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Changes of ginsenosides in Korean red ginseng (*Panax ginseng*) fermented by *Lactobacillus plantarum* M1

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ABSTRACT

To obtain microorganisms for the microbial conversion of ginsenosides in red ginseng powder (RGP), *Lactobacillus* species (M1–M4 and P1–P4) were isolated from commercial ginseng products. Strain M1 was determined to be *L. plantarum* by 16S rRNA sequencing. Red ginseng powder (RGP) fermented by *L. plantarum* M1 had a high total content of ginsenosides (142.4 mg/g) as compared to the control (121.8 mg/g). In particular, the ginsenoside metabolites Rg3, Rg5, Rk1, Compound K (CK), Rh1, and Rg2 showed a high level in the fermented RGP (65.5 mg/g) compared to the control (32.7 mg/g). During fermentation for 7 days, total sugar content decreased from 8.55 mg/g to 4 mg/g, uronic acid content reached its maximum (53.43 μg/g) at 3 days, and total ginsenoside content increased to 176.8 mg/g at 4 days. In addition, ginsenoside metabolites increased from 38.0 mg/g to 83.4 mg/g at 4 days of fermentation. Using everted instestinal sacs of rats, the fermented red ginseng showed a high transport level (10.3 mg of polyphenols/g sac) compared to non-fermented red ginseng (6.67 mg of polyphenols/g sac) after 1 h. These results confirm that fermentation with *L. plantarum* M1 is very useful for preparing minor ginsenoside metabolites while being safe for foods.

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1. Introduction

Ginseng (the roots of *Panax ginseng* C.A. Meyer, Araliaceae) is frequently taken orally as a traditional herbal medicine in Asian countries [1,2], and its use is increasing worldwide. Ginseng contains various pharmaceutical components such as ginsenosides (saponins), polyacetylenes, polyphenolic compounds, and acidic polysaccharides, and among them, the major components are the ginsenosides, which are glycosides with steroids or triterpenes as aglycons. So far over 30 different ginsenosides have been isolated and identified from ginseng saponins. The main ginsenosides are glycosides that contain an aglycone with a dammarane skeleton, and include protopanaxadiol-type saponins such as ginsenosides Rb1, Rb2, Rc, and Rd, as well as protopanaxatriol-type saponins such as ginsenosides Re and Rg1, constituting more than 80% of the total ginsenosides [3]. These ginsenosides are an important class of physiologically active compounds that are found in many herbs, which possess anti-inflammatory activity [4] and antitumor activity such as the inhibition of tumor-induced angiogenesis and the prevention of tumor invasion and metastasis [5,6].

However, the oral bioavailabilities of these major ginsenosides are reported to be extremely low (Rb1, 0.1–4.4%; Rb2, 3.7%; Rg1, 1.9–18.4%) [7–9]. Ginsenoside sugar chains are closely related to their biological activity and modification of their sugar chains may markedly change their biological activity [10–12]. Recently, it was suggested that orally consumed ginsenosides are metabolized by human intestinal bacteria, and deglycosylated ginsenoside metabolites are known to be more readily absorbed into the bloodstream and act as active compounds [13–15]. For example, the protopanaxadiol-type saponins including Rb1, Rb2, and Rc are metabolized to compound K by human intestinal bacteria [2,16]. In addition, Re and Rg1, belonging to protopanaxatriol-type ginsenosides, are metabolized to Rh1 or F1 by intestinal bacteria [2,17].

Intestinal bacteria are known to cleave the oligosaccharides connected to the C-3 or C-20 hydroxyl group of the aglycone stepwise from the terminal sugar [5,10]. The main metabolic pathways are proposed to be as follows: protopanaxadiol-type, Rb1 → [M10 (Rd) → M5 (F2) or M9 → M13] → M1, Rb2 → M6 → M2 → M1, and Rc → M7 → M3 → M1 (M1 is gradually hydrolyzed to M12); protopanaxatriol-type, Re → Rg1 → M11 (F1) or M8 (Rh1) → M4. Many kinds of bacteria including *Prevotella oris* [11], *Eubacterium* A-44 [18], *Bifidobacterium* K506 [5], *Bacteroides* JY6 [5], and *Fusobac-*

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terium K-60 [5] seem to cooperatively metabolize ginsenosides. However, most microorganisms used for the transformation of ginsenosides are not of a food-grade standard. In addition, the efficiency of the conversion and transformation pathways differs greatly owing to the diversity of the resident microflora between individuals. Therefore, the production of specifically transformed ginsenosides with more uniform and targeted biological functions using selected microorganisms is needed.

In seeking to utilize the beneficial properties of ginsenoside metabolites using food-compatible microorganisms, we screened edible *Lactobacillus* species capable of metabolizing ginsenosides from ginseng and investigated changes in levels of total sugars, uronic acid, polyphenols, and ginsenoside metabolites during fermentation.

2. Materials and methods

2.1. Materials

Six-year-old red ginseng powder (RGP) was purchased at a ginseng market in Geumsan, Korea. Standard ginsenoside materials including Compound K (CK), Rb1, Rb2, Rc, Rd, Re, Rf, Rg1, Rg2, Rg3, Rg5, Rh1, Rh2, and Rk1 were purchased from Embo Laboratory (Daejeon, Korea). All other chemicals were of reagent grade and were obtained from local suppliers.

2.2. Screening and identification of microorganisms for fermentation using red ginseng

Microorganisms were isolated from colonies cultured on Potato Dextrose Agar (PDA) and Man, Rogasa, and Sharpe (MRS) agar plates by the inoculation of the commercial ginseng products. Highly grown strains on both medium substrates were subjected to further selection by including the use of the total ginsenosides in the ginseng as a sole carbon source. To obtain the most efficient strains for fermentation using red ginseng, red ginseng agar plates (5 g of RGP and 2 g of agar in 100 mL) were used. The strains were serially diluted from 10^5 to 10^7 and spread onto the red ginseng agar plates and incubated at 37 °C for 48 h. After isolating the strains from colonies, their 16S rRNA sequences were determined using a Prism™ Bigdye™ Terminator Cycle Sequencing Ready Reaction Kit V.3.1 (Applied Biosystems, Foster City, CA, USA) in combination with an Applied Biosystems 3730XL Capillary DNA Sequencer, with primer sets p27F and p1492R [19] by Genotech Co. Ltd (Daejeon, Korea). The phylogenic relationship of the isolates was determined by comparing the sequencing data with the related 16S rRNA gene sequences in the GeneBank database of the National Center for Biotechnology Information, via a BLAST search. Phylogenetic trees were constructed by using the Jukes and Cantor algorithm [8] and neighbor-joining method [20]. To determine the stability of our phylogenetic tree, the sequence data were sampled 1000 times for bootstrap analysis using Mega version 3.1 with Kimura 2-parameter distances.

2.3. Fermentation conditions using red ginseng

A 2.5 g amount of RGP was poured into a 100 mL flask, dissolved with 50 mL of distilled water, and sterilized at 121 °C for 15 min (RGP medium). The bacterial strains isolated from the red ginseng plates were pre-cultured in MRS broth (Difco, Detroit, MI, USA) containing 0.05% (w/v) L-cysteine·HCl under mild aerobic conditions overnight at 37 °C, and were then sub-cultured in MRS broth again. The sub-cultured broths were inoculated into the RGP medium at 2% (v/v, 10^8 cfu/mL) and incubated at 37 °C for 7 days with mild shaking.

2.4. Laboratory-scale fermentation using red ginseng

Laboratory-scale fermentation using red ginseng was carried out in a 5 L vertical glass fermenter (Fermentec, Seoul, Korea) at 37 °C with a 3 L working volume of medium (pH 6.0) including 150 g of RGP. A 4% (v/v) culture of *L. plantarum* M1 grown in MRS broth was used as an inoculum. The cultures were stirred at 20 rpm throughout the fermentation. Samples (20 mL) were withdrawn through the sampling port every 24 h and were used in assays to determine various components such as polyphenols, total sugars, uronic acid, and ginsenosides.

2.5. High pressure liquid chromatography (HPLC) analysis of ginsenosides

Extraction of the ginsenoside was carried out with 70% ethanol for 8 h at 70 °C using a round-bottom flask fitted with a cooling condenser [14]. Each extraction was performed three times. The obtained extracts were evaporated using a rotary evaporator under vacuum at 45 °C. Then, the evaporated residues were dissolved in 100 mL of distilled water and washed with 100 mL of diethyl ether. The aqueous layer was extracted three times with 100 mL of water-saturated *n*-butanol. The butanol extract was washed with 100 mL of distilled water to remove impurities and yield the crude saponins. The remaining butanolic solution was transferred to a tared round-bottom flask for evaporation using a rotary evaporator under vacuum at 60 °C. The levels of 14 major ginsenosides were analyzed using an HPLC-based technique [9].

The HPLC system was an ACME 9000 HPLC (Young Lin Instrument, Anyang, Korea) with an ELSD detector. A Prevail carbohydrate ES column (4.6 × 250 mm, Alltech Associates, Dearfield, IL, USA) was also used. The solvent flow rate was held constant at 0.8 mL/min. A column oven was used to fix the column temperature at 35 °C. The mobile phase used for the separation consisted of solvent A (acetonitrile:water:isopropyl alcohol = 80:5:15) and solvent B (acetonitrile:water:isopropyl alcohol = 67:21:12). A gradient elution procedure was used as follows: 0–28 min 90% A, 28–35 min 15% A, 35–45 min 20% A, 45–50 min 25% A, 50–51 min 10% A, 51–57 min 0% A, 57–58 min 75% A, and 58–65 min 90% A. The injection volume used for analysis was 20 μL. The peak identifications were based on retention times and comparisons with injected standard samples. All solutions were filtered through 0.45 μm membrane syringe filters (Millipore, Bedford, MA, USA) prior to analysis. To determine the calibration curves, the ginsenoside standards CK, Rh2, Rh1, Rg5, Rk1, Rg2, Rg3, Rg1, Rf, Re, Rd, Rb2, Rc, and Rb1 were dissolved individually in HPLC-grade methanol and adjusted to the appropriate concentrations and quantities. The level of total ginsenosides was determined by summing the levels of the 14 ginsenosides.

2.6. Analytical methods

Total polyphenol (TP) content was determined using the Folin-Ciocalteu method [21] adapted to a microscale using gallic acid as standard (50–800 μg/L). Total sugar and uronic acid levels were determined using the phenol–sulfuric acid [22] and *m*-hydroxydiphenyl methods [23], respectively, using glucose and galacturonic acid as the respective standards. In all cases, the analyses were performed in triplicate unless otherwise specified. These values were averaged and standard deviations (SD) were calculated. All data were analyzed by one-way analysis of variance and Duncan's multiple range tests using SPSS version 10.0 software (SPSS, Chicago, IL, USA). The results were considered significant at $p < 0.05$.

2.7. Intestinal transport across everted intestinal sacs

Everted intestinal sac experiments were performed according to the method of Tandon et al. [24] with some modifications. Male Sprague–Dawley rats, weighing 220–250 g (Nara Biotech., Deajon, Korea) were fasted overnight with free access to water until they were anaesthetized with urethane. Then, a middle abdominal incision was made and the jejunum was quickly taken. After the underlying mesenterium was removed, the jejunum was flushed with ice-cold Krebs–Henseleit bicarbonate (KHB) buffer to remove the intestinal contents. The jejunum was gently stretched and cut into segments (10 cm-long each). Each of the sacs was carefully everted with a glass rod. One end was ligated, and the other end was ligated with a conical rubber stopper that housed one port for the removal and addition of serosal fluid, and another port for the continuous supply of 5% CO_2 and 95% O_2 throughout the experiment. After being filled with 1 mL of KHB buffer (inner compartment), the sacs were incubated in 29.5 mL of the same buffer (outer compartment) that contained 0.5 mL of ginseng sample at 37 °C in a water bath. Two-hundred microliters of the serosal fluid inside the sacs was taken at 30 min and 1 h, followed by immediate replacement with fresh KHB buffer of the same volume (200 μL). The transfer of the serosal fluid was reflected by an increase in volume inside the sac, and gut fluid uptake was determined by measuring the increase in fluid volume in the gut. Intestinal transport of the ginseng sample was expressed as mg of polyphenols/g tissue dry weight. The polyphenol contents of the transports were determined according to the Folin-Ciocalteau method [21].

3. Results

3.1. Isolation and identification of microorganisms for fermentation using red ginseng

To screen the strains capable of fermentation using red ginseng, 34 colonies were isolated from commercial ginseng products and 8 of these isolates were capable of growing on red ginseng agar plates. The 8 isolated strains (M1–M4 and P1–P4) were Gram-positive cocci and formed creamy, opaque, and circular colonies. All isolates were determined to be *Lactobacillus* species by the examination of their metabolic characteristics (data not shown) and 99–100% homology of their 16S rRNA gene sequences with sequences of *Lactobacillus* species. The strains M1 and P2 were identified as *L. plantarum* and strains M2, M3, M4, P1, P3, and P4 were *L. brevis*.

The 16S rDNA sequencing allowed for identification at the species level with identification at 99% when compared to sequences in the GenBank database, and was used to perform multiple alignments using the ClustalX program. The sequencing of the

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