

# Complete Biotransformation of Protopanaxatriol-Type Ginsenosides in *Panax ginseng* Leaf Extract to Aglycon Protopanaxatriol by $\beta$ -Glycosidases from *Dictyoglomus turgidum* and *Pyrococcus furiosus*<sup>S</sup>

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Aglycon protopanaxatriol (APPT) has valuable pharmacological effects such as memory enhancement and tumor inhibition.  $\beta$ -Glycosidase from the hyperthermophilic bacterium *Dictyoglomus turgidum* (DT-bgl) hydrolyzes the glucose residues linked to APPT, but not other glycoside residues.  $\beta$ -Glycosidase from the hyperthermophilic bacterium *Pyrococcus furiosus* (PF-bgl) hydrolyzes the outer sugar at C-6 but not the inner glucose at C-6 or the glucose at C-20. Thus, the combined use of DT-bgl and PF-bgl is expected to increase the biotransformation of PPT-type ginsenosides to APPT. We optimized the ratio of PF-bgl to DT-bgl, the concentrations of substrate and enzyme, and the reaction time to increase the biotransformation of ginsenoside Re and PPT-type ginsenosides in *Panax ginseng* leaf extract to APPT. DT-bgl combined with PF-bgl converted 1.0 mg/ml PPT-type ginsenosides in ginseng leaf extract to 0.58 mg/ml APPT without other ginsenosides, with a molar conversion of 100%. We achieved the complete biotransformation of ginsenoside Re and PPT-type ginsenosides in ginseng leaf extract to APPT by the combined use of two  $\beta$ -glycosidases, suggesting that discarded ginseng leaves can be used as a source of the valuable ginsenoside APPT. To the best of our knowledge, this is the first quantitative production of APPT using ginsenoside Re, and we report the highest concentration and productivity of APPT from ginseng extract to date.

**Keywords:** Aglycon protopanaxatriol, *Dictyoglomus turgidum*,  $\beta$ -glycosidase, *Panax ginseng* leaf extract, *Pyrococcus furiosus*

## Introduction

Ginsenosides, the main substances with pharmacological effects in ginseng (*Panax ginseng* Meyer), are divided into protopanaxadiol (PPD) and protopanaxatriol (PPT) types according to the number and position of the hydroxyl groups. PPD-type ginsenosides include different sugars linked to C-3 and C-20 of the aglycon PPD skeleton, whereas PPT-type ginsenosides contain different sugars linked to C-6 and C-20 of the aglycon PPT (APPT) skeleton. The C-6 outer sugar of the PPT-type ginsenosides is glucose, rhamnose, or xylose, whereas the C-6 inner sugar and the C-20 sugar are always glucose [1]. PPT-type ginsenosides consist of

Re, R<sub>1</sub>, R<sub>2</sub>, Rg<sub>1</sub>, Rg<sub>2</sub>, Rh<sub>1</sub>, Rf, F<sub>1</sub>, and APPT according to the presence or absence of different types of sugars. PPT-type ginsenosides have anti-diabetic, anti-inflammatory, and anti-amnesic activities, and may protect against Alzheimer's disease and the UVB-induced apoptosis of keratinocyte [2–7].

Ginsenosides with one sugar or no sugars are more rapidly absorbed into the bloodstream and have better pharmacological effects than ginsenosides with three or four sugars [8, 9]. APPT, a ginsenoside with no linked sugars, has excellent pharmacological effects such as memory enhancement and antitumor activities [10, 11]. APPT can be transformed from PPT-type ginsenosides, including R<sub>1</sub>, Re, Rg<sub>1</sub>, Rg<sub>2</sub>, and Rf, by the intestinal bacterium *Bacteroides* JY-6

[9], and by  $\beta$ -glucosidases from *Acinosynnema mirum* [12], *Aspergillus niger* [13], *Gordonia terrae* [1], and *Penicillium aculeatum* [14], ginsenosidase type IV from *Aspergillus* sp. 39g [15], naringinase from *Penicillium decumbens* [6], and  $\beta$ -glycosidase from *Dictyoglomus turgidum* (DT-bgl) [16]. Although ginsenoside Re can be transformed to APPT by human intestinal microflora [9], the quantitative production of APPT from ginsenoside Re and *P. ginseng* leaf extract has not been attempted. Moreover, the complete biotransformation of ginseng extract to APPT has not yet been achieved.

Ginsenosides are present in the leaves, stems, flowers, and fruit as well as the roots of ginseng plants [17–19]. The content of ginsenoside Re in the leaves and fruit is higher than that in the stems, flowers, and roots of ginseng plants, regardless of the extraction solvent used, extraction time, and age of the plant [18, 20]. Ginseng leaf extract is a cheap substrate for APPT production because ginseng leaves are discarded during ginseng harvesting. In this study, we optimized the ratio of DT-bgl to  $\beta$ -glycosidase from *Pyrococcus furiosus* (PF-bgl) and the concentrations of enzyme and substrate. Under the optimized conditions, the PPT-type ginsenosides in *P. ginseng* leaf extract were completely converted to the valuable ginsenoside APPT by DT-bgl combined with PF-bgl.

## Materials and Methods

### Materials

We purchased the ginsenoside standards Re, Rg<sub>1</sub>, Rg<sub>2</sub>, Rf, F<sub>1</sub>, Rh<sub>1</sub>, and APPT from Ambo Laboratories (Korea) and BTGin (Korea).

### Preparation of Ginseng Leaf Extract

Six-year-old *P. ginseng* leaf extract was prepared at the National Institute of Horticultural and Herbal Science (Korea). The *P. ginseng* leaves were dried for 48 h at 40°C in a forced air convection oven. The dried leaves were ground to less than 0.5 mm diameter using a mixer, and thoroughly blended and homogenized using a mixer mill (Retsch MM400; Haan, Germany). The fine leaf powder (100 g) was suspended in 1 L of 80% (v/v) methanol. The mixture was ultrasonically extracted for 1 h at 50°C. The leaf extract was filtered through a 0.45- $\mu$ m pore filter, methanol in the filtrate was removed using an evaporator, the residue was dissolved in 500 ml of water, and 20% (w/v) of the solvent-free solution was used as the ginseng leaf extract.

### Preparation of Enzymes

DT-bgl and PF-bgl were expressed as described previously [20, 21]. Recombinant *Escherichia coli* ER2566 was cultivated in LB medium mixed with 20  $\mu$ g/ml kanamycin in a 2-L flask at 37°C. As the OD<sub>600</sub> (the optical density measured at 600 nm) of the bacterial

broth reached 0.6, 0.1 mM isopropyl- $\beta$ -D-thiogalactopyranoside was supplemented to induce enzyme expression. The bacterial cells were cultivated for a further 16 h at 16°C and harvested. The suspended cells in 50 mM citrate/phosphate buffer (pH 6.0) were disrupted by sonication on ice. The intact cells and cell debris were eliminated by centrifugation at 12,000  $\times$ g for 30 min, and the supernatant was heated at 75°C for 30 min. The insoluble proteins of the heat-treated suspension were eliminated by centrifugation at 12,000  $\times$ g for 30 min. The obtained supernatant was used as an enzyme for the biotransformation of ginsenosides.

### Hydrolytic Activity

One unit (U) of DT-bgl or PF-bgl activity was defined as the amount of enzyme required to liberate 1  $\mu$ mol *p*-nitrophenol (*p*NP) from *p*NP- $\beta$ -D-glucopyranoside per minute at 80°C and pH 6.0. The substrate specificity was determined after incubation at 80°C for 10 min in 50 mM citrate/phosphate buffer (pH 6.0) containing 0.4 mg/ml Re, Rg<sub>1</sub>, Rg<sub>2</sub>, Rh<sub>1</sub>, F<sub>1</sub>, DT-bgl, or PF-bgl, and 4% (v/v) dimethyl sulfoxide. The DT-bgl concentration was 0.83 U/ml for Re, Rf, and F<sub>1</sub>; and 4.2 U/ml for Rh<sub>1</sub> and Rg<sub>1</sub>. The PF-bgl enzyme concentration was 104.0 U/ml for Re and Rg<sub>2</sub>; and 312.5 U/ml for Rf. The specific activity was calculated from the substrate consumption rate.

### Optimization of Reaction Conditions Using Ginsenoside Re

The effect of the ratio of PF-bgl to DT-bgl on the biotransformation of ginsenoside Re to APPT was investigated with 0.4 mg/ml Re as a substrate by varying the concentration of DT-bgl from 0.0 to 6.0 mg/ml (0.0 to 50 U/ml) and that of PF-bgl from 1.0 to 0.0 mg/ml (1,040 to 0.0 U/ml). The optimal concentration of ginsenoside Re required for APPT production was determined by varying the concentration of Re from 0.5 to 2.0 mg/ml with 1.2 mg/ml (5.5 U/ml) DT-bgl and 0.8 mg/ml (832 U/ml) PF-bgl. To determine the optimal concentrations of the two enzymes for APPT production, the concentrations of DT-bgl and PF-bgl at the constant weight ratio of 1.2:0.8 were varied from 0.6 and 0.4 mg/ml (5.0 and 416 U/ml) to 4.8 and 3.2 mg/ml (40 and 3,328 U/ml), respectively, with 1.0 mg/ml Re. The optimization reactions were performed in 50 mM citrate/phosphate buffer (pH 6.0) at 80°C for 30 min. The time-course reactions for the biotransformation of ginsenoside Re to APPT were carried out with 1.0 mg/ml Re, 2.4 mg/ml (20 U/ml) DT-bgl, and 1.6 mg/ml (1,664 U/ml) PF-bgl in 50 mM citrate/phosphate buffer (pH 6.0) at 80°C for 4 h.

### Optimization of Reaction Conditions Using Ginseng Leaf Extract

The optimal ratio of DT-bgl to PF-bgl for the conversion of ginseng leaf extract to APPT was determined with ginseng leaf extract containing 1.0 mg/ml PPT-type ginsenosides by varying the concentration of DT-bgl from 0.0 to 7.2 mg/ml (0.0 to 60 U/ml) and the concentration of PF-bgl from 2.25 to 0.0 mg/ml (2,340 to 0.0 U/ml). The effect of substrate concentration on APPT production from ginseng leaf extract was investigated with

5.6 mg/ml (47 U/ml) DT-bgl and 0.5 mg/ml (520 U/ml) PF-bgl by varying the concentration of PPT-type ginsenosides in the leaf extract from 0.5 to 2.0 mg/ml. To determine the optimal enzyme concentration, the concentrations of DT-bgl and PF-bgl at the constant weight ratio of 5.6:0.5 were varied from 2.8 and 0.25 mg/ml (23 and 260 U/ml) to 14.0 and 1.25 mg/ml (117 and 1,300 U/ml), respectively, with the leaf extract containing 1.0 mg/ml PPT-type ginsenosides. The optimization reactions were performed in citrate/phosphate buffer (pH 6.0) at 80°C for 3 h. The time-course reactions for the biotransformation of ginseng leaf extract to APPT were performed with the leaf extract containing 1.0 mg/ml PPT-type ginsenosides, 14.0 mg/ml (117 U/ml) DT-bgl, and 1.25 mg/ml (1,300 U/ml) PF-bgl in 50 mM citrate/phosphate buffer (pH 6.0) at 80°C for 11 h.

### Analytical Methods

The ginsenosides were analyzed using a high-performance liquid chromatography (HPLC) system (Agilent 1100; Agilent, USA) with a UV detector set to detect at 203 nm and an octadecyl-silica column (250 × 4.6 mm, S-5 μm, and 12 nm; YMC; Dinslaken, Germany). The ginsenosides mixed with the internal standard digoxin were eluted in the column at 30°C by pumping with a linear gradient of water and acetonitrile (70:30 to 30:70 of volume ratio) for 45 min at a flow rate of 1 ml/min.

## Results and Discussion

### Substrate Specificity of DT-bgl and PF-bgl

The substrate specificity of DT-bgl and PF-bgl toward PPT-type ginsenosides is shown in Table 1. The specific activity of DT-bgl followed the order of  $F_1 > Re > R_{g_1} > R_f > Rh_1$ , and the specific activity of PF-bgl followed the order of  $R_f > Re > R_{g_2}$ . The specific activity of PF-bgl for ginsenosides  $R_f$  and  $Re$  was 65- and 6-fold higher than that of DT-bgl, respectively.

### Biotransformation of PPT-Type Ginsenosides to APPT by DT-bgl Combined with PF-bgl

DT-bgl has the hydrolytic pathways of PPT-type ginsenosides of  $Rh_1 \rightarrow APPT$ ,  $R_{g_1} \rightarrow Rh_1 \rightarrow APPT$ ,  $R_f \rightarrow Rh_1 \rightarrow APPT$ ,  $F_1 \rightarrow APPT$ , and  $Re \rightarrow R_{g_2}$  because it hydrolyzes the glucose and xylose residues in PPT-type ginsenosides but not the rhamnose residue at C-6 in ginsenosides  $Re$  and  $R_{g_2}$  [16], which can be converted to APPT with the aid of PF-bgl. PF-bgl hydrolyzes the outer sugar at C-6 in PPT-type ginsenosides but not the inner glucose at C-6 or the glucose at C-20. Thus, PF-bgl converts  $Re$ ,  $R_{g_2}$ , and  $R_f$  to  $R_{g_1}$ ,  $Rh_1$ , and  $Rh_1$ , respectively [21], which can be transformed to APPT by DT-bgl. Although PF-bgl cannot produce APPT using PPT-type ginsenosides, DT-bgl combined with PF-

**Table 1.** Substrate specificity of β-glycosidases from *D. turgidum* (DT-bgl) and *P. furiosus* (PF-bgl) for PPT-type ginsenosides.

Enzyme	Substrate	Product	Specific activity (U/mg)
DT-bgl	Re	$R_{g_2}$	58.8
	$R_{g_1}$	$Rh_1$	45.5
	$R_f$	$Rh_1$	17.2
	$Rh_1$	APPT	6.6
	$F_1$	APPT	244
PF-bgl	Re	$R_{g_1}$	354
	$R_{g_2}$	$Rh_1$	31.9
	$R_f$	$Rh_1$	1,120

One unit (U) of DT-bgl or PF-bgl activity was defined as the amount of enzyme required to liberate 1 μmol *p*-nitrophenol (*p*NP) from *p*NP-β-D-glucopyranoside per minute at 80°C and pH 6.0.

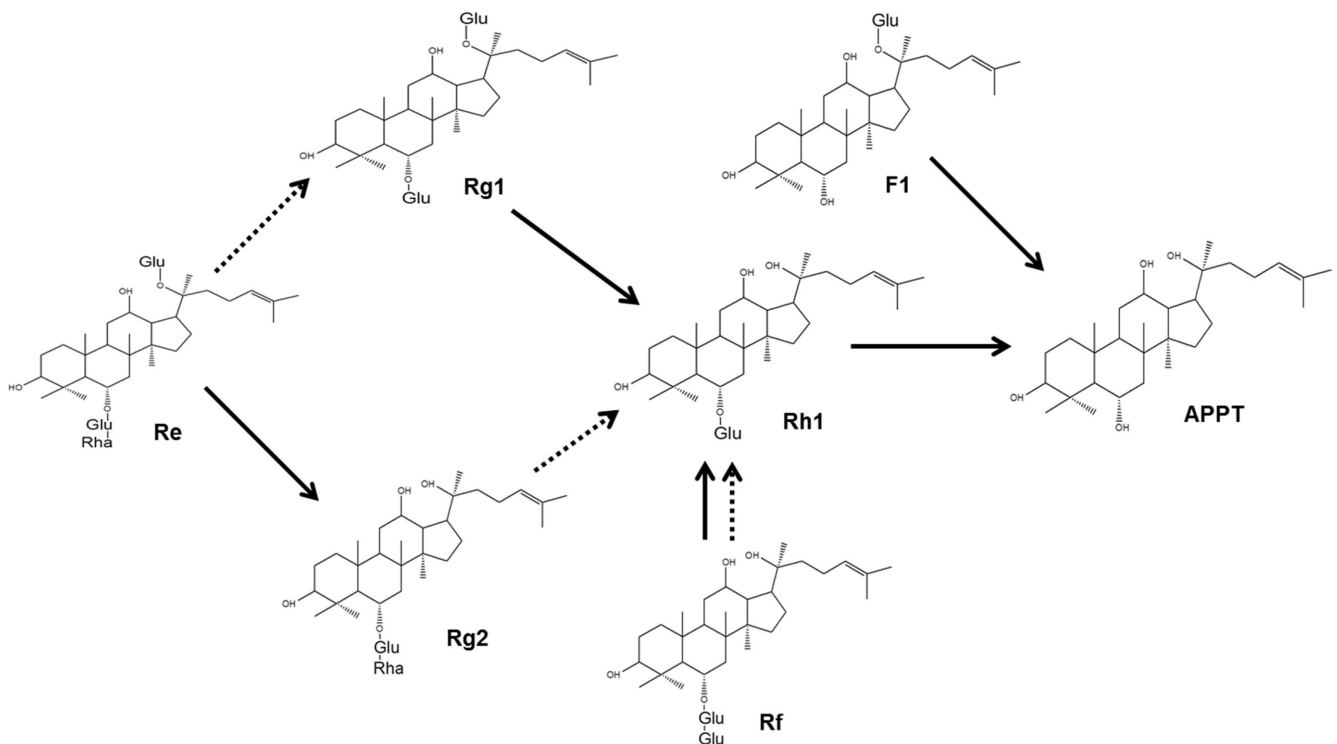
bgl can convert all the PPT-type ginsenosides in ginseng leaf extract to APPT via the four transformation pathways  $Re \rightarrow R_{g_2} \rightarrow Rh_1 \rightarrow APPT$ ,  $Re \rightarrow R_{g_1} \rightarrow Rh_1 \rightarrow APPT$ ,  $F_1 \rightarrow APPT$ , and  $R_f \rightarrow Rh_1 \rightarrow APPT$  (Fig. 1).

### Optimization of Reaction Conditions for the Biotransformation of Ginsenoside Re to APPT by DT-bgl Combined with PF-bgl

The maximal concentrations of DT-bgl and PF-bgl used for the ratio experiments for the biotransformation of ginsenoside  $Re$  to APPT were 6.0 and 1.0 mg/ml, respectively, because the specific hydrolytic activity of PF-bgl for ginsenoside  $Re$  was 6-fold higher than that of DT-bgl. The biotransformation of  $Re$  to APPT was conducted with 0.4 mg/ml  $Re$  by varying the concentration of DT-bgl from 0.0 to 6.0 mg/ml and the concentration of PF-bgl from 1.0 to 0.0 mg/ml. After 30 min, the maximum biotransformation of  $Re$  to APPT was observed at 1.2 mg/ml DT-bgl and 0.8 mg/ml PF-bgl at a concentration ratio of 3:2 (Fig. 2). The optimum concentrations of  $Re$ , DT-bgl, and PF-bgl were 1.0, 2.4, and 1.6 mg/ml, respectively (Fig. S1).

### Biotransformation of Ginsenoside Re to APPT by DT-bgl Combined with PF-bgl

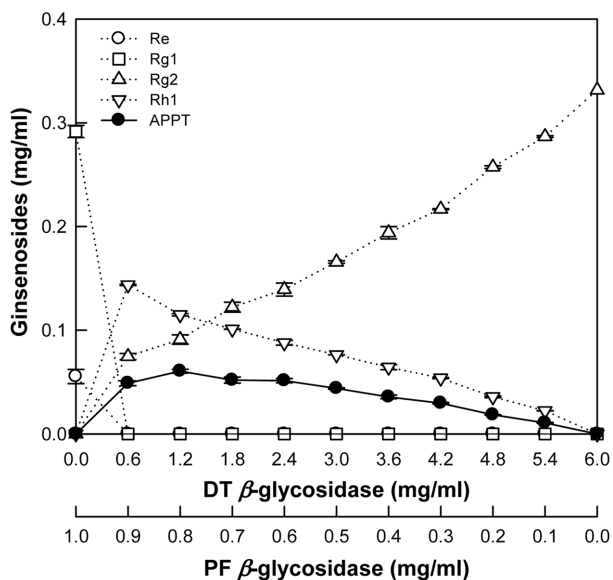
The optimal reaction conditions for the biotransformation of ginsenoside  $Re$  to APPT were 2.4 mg/ml DT-bgl, 1.6 mg/ml PF-bgl, and 1.0 mg/ml  $Re$  at pH 6.0 and 80°C. Under these optimized conditions, the time-course reactions for the biotransformation of  $Re$  to APPT were performed using ginsenoside  $Re$  for 4 h. DT-bgl combined with PF-bgl converted 1.0 mg/ml  $Re$  to 0.5 mg/ml APPT with a productivity of 126 mg/l/h and a molar conversion of



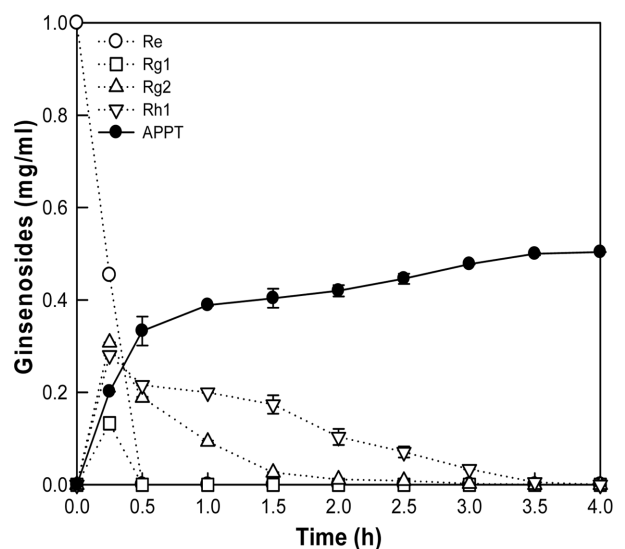
**Fig. 1.** Hydrolytic pathway from protopanaxatriol-type ginsenosides to aglycon protopanaxatriol (APPT) by *Dictyoglomus turgidum*  $\beta$ -glycosidase (solid line) supplemented with *Pyrococcus furiosus*  $\beta$ -glycosidase (dotted line).

100% after 4 h (Fig. 3). The HPLC profiles during the biotransformation of ginsenoside Re to APPT by DT-bgl

supplemented with PF-bgl at 0, 2, and 4 h are presented in Fig. S3.



**Fig. 2.** Effect of the combination of *Dictyoglomus turgidum* (DT)  $\beta$ -glycosidase (DT-bgl) with *Pyrococcus furiosus* (PF)  $\beta$ -glycosidase on the biotransformation of ginsenoside Re to aglycon protopanaxatriol (APPT).



**Fig. 3.** Time-course reactions for the biotransformation of ginsenoside Re substrate to aglycon protopanaxatriol (APPT) using *Dictyoglomus turgidum*  $\beta$ -glycosidase combined with *Pyrococcus furiosus*  $\beta$ -glycosidase.

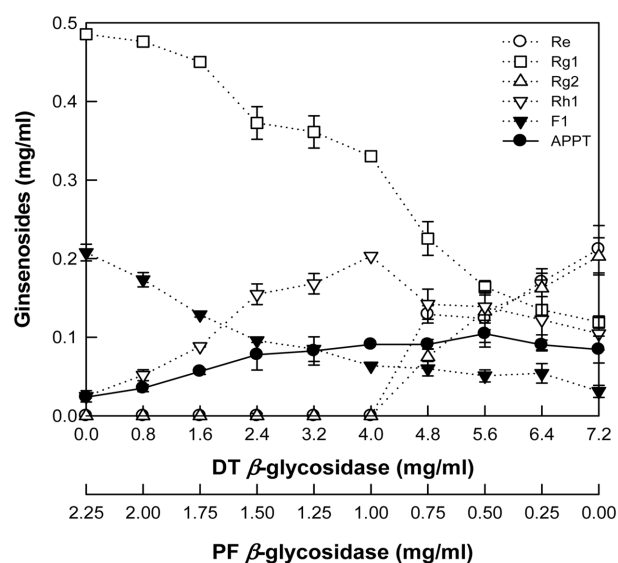
**Table 2.** Contents of protopanaxatriol (PPT)-type and protopanaxadiol (PPD)-type ginsenosides in 20% (w/v) *Panax ginseng* leaf extract.

Ginsenoside	Concentration (mg/ml (w/v))	Content (% (w/w))
Rg <sub>1</sub>	0.42	12.9
Re	1.12	34.4
Rf	0.20	6.1
F <sub>1</sub>	0.28	8.6
PPT sum	2.02	62.0
Rb <sub>1</sub>	0.08	2.5
Rc	0.12	3.7
Rb <sub>2</sub>	0.14	4.3
Rb <sub>3</sub>	0.00	0.0
Rd	0.50	15.3
F <sub>2</sub>	0.40	12.3
PPD sum	1.24	38.0
Total	3.26	100.0
PPT/PPD	1.60	

#### Optimization of Reaction Conditions for the Biotransformation of PPT-Type Ginsenosides in Ginseng Leaf Extract to APPT by DT-bgl Combined with PF-bgl

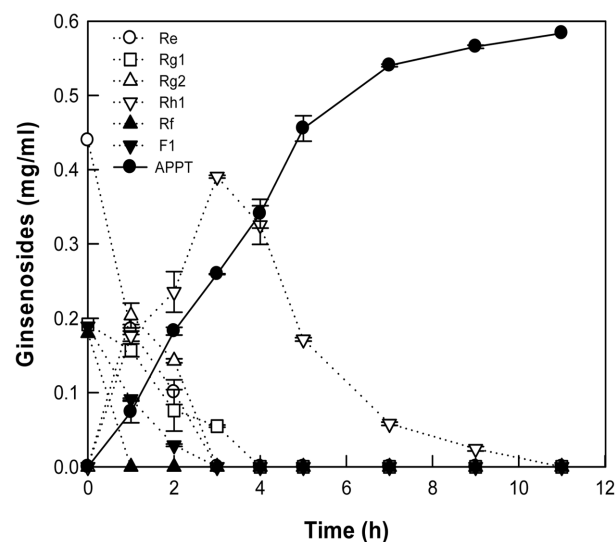
The *P. ginseng* leaf extract (20% (w/v)) obtained by extraction with 80% (v/v) methanol contained 2.02 mg/ml PPT-type ginsenosides, which constituted 62.0% (w/w) of the total ginsenosides in ginseng leaf extract (Table 2). The content of specific PPT-type ginsenosides in the total PPT-type ginsenosides in the leaf extract followed the order Re (55.4% (w/w)) > Rg<sub>1</sub> (20.8%) > F<sub>1</sub> (13.9%) > Rf (9.9%), indicating that the efficient hydrolysis of ginsenoside Re is essential for the increased biotransformation of PPT-type ginsenosides in ginseng leaf extract to APPT.

The maximum concentrations of DT-bgl and PF-bgl used for the ratio experiments for the biotransformation of PPT-type ginsenosides in ginseng leaf extract to APPT from ginsenoside Re were 7.2 and 2.25 mg/ml, respectively, because the specific hydrolytic activity of PF-bgl for PPT-type ginsenosides in the leaf extract was approximately 3.2-fold higher than that of DT-bgl. The biotransformation of PPT-type ginsenosides in ginseng leaf extract to APPT was performed with the leaf extract containing 1.0 mg/ml PPT-type ginsenosides by adding PF-bgl to DT-bgl at ratios ranging from 2.25:0 to 0:7.2 mg/ml. After 3 h, the maximum biotransformation of PPT-type ginsenosides in ginseng leaf extract to APPT was observed at 5.6 mg/ml DT-bgl and



**Fig. 4.** Effect of combination of *Dictyoglomus turgidum*  $\beta$ -glycosidase with *Pyrococcus furiosus*  $\beta$ -glycosidase on the biotransformation of protopanaxatriol-type ginsenosides in *Panax ginseng* leaf extract as a substrate to aglycon protopanaxatriol (APPT).

0.5 mg/ml PF-bgl (Fig. 4). The optimum concentrations of PPT-type ginsenosides in the leaf extract, DT-bgl, and PF-bgl were 1.0, 14, and 1.25 mg/ml (Fig. S3).



**Fig. 5.** Time-course reactions for the biotransformation of protopanaxatriol-type ginsenosides in *Panax ginseng* leaf extract as a substrate to aglycon protopanaxatriol (APPT) using *Dictyoglomus turgidum*  $\beta$ -glycosidase combined with *Pyrococcus furiosus*  $\beta$ -glycosidase.

**Table 3.** Production of aglycon protopanaxatriol (APPT) from protopanaxatriol-type ginsenosides and *Panax ginseng* extracts.

Microorganism	Enzyme	Substrate (mg/ml)	APPT (mg/ml)	Molar conversion (%)	Productivity (mg/l/h)	Reference
<i>Aspergillus niger</i>	$\beta$ -Glucosidase (crude)	Rf	NC	90.4	NC	[13]
<i>Dictyoglomus turgidum</i>	$\beta$ -Glycosidase	R <sub>1</sub> (1.0)	0.3	75.6	15.0 <sup>a</sup>	[18]
		Rg <sub>1</sub> (1.0)	0.6	100.0	90.2 <sup>a</sup>	
		Rf (1.0)	0.6	100.0	90.2 <sup>a</sup>	
		<i>P. ginseng</i> root extract (2.3)	0.5	43.2	23.0	
		<i>P. notoginseng</i> root extract (1.4)	0.6	81.0	31.0	
<i>Gordonia terrae</i>	$\beta$ -Glucosidase	Rg <sub>1</sub> (4.0)	1.1	65.8	224.0 <sup>a</sup>	[14]
<i>Penicillium aculeatum</i>	$\beta$ -Glucosidases	Rf (0.4)	0.2	90.0	33.3	[15]
<i>Dictyoglomus turgidum</i>	$\beta$ -Glycosidase	Re (1.0)	0.5	100.0	125.9	This study
and <i>Pyrococcus furiosus</i>	and $\beta$ -Glycosidase	<i>P. ginseng</i> leaf extract (1.0)	0.6	100.0	53.0	

NC, Not calculated.

The numbers in parentheses after the substrates represent the concentrations of substrates.

<sup>a</sup>The value calculated, based on data from the reference.

### Biotransformation of PPT-Type Ginsenosides in Ginseng Leaf Extract to APPT by DT-bgl Combined with PF-bgl

The optimal reaction conditions for APPT production from ginseng leaf extract were 14 mg/ml DT-bgl, 1.25 mg/ml PF-bgl, and the leaf extract containing 1.0 mg/ml PPT-type ginsenosides at pH 6.0 and 80°C. Time-course reactions for the biotransformation of PPT-type ginsenosides in ginseng leaf extract to APPT were performed using the leaf extract containing 1.0 mg/ml PPT-type ginsenosides for 11 h. DT-bgl supplemented with PF-bgl converted 1.0 mg/ml PPT-type ginsenosides in ginseng leaf extract to 0.58 mg/ml APPT without other ginsenosides, with a productivity of 53 mg/l/h and a molar conversion of 100% after 11 h (Fig. 5). The HPLC profiles during the conversion of PPT-type ginsenosides in ginseng leaf extract to APPT by DT-bgl supplemented with PF-bgl at 0, 6, and 11 h are presented in Fig. S4.

The quantitative production of APPT from PPT-type ginsenosides and ginseng extracts by enzymes is presented in Table 3.  $\beta$ -Glucosidase from *G. terrae* converted 4.0 mg/ml Rg<sub>1</sub> to 1.1 mg/ml APPT [20];  $\beta$ -glucosidase from *A. niger* converted Rf to APPT with a molar conversion of 90.4% [13];  $\beta$ -glucosidase from *Pen. aculeatum* converted 0.4 mg/ml Rf to 0.2 mg/ml APPT [14]; and DT-bgl converted 1 mg/ml R<sub>1</sub>, Rg<sub>1</sub>, and Rf to 0.3, 0.6, and 0.6 mg/ml APPT, respectively [16].  $\beta$ -Glucosidase from *G. terrae* had the highest previously reported productivity of APPT owing to high substrate concentration [20]. However,  $\beta$ -glucosidase from *G. terrae* exhibited low molar conversion and could not be used to produce APPT from ginseng extract because the

enzyme did not hydrolyze Rg<sub>2</sub> or R<sub>2</sub>.  $\beta$ -Glucosidase from *G. terrae* and *Pen. aculeatum* incompletely converted Rg<sub>1</sub> and Rf to APPT with molar conversions of 65.8% and 90.0%, respectively, whereas DT-bgl completely converted Rg<sub>1</sub> and Rf to APPT [14, 16, 20]. DT-bgl did not convert Rg<sub>2</sub> and Re to APPT, and did not therefore achieve the complete conversion of ginseng leaf extract to APPT. In contrast, DT-bgl supplemented with PF-bgl completely converted ginsenoside Re and PPT-type ginsenosides in the leaf extract to APPT. In the current study, we enzymatically produced APPT using ginsenoside Re for the first time, and the results represent the highest concentration and productivity of APPT from ginseng extract to date.

In conclusion, DT-bgl completely converted Rg<sub>1</sub>, Rh<sub>1</sub>, and Rf to APPT, but did not convert Rg<sub>2</sub> and Re to APPT. PF-bgl converted Rg<sub>2</sub> and Re to Rh<sub>1</sub> and Rg<sub>2</sub>, but did not convert Rg<sub>1</sub> and Rh<sub>1</sub> to APPT. Thus, DT-bgl supplemented with PF-bgl completely converts PPT-type ginsenosides in ginseng leaf extract to APPT. To the best of our knowledge, this is the first complete conversion of ginseng extract to APPT, and the reaction system achieved the highest productivity of APPT from ginseng extract to date. Our results demonstrate that DT-bgl supplemented with PF-bgl is an efficient enzyme system for the production of APPT using ginseng leaf extract, and discarded ginseng leaves can be used as a source of the valuable ginsenoside APPT.

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## Conflict of Interest

The authors have no financial conflicts of interest to declare.

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