INTRODUCTION

Panax ginseng Meyer is one of the most valuable Oriental medicinal plants. Ginseng saponin (ginsenoside) is the major pharmacological component in ginseng. More than 100 ginsenosides have been isolated from ginseng plant [1,2]. Among these, six major ginsenosides, such as Rb1, Rb2, Rc, Rd, Re, and Rg1, constitute more than 90% of the total ginsenosides in white and red ginseng [3]. In recent decades, many studies have been reported the successful transformation of these major forms of ginsenoside into the more active minor forms of ginsenosides, such as Rg3, Rh2, and compound K (anti-tumor, antidiabetic, antistress, and antioxidative) [4]. Minor saponins have high pharmacological activity, however the natural availability of these saponins are very less in ginseng. Studies have been reported the success of converting major ginsenosides to the minor saponin varieties using methods such as heating [5,6], acid hydrolysis [7,8], and microbial enzymatic conversion [9-11]. Ginsenoside Rb1 has four glucose molecules with (1→2)-β-glycosidic linkages at C-3 and (1→6)-β-glycosidic linkages at C-20.

Kimchi is a traditional fermented food in Korea that accompanies many meals. The ingredients include Korean cabbage, vegetables, garlic, and other seasonings. During Kimchi fermentation, lactic acid bacteria (LAB) produce lactic acid by hydrolyzing carbohydrates (glucose and lactose). LAB is not only widely recognized, but is also well known for its physiological effects such as drug metabolism, antimutation, anticancer, gastric sec-
retomotor activity, and radiation resistance [12-14].

In this study, we isolated \( \beta \)-glucosidase producing bacteria from Kimchi and tested the bio conversion ability of ginsenoside Rb1. The crude enzyme from strain 6105 was reacted with ginsenoside Rb1 to convert it into specific components. Finally, the results were confirmed with TLC and HPLC.

**MATERIALS AND METHODS**

**Materials**

Ginsenoside Rb1 was obtained from the Ginseng Genetic Resource Bank at Kyung Hee University, Korea. Standard ginsenosides including 20(S)-Rb1, 20(S)-Rd, 20(S)-Rg3, 20(S)-Rh2, and compound-K were obtained from KT&G in Daejeon, Korea. De Man, Rogosa and Sharpe (MRS) broth was purchased from Difco (Detroit, MI, USA). \( p \)-Nitrophenyl-\( \beta \)-D-glucopyranoside (\( p \)-NPG) and \( p \)-nitrophenol (\( p \)-NP) were purchased from Sigma Aldrich (St. Louis, MO, USA). The 60 F254 silica gel plate (Merck, Darmstadt, Germany) was used for TLC and silica gel 60 (Merck) was used for column chromatography. An HPLC (NS 3000i system, Futecs Co., Daejeon, Korea) was used with a UV detector and gradient pump.

**Isolation of \( \beta \)-glucosidase producing bacteria from Kimchi**

We collected many kinds of Kimchi from various regions in Korea, and \( \beta \)-glucosidase producing bacteria were isolated using a esculin agar medium. Single colonies from the plates were purified onto new plates. The isolated strains were incubated at 37°C for 24 h in MRS agar (Difco). We suggested that the bacteria could convert the major ginsenoside Rb1 to the minor ginsenoside Rg3.

**Culture conditions**

One micro liter of cells were grown in 100 mL Erlenmeyer flasks containing 50 mL of MSR broth and incubated at 30°C on a rotary shaker (160 rpm) until it reaches the absorbance 0.8 to 1.0. Later the cells were harvested and re-inoculated onto the MRS broth containing 1% (w/v) cellobiose, glucose, lactose, sucrose, hesperidin and soluble starch. Growth was monitored using spectrophotometer at O.D. 600.

**Preparation of crude enzyme**

Bacterial cells were removed by centrifugation at 10,000 rpm for 20 min at 4°C. The supernatant containing the extracellular \( \beta \)-glucosidases was used as the crude enzyme and was precipitated using acetone. The precipitates were dissolved in 20 mM sodium phosphate buffer, pH 7.0. Later, their activity was checked using 1 mM ginsenoside Rb1.

**Assay of ginsenoside Rb1 transformation activity**

To determine the \( \beta \)-glucosidase activity, 50 \( \mu \)L of crude enzyme were added to 50 \( \mu \)L of 10 mM \( p \)-NPG suspended in 20 mM sodium phosphate buffer (pH 7.0). Then the mixture was incubated at 37°C for 10 min. The reaction was stopped by adding 100 \( \mu \)L of 1 M Na\(_2\)CO\(_3\), and the \( p \)-NP produced was measured at 405 nm by a UV spectrophotometer (Ultrospec 2100 Pro; Amersham Biosciences, Cambridge, UK). One unit of enzyme activity was defined as the amount released 1 nmol \( p \)-NP per min from \( p \)-NPG under the above reaction conditions.

**Effect of temperature and pH on enzyme activity**

The optimal temperature for enzyme activity was determined over a temperature ranges between 25°C to 70°C using the standard assay conditions. To determine the optimal pH the enzyme activity was measured over a pH range of 4.5 to 8.0. The buffers used for the assay are 20 mM acetate buffer (pH 4.5-5.5), 20 mM sodium phosphate buffer (pH 6-7.5), and 20 mM Tris-HCl buffer (pH 8.0).

**Biotransformation**

The reaction mixture, containing 200 \( \mu \)L of 1 mM ginsenoside Rb1 and 200 \( \mu \)L of bacterial suspension were cultured in MRS broth, was incubated for 72 h at 37°C. Then extracted twice with 200 \( \mu \)L of \( n \)-butanol. The \( n \)-butanol fraction of the reaction mixture was evaporated in vacuo, and the residue was dissolved in CH\(_3\)OH.

**Analysis of ginsenosides by thin-layer chromatography**

TLC was performed using a silica gel 60 F254 plate. CHCl\(_3\)-CH\(_2\)OH-H\(_2\)O (65:35:10, v/v/v, lower phase) mixture was used as the developing solvent for TLC. The spots on the TLC plates were detected by spraying 10% H\(_2\)SO\(_4\) followed by heating at 110°C for 5 min.

**Analysis of ginsenosides by high pressure layer chromatography**

HPLC was performed using a NS 3000 system (Futecs Co.), equipped with a UV detector and a gradient pump. The C18 column (YMC c18 pro, 250×4.6 mm, 5 \( \mu \)m) was used for this analysis with 20 \( \mu \)L sample volume. The mobile phase consisted of CH\(_3\)CN (A) with distilled H\(_2\)O (B). The solvent A/solvent B form the gradient in the ratios as follows: 15:85, 21:79, 58:42, 90:10, 90:10,
15:85, and 15:85, with run times of 0-5, 5-25, 70-72, 72-82, 82-84, and 84-100 min, respectively, at a 1.6 mL/min flow rate. Detection was done at 203 nm.

Analysis of nuclear magnetic resonance spectrum

An 80 mL suspension of strain 6105 from the MRS broth was mixed with the same volume of 1 mM ginsenoside Rb1 and incubated at 30°C, in 160 rpm for 60 h. The reaction mixture was extracted with 150 mL of n-butanol twice and was evaporated in vacuo at 45°C. The residue was applied to the silica gel 60 column and eluted with CHCl3-CH3OH-H2O (65:35:10, v/v, lower phase). Twenty milliliter of fraction was collected, separated and the metabolite transformed from ginsenoside Rb1 was dissolved in pyridine-d5 and identified by 1H-NMR and 13C-NMR using FT-NMR spectrometry (400 MHz; Varion Inova AS 400, Varion, Palo Alto, CA, USA)

RESULTS

Phylogenetic study

β-glucosidase producing bacteria was isolated from Kimchi using esculin-MRS agar. The 16S rRNA gene sequences of the strain 6105 were aligned with type strains found to have the closest taxonomic relationships. Strain 6105 was grouped with a Lactobacillus species, and the highest degrees of 16S rRNA gene sequence identities were to L. plantarum JCM 11499 (D79210) (99.1%), L. pentosus JCM 15581 (D79211) (99.2%) (Fig. 1).

Characterization of the crude enzyme of strain 6105

The effect of temperature and pH on the activity of β-glucosidase was shown in Fig. 2. The optimum temperature for maximal activity was 37°C. At 37°C ginsenoside Rb1 was converted into compound K, however, the crude enzyme activity was decreased over 40°C dramatically and not converted any compounds at 50°C to 70°C. Under optimal temperature conditions, the crude enzyme activity was high at the pH ranges from 6.0 to 8.0. Ginsenoside Rb1 was almost hydrolyzed on pH 6.0 to 7.5 and the crude enzyme shows maximum activity at the pH 7.0 which converts ginsenoside Rb1 into ginsenoside Rd, F2 and compound K.

The HPLC profile of the enzymatic transformation of ginsenoside Rb1 by crude enzyme of strain 6105 is shown in Fig. 3. The identities of the peaks were established by comparison of retention time values with those of standards. In the HPLC chromatogram, the peaks with retention time 37.23, 41.68, 56.01, and 61.56 min correspond to ginsenoside Rb1, gypenoside XVII, F2, and compound K.

Effect of carbon source supplements on β-glucosidase activity

The relative rates of hydrolysis of various substrates by crude enzyme are shown in Fig. 4. Strains were cultured on MRS broth and inoculated on MRS broth with 1% carbon source such as glucose, cellobiose, lactose, su-
crose, hesperidin, and soluble starch to prepare crude enzyme (supernatant). The highest yields of β-glucosidase activity (0.95 U/mL) were obtained in lactose culture broth (Table 1). We hypothesized that carbon sources cause an increase β-glucosidase activity. The ginsenoside Rb1 was converted in the following sequence: Rb1→Rd and gypenoside XVII→F2→compound K (Fig. 5) by the
crude enzyme from strain 6105, consecutively hydrolyzing of ginsenoside Rb1.

**Structural identification of metabolites**

We identified the metabolites of ginsenoside Rb1 by $^1$H-NMR and $^{13}$C-NMR spectroscopy, because the $R_f$ value and retention time of the metabolites were the same as the standard compound K in TLC and HPLC analysis.

**Metabolite 1**

In the $^1$H-NMR spectrum of metabolite 1, the proton signals for the H-1 of the 3-O-inner-glucopyranosyl moiety, 3-O-outer-glucopyranosyl moiety, and 20-glucopyranosyl moiety appeared respectively, at $\delta$ 4.87 ppm (1H, $d$, $J$=8.2 Hz, H-3-glc-1H$'$), $\delta$ 5.05 ppm (1H, $d$, $J$=7.8 Hz, H-20-glc-1H$''$), $\delta$ 5.08 ppm (1H, $d$, $J$=7.6 Hz, H-20-glc-1H$'''$), showing that the aglycon of metabolite 1 harbored three $\beta$-D-glucoses. In the $^{13}$C NMR (pyridine-d$_5$, 100 MHz) spectrum of metabolite 1, the signals for C-2 of the 3-inner-glucose was shifted up field, from 83.5 ppm to 78.4 ppm, and other signals were similar to those of metabolite 1. Compared with as previous reported[15], metabolite 1 was identified as 3-O-$[\beta$-D-glucopyranosyl]-20-O-$[\beta$-D-glucopyranosyl]-20(S)-protopanaxadiol, identical to gypenoside XVII.

**Metabolite 2**

In the $^1$H-NMR spectrum of metabolite 2, the proton signals for the H-1 of the 3-O-inner-glucopyranosyl moiety, 3-O-outer-glucopyranosyl moiety, and 20-glucopyranosyl moiety appeared respectively, at $\delta$ 4.94 ppm (1H, $d$, $J$=7.7 Hz, H-20-glc-1$''$), $\delta$ 4.87 ppm (1H, $d$, $J$=8.2 Hz, H-3-glc-1H$'$), $\delta$ 5.19 ppm (1H, $d$, $J$=7.4 Hz, H-3-glc-1H$''$); Compared with as previous reported[16], metabolite 2 was identified as 3-O-$[\beta$-D-glucopyranosyl]-20(S)-protopanaxadiol, identical to ginsenoside Rd.

**Metabolite 3**

In the $^1$H-NMR spectrum of metabolite 3, the proton signals for the H-1 of the 3-O-inner-glucopyranosyl moiety, 3-O-outer-glucopyranosyl moiety, and 20-glucopyranosyl moiety appeared respectively, at $\delta$ 4.87 ppm (1H, $d$, $J$=8.2 Hz, H-3-glc-1H$'$), $\delta$ 5.05 ppm (1H, $d$, $J$=7.8 Hz, H-20-glc-1H$''$), $\delta$ 5.08 ppm (1H, $d$, $J$=7.6 Hz, H-20-glc-1H$'''$), showing that the aglycon of metabolite 1 harbored three $\beta$-D-glucoses. In the $^{13}$C NMR (pyridine-d$_5$, 100 MHz) spectrum of metabolite 3 with that of metabolite 2, the signal for the C-2 of the 3-inner-glucopyranosyl moiety was shifted up field, from 83.2 ppm to 78.4 ppm, and other signals were similar to those of metabolite 2. Additionally, based on the fact that the anomeric carbon signals at $\delta$ 106.9 and $\delta$ 98.2 ppm showed to be similar to signals for C-1 of the 3-O-inner-glucopyranosyl moiety and 20-O-inner-glucopyranosyl moiety of metabolite 2, it was confirmed that two glucoses connected with the C-3 and C-20 sites of the aglycon moiety. Compared with as previous reported[17], metabolite 3 was identified as 3-O-$[\beta$-D-glucopyranosyl]-20-O-$[\beta$-D-glucopyranosyl-(6,1)-$\beta$-D-gluco-pyranosyl]-20(S)-protopanaxadiol, identical to ginsenoside F2.

**Metabolite 4**

In the $^1$H-NMR spectrum of metabolite 4, the anomer-
Comparing with others reported strain optimum pH, such as Paecilomyces thermophila [21], Strain 6105 has optimum activity at pH 6.0 to 7.5. On the MRS medium containing hesperidin and soluble starch, there was less activity. These results suggest that strain 6105 can increase β-glucosidase activity on the MRS medium including glucose, cellubiose, lactose, sucrose like C. peltata [24].

We found that compound K has a highly efficient function through this study. Compound K has many pharmacological activities including protection of the liver from injury [27], inhibition of the P450-mediated metabolism [28], and inhibition at the a(1G)-type of Ca$^{2+}$ channel [29]. It also triggers apoptosis in activated hepatic stellate cells via caspase-3 activation [30] and potent cytotoxic activity against tumor cell lines [31]. However, minor saponins like ginsenoside F2 and compound K are found in small amounts in ginseng roots. Therefore, it is desirable to produce a greater amount of minor ginsenoside such as compound K. Our experiment demonstrates that strain 6105 is capable of producing the minor ginsenoside F2 and compound K using crude enzymes.

**ACKNOWLEDGEMENTS**

This research was supported by the Ministry of Knowledge Economy, Korea, under the Information Technology Research Center support program supervised by the National IT Industry Promotion Agency (NIPA-2011-C1090-1121-0003).

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