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The bioavailability of red ginseng extract fermented by *Phellinus linteus*

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For the improvement of ginsenoside bioavailability, the ginsenosides of fermented red ginseng by *Phellinus linteus* (FRG) were examined with respect to bioavailability and physiological activity. The polyphenol content of FRG (19.14±0.50 mg/g) was significantly higher (p<0.05) compared with that of non-fermented red ginseng (NFRG, 11.31±1.15 mg/g). The antioxidant activities in FRG, such as 2,2'-diphenyl-1-picrylhydrazyl, 2,2-azino-bis-3-ethylbenzothiazoline-6-sulphonic acid, and ferric reducing antioxidant power, were significantly higher (p<0.05) than those in NFRG. The HPLC analysis results showed that the FRG had a high level of ginsenoside metabolites. The total ginsenoside contents in NFRG and FRG were 41.65±1.53 mg/g and 50.12±1.43 mg/g, respectively. However, FRG had a significantly higher content (33.90±0.97 mg/g) of ginsenoside metabolites (Rg3, Rg5, Rk1, compound K, Rh1, F2, and Rg2) compared with NFRG (14.75±0.46 mg/g). The skin permeability of FRG was higher than that of NFRG using Franz diffusion cell models. In particular, after 3 h, the skin permeability of FRG was significantly higher (p<0.05) than that of NFRG. Using a rat everted intestinal sac model, FRG showed a high transport level compared with NFRG after 1 h. FRG had dramatically improved bioavailability compared with NFRG as indicated by skin permeation and intestinal permeability. The significantly greater bioavailability of FRG may have been due to the transformation of its ginsenosides by fermentation to more easily absorbable forms (ginsenoside metabolites).

Keywords: *Panax ginseng*, Fermented red ginseng, Bioavailability

INTRODUCTION

Ginseng (*Panax ginseng*) is one of the most valuable oriental herbs. The dried root of the plant has been used as a healing drug and health tonic in Asian countries, such as China, Japan, and Korea, since ancient times [1]. Ginseng ginsenosides (saponins) are regarded as the principal components responsible for the pharmacological activities of ginseng, and more than 40 ginsenosides have been isolated and identified in Korean ginseng (*P. ginseng*) and other *Panax* spp. [2]. Red ginseng, the steamed and dried root, contains polysaccharides and ginsenosides, such as Rg3, Rb1, Rb2, and Rc, as its main constituents [3]. Various biological activities of ginsenosides have been reported, such as anti-senescence, immunomodulatory, anti-tumour, anti-inflammatory, and anti-diabetic [4-7].

Ginseng is typically administered orally, after which the ingredients are exposed to gastric juices, digestive enzymes, and bacterial enzymes. Intact ginsenosides are

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absorbed only from the intestines (the absorption rate is as low as 0.1% to 3.7%), and their intestinal bacterial metabolites are absorbed into the blood. The pharmacological actions of these ginsenosides have been explained through their biotransformation by human intestinal bacteria [8-10]. Ginsenosides act as precursors that are metabolised to bioactive forms by intestinal bacterial deglycosylation and fatty acid esterification in the body [11-14]. Intestinal microfloras are very changeable depending on host conditions (such as diet, health, and even stress). Therefore, it is hypothesised that individual differences in ginseng efficacy may be partly associated with the intestinal microflora of patients. The efficiency of conversion and transformation pathways may differ greatly due to the diversity of resident microflora between individuals [15].

To improve the bioavailability of these compounds, many different strategies have been used. Several studies have shown that the transformation of ginsenosides into deglycosylated ginsenosides (metabolites) was required for them to provide more effective *in vivo* physiological action [16]. Various transformation methods, including mild acid hydrolysis, enzymatic conversion, and microbial conversion, have been used [9,17,18]. Chemical methods, however, accompany side reactions, such as epimerisation, hydration, and hydroxylation. Futhermore, most of the microorganisms used for the transformation of ginsenosides are not of food-grade.

The purpose of this study was to enhance the beneficial properties of ginsenosides by using food-compatible microorganisms. We screened edible mushroom species which are capable of metabolising ginsenoside from ginseng. *Phellinus linteus* was proven to be a very useful organism by which ginseng can be processed with both selectivity and efficiency, in terms of a structural modification and metabolic study of red ginseng, processing the ginseng with both selectivity and efficiency. In the present study, the physiological activities of fermented red ginseng by *P. linteus* (FRG) and non-fermented red ginseng (NFRG) were investigated, and their bioavailabilities were compared *in vitro*.

MATERIALS AND METHODS

Materials

The red ginseng extract (RGE, 60 degrees Brix) was kindly donated by Serombio Co. (Gunpo, Korea). Standard ginsenosides, including the compounds Rg1, Re, Rf, Rh1(s), Rg2(s), Rg2(r), Rb1, Rc, Rb2, Rd, F2, Rg3(s), Rg3(r), compound K (CK), Rg5, Rk1, Rh2(s), and Rh2(r), were purchased from Ambo Institute (Daejeon, Korea). All of the other chemicals were reagent grade, and were obtained from local suppliers.

Microorganism and fermentation

The strains of *P. linteus* was received from Chungju University (Jeongpyung, Korea) and maintained on potato dextrose agar slants. The slants were inoculated and incubated at 25°C for 7 d and then stored at 4°C for approximately 2 wk. For the preculture, potato dextrose broth (Difco Laboratories, Detroit, MI, USA) of an initial pH of 6.0 was prepared and inoculated with a mycelial suspension from a broth culture, followed by 7 d of incubation at 25°C on a rotary shaker (150 rpm). RGE was poured into a 500-L fermenter (Kobiotech Co., Pyeongtaek, Korea), dissolved with distilled water to a concentration of 20 degrees Brix, and sterilized at 121°C for 15 min (RGE medium). The precultured broths were inoculated into the RGE medium at 6 L and incubated at 25°C, with an aeration rate of 1.0 vvm for 5 d with mild shaking (150 rpm).

Determination of chemical composition and antioxidant activity

The amount of total carbohydrate was measured by the phenol-sulphuric acid method, using glucose as a standard [19]. Uronic acid was estimated by the 3-phenylphenol method, using galacturonic acid as a standard [20]. The polyphenol contents of the extracts were determined by employing the Folin-Ciocalteu method, using protocols reported elsewhere, and then the polyphenol results were expressed as the amount of gallic acid equivalents [21].

Stable 2,2'-diphenyl-1-picrylhydrazyl (DPPH) and 2,2-azino-bis-3-ethylbenzothiazoline-6-sulphonic acid (ABTS) were used to determine the free radical scavenging activities of Korean ginseng leaf extracts. The DPPH scavenging activity was measured by a method described by Quang et al. [22], with certain modifications. The ABTS radical scavenging activity was determined as described by Wang et al. [23] and Almajano et al. [24], with slight modifications. The antioxidant activities of the test samples were expressed as IC_{50}, i.e., the amount of tested extract required for a 50% decrease in the absorbance of the DPPH and ABTS radicals. The ferric reducing antioxidant power (FRAP) method was performed as described by Benzie and Strain [25]. ΔA is proportional to the combined (total) ferric reducing/antioxidant power (FRAP value) of the antioxidants in the sample. The final results were expressed as micromole 2,4,6-tripyridyl-s-triazine (TPTZ) equivalents per gram on a dried basis (μM TPTZ/g).

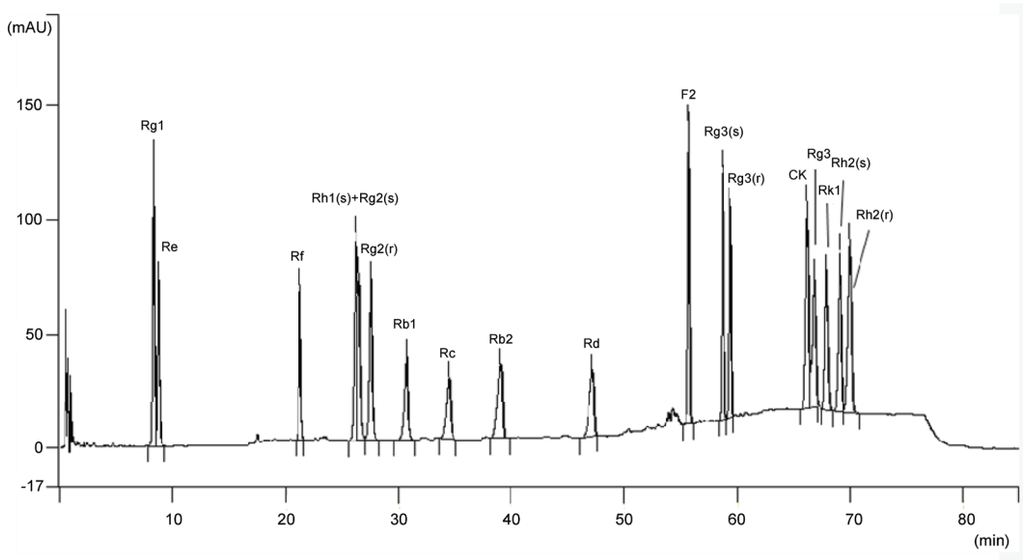

Fig. 1. A chromatogram of standard ginsenosides using HPLC assay. The column configuration consisted of an IMtakt Cadenza CD-C18 (4.6×75 mm). The UV absorption was measured at 203 nm. Gradient elution was employed, using solvent A (10% acetonitrile) and solvent B (90% acetonitrile) at 40°C. CK, compound K.

HPLC analysis of ginsenosides

A purified sample for HPLC analysis was prepared by C18 ODS cartridge (Waters Associates, Milford, MA, USA) solid-phase extraction according to a method described previously [26]. The levels of major 16 ginsenosides were analyzed by using an HPLC-based technique developed by Lee et al. [27]. The utilized HPLC system was a Varian Prostar 200 equipped with a quaternary solvent delivery system, an autosampler, and a UV detector (203 nm). The column was IMtakt Cadenza CD-C18 column (4.6×75 mm; Imtakt Co., Kyoto, Japan). Gradient elution was employed, using 10% acetonitrile and 90% acetonitrile at 40°C. The flow rate was maintained at 1.3 mL/min, and the sample injection volume was 5 μL. Representative HPLC chromatograms of the 18 standard ginsenosides is shown in Fig. 1.

Skin permeability test across Franz-type diffusion cell models

Skin permeation was determined by the method of Sonavane et al. [28], with certain modifications. Male Sprague-Dawley (SD) rats, weighing 250 to 300 g (Nara Bio Animal Center, Seoul, Korea), were used for the study. The excised skin was mounted in a Franz-type diffusion cell. Then, 4.9 mL of 0.1 M sodium phosphate buffer (pH 7.4) was used as a receptor medium, and 100 μL of ginseng sample was placed on the donor side. The receptor medium was kept at 37°C and stirred with a magnetic stirrer at 400 rpm. The polyphenol content of the transports was determined by the Folin-Ciocalteau method [21].

Intestinal permeability test across everted intestinal sac models

Everted intestinal sac experiments were performed according to the method of Tandon et al. [29], with certain modifications. Male SD rats, weighing 220 to 250 g, were fasted overnight with free access of water until sacrifice. Intestine was obtained after urethane euthanasia. After being everted and filled with 1 mL of Krebs-Henseleit bicarbonate (KHB) buffer (inner compartment), the sacs were incubated in 29.5 mL of the same buffer (outer compartment) with 0.5 mL of ginseng sample at 37°C in a water bath. The serosal fluid from the inside of the sac was sampled at 10, 20, 30, and 60 min. The intestinal transport of the ginseng sample was expressed as mg of polyphenol and mg of total and metabolite ginsenoside/g tissue dry weight. The polyphenol content of the transports was determined by the Folin-Ciocalteau method [21]. The total and metabolite ginsenoside of the transports was determined according to the HPLC analysis of ginsenosides. Skin and intestinal permeability studies conformed to the Korea University (Seoul, Korea) guidelines for the ethical treatment of laboratory animals.

Table 1. General composition and antioxidant activities of NFRG and FRG

	NFRG	FRG
Dry weight (mg/mL)	927.00±23.30	827.33±25.42[1)]
Total sugar (mg/g)	436.71±10.17	432.76±14.69
Uronic acid (mg/g)	71.38±2.74	45.44±1.30[1)]
Polyphenol (mg/g)	11.31±1.15	19.14±0.50[1)]
DPPH (IC_{50}, mg/mL)	3.58±0.07	1.57±0.06[1)]
ABTS (IC_{50}, mg/mL)	5.21±0.08	2.61±0.12[1)]
FRAP (mM TPTZ/g)	44.21±0.84	83.52±5.04[1)]

NFRG, non-fermented red ginseng; FRG, fermented red ginseng by *Phellinus linteus*; DPPH, 2,2'-diphenyl-1-picrylhydrazyl; ABTS, 2,2-azino-bis-3-ethylbenzothiazoline-6-sulphonic acid; FRAP, ferric reducing antioxidant power; TPTZ, 2,4,6-tripyridyl-s-triazine.
[1)]The FRG shows a significant difference compared with the NFRG (p<0.05).

Statistical analysis

All of the expressed values are the means of triplicate determinations. All of the statistical analyses were performed using the SPSS ver. 12.0 (SPSS Inc., Chicago, IL, USA). The t-test was used to assess the differences between two samples. All of the data were two-sided at the 5% significance level and are reported as the means±standard deviations.

RESULTS AND DISCUSSION

General compositions and antioxidant activities of non-fermented red ginseng and fermented red ginseng by *Phellinus linteus*

Table 1 shows the general compositions and antioxidant activity of the NFRG and FRG. The total sugar contents of NFRG and FGR were 436.71±10.17 mg/g and 432.76±14.69 mg/g, respectively, but these levels were not significantly different. The uronic acid (an acidic polysaccharide) content was significantly higher (p<0.05) in the NFRG (71.38±2.74 mg/g) compared with the FRG (45.44±1.30 mg/g). Additionally, the polyphenol content of FRG (19.14±0.50 mg/g) was significantly higher (p<0.05) compared with that of NFRG (11.31±1.15 mg/g). The antioxidant activities in the FRG, such as DPPH, ABTS and FRAP were significantly higher (p<0.05) than those in the NFRG.

The major component of Korean ginseng was carbohydrates, which include starch, polysaccharides, cellulose, and glycosides. Ginseng mainly consists of carbohydrates (60 to 70 g carbohydrate/100 g solid), and starch is a major component of ginseng carbohydrates [30]. Ginseng might contains pectin-type polysaccharide that is mainly composed of galacturonic and glucuronic acids (93%). Uronic acids obtained from plant sources have been shown to exhibit a variety of biological activities, including immunostimulatory, antioxidant, antitumour, and antiviral properties [31].

Table 2. Ginsenosides content of NFRG and FRG

Ginsenoside	NFRG (mg/g)	FRG (mg/g)
Rg1	1.50±0.06	0.35±0.02[1)]
Re	3.86±0.15	0.61±0.02[1)]
Rf	1.53±0.06	1.46±0.05
Rh1(s)+Rg2(s)	0.98±0.02	1.44±0.04[1)]
Rg2(r)	1.31±0.05	2.24±0.07[1)]
Rb1	8.75±0.44	1.98±0.06[1)]
Rc	4.33±0.13	1.77±0.06[1)]
Rb2	4.09±0.12	2.70±0.08[1)]
Rd	2.83±0.10	7.34±0.21[1)]
F2	1.48±0.05	4.00±0.11[1)]
Rg3(s)	2.69±0.08	6.21±0.18[1)]
Rg3(r)	1.74±0.06	3.62±0.11[1)]
CK+Rg5	1.72±0.06	3.92±0.11[1)]
Rk1	4.82±0.14	12.20±0.36[1)]
Rh2(s)	ND	0.28±0.01
Rh2(r)	ND	ND
Total	41.65±1.53	50.12±1.43[1)]
Rg1+Rb1	10.25±0.51	2.33±0.07[1)]
Panaxdiols	24.43±0.94	23.62±0.67
Panaxtriols	9.19±0.36	6.11±0.17[1)]
Metabolite[2)]	14.75±0.46	33.90±0.97[1)]

NFRG, non-fermented red ginseng; FRG, fermented red ginseng by *Phellinus linteus*; CK, compound K; ND, not detected,
[1)]The FRG shows a significant difference compared with the NFRG (p<0.05).
[2)]Sum of Rg2, Rg3, Rg5, Rk1, CK, Rh1, Rh2, and F2.

Both DPPH and ABTS were assessed in radical-scavenging assays, which have been widely used to measure the antioxidant capacities of natural extracts based on their ability to reduce the radical cation. The chelation power of RGE was examined against Fe^{2+}, as this ion is the most effective pro-oxidant that is found in the food system [32]. Metal chelation agents thereby exert dramatic effects in blocking the formation of chain initiators by preventing the metal-assisted homolysis of hydroperoxides. Many natural substances, such as polyphenols, are able to act as antioxidants and are widely spread in food and plants. Polyphenols possess antioxidant activity, and therefore can delay lipid oxidation [33]. Their action is associated with the number and substitution pattern of phenolic hydroxyl groups [34].

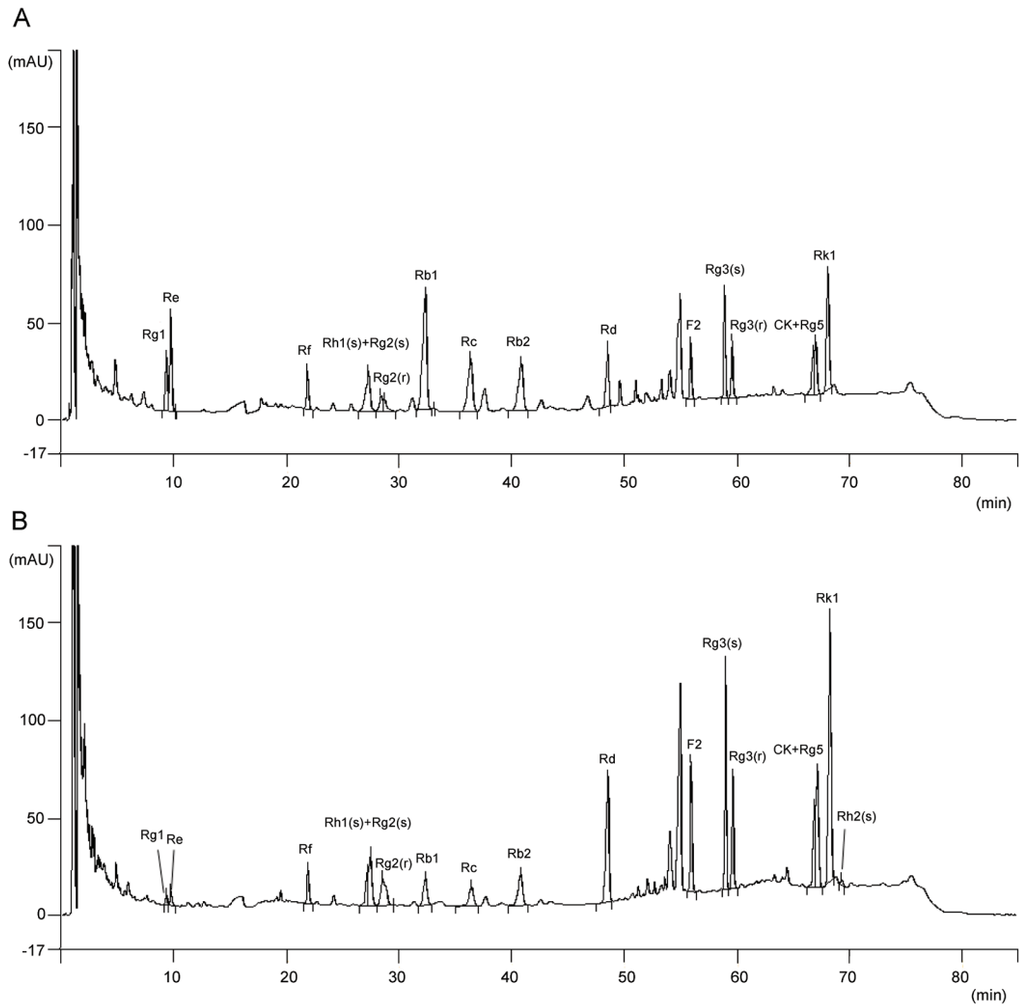

Fig. 2. A chromatogram of (A) non-fermented red ginseng and (B) fermented red ginseng by *Phellinus linteus* ginsenosides using HPLC assay. The column configuration consisted of an IMtakt Cadenza CD-C18 (4.6×75 mm). The UV absorption was measured at 203 nm. Gradient elution was employed, using solvent A (10% acetonitrile) and solvent B (90% acetonitrile) at 40°C.

Ginsenoside content of non-fermented red ginseng and fermented red ginseng by *Phellinus linteus*

The ginsenoside composition of the NFRG and FRG was compared by HPLC (Table 2 and Fig. 2). The total ginsenoside contents of the NFRG and FRG were 41.65±1.53 mg/g and 50.12±1.43 mg/g, respectively, and these levels were significantly different (p<0.05). Rb1 and Rg1 are the two main ginsenosides contained in red ginseng. The sum of Rb1 and Rg1 in the NFRG (10.25±0.51 mg/g) was significantly higher (p<0.05) than that of the FRG (2.33±0.07 mg/g). However, the level of ginsenoside metabolites (Rg2, Rg3, Rg5, Rk1, CK, Rh1, Rh2, and F2) was significantly higher (p<0.05) in the FRG (33.90±0.97 mg/g) compared to that of NFRG (14.75±0.46 mg/g).

Ginsenosides are classified into several categories according to their chemical constitutions, and more than 40 ginsenoside variants have been reported [35]. Among these variants, six major ginsenosides, including Rb1, Rb2, Rc, Rd, Re, and Rg1, account for 90% (w/w) of the total saponins in white and red ginseng [2]. In recent decades, many studies have focused on the pharmaceutical activities of minor ginsenosides, such as Rd, Rg^3, Rh^2, and CK, as their activities are found to be superior to those of major ginsenosides. These minor ginsenosides are present in ginseng only in a small amount and are known to be produced by the hydrolysis of sugar moieties of the major ginsenosides. Recently, several investigators have reported that Rb1, Rb2, and Rc are metabolized by intestinal bacteria in rats and humans after oral

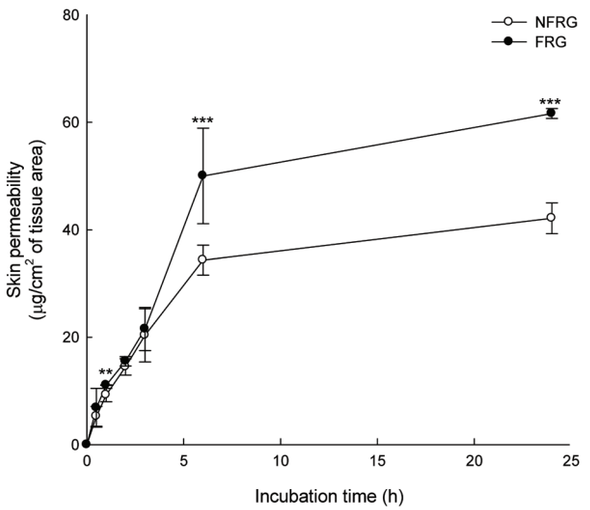

Fig. 3. The skin permeability test using the Franz diffusion cell model of non-fermented red ginseng (NFRG) and fermented red ginseng by *Phellinus linteus* (FRG). A 100 μL sample of ginseng was placed on the donor side of a Franz cell. The receptor medium was kept at 37°C and stirred with a magnetic stirrer at 400 rpm. Aliquots (0.5 mL) of the receptor medium were withdrawn at 0.5 h, 1 to 3 h, 6 h, and 24 h. The skin permeability of the ginseng sample is expressed as mg of polyphenol/g tissue dry weight. The FRG shows a significant difference compared with the NFRG (***p*<0.01, ****p*<0.001).

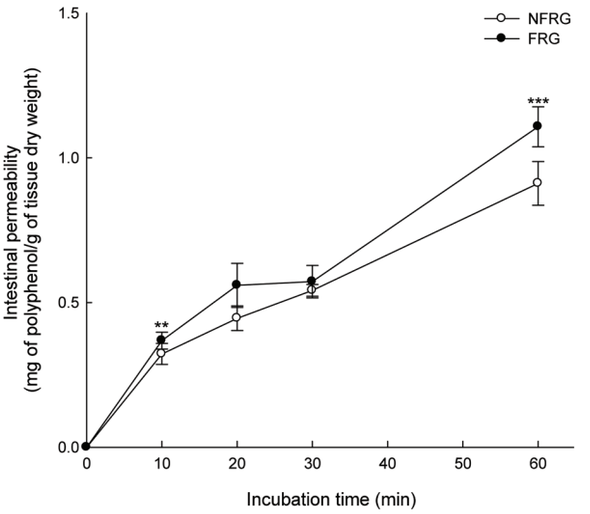

Fig. 4. The intestinal permeability test using the everted intestinal sac model of non-fermented red ginseng (NFRG) and fermented red ginseng by *Phellinus linteus* (FRG). After an everted sac was filled with 1 mL of Krebs-Henseleit bicarbonate buffer (inner compartment), the sacs were incubated in 29.5 mL of the same buffer (outer compartment) that contained 0.5 mL of ginseng sample at 37°C in a water bath. The serosal fluid inside the sacs was sampled at 10 min, 20 min, 30 min, and 1 h. The intestinal transport of the ginseng sample is expressed as mg of polyphenols/g tissue dry weight. The FRG shows a significant difference compared with the NFRG (***p*<0.01, ****p*<0.001).

administration and the main metabolite of protopanaxadiol (PPD)-type ginsenoside is CK [10,11]. In addition, ginseng and its derived products are orally administered in most cases, and a number of metabolites are produced via degradation of ginsenosides by acid or intestinal bacteria in the gastrointestinal tract. The intestinal bacteria population is variable, depending on the conditions of the host including diet, health, and even stress. To overcome such variations, a microorganism that can produce ginsenoside metabolites would be deemed valuable. Therefore, the goal in the processing of FRG by *P. linteus* was the production of ginsenoside metabolites.

Skin permeability test using the Franz diffusion cell model of non-fermented red ginseng and fermented red ginseng by *Phellinus linteus*

A permeability study of NFRG and FRG through rat skin was conducted by using the Franz diffusion cells. The polyphenol contents of the samples transported through the rat skin was significantly increased over time (Fig. 3). The skin permeability of the FRG was higher than that of the NFRG. In particular, after 3 h, the skin permeability of the FRG was significantly higher ($p<0.05$) compared with that of the NFRG.

Recent studies on exploiting natural compounds for cosmetics industry have drawn much attention to the maximization of the bioactivity of extracts [36]. And then, in commonly used bioactive ingredients of plants, there are always mixtures of many compounds belonging to this group that are present in the form of aglycones and hydrophilic glycosides. However, glycosides are disadvantageous ingredients for skin cosmetics due to their low skin permeability. In contrast, aglycone ingredients are hydrophobic and can permeate human skin [37]. Wiechers [38] reported that low molecular weight contributes to easier skin penetration, as there is an upper molecular weight limit for chemical compounds and drugs to be absorbed across the human skin barrier. Therefore, Bos and Meinardi [39] reported that certain skin penetration enhancers have low molecular weight. Wisuitiprot et al. [40] studied the skin permeability of various catechins from tea extract. Their results showed that epicatechin (EC) and epigallocatechin (EGC) have low molecular weight, which enables easier skin penetration. However, EGC contains more hydroxyl groups than EC, which may also reduce the ability to permeate the skin. For epigallocatechingallate and epicatechingallate, skin permeation was limited because of their molecular weight and molecular structures. Thus, the hydrolysis of glycoside ingredients into their aglycone forms has

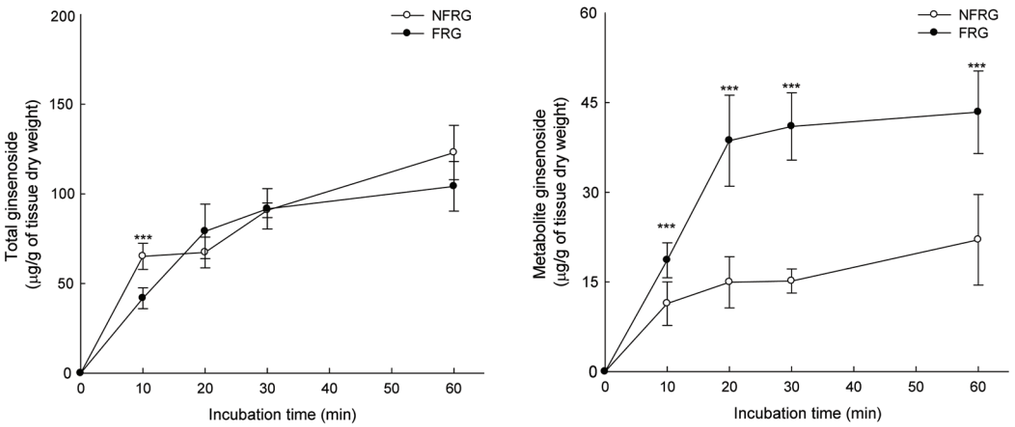

Fig. 5. The ginsenoside intestinal permeability test using the everted intestinal sac model of non-fermented red ginseng (NFRG) and fermented red ginseng by *Phellinus linteus* (FRG). After an everted sac was filled with 1 mL of Krebs-Henseleit bicarbonate buffer (inner compartment), the sacs were incubated 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. The serosal fluid inside the sacs was sampled at 10 min, 20 min, 30 min, and 1 h. The intestinal transport of the ginseng sample is expressed as ug of total and metabolite ginsenoside/g tissue dry weight. The FRG show a significant difference compared with the NFRG (***p<0.001).

attracted attention as an effective means of enhancing the permeability and, consequently, the bioactivity of extracts [36].

Intestinal permeability test using the everted intestinal sacs model of non-fermented red ginseng and fermented red ginseng by *Phellinus linteus*

We tested intestinal permeability of NFRG and FRG by everted intestine sac method. The everted intestine (gut) of rats has been widely used as a suitable *in vitro* model to study the intestinal transference of nutrients and drugs [29]. After 10 min, the polyphenol transport level of the FRG was significantly higher (p<0.05) than that of the NFRG (Fig. 4). After 20 and 30 min, the polyphenol transport level of the FRG was slightly higher than that of the NFRG, but was not significantly different. After 60 min, the FRG showed a significantly higher (p<0.05) transport level compared to that of the NFRG. The ginsenoside transport levels of the NFRG and FRG were compared using HPLC (Fig. 5). After 10 min, total ginsenoside transport level of the FRG was significantly higher (p<0.05) than that of the NFRG. However, after other amounts of time, there was no significant difference. The ginsenoside metabolite transport level of FRG was significantly higher (p<0.05) than that of NFRG.

The mechanism of the absorption of ginsenosides across the intestinal membranes *in vivo* is not yet clearly understood. Orally administered ginsenosides are absorbed only from the intestines (the absorption rate is as low as 0.1% to 3.7%), and their intestinal bacterial metabolites are absorbed into the blood. Similar to other plant glycosides, ginsenosides are hydrolyzed by intestinal bacteria, followed by absorption. Investigations of bacterial fermentation have shown that the intestinal degradation of PPD ginsenosides proceeds stepwise via the cleavage of sugar moieties, mainly liberating the monoglycosylated ginsenoside CK [10,41]. However, these intestinal microfloras are very changeable depending on host conditions (such as diet, health, and even stress) [42,43]. Therefore, it is hypothesised that individual differences in ginseng efficacy may be partially associated with the intestinal microflora of patients. The efficiency of the conversion and transformation pathways may differ greatly due to the diversity of resident microflora among individuals [15]. Liu et al. [44] reported that poor membrane permeability was a major factor limiting the intestinal absorption of ginsenosides. Ginsenosides can be transformed into more easily absorbable forms (ginsenoside metabolites) by fermentation. Our results showed that, by fermentation, the ginsenoside metabolite contents could be enhanced by approximately 260%, leading to a high level of intestinal permeability compared to NFRG.

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