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RESEARCH ARTICLE

β-Glycosidase-Assisted Bioconversion of Ginsenosides in Purified Crude Saponin and Extracts from Red Ginseng (*Panax ginseng* C. A. Meyer)

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Abstract Major ginsenosides in ginseng (*Panax ginseng*) and its products are highly glycosylated, hence poorly absorbed in the gastrointestinal tract. β-Glycosidase-assisted deglycosylation of pure ginsenosides was peformed to study bioconversion mechanisms. Ginsenoside standard compounds, crude saponin, and red ginseng extracts were incubated with β-glycosidase (0.05% w/v, 55ºC). β-Glycosidase has a broad specificity for β-glycosidic bonds, hydrolyzing the β-(1→6), α-(1→6), and α-(1→2) glycosidic linkages. The final metabolite of protopanaxadiol ginsenosides was Rg3 while the metabolite of protopanaxatriol ginsenosides was Rh1. β-Glycosidase treatment of red ginseng extracts resulted in a decrease in the amounts of Rb1, Rc, Re, and Rg2 after 24 h, whereas levels of the less glycosylated Rd, Rb1, Rg, Rg3, Rg1, and Rh1 forms increased. When crude saponin was incubated with β-glycosidase for 24 h, levels of Rb1, Rc, Re, and Rg1 decreased while levels of Rd, Rg3, and Rh1 increased as deglycosylated ginsenosides.

Keywords: ginsenosides, β-glycosidase, bioconversion, saponin, red ginseng, extract

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Introduction

Roots of ginseng (*Panax ginseng* C.A. Meyer) have been used as a traditional medicine for preventive and therapeutic purposes in Asian countries for thousands of years (1). Ginsenosides are triterpenoid saponins that are found almost exclusively in ginseng and are believed to be the main active compounds behind the reported health benefits of ginseng. Ginsenosides, with few exceptions, share a similar basic structure containing a dammarane triterpenoidal skeleton with a modification at C-20 (2,3). Ginsenosides differ from each other in the types of sugar moieties, their number, and site of attachment (1). Nearly 50 ginsenosides have been identified in ginseng (4). The major ginsenosides in the roots of ginseng are the 20(S)-protopanaxadiol (PPD) ginsenosides Rb1, Rb2, Rc, and Rd, and the 20(S)-protopanaxatriol (PPT) ginsenosides Re, Rg1, Rf, and Rg2. The Re and Rg1 ginsenosides usually account for more than 90% of the total ginsenoside content of ginseng roots (5). Ginseng roots are usually processed to red ginseng using steaming and drying, whereas dried roots (without the steaming process) are categorized as white ginseng (1,5). The partly deglycosylated ginsenosides Rh1, Rh2, and Rg3 are present in red ginseng as artifacts produced during steaming, along with all the ginsenosides detected in white ginseng (6). Transformation of ginsenosides into deglycosylated ginsenosides is required for more effective *in vivo* physiological actions (7). For instance, the anticancer activities of ginsenosides after oral administration are based on metabolites formed due to deglycosylation by intestinal bacteria (8). The pharmaceutical activities of the less glycosylated minor ginsenosides Rd, Rg3, Rh2, and compound-K are superior to the activities of the major ginsenosides. These minor ginsenosides are present in ginseng in small percentages and are produced by

hydrolysis of the sugar moieties of the major ginsenosides (9,10). Once administered orally, ginsenosides are exposed to gastric juice, digestive, and bacterial enzymes in the stomach and intestines. However, ginsenosides are not affected by gastric juice, with the exception of a slight oxygenation. In addition, the oral bioavailability of intact ginsenosides from the intestines is extremely low (0.1-4.4% of Rb1, 3.7% of Rb2, and 1.9-18.4% of Rg1) (8,11) probably due to a high Mw and hydrophilicity (12).

Different studies have focused on conversion of major ginsenosides to the more active minor ginsenosides using heat, mild acid hydrolysis (13), alkaline cleavage (14), and microbial (15) and enzymatic conversions (10). Chemical methods result in undesirable side reactions, such as epimerization, hydration, and hydroxylation (16). In addition, heat and acid treatments can randomly hydrolyze glycosidic linkages in other, minor active ginsenosides and acidic polysaccharides, which can decrease the pharmaceutical efficacy of ginseng (9). The effects of various microorganisms on conversion of ginsenosides to their aglycones have been extensively studied during (4,9,16). However, investigations of the microbial conversion of ginsenosides have been limited to only a few ginsenoside types (17-21) and most of the microorganisms used for transformation of ginsenosides are not suitable for production of edible products due to health and safety considerations (16). Moreover, different studies have used organic chemicals, which are not suitable for commercial ginseng products, to obtain ginseng extracts (19), Therefore, enzyme-assisted bioconversion to hydrolyze sugar moieties at specific positions can be used as an alternative production process for active minor ginsenosides. β-Glycosidase is an enzyme capable of catalyzing the bioconversion of plant glycosides to aglycones (22) via action upon monoglycosides, diglycosides, and modified glycosides (23). Despite these useful actions, the effects of β-glycosidase on hydrolysis of ginsenosides have not been extensively studied.

The objectives of this study were to investigate the effects of β-glycosidase on deglycosylation of 10 major ginsenosides in red ginseng, and to determine the conversional pathways of ginsenosides after β-glycosidase treatment. The effects of β-glycosidase on both PPD and PPT ginsenosides were determined either in pure forms or in complexes, such as crude saponin and red ginseng extracts. Red ginseng was extracted using water to test the applicability of a β-glycosidase treatment to the production process of commercial red ginseng products.

Materials and Methods

Materials Freshly harvested 4 year old Korean ginseng roots from Keumsan (Korea) were provided by Nutrex Technology Co., Ltd. (Naju, Korea). β-Glycosidase derived from *Aspergillus fumigatus* was purchased from Amano Enzyme Inc. (Nagoya, Japan). Membrane syringe filters (13 mm diameter, 0.45 μm pore size) were purchased from Millipore (Bedford, MA, USA). Ethanol and ether were purchased from Samchun Pure Chemical Co., Ltd. (Pyeongtaek, Korea). Butanol was purchased from J. T. Baker (Phillisburg, NJ, USA). HPLC-grade methanol, water, and acetonitrile were purchased from Burdick & Jackson (Muskegon, MI, USA). Ginsenoside standards (Rb1, Rb2, Rc, Rd, Rg3, Re, Rg1, Rg2, Rf, and Rh1) were obtained from ChromaDex Inc. (Irvine, Canada).

β-Glycosidase treatment of ginsenosides Each ginsenoside standard was treated with β-glycosidase and analyzed using high performance liquid chromatography (Dionex; Sunnyvale, CA, USA) after enzyme treatment. Individual ginsenoside standards were dissolved in analytical grade methanol and diluted with water (analytical grade) to produce a 250 ppm solution. The solution was mixed with β-glycosidase (0.05% w/v) and incubated at 55ºC in a water bath (SB-651; Eyela, Tokyo, Japan) for 6 h. These reaction conditions were selected based on a previous study relating to the use of β-glycosidase for bioconversion of bioactive compounds (24). A 1 mL aliquot from the solution was taken every 1 h and analyzed using HPLC. A control sample was also taken at 0 h just after addition of β-glycosidase.

Preparation of red ginseng extracts Fresh ginseng roots were washed with tap water and steamed at 98ºC for 3 h using an autoclave (SS-325; Tomy Kogyo Co., Tokyo, Japan). Steamed roots were dried at 60ºC for 3 days using a forced convection type dryer (OF-22GW; Jeio Tech Co., Daejeon, Korea) to form red ginseng after Maillard reactions (25). Dried red ginseng was extracted with water for 3 h at 80ºC 3 times. Extracts were concentrated using a rotary evaporator (N-1000; Eyela) under vacuum at 70ºC until the sugar content of the extract was 70ºBx.

β-Glycosidase treatment of crude saponin For comparison of a ginsenoside bioconversion to a ginsenoside complex, crude saponin was purified (26) from a red ginseng extract and treated with β-glycosidase. An amount of 7 g of red ginseng extract was transferred into a 250 mL round bottom flask and evaporated using a rotary evaporator (N-1000; Eyela) under vacuum at 70ºC. The dried residue was extracted three times with 50 mL of water saturated butanol at 80ºC for 1 h. The butanol solution was filtered, transferred to a separatory funnel, and washed with 20 mL of distilled water to remove impurities. Afterwards, the butanol solution was transferred to a round bottom flask for evaporation. The dried residue was extracted with ether at

36ºC for 30 min and cooled for 30 min. After cooling, ether was discarded and the flask was dried at 105ºC for 20 min, cooled in a desiccator for 30 min, and weighed until a constant weight was obtained. The weight was then compared to the weight of the empty flask. The difference in weights corresponded to the crude saponin extracted from the red ginseng extract. Purified crude saponin was dissolved in distilled water at the ratio of 1/200 (w/v), then mixed with β-glycosidase (0.05% w/v). A β-glycosidase treated crude saponin solution was incubated at 55ºC in a water bath (SB-651; Eyela) for 24 h. A 20 mL aliquot of the solution was taken every 4 h, transferred to a round bottom flask maintained until a constant weight was achieved, and evaporated. The dried crude saponin was dissolved in methanol for HPLC analysis. A control sample was taken at 0 h after enzyme addition.

β-Glycosidase treatment of red ginseng extracts An amount of 20 g of a red ginseng extract was dissolved in 180 mL of distilled water and mixed with β-glycosidase (0.05% w/v) to achieve a homogenous solution. This solution was placed at 55ºC in a water bath (SB-651; Eyela) for 24 h. A 20 mL sample was taken at 0 h as a control, and thereafter every 4 h for further analysis. Samples were transferred to a 250 mL round-bottom flask and evaporated using a rotary evaporator (N-1000; Eyela) under vacuum at 70ºC. The dried residue was extracted three times with 50 mL of water saturated butanol at 80ºC for 1 h. The proceeding steps for purification of crude saponin were the same as described above. Purified crude saponin was dissolved in methanol before HPLC analysis.

HPLC analysis of ginsenosides The 10 ginsenosides Rb1, Rb2, Rc, Rd, Rg3, Rg1, Rg2, Re, Rf, and Rh1 were analyzed using a chromatographic method (27). The HPLC system consisted of a P680 pump, an ASI-100 auto-sampler, a UVD340U UV detector, a TCC100 column oven (Dionex) and a Capcell Pak C18 MG column (4.6 mm×250 mm; Shiseido, Tokyo, Japan). The detection wavelength was set at 203 nm and the solvent flow rate was kept constant at 1.6 mL/min. The mobile phase used for separation consisted of solvent A (water) and solvent B (acetonitrile). The gradient elution procedure was 0-10 min with 80% A, 10-40 min with 68% A, 40-48 min with 58% A, 48-50 min with 0% A, 50-60 min with 0% A, 60-62 min with 80% A, and 62-70 min with 80% A. The injection volume was 20 μL for analysis and all samples were filtered using 0.45 μm membrane syringe filters (Millipore) before analysis. Peak identifications were based on the retention times and comparisons with calibration curves obtained using injected standard samples prepared by dissolving pure ginsenosides in analytical grade methanol.

Statistical analysis Experiments and analytical measurements were carried out in triplicate. Data are represented as mean±standard deviation (SD). Statistical analysis was performed using the GraphPad Prism 5.0 software (GraphPad Software Inc., La Jolla, CA, USA). Data were analyzed using a one-way analysis of variance (ANOVA). Significance was defined at $p<0.05$ and significant results were further analyzed using Tukey's post-hoc test.

Results and Discussion

Effects of β-glycosidase on ginsenoside compounds The protopanaxadiol (PPD) ginsenosides Rb1, Rb2, and Rc were converted to Rd, which was subsequently converted to Rg3. Rb1 was almost completely converted to Rd using a β-glycosidase treatment in 1 h (Fig. 1A). Rd was further hydrolyzed to Rg3; however, the conversion rate of Rb1 to Rd was much faster than the conversion rate of Rd to Rg3 as Rd was detected even after 6 h. Rb2 (Fig. 1B) and Rc (Fig. 1C) were also converted to Rd and Rg3. However, their conversion patterns were different from the pattern of Rb1. Both were detected even after 6 h of enzyme treatment, and the concentration of Rg3 kept increasing, suggesting that β-glycosidase can hydrolyze not only the β-(1→6) glycosidic linkage at C-20 of Rb1, but also the α-(1→6) glycosidic linkage at C-20 of Rb2 and Rc, producing Rd. β-Glycosidase may release saccharides in a disaccharide unit, thus producing Rg3 directly from Rb1, Rb2, and Rc since β-glycosidase is known to act mainly on disaccharide glycosides (23). The conversion rate of each ginsenoside was probably dependent on the affinity of β-glycosidase to the specific ginsenoside. This enzyme is more efficient in hydrolyzing the β-(1→6) glycosidic linkage of Rb1 than the α-(1→6) glycosidic linkages of Rb2 and Rc. Furthermore, hydrolysis of Rc was faster than hydrolysis of Rb2. Other enzymes may also produce different rates of ginsenoside bioconversion (10).

The protopanaxatriol (PPT) ginsenosides Re, Rg1, Rf, and Rg2 were hydrolyzed to Rh1 using a β-glycosidase treatment. Rg1 was rapidly produced from Re (Fig. 2A) after 1 h of incubation. After 6 h of incubation, Re was almost entirely converted to Rg1, which was later partly converted to Rh1. Similar results were observed for hydrolysis of Rg1 alone, although most of the Rg1 remained intact after 6 h of incubation (Fig. 2B). Rf was also partly hydrolyzed to Rh1 after 6 h of treatment with β-glycosidase (Fig. 2C). Conversion of Rg2 to Rh1 was relatively faster than conversion of Rg1 and Rf since Rg2 was completely converted to Rh1 within 6 h (Fig. 2D). These results indicate that β-glycosidase probably hydrolyzes the α-(1→2) glycosidic linkage at C-6 of Re and Rg2, and

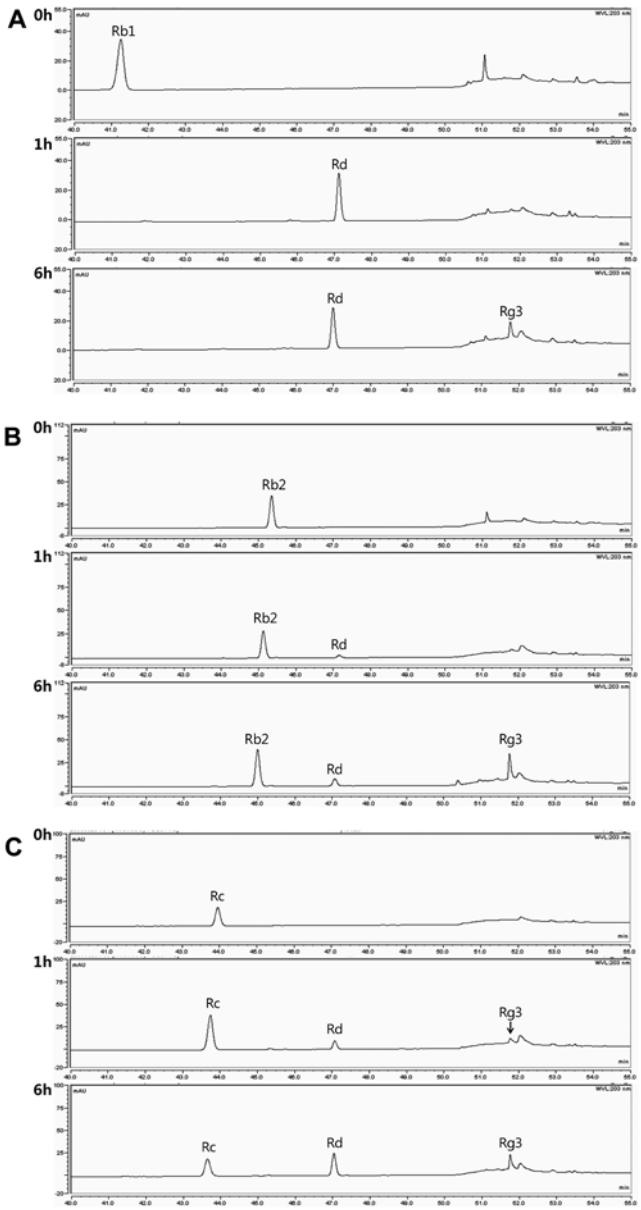

Fig. 1. Chromatograms showing conversion of the protopanaxadiol ginsenosides Rb1 (A), Rb2 (B), and Rc (C) using β-glycosidase treatment. Ginsenoside solutions (250 ppm) were incubated with β-glycosidase (0.05% w/v) at 55ºC in a water bath for 6 h.

probably favors the α-rhamnopyranosyl β-(1→2) glucopyranosyl bond of Re and Rg2, compared to α-glucopyranosyl β-(1→2) glucopyranosyl linkage of Rf.

Zhao *et al*. (28) observed that the fungal enzymes of *Cylindrocarpon destructans* were able to transform the PPD ginsenosides Rb1, Rb2, Rc, and Rd, whereas the PPT ginsenosides Re and Rg1 remained unaffected after 48 h of reaction with these fungal enzymes. The bioconversion products from PPD ginsenosides in this study included Rd, ginsenoside XVII, compound O, compound Mb, and ginsenoside F2, suggesting that enzymes from *C. destructans* had a higher specicity for the glycosidic linkage at C3 than the glycosidic linkage at C20 (28). On the other hand, Wang *et al*. (24) reported that a ginsenosidase type IV from an *Aspergillus* strain hydrolyzed the PPT ginsenosides Rg2 and Rf to Rh1, and further hydrolyzed Rh1 to aglycone, while the enzyme was not able to hydrolyze PPD ginsenosides. In the present study, β-glycosidase showed broader specificities than the fungal enzymes as it transformed both PPD and PPT ginsenosides. Based on these findings, conversional pathways for PPD and PPT ginsenosides using a β-glycosidase treatment can be proposed. The final product of β-glycosidase-assisted bioconversion of PPD ginsenosides was Rg3, whereas the final product of PPT ginsenosides was Rh1 (Fig. 3). Rb1, Rb2, and Rc may either be converted to Rg3 directly, or first to Rd, which is subsequently converted to Rg3 in the presence of β-glycosidase at a low concentration with moderate heating. The 2 β-D-glucopyranosyl moieties are hydrolyzed from Rb1 to form Rg3, and α-L-arabinopyranosyl and β-D-glucopyranosyl are cleaved from Rb2 to form Rg3. Rc is converted to Rd due to removal of 1 α-D-arabinofuranosyl, and subsequent removal of 1 β-D-glucopyranosyl from Rd via β-glycosidase hydrolysis results in Rg3 (Fig. 3A).

Among PPT ginsenosides, Re is first converted to Rg1 via hydrolysis of the α-L-rhamnopyranosyl moiety, then a β-D-glucopyranosyl moiety is further removed to form Rh1. Conversion of Rf and Rg2 to Rh1 requires hydrolysis of β-D-glucopyranose and α-L-rhamnopyranose, respectively. Hence no intermediate is produced in these bioconversions (Fig. 3B).

Rg3 and Rh1 probably have more bioavailability, compared to the parent ginsenosides, due to lower Mw (24). *In vivo* studies have shown that Rg3 can be metabolized to Rh2 and/or 20(S)-PPD by human intestinal bacteria (29). This is consistent with a pharmacokinetic study in rats showing that intra-gastric administration of 10 mg/kg of Rg3 resulted in plasma concentrations of approximately 40 and 100 ng/mL of Rh2 and 20(S)-PPD, respectively, 4 h after administration (30). Rh2 and 20(S)-PPD are probably more physiologically active than the intact ginsenosides Rb1, Rd, Rb2, and Rc. Rh2 and 20(S)-PPD have been shown to be more cytotoxic against tumor cells, compared with the intact ginsenosides (29). However, intestinal bacterial strains may vary from individual to individual, and the capacity to metabolize ginsenosides to more bioactive forms may also vary (9). Therefore, transformation of ginsenosides prior to ingestion could be beneficial to maximize absorption and pharmacological activities. For example, transformation of PPT ginsenosides into Rh1 using β-glycosidase is expected to increase the bioactivity of the intact ginsenosides and to aid in further transformation to ginsenoside F1, and then finally to 20(S)-

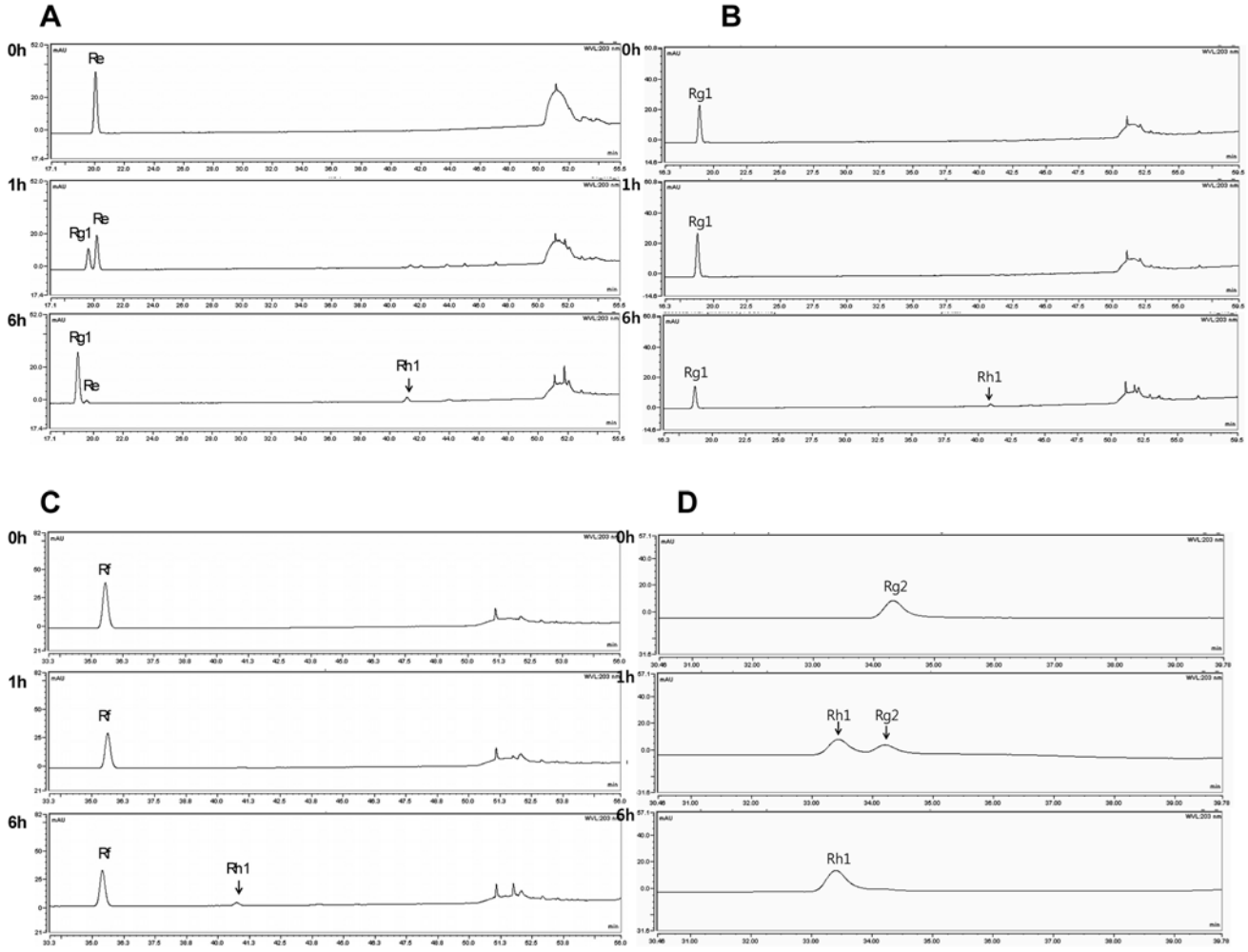

Fig. 2. Chromatograms showing the conversion of the protopanaxatriol ginsenosides Re (A), Rg1 (B), Rf (C), and Rg2 (D) using β-glycosidase treatment. Ginsenoside solutions (250 ppm) were incubated with β-glycosidase (0.05% w/v) at 55ºC in a water bath for 6 h.

PPT by intestinal bacteria (7). It is also recommended to treat ginsenosides with β-glycosidase prior to *in vivo* trials to assess the health effects. Hence, it should be feasible to develop a specific bioconversion processes to obtain specifically designed functional products using appropriate combinations of ginsenoside substrates and suitable enzymes (21).

Effects of β-glycosidase on red ginseng extracts Red ginseng, which is commonly sold in the form of a concentrated extract, is preferred over white ginseng because of more health benefits due to increased bioavailability of phenolic compounds and ginsenosides (31). Although different studies have reported microbial enzyme treatments of ginseng extracts for producing compound K and ginsenoside aglycon (19), all such studies used organic solvents in preparation of ginseng extracts. Therefore, to evaluate the industrial applications of β-glycosidase treatments, red ginseng extracts prepared using water were treated with β-glycosidase for 24 h and changes in 10 ginsenosides were analyzed every 4 h. Levels of each ginsenoside were normalized to relative compositions by comparison to the total ginsenoside content (sum of 10 ginsenosides) at each time interval to compensate for sampling error (Table 1). The levels of the PPD ginsenosides Rb1, Rb2, and Rc decreased over time due to β-glycosidase treatment, but this decrease was not significant. The relative proportion of Rc was significantly (p<0.05) decreased by β-glycosidase treatment from a beginning concentration of 17.37% to 12.94% after 12 h, 11.43% after 16 h, 10.72% after 20 h, and 8.64% after 24 h. The content and relative proportion of Rd was significantly (p<0.05) increased by treatment with β-glycosidase for 24 h from 0.77 mg/g (8.92%) to 2.37 mg/g (25.99%). A significant increase in the relative proportion of Rd was evident even after 12 h of treatment. The level of Rg3 was increased from 0.81 to 1.65% after 24 h due to hydrolysis; however the increase was not consistent.

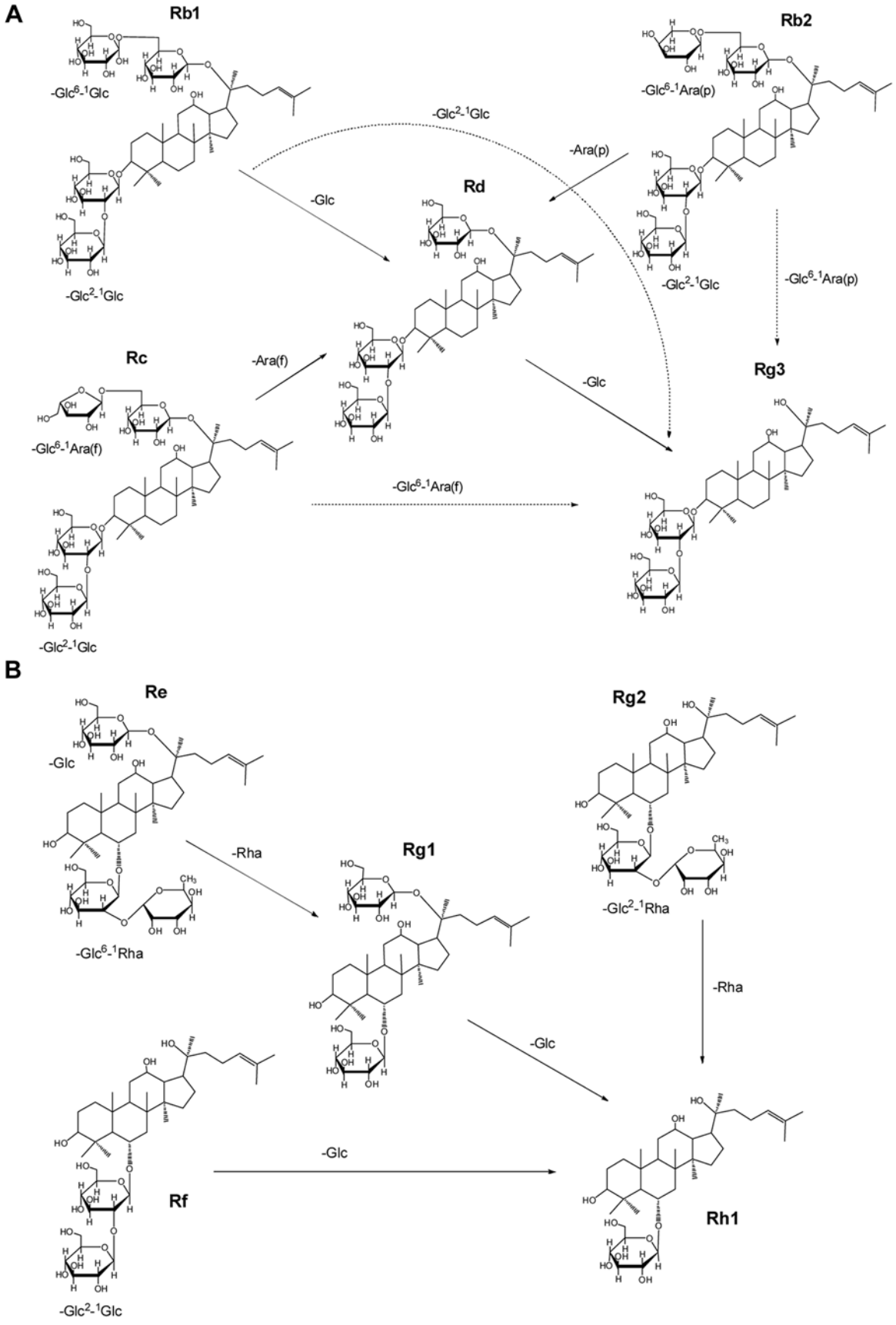

Fig. 3. Suggested conversional pathways of protopanaxadiol ginsenosides (A) and protopanaxatriol ginsenosides (B) using β-glycosidase treatment. Glc, β-D-glucopyranosyl; Rha, α-L-rhamnopyranosyl; Ara(f), α-D-arabinofuranosyl; Ara(p), α-L-arabinopyranosyl

Table 1. Changes in ginsenoside content (mg/g red ginseng extract) and percentage (%) changes in the relative proportions of individual ginsenosides in a red ginseng extract using β-glycosidase treatment

Ginsenoside	β-Glycosidase reaction time													
	0 h		4 h		8 h		12 h		16 h		20 h		24 h	
	mg/g	%	mg/g	%	mg/g	%	mg/g	%	mg/g	%	mg/g	%	mg/g	%
Rb1	2.8 ±0.27	32.37 ±4.42	2.32 ±0.7	32.69 ±2.9	2.44 ±0.44	33.66 ±3.88	2.36 ±0.35	32.71 ±3.8	2.45 ±0.4	31.26 ±3.38	2.63 ±0.28	27.67 ±0.16	2.37 ±0.59	26.46 ±0.11
Rb2	0.73 ±0.3	8.57 ±4.31	0.95 ±0.39	9.98 ±5.31	0.89 ±0.24	9.19 ±4.52	0.64 ±0.27	8.76 ±3.44	0.68 ±0.21	8.57 ±2.2	0.77 ±0.11	8.1 ±0.39	0.66 ±0.13	7.4 ±0.34
Rc	1.52 ±0.28	17.37 ±1.09	1.54 ±0.63	15.16 ±1.7	1.58 ±0.44	15.08 ±1.09	0.93 ±0.08	12.94 ±0.38*[1)]	0.9 ±0.22	11.43 ±1.3*	1.03 ±0.21	10.72 ±1.27*	0.76 ±0.12	8.64 ±0.69*
Rd	0.77 ±0.04	8.92 ±0.71	1.44 ±0.25	15.05 ±3.74	1.68 ±0.23	16.51 ±2.39	1.42 ±0.11	19.82 ±2.74*	1.73 ±0.09	22.25 ±2.75*	2.17 ±0.22*	22.94 ±2.44*	2.37 ±0.91*	25.99 ±3.27*
Rg3	0.07 ±0.06	0.81 ±0.57	0.07 ±0.04	0.65 ±0.43	0.13 ±0.1	1.09 ±0.79	0.09 ±0.08	1.19 ±1.04	0.07 ±0.07	0.91 ±0.68	0.12 ±0.04	1.21 ±0.24	0.14 ±0.05	1.65 ±0.84
Re	1.59 ±0.38	18.17 ±3.45	0.84 ±0.57	7.89 ±3.07*	0.67 ±0.36	6.15 ±2.36*	0.25 ±0.15*	3.37 ±1.87*	0.17 ±0.08*	2.15 ±0.68*	0.16 ±0.07*	1.64 ±0.77*	0.15 ±0.05*	1.64 ±0.11*
Rg1	0.69 ±0.38	7.72 ±3.61	1.26 ±0.7	12.26 ±5.28	1.32 ±0.71	12.1 ±4.58	1.07 ±0.39	14.96 ±5.35	1.31 ±0.55	16.47 ±5.18	1.94 ±0.23	20.42 ±1.09*	1.85 ±0.31	20.92 ±1.46*
Rf	0.22 ±0.01	2.51 ±0.18	0.28 ±0.05	2.87 ±0.53	0.29 ±0.1	2.74 ±0.63	0.18 ±0.01	2.49 ±0.18	0.21 ±0.01	2.76 ±0.42	0.25 ±0.05	2.66 ±0.59	0.25 ±0.12	2.65 ±0.66
Rg2	0.19 ±0.09	2.13 ±0.84	0.09 ±0.06	0.82 ±0.28*	0.05 ±0.03	0.43 ±0.2*	0.02 ±0.02*	0.28 ±0.27*	0.01 ±0.01*	0.09 ±0.08*	0.02 ±0.01*	0.18 ±0.1*	0.01 ±0.00*	0.05 ±0.05*
Rh1	0.13 ±0.06	1.45 ±0.52	0.27 ±0.17	2.58 ±1.05	0.33 ±0.17	3.05 ±1.14	0.25 ±0.06	3.49 ±0.62	0.33 ±0.1	4.13 ±0.84*	0.42 ±0.04	4.45 ±0.04*	0.4 ±0.02	4.6 ±0.79*
Rg1+Rb1	3.49 ±0.65	40.09 ±1.31	4.46 ±1.4	44.96 ±3.05*	4.76 ±1.15	45.76 ±0.71*	3.43 ±0.74	47.67 ±1.65*	3.76 ±0.95	47.73 ±1.81*	4.57 ±0.51	48.09 ±1.25*	4.22 ±0.91	47.38 ±1.36*

[1)]*Significantly different from the control at 0 h (p<0.05) within a row. Values are expressed as the mean±SD (n=3).

Among PPT ginsenosides, the levels of Re and Rg2 showed significant decrease from 1.59 to 0.15 mg/g, and from 0.19 to 0.01 mg/g, respectively, after 24 h of incubation with β-glycosidase. The relative proportion of Rg1 increased from 7.72 to 20.42% and to 20.92% after 20 and 24 h, respectively, due to conversion of Re to Rg1. The relative proportion of Rh1 increased from 1.45 to 4.13% after 16 h of incubation with β-glycosidase. The level and relative proportion of Rf did not show significant change.

The sum of Rb1 and Rg1 (Rb1+Rg1) is a critical parameter for commercial red ginseng products in Korea. According to the Korean Food Code (32), red ginseng powder or extract must contain 0.8-34 mg/g of Rb1+Rg1. For immune-stimulating and invigorating purposes, 3-80 mg of Rb1+Rg1 should be ingested, while 2.4-23 mg of Rb1+Rg1 should be ingested to inhibit platelet aggregation (31). The results of this study have shown that the Rb1+Rg1 amount increased by 130% after 4 h and by 137% after 8 h of incubation with β-glycosidase (Table 1). The relative proportion of Rb1+Rg1 showed significant increase in comparison to other ginsenosides after β-glycosidase treatments for 4, 8, 12, 16, 20, and 24 h. Fast conversion of Re to Rg1 contributed to the increased level of Rb1+Rg1, although the level of Rb1 was decreased slightly over time due to hydrolysis by β-glycosidase. For incubation times longer than 24 h, the Rb1+Rg1 total might be decreased due to hydrolysis of Rb1.

Effects of β-glycosidase on crude saponin Crude saponin purified from red ginseng extracts was incubated with β-glycosidase (0.05% w/v) at 55ºC for 24 h. Samples were taken every 4 h over a 24 h period. The levels and relative proportions of Rb1, Rc, Re, and Rg1 decreased significantly (p<0.05) after 24 h of treatment with β-glycosidase (Table 2). Significant decrease in the amount of Rb1, Rc, and Re were prominent even after 4 h of enzyme reaction. The level and relative proportion of Rd started to increase significantly after 4 h of reaction time, and kept increasing afterwards. The relative proportions of Rg3, Rg2, and Rh1 also started to show significant increase after 4 h. Neither the amounts nor the relative proportions of Rb2 and Rf showed significant changes during 24 h of enzyme reaction. These results are consistent with results from the β-glycosidase treatment of individual ginsenosides since increases in the amounts Rg3 and Rh1 were observed. Rg3 and Rh1 are the end products in β-glycosidase-assisted

Table 2. Changes in ginsenoside contents (mg/g crude saponin) and percentage (%) changes in relative proportions of individual ginsenosides in a crude saponin solution using β-glycosidase treatment

Ginsenoside	β-Glycosidase reaction time													
	0 h		4 h		8 h		12 h		16 h		20 h		24 h	
	mg/g	%	mg/g	%	mg/g	%	mg/g	%	mg/g	%	mg/g	%	mg/g	%
Rb1	54.54 ±4.69	31.94 ±2.09	6.68 ±1.47*[1)]	5.05 ±0.35*	3.64 ±0.92*	3.32 ±0.41*	3.92 ±0.56*	2.93 ±0.35*	4.21 ±1.33*	3.16 ±0.27*	3.04 ±1.27*	3.08 ±0.93*	2.26 ±0.86*	2.27 ±0.48*
Rb2	24.8 ±7.24	14.26 ±2.3	20.41 ±2.74	15.58 ±0.59	16.46 ±3.42	14.98 ±0.42	20.26 ±3.73	14.96 ±0.71	20.68 ±6.54	15.37 ±0.5	14.79 ±6.09	14.27 ±0.55	15.61 ±4.64	15.46 ±1.08
Rc	24.21 ±7.44	13.88 ±2.11	12.83 ±2.4*	9.73 ±0.52*	7.41 ±1.92*	6.68 ±0.42*	6.52 ±1.43*	4.8 ±0.5*	5.03 ±1.47*	3.89 ±0.72*	3.1 ±1.11*	3.12 ±0.57*	3.03 ±1.03*	3.05 ±0.59*
Rd	13.69 ±2.55	7.94 ±0.51	53.22 ±7.34*	40.58 ±1.00*	49.83 ±9.53*	45.47 ±0.88*	64.84 ±8.99*	48.23 ±1.88*	65.99 ±20.83*	49.02 ±0.77*	52.84 ±21.42*	50.76 ±1.79*	51.53 ±15.38*	51.01 ±0.95*
Rg3	1.97 ±0.76	1.13 ±0.37	2.28 ±0.37	1.73 ±0.11*	2.43 ±0.63	2.19 ±0.22*	3.1 ±0.54	2.3 ±0.11*	3.39 ±0.98	2.57 ±0.29*	3.14 ±1.23	3.07 ±0.23*	3.11 ±1.01	3.1 ±0.33*
Re	33.04 ±5.15	19.46 ±4.01	17.44 ±3.81*	13.19 ±0.96*	12.79 ±3.33*	11.54 ±0.65*	14.1 ±3.04*	10.38 ±0.83*	11.74 ±3.45*	8.88 ±0.61*	8.33 ±3.13*	8.13 ±0.6*	7.21 ±1.63*	7.34 ±0.71*
Rg1	10.02 ±1.43	5.84 ±0.15	8.68 ±1.47	6.6 ±0.3	7.26 ±1.68	6.59 ±0.34	8.67 ±1.73	6.4 ±0.39	8.27 ±2.38	6.24 ±0.3	5.8 ±2.21*	5.67 ±0.37	5.46 ±1.65*	5.46* ±0.47
Rf	3.01 ±0.53	1.75 ±0.08	2.22 ±0.45	1.69 ±0.14	1.94 ±0.52	1.75 ±0.11	2.38 ±0.54	1.75 ±0.12	2.27 ±0.68	1.7 ±0.05	1.8 ±0.73	1.75 ±0.07	1.78 ±0.55	1.77 ±0.14
Rg2	3.66 ±0.28	2.15 ±0.18	3.86 ±0.58	2.95 ±0.16*	3.67 ±0.68	3.35 ±0.17*	4.79 ±0.7	3.56 ±0.26*	5.29 ±1.68	3.94 ±0.16*	4.24 ±1.72	4.09 ±0.28*	4.28 ±1.32	4.24 ±0.2*
Rh1	2.9 ±0.86	1.67 ±0.24	3.76 ±0.35	2.9 ±0.29*	4.52 ±1.03	4.12 ±0.34*	6.35 ±1.33	4.69 ±0.33*	7.28 ±2.71*	5.24 ±0.75*	6.38 ±2.91	6.06 ±0.57*	6.38 ±2.08	6.3 ±0.38*
Rg1+Rb1	64.56 ±6.12	37.78 ±2.25	15.36 ±2.94*	11.65 ±0.65*	10.9 ±2.6*	9.9 ±0.75*	12.59 ±2.29*	9.33 ±0.73*	12.48 ±3.71*	9.39 ±0.56*	8.84 ±3.48*	8.75 ±1.3*	7.72 ±2.51*	7.73 ±0.95*

[1)]*Significantly different from a control at 0 h ($p<0.05$) within a row. Values are expressed as the mean±SD ($n=3$).

deglycosylation of pure PPD and PPT ginsenosides, respectively. Furthermore, crude saponin may be a more favorable substrate for enzyme reaction, compared with original red ginseng extracts, due to fewer impurities after the purification processes because ginseng contains saponins, polysaccharides, and flavonoids (33).

The initial conversion rate of Rb1 was fast so that the level of Rb1 decreased from 54.54 to 6.68 mg/g after 4 h, and then decreased to 2.26 mg/g after 24 h. A marked decrease (34.21 to 5.03 mg/g) in the Rc amount was observed after 16 h. The level of Re was decreased by 65% during the first 8 h of incubation, and then decreased gradually thereafter. The decrease in reaction rates might be due to saturation of β-glycosidase with an excess of substrate, or there might not be sufficient available substrate for enzymatic hydrolysis due to the fast initial reaction. The level of Rd was increased rapidly after 4 h of incubation; however, it did not show significant change afterwards, probably due to β-glycosidase saturation with substrate, or some of the newly synthesized Rd was further hydrolyzed to Rg3.

The level of Rg1 started to show significant decrease (from 10.02 to 5.80 mg/g) after 20 h of reaction. The relative contents of Rg3 and Rh1 to the total ginsenoside content showed significant increases after 4 h of incubation with β-glycosidase. Ko *et al*. (10) reported synthesis of Rd from PPD ginsenosides in a crude saponin mixture when treated with the glycoside hydrolases lactase and β-galactosidase from *Aspergillus oryzae,* and cellulase from *Trichoderma viride*. Synthesis of Rd and Rg3 from PPT ginsenosides was also reported in a saponin mixture treated with lactase from *Penicillium* sp. β-glycosidase assisted bioconversion was comparable with other methods of ginsenoside conversion, such as puffing (use of heating and pressure). An *et al*. (26) observed that the Rg3 content of crude saponin prepared from puffed red ginseng increased up to 3.35 mg/g after an increase in the puffing pressure. The β-glycosidase-assisted reaction produced 3.13 mg/g of Rg3 after 24 h of incubation. Enzymes play important roles in liberation of ginsenosides from ginseng leaves when used at an appropriate stage in the extraction process (34). β-glycosidase favors hydrolysis of the PPD ginsenosides Rb1, Rc, and Re over the PPT ginsenosides Rg1, Rg2, Rf, and Rb2. The PPD ginsenosides changed more dramatically after β-glycosidase treatment in red ginseng extract (Table 1) and crude saponin (Table 2) as discussed earlier. The

PPD ginsenosides Rb1, Rb2, and Rc comprise major portions of the total ginsenoside content in crude saponin. Lower amounts of PPT ginsenosides may limit the probability of interaction with β-glycosidase and, thus, hydrolyzation by the enzyme. Shehzad *et al*. (35) reported that the 6 major ginsenosides Rb1, Rb2, Rc, Rd, Re, and Rg1 comprise approximately 90% w/w of the total weight of saponin, which is consistent with the findings of this report. At the start of enzyme treatment, these ginsenosides were present in higher proportions, compared to other ginsenosides in red ginseng extracts and a purified crude saponin solution. Conversional pathways for ginsenosides using various microbial enzymes have been reported (17,19,21). However, complete transformational pathways were not developed in these studies, suggesting the presence of partial pathways for PPD and PPT ginsenosides, and other ginsenosides. This study is useful for providing conversional pathways for both PPD and PPT ginsenosides using β-glycosidase. Further research will, however, be necessary to arrive at more in-depth conclusions regarding ginsenoside bioconversion using β-glycosidase.

The effects of β-glycosidase on both PPD and PPT ginsenosides are shown and activities of the β-glycosidase enzyme in different settings using pure ginsenosides, crude saponin, and red ginseng extracts are compared. Determination of conversional pathways of ginsenosides was accomplished by incubating ginsenoside standards with β-glycosidase, followed by HPLC analysis. β-Glycosidase has a broad specificity for various β-glycosidic bonds, including β-(1→6), α-(1→6), and α-(1→2)-glycosidic linkages. The final metabolite of the PPD ginsenosides Rb1, Rb1, Rc, and Rd was Rg3, while the metabolite of the PPT ginsenosides Rg1, Rg2, Re, and Rf was Rh1 using β-glycosidase treatment. β-Glycosidase treatment can raise the bioactive potential of pure ginsenosides by conversion to deglycosylated and more active forms. When crude saponin was incubated with β-glycosidase for 24 h, the levels of Rb1, Rc, Re, and Rg1 decreased due to hydrolysis of ginsenoside sugar moieties, while the levels of the deglycosylated ginsenosides Rd, Rg3, and Rh1 increased. When β-glycosidase was applied to red ginseng extract, the levels of Rb1, Rc, Re, and Rg2 decreased after 24 h of incubation, while the levels of the less glycosylated Rd, Rg3, Rg1, and Rh1 forms increased. The total amount of Rb1 and Rg1 (Rb1+Rg1), which is an important criterion for commercially available red ginseng extracts in Korea, was increased by 130-140% after β-glycosidase treatment. Therefore, the β-glycosidase treatment of red ginseng extract can be an inexpensive and effective way to improve the quality of red ginseng products with a higher Rb1+Rg1 content. Furthermore, higher amounts of ginsenoside aglycones in enzyme-treated red ginseng extracts with increased bioavailability and pharmacological activities of ginsenosides can benefit consumers.

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References

1. Park JD, Rhee DK, Lee YH. Biological activities and chemistry of saponins from *Panax ginseng* C.A. Meyer. Phytochem. Rev. 4: 159-175 (2005)
2. Huang KC. The Pharmacology of Chinese Herbs. CRC Press, Boca Raton, FL, USA (1999)
3. Shibata S, Tanaka O, Shoji J, Saito H. Chemistry and pharmacology of *panax*. pp. 217-284. In: Economic and Medicinal Plants Research. Wagner H, Farnsworth NR, Hikino H (eds). Academic Press, London, UK (1985)
4. Cheng LQ, Na JR, Bang MH, Kim MK, Yang DC. Conversion of major ginsenoside Rb1 to 20(S)-ginsenoside Rg3 by *Microbacterium* sp. GS514. Phytochemistry 69: 218-224 (2008)
5. Christensen LP. Ginsenosides chemistry, biosynthesis, analysis, and potential health effects. Adv. Food Nutr. Res. 55: 1-99 (2009)
6. Kitagawa I, Taniyama T, Shibuya H, Noda T, Yoshikawa M. Chemical studies on crude drug processing. V. On the constituents of ginseng radix rubra (2): Comparison of the constituents of white ginseng and red ginseng prepared from the same *Panax ginseng*. Yakugaku. Zasshi. 107: 495-505 (1987)
7. Tawab MA, Bahr U, Karas M, Wurglics M, Schubert-Zsilavecz M. Degradation of ginsenosides in humans after oral administration. Drug Metab. Dispos. 31: 1065-1071 (2003)
8. Hasegawa H. Proof of the mysterious efficacy of ginseng: Basic and clinical trials: metabolic activation of ginsenoside: Deglycosylation by intestinal bacteria and esterification with fatty acid. J. Pharmacol. Sci. 95: 153-157 (2004)
9. Kim MK, Lee JW, Lee KY, Yang DC. Microbial conversion of major ginsenoside Rb(1) to pharmaceutically active minor ginsenoside rd. J. Microbiol. 43: 456-462 (2005)
10. Ko SR, Suzuki Y, Suzuki K, Choi KJ, Cho BG. Marked production of ginsenosides Rd, F2, Rg3, and compound K by enzymatic method. Chem. Pharm. Bull. 55: 1522-1527 (2007)
11. Hu Y, Luan H, Hao D, Xiao H, Yang S, Yang L. Purification and characterization of a novel ginsenoside-hydrolyzing β-D-glucosidase from the China white jade snail (*Achatina fulica*). Enzyme Microb. Tech. 40: 1358-1366 (2007)
12. Karikura M, Miyase T, Tanizawa H, Taniyama T, Takino Y. Studies on absorption, distribution, excretion and metabolism of ginseng saponins. VI. The decomposition products of ginsenoside Rb2 in the stomach of rats. Chem. Pharm. Bull. 39: 400-404 (1991)
13. Han BH, Park MH, Han YN, Woo LK, Sankawa U, Yahara S, Tanaka O. Degradation of ginseng saponins under mild acidic conditions. Planta Med. 44: 146-149 (1982)
14. Chen YJ, Nose M, Ogihara Y. Alkaline cleavage of ginsenosides. Chem. Pharm. Bull. 35: 1653-1655 (1987)
15. Bae EA, Han MJ, Choo MK, Park SY, Kim DH. Metabolism of 20(S)- and 20(R)-ginsenoside Rg3 by human intestinal bacteria and its relation to *in vitro* biological activities. Biol. Pharm. Bull. 25: 58-63 (2002)
16. Chi H, Ji GE. Transformation of ginsenosides Rb1 and Re from *Panax ginseng* by food microorganisms. Biotechnol. Lett. 27: 765-771 (2005)
17. Ko SR, Choi KJ, Suzuki K, Suzuki Y. Enzymatic preparation of ginsenosides Rg2, Rh1, and F1. Chem. Pharm. Bull. 51: 404-408 (2003)
18. Wang DM, Yu HS, Song JG, Xu YF, Liu CY, Jin FX. A novel ginsenosidase from an Aspergillus strain hydrolyzing 6-O-multi-glycosides of protopanaxatriol-type ginsenosides, named ginsenosidase type IV. J. Microbiol. Biotechnol. 21: 1057-1063 (2011)
19. Yoo MH, Yeom SJ, Park CS. Production of aglycon protopanaxadiol

via compound K by a thermostable beta-glycosidase from *Pyrococcus furiosus*. Appl. Microbiol. Biotechnol. 89: 1019-1028 (2011)
20. Yu H, Zhang C, Lu M, Sun F, Fu Y, Jin F. Purification and characterization of new special ginsenosidase hydrolyzing multi-glycisides of protopanaxadiol ginsenosides, ginsenosidase type I. Chem. Pharm. Bull. 55: 231-235 (2007)
21. Chi H, Kim DH, Ji GE. Transformation of ginsenosides Rb2 and Rc from Panax ginseng by food microorganisms. Biol. Pharm. Bull. 28: 2102-2105 (2005)
22. Pyo YH, Lee TC, Lee YC. Enrichment of bioactive isoflavones in soymilk fermented with beta-glucosidase-producing lactic acid bacteria. Food Res. Int. 38: 551-559 (2005)
23. Yamamoto S, Okada M, Usui T, Sakada K, Toumoto A, Tsuruhami K. Diglycosidase isolated from microorganisms. U.S. Patent 7,109,014 B1 (2006)
24. Ryu YG, Won B, Park HR, Ghafoor K, Park J. Effects of the beta-glycosidase reaction on bio-conversion of isoflavones and quality during tofu processing. J. Sci. Food Agr. 90: 843-849 (2010)
25. Ghafoor K, Kim SO, Lee DU, Seong K, Park J. Effects of high hydrostatic pressure on microstructure and color of red ginseng (*Panax ginseng*). J. Sci. Food Agr. 92: 2975–2982 (2012)
26. An YE, Ahn SC, Yang DC, Park SJ, Kim BY, Baik MY. Chemical conversion of ginsenosides in puffed red ginseng. LWT-Food Sci. Tech. 44: 370-374 (2011)
27. Kong BM, Park MJ, Min JW, Kim HB, Kim SH, Kim SY. Physico-chemical characteristics of white, fermented and red ginseng extracts. J. Ginseng Res. 32: 238-243 (2008)
28. Zhao X, Gao J, Song C, Fang Q, Wang N, Zhao T, Liu D, Zhou Y. Fungal sensitivity to and enzymatic deglycosylation of ginsenosides. Phytochemistry 78: 65-71 (2012)
29. Bae E, Han MJ, Kim EJ, Kim DH. Transformation of ginseng saponins to ginsenoside Rh2 by acids and human intestinal bacteria and biological activities of their transformations. Arch. Pharm. Res. 27: 61-67 (2004)
30. Xie HT, Wang GJ, Sun JG, Tucker I, Zhao XC, Xie YY, Li H, Jiang X, Wang R, Xu MJ, Wang W. High-performance liquid chromatographic-mass spectrometric determination of ginsenoside Rg3 and its metabolites in rat plasma using solidphase extraction for pharmacokinetik studies. J. Chromatogr. B 818: 167-173 (2005)
31. Kim KT, Yoo KM, Lee JW, Eom SH, Hwang IK, Lee CY. Protective effect of steamed American ginseng (*Panax quinquefolius* L.) on V79-4 cells induced by oxidative stress. J. Ethnopharmacol. 111: 443-450 (2007)
32. KFDA. Food Code. Health Functional Food Division, Food and Drug Administration, Korea. II.2.1.2-1 (2008)
33. Kim DH. Chemical diversity of Panax ginseng, *Panax quinquifolium*, and *Panax notnoginseng*. J. Ginseng Res. 36: 1-15 (2012)
34. Lee HJ, Lee HS, Cho HJ, Kim SY, Suh HJ.Utilization of hydrolytic enzymes for the extraction of ginsenosides from Korean ginseng leaves. Process Biochem. 47: 538-543 (2012)
35. Shehzad O, Ha IJ, Park Y, Ha YW, Kim YS. Development of a rapid and convenient method to separate eight ginsenosides from *Panax ginseng* by high-speed countercurrent chromatography coupled with evaporative light scattering detection. J. Sep. Sci. 34: 1116-1122 (2011)