides were formed when the reactions were performed in the absence of enzyme or in the presence of *E. coli* cells, which lack the α-L-arabinofuranosidase gene from *C. saccharolyticus*. The maximal hydrolytic activity of the recombinant α-L-arabinofuranosidase from *C. saccharolyticus* for the production of ginsenoside Rd from ginsenoside Rc was observed at pH 5.5 and 85°C (Fig. 1). The hydrolytic activities of α-L-arabinofuranosidases from *B. longum*, [5] *B. breve*, [9] and *R. ginsenosidimutans* [1] for ginsenoside Rc were maximal at pH 4.7 and 57°C, pH 4.5 and 45°C, and pH 7.5 and 37°C, respectively. The ginsenoside Rd-producing activity of β-glucosidase from *T. caldophilus* from ginsenoside Rb_1 was maximal at 90°C [11].

Thermal inactivation of α-L-arabinofuranosidase from *C. saccharolyticus* followed first-order kinetics with half-lives of 130, 41, 2.5, and 0.12 h at 70°C, 75°C, 80°C, and 85°C, respectively (Fig. 2). The half-lives of β-glucosidase from *T. caldophilus* at these temperature were 12.5, 7.1, 4.9, and 2.6 h, respectively [11].

Effect of Solvent on the Activity of *C. saccharolyticus* α-L-Arabinofuranosidase for Ginsenoside Rd Production

The effect of organic solvent on the hydrolytic activity for ginsenoside Rd production by α-L-arabinofuranosidase from *C. saccharolyticus* was examined at concentrations of 5% and 10% (v/v). Among the solvents tested, *n*-hexane

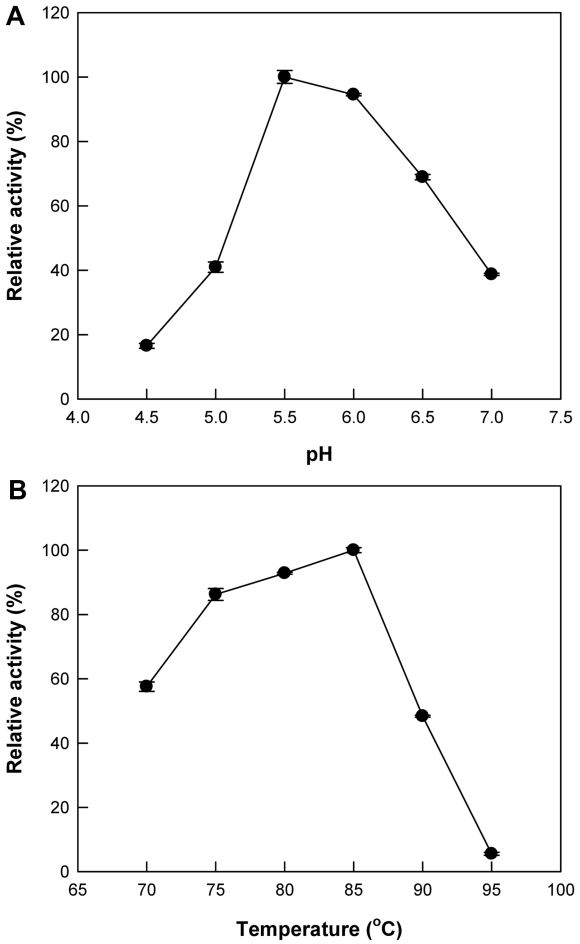

Fig. 1. Effects of pH and temperature on the activity of α-L-arabinofuranosidase from *C. saccharolyticus* for ginsenoside Rc.
(**A**) Effect of pH on the activity of α-L-arabinofuranosidase from *C. saccharolyticus*. (**B**) Effect of temperature on the activity of α-L-arabinofuranosidase from *C. saccharolyticus*. At the relative activity of 100%, 0.06 mg/ml (0.064 mM) ginsenoside Rd was produced.

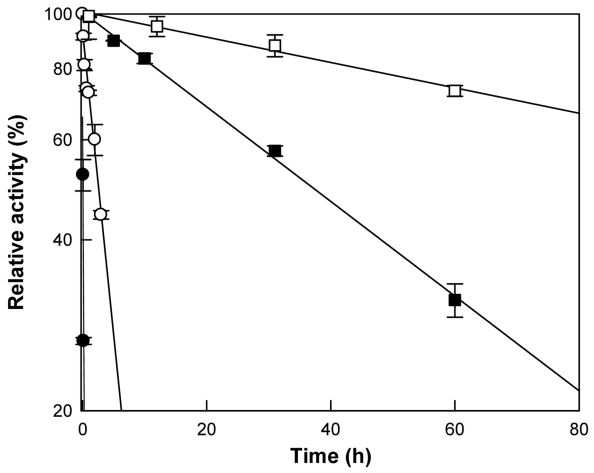

Fig. 2. Thermal inactivation of the activity of α-L-arabinofuranosidase from *C. saccharolyticus* at temperatures of 70°C (□), 75°C (■), 80°C (○), and 85°C (●) for varying time periods.

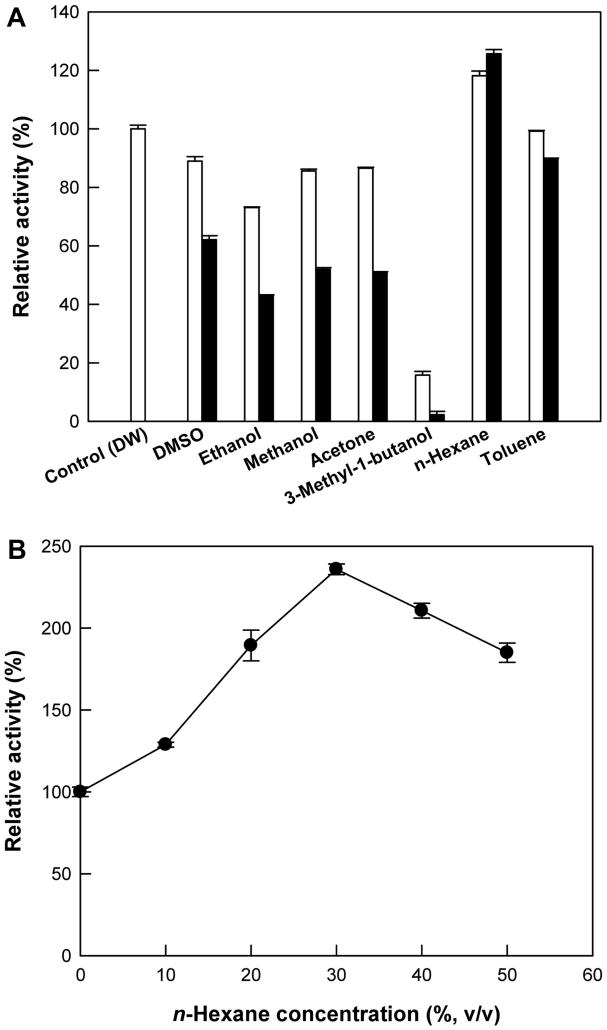

Fig. 3. Effect of solvent on the activity of α-L-arabinofuranosidase from *C. saccharolyticus* for ginsenoside Rc.
(**A**) Effect of 5% (v/v) (*empty bar*) or 10% (v/v) (*filled bar*) solvent. (**B**) Effect of *n*-hexane concentration. At the relative activity of 100%, 0.06 mg/ml (0.064 mM) ginsenoside Rd was produced. Data present the means of three experiments and error bars represent standard deviation.

was the most effective solvent for the hydrolysis of ginsenoside Rc (Fig. 3A). Maximum enzyme activity occurred at 30% (v/v) *n*-hexane (Fig. 3B). *n*-Hexane, a water-immiscible and nonpolar solvent, is a proper solvent for ginsenoside Rc hydrolysis, because the conformation of enzyme is relatively stable in a water-immiscible and less-polar solvent [10].

Substrate Specificity of *C. saccharolyticus* α-L-Arabinofuranosidase for Aryl-Glycosides and Ginsenosides

α-L-Arabinofuranosidase from *C. saccharolyticus* exhibited hydrolytic activity for *p*-nitrophenyl(NP)-α-L-arabinofuranoside, and ginsenosides Rc and compound Mc, which contained α-L-arabinofuranoside at the 20-C in the ginsenosides.

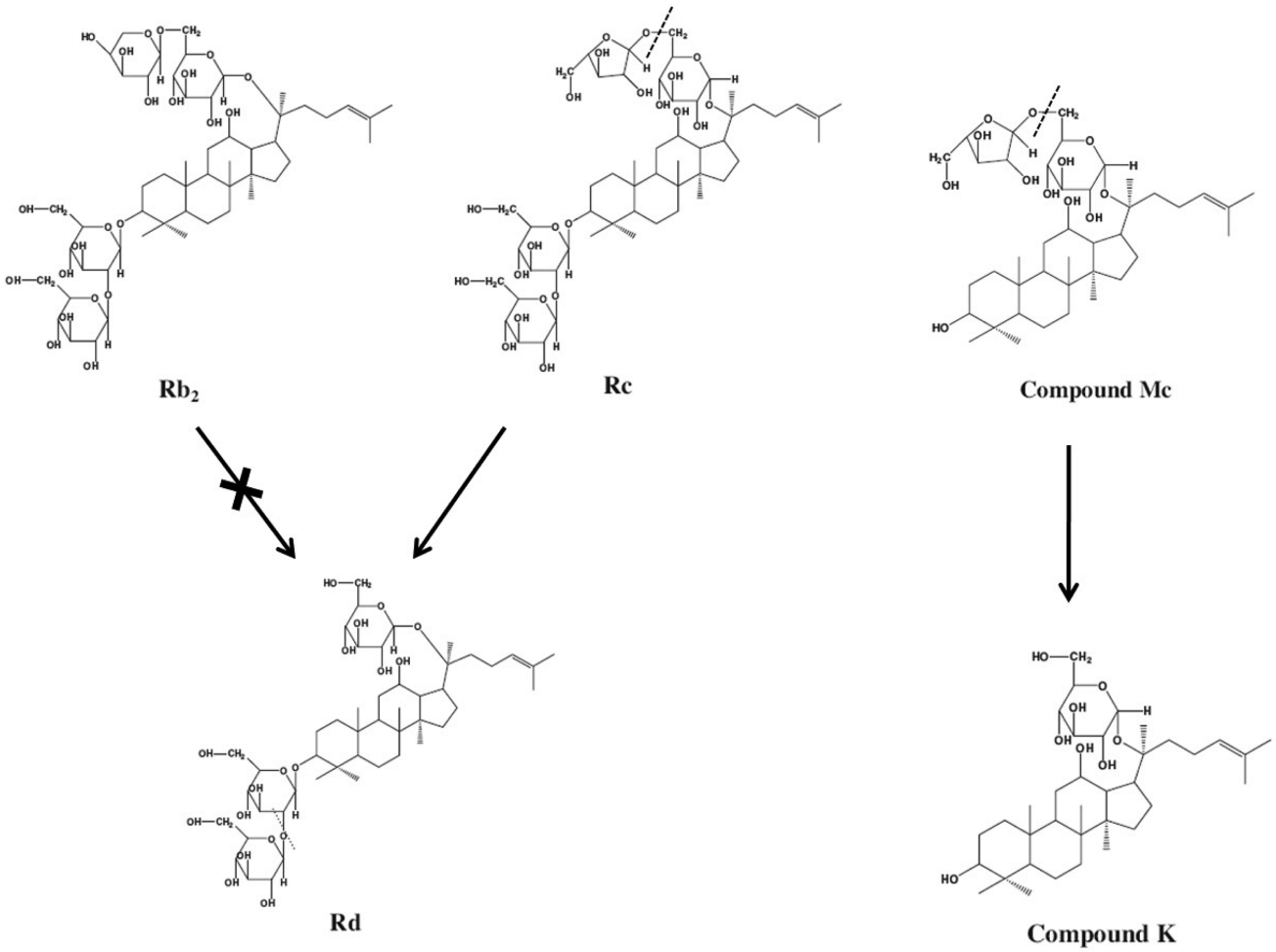

Fig. 4. Biotransformations to ginsenoside Rd and compound K from ginsenoside Rc and compound Mc, respectively, by α-L-arabinofuranosidase from *C. saccharolyticus*.

Based on the analytical methods used in this study, activity was not detected for *p*NP-α-L-arabinopyranoside, *p*NP-α-D-glucopyranoside, *p*NP-α-D-galactopyranoside, *p*NP-β-L-arabinofuranoside, *p*NP-β-L-arabinopyranoside *p*NP-β-D-glucopyranoside, *p*NP-β-D-galactopyranoside, *p*NP-β-D-fucopyranoside, *p*NP-β-D-xylopyranoside, ginsenoside Rb_2, which contained α-L-arabinopyranoside at the 20-C, or ginsenoside Rb_1, which contained β-D-glucopyranoside

Table 1. Enzymatic conversion of ginsenoside Rb_1, Rb_2, or Rc to ginsenoside Rd.

Substrate	Organism	Enzyme	Product (g/l)	Molar yield (%)	Productivity (g l^{-1} h^{-1})	Reference
Ginsenoside Rb_1	*Cladosporium fulvum*	β-Glucosidase	0.19	86	0.001	[15]
	Thermus caldophilus	β-Glucosidase	0.70	80	1.6	[11]
Ginsenoside Rb_2	*Bifidobacterium longum*	α-L-Arabinopyranosidase				[5]
	Bifidobacterium breve	α-L-Arabinopyranosidase				[9]
Ginsenoside Rc	*Bifidobacterium longum*	α-L-Arabinofuranosidase				[5]
	Bifidobacterium breve	α-L-Arabinofuranosidase				[9]
	Rhodanobacter ginsenosidimutans	α-L-Arabinofuranosidase				[1]
	Caldicellulosiruptor saccharolyticus	α-L-Arabinofuranosidase	7.0	100	14	This study

at the 20-C. Thus, the enzyme was only active for the α-linked arabinofuranose in ginsenosides and aryl-glycosides. The hydrolytic activity for compound Mc was higher than ginsenoside Rc. Thus, α-L-arabinofuranosidase from *C. saccharolyticus* converted ginsenoside Rc and compound Mc to ginsenoside Rd and compound K, respectively, but did not convert ginsenoside Rb_2 to ginsenoside Rd (Fig. 4).

Production of Ginsenoside Rd from Ginsenoside Rc by α-L-Arabinofuranosidase from *C. saccharolyticus*

The optimum temperature for the production of ginsenoside Rd by α-L-arabinofuranosidase from *C. saccharolyticus* was 80°C, because the half-life of the enzyme at 85°C was less than the reaction time. The effect of enzyme concentration on the production of ginsenoside Rd was investigated at 80°C. The reactions were performed with 4 g/l ginsenoside Rc as a substrate by varying the enzyme concentration from 76 to 379 U/ml (Fig. 5A). The production of ginsenoside Rd increased with increasing concentrations of the enzyme; however, the production of ginsenoside Rd per the enzyme concentration decreased at concentrations above 227 U/ml that corresponded to 0.013 mg/ml enzyme. The production of ginsenoside Rd from ginsenoside Rc was assessed with 227 U/ml enzyme for 30 min by varying the concentration of ginsenoside Rc from 0.4 to 8.0 g/l. Regardless of the substrate concentration, ginsenoside Rc was completely converted to ginsenoside Rd (data not shown). Thus, the optimal reaction conditions for the production of ginsenoside Rd from ginsenoside Rc by α-L-arabinofuranosidase from *C. saccharolyticus* were pH 5.5, 80°C, 227 U/ml enzyme, and 8.0 g/l ginsenoside Rc in the presence of 30% (v/v) *n*-hexane. Under these conditions, the enzyme produced 7.0 g/l ginsenoside Rd after 30 min, with a molar yield of 100% and a productivity of 14 g l^{-1} h^{-1} (Fig. 5B).

Although ginsenoside Rd-producing activity from ginsenosides Rb_2 and Rc has been reported, the quantitative production of ginsenoside Rd from ginsenosides Rb_2 and Rc using enzyme has not been attempted. The productivity of ginsenoside Rd from ginsenoside Rc by α-L-arabinofuranosidase from *C. saccharolyticus* was 9- and 14,000-fold higher than those from ginsenoside Rb_1 by β-glucosidases from *C. fulvum* [15] and *T. caldophilus* [11], respectively (Table 1). Moreover, *C. saccharolyticus* α-L-arabinofuranosidase completely hydrolyzed ginsenoside Rc to ginsenoside Rd, whereas the previously reported enzymes did not completely hydrolyze ginsenosides Rb_1 and Rc to ginsenoside Rd. These results suggest that α-L-arabinofuranosidase from *C. saccharolyticus* is an effective ginsenoside Rd producer.

In conclusion, the production of ginsenoside Rd from ginsenoside Rc was optimized by the recombinant α-L-arabinofuranosidase from *C. saccharolyticus*. Under optimum conditions, the enzyme completely converted ginsenoside Rc to ginsenoside Rd with the highest productivity ever reported. Thus, α-L-arabinofuranosidase from *C. saccharolyticus* is an effective enzyme for the production of ginsenoside Rd using ginsenoside Rc as a substrate.

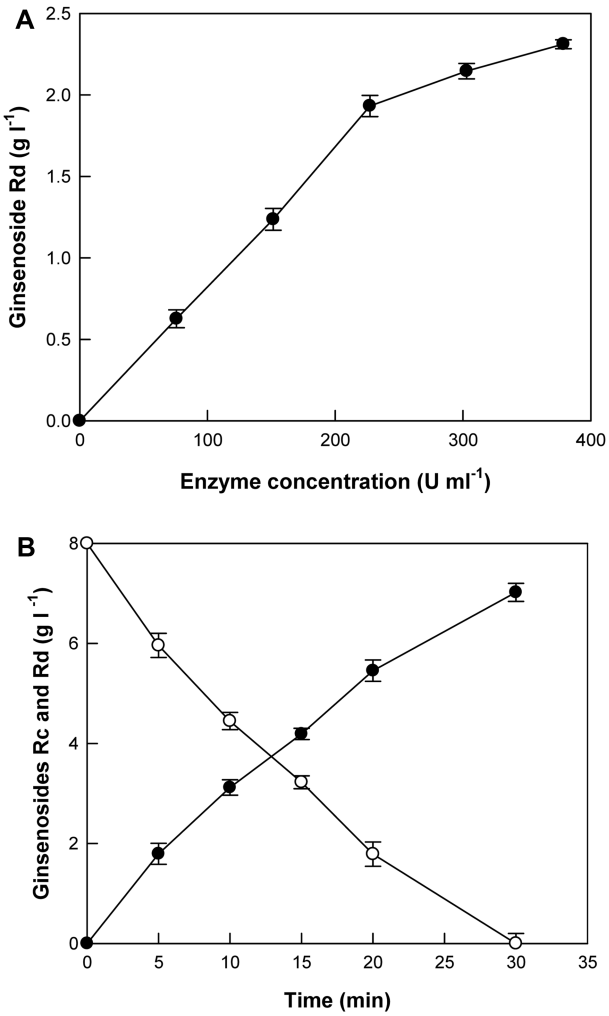

Fig. 5. Effect of enzyme concentration on ginsenoside Rd production from ginsenoside Rc by α-L-arabinofuranosidase from *C. saccharolyticus*.
(**A**) Effect of enzyme concentration on ginsenoside Rd production. (**B**) Ginsenoside Rd (●) production from ginsenoside Rc (○) under the optimum conditions. Data represent the means of three experiments and error bars represent standard deviation.

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