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Fermentative transformation of ginsenosides by a combination of probiotic *Lactobacillus helveticus* and *Pediococcus pentosaceus*

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프로바이틱스 *Lactobacillus helveticus*와 *Pediococcus pentosaceus*의 조합에 의한 진세노사이드의 발효적 형질전환

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Ginseng are native traditional herbs, which exhibit excellent pharmacological activities. Probiotic *Lactobacillus helveticus* KII13 and *Pediococcus pentosaceus* strain KID7 were used for ginsenoside transformation by fermenting crude ginseng extract to enhance minor gisenoside content. Thin-layer chromatography (TLC) analysis of fermented ginseng extract showed that the minor ginsenosides Rg3, Rh1, and Rh2 were main products after 5 days of fermentation. HPLC analysis was performed to quantify the major and minor ginsenosides. The Rg3 peak appeared on the 3rd day while the appearance of Rh2 peak and Rh1 peak were observed on the 5th day. The co-culture of *L. helveticus* KII13 and *P. pentosaceus* KID7 converted major ginsenosides (Rb1 and Rg1) into minor ginsenosides (Rg3, Rh2, and Rh1).

Keywords: *Lactobacillus helveticus*, *Pediococcus pentosaceus*, ginseng extract, ginsenoside, probiotics

Panax ginseng C. A. Meyer, *Panax quinquefolius* L., and *Panax notoginseng* F. H. Chen are the most common species of ginseng. Korean ginseng (*Panax ginseng* C. A. Meyer) is native to the Asian Far East (33~48 latitude in Korea, northern Manchuria, and parts of Russia) and has excellent pharmacological efficacy (Choi, 2008). Increasing research on the major components and pharmacological effects of ginseng resulted in its recognition as a natural health food. In addition, ginseng has been mainly used in Asian countries such as Korea in the form of herbal medicine for various diseases such as psychiatric disorders, nervous system diseases and diabetes (Tang and Eisenbrand, 1992; Lee *et al*., 2008).

Saponins, which are the main components of ginseng has the form of triterpenoid glycosides and are called ginsenosides. Minor saponins (Rg3, Rh2, Rh1, etc.) produced by hydrolysis of major saponins (Rg1, Re, Rb1, Rc, Rb2, and Rd) contained in ginseng, has been reported to exhibit excellent pharmacological activities such as inhibition of invasion of tumour cells *in vitro*, induction of cancer cell apoptosis, inhibition of cancer cell metastasis, hypotension, suppression of catecholamine secretion, analgesic activity and immunity (Christensen, 2008; Qi *et al.*, 2011; Yang *et al.*, 2015). Studies on the production of minor saponins have been actively pursued (Chi and Ji, 2005; Chi *et al.*, 2005; Han *et al.*, 2010; Kim *et al.*, 2010; Hong *et al.*, 2012;

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Lim *et al.*, 2015; Huq *et al.*, 2016) and their pharmacological activities have been verified (Qi *et al*., 2010; Lim *et al*., 2015).

The present study demonstrates that fermentation with probiotic lactic acid bacteria (LAB) could result in enrichment of minor ginsenosides such as Rg3, Rh2, and Rh1. The ginseng extract was fermented with a mixture of our previously reported probiotic lactic acid bacteria such as *Lactobacillus helveticus* KII13 (Damodharan *et al*., 2016) and *Pediococcus pentosaceus* strain KID7 (Damodharan *et al*., 2015).

One hundred gram of ginseng roots were washed and freeze dried to remove moisture. Dried roots were powdered and extracted with 30 volumes of 80% ethanol at 80°C for 1 h in water bath with shaking followed by filtering with 3 mm filter paper (Whatman). The second extraction used the remainder with 20 volumes of 80% ethanol. After an additional extraction, ginseng extract was concentrated using a vacuum evaporator and then dissolved in 10 volumes of water.

The fermentation medium contained skim milk powder, tryptic soy broth powder and ginseng extract concentrate in the ratio of 1:1.5:2.5 (W%), respectively per 100 ml of distilled water and sterilized by autoclaving. *L. helveticus* KII13 and *P. pentosaceus* strain KID7 were cultured in 10 ml MRS liquid broth at 37°C for 24 h. After incubation, cells were harvested by centrifugation at 8,000 × *g* for 10 min and washed with sterile phosphate buffered saline (PBS) twice (at the final concentration of 10^8 cells/ml). Both the prepared LAB cells (in a ratio of 1:1) were inoculated at a concentration of 10% (v/v) of fermentation medium for transformation of ginsenoside. After inoculation, the fermentation medium was incubated statically at 37°C for 7 days. Samples were retrieved from the fermentation medium each day and analyzed for ginsenoside transformation by TLC and HPLC analysis.

A 1 ml aliquot of the fermentate was collected each day and extracted with equal volume of *n*-butanol by shaking the mixture for 1 h followed by incubation at room temperature for an additional 12 h. The *n*-butanol fraction was evaporated to dryness with a rotary vacuum evaporator (N-1000V, EYELA). Crude extract was dissolved in 50 µl of methanol, which was subjected to thin layer chromatography (TLC) analysis. TLC was conducted on silica gel 60 F_{254} pre-coated plates (Merck) with chloroform: methanol: water (65:35:10, lower phase) as the developing solvent. The spots were visualized by spraying with 10% sulfuric acid, followed by heating at 110°C for 10 min (Palaniyandi *et al*., 2015).

The ginsenoside compositions were identified through comparison with standard ginsenoside according to our previous report (Palaniyandi *et al.*, 2015, 2017). Analysis was performed in a Waters Alliance 2695 HPLC system equipped with a Sunfire C18 column (4.5 mm × 25 cm) maintained at a constant temperature of 40°C using a column incubator. HPLC-grade acetonitrile (A) and water (B) were used as mobile phase. The analysis was performed at mobile phase flow rate of 1 ml/min using the following conditions: 0~8 min, 20~30% A; 8~12 min, 30~40% A; 12~15 min, 40~65% A; 15~20 min, 65~100% A, 20~30 min, 100% A; 30~35 min, 100~30% A; 35~40 min, 30~20% A, and column equilibration for 5 min with 20% A. Samples (20 µl) was injected into the column using an automated sample injector (Waters 2707 Autosampler) and it was monitored at wavelength of 203 nm using Waters 2996 PDA Detector. A mixture of ginsenosides Re, Rg1, Rb1, Rf, Rc, Rb2, Rg2, Rh1, Rd, Rg3, Ck, and Rh2 was used as standard for HPLC analysis. Ginsenosides such as Rb1, Rb2, Rc, Re, Rg2, and Rg3 were a kind gift from Prof. Nam-In Baek, Natural Products Chemistry Lab, Kyung Hee University, Korea. Other ginsenosides were obtained from Chromadex.

Screening of fermented ginseng extract by TLC analysis showed that fermentation of ginseng extract with strain KII13 and KID7 decreased ginsenosides Rb1 and Rg1, whereas ginsenosides Rg3, Rh2, and Rh1 were increased over an incubation period of 7 days (Fig. 1). The ginsenoside Rb1 could be hydrolysed by β-glucosidase through a series of hydrolytic pathway (Park *et al*., 2010). Rb1 is transformed to ginsenoside Rg3, which gradually increased on 3rd day (Fig. 1). Rg3 was produced by hydrolysing the outer glycosidic linkage at C-20 position of ginsenoside Rd, which is an intermediate in the conversion of Rb1→Rg3. The content of Rg3 was increased by the 5th day with a slight increase on the 7th day (Fig. 1), as it is converted to minor ginsenoside Rh2 (Fig. 1) and other compounds by the probiotic strains. The individual and co-cultures of *Lactobacillus* and *Pediococcus* genus have been used to enhance biological efficiency of fermented ginseng. Lin *et al*. (2010) have reported that *L. helveticus* converted major ginsenoside to minor gisenosides Rg3, Rh1 and protopanaxatriol, which can enhance anti-hepatoma and anti-cancer activities. Eom *et al*.

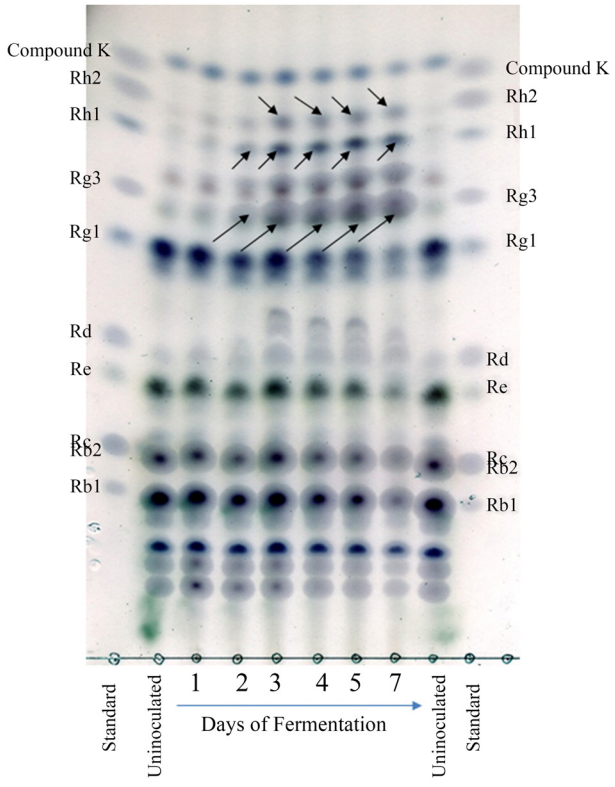

Fig. 1. TLC analysis of the fermented ginseng extract.

(2018) dertermined that the ginseng marc fermented with *Pediococcus acidilactici* enhanced its antioxidant and nitric oxide scavenging activites. The co-culture of *P. pentosaceus* and *L. mesentoroides* also showed that Rb1 transfered to Rg3 (Park *et al*., 2017). In the present, a co-culture of probiotic strains *L. helveticus* KII13 and *P. pentosaceus* KID7 converted ginsenoside Rb1, Rg1 into ginsenosides Rg3, Rh1 and Rh2.

HPLC analysis of ginseng extract fermented with the combination of probiotic *L. helveticus* KII13 and *P. pentosaceus* KID7 (Fig. 2) to confirm the results of the TLC analysis. The Rb1 peak decreased but the peak of Rg3 increased on the 3rd day compared to zero day (Fig. 2). The appearance of Rh2 peak and an increase in the Rh1 peak were observed on the 5th day (Fig. 2). On the 5th day, the relative amounts of ginsenosides Rb1, Rg3, and Rh2 were 1.2%, 76.1%, and 14.7%, respectively, whereas, on the 7th day amounts were 0.7%, 75.2%, and 12.4%, respectively. Additionally, the amounts of Rg1 and Rh1 were 0.8% and 13.6%, respectively on 5th day and were 0.4% and 14.2%, respectively on the 7th day. The hydrolytic pathway of major protopanaxadiol (PPD)-type ginsenosides to minor PPD-type ginsenosides conversion happens through several intermediates as follows: Rb1→Rd→Rg3→Rh2 or Rb1→Rd→F2→Ck and/or Rh2; Rb2→Rd→Rg3 or F2→Ck or Rh2, Rb2→C-O→F2 or C-Y→Ck or Rh2; Rc→Rd→Rg3 or F2→Ck or Rc→C-Mc_1→F2 or C-Mc→Ck or Rh2 (Park *et al*., 2010). Similarly, the hydrolytic pathway of major protopanaxatriol (PPT)-type ginsenosides to minor PPT-type ginsenosides conversion also happens through several intermediates as follows: Re→Rg1 or Rg2→F1 or Rh1; R1→R2 or Rg1→F1 or Rh1; and Rf→Rh1 (Park *et al*., 2010). Various microbial enzymes have been shown to hydrolyze the glycosides of major PPD and PPT-type ginsenosides and produce a variety of minor compounds, which has been reviewed by Park *et al*. (2010). Since, we used a combination of probiotic strains and crude ginseng extract for fermentation, it is observed in the HPLC chromatogram of 5th and 7th day the major ginsenoside peaks disappeared and several new peaks appeared, indicating hydrolysis of major ginsenosides to form minor ginsenosides (Fig. 2). The results of our study suggested that hydrolytic pathway of ginsenoside Rb1 and Rg1 by the probiotic strains is Rb1→Rg3→Rh2 and Re→Rg1→Rh1 (Fig. 3). A similar pathway was observed for a *L. paracasei* subsp. *tolerans* MJM60396 in our previous report (Palaniyandi *et al*., 2016). The peak of Rg3 appeared on the 3rd day while Rh2 appeared on the 7th day. The recombinant *L. lactis* produced minor ginsenoside Rg3 as follows: Rb1→Rd→Rg3 and Rg3 was produced after 24 h (Li *et al*., 2017). Production of minor ginsenosides using β-glucosidase-producing bacteria has been described in previous reports. *P. pentosaceus* WiKim20 and *L. mesenteroides* WiKim19 transformed ginsenoside Rb1 into the ginsenoside Rg3, although *P. pentosaceus* WiKim20 lacked β-glucosidase activity (Park *et al*., 2017). *Weissella hellenica* DC06 could also convert major ginsenoside Rb1 into pharmacologically active ginsenoside Rg3 (Huq *et al*., 2016). The peak for ginsenoside Rg3 was observed on the 3rd day and Rb1 was no longer present within 7 days fermentation.

To our knowledge, this is the first report on biotransformation of ginsenoside Rb1 to non-natural ginsenosides Rg3, Rh2 and Rh1 using a combination of two different probiotic strains. By using food grade bacteria (GRAS), there is potential of preparing a higher biofunctional ginseng extract by fermentation for food and pharmaceutical-applications.

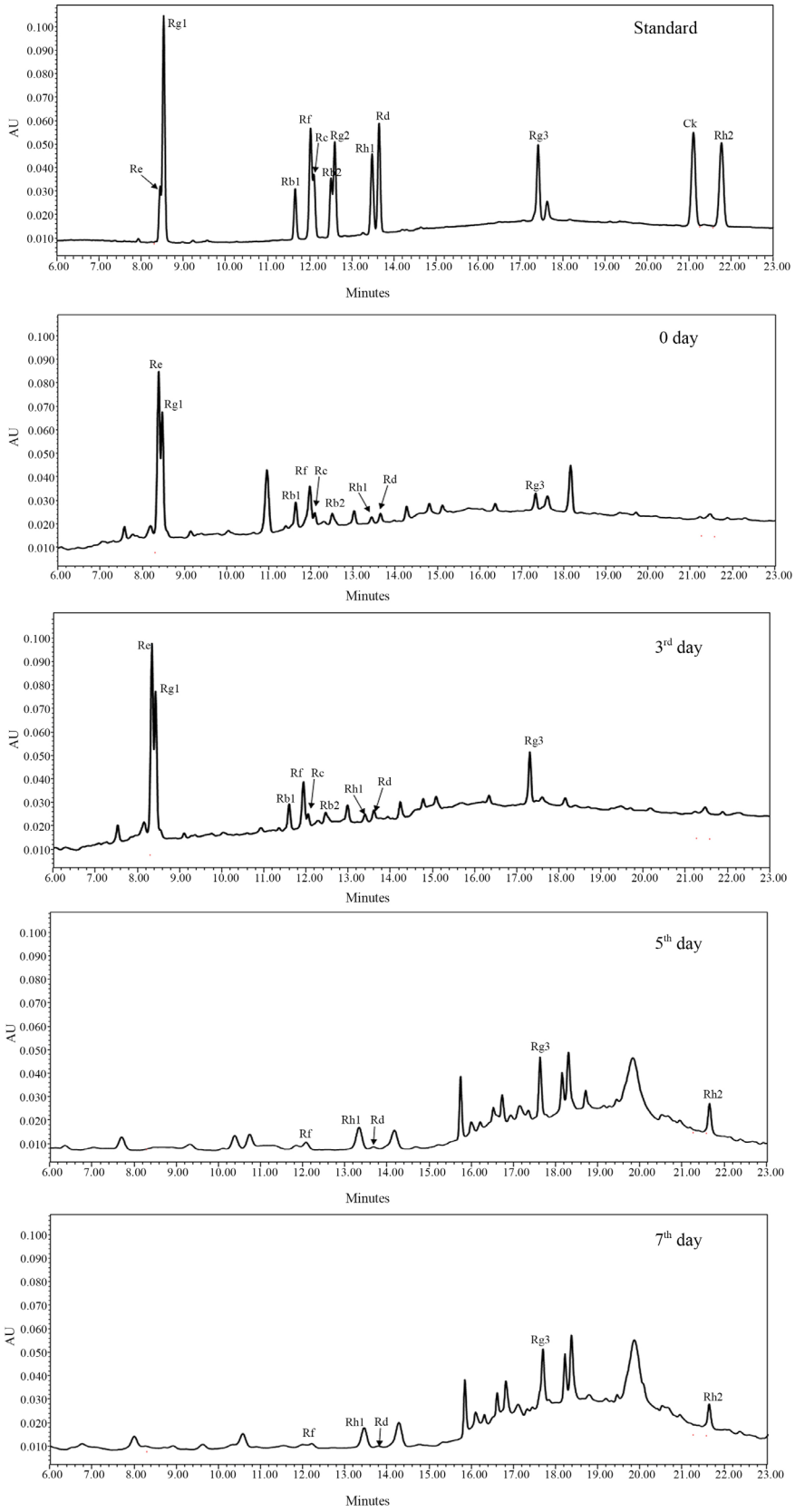

Fig. 2. Time course HPLC analysis of the composition of major and minor ginsenoside in ginseng extract fermented by the probiotic strains combination.

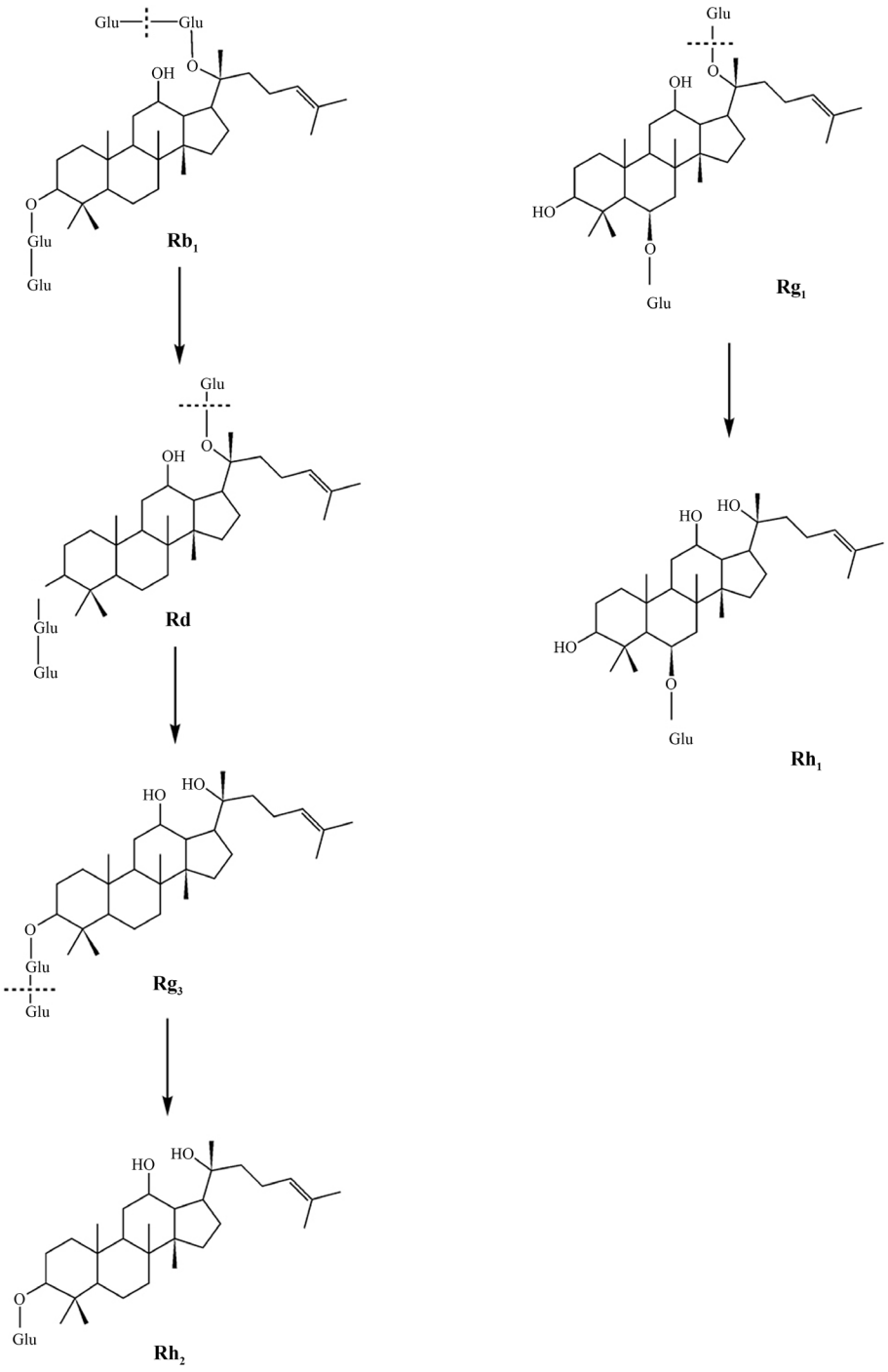

Fig. 3. Schematic representation of the possible hydrolytic pathway of ginsenoside Rg3, Rh2, and Rh1 production from Rb1 and Rg1.

적 요

인삼은 우수한 약리 활성 작용을 보이는 전통적인 약초이다. 본 연구에는 프로바이오틱스 *Lactobacillus helveticus* KII13과 *Pediococcus pentosaceus* KID7 균주를 진세노사이드(ginsenoside) 함량을 증가시키기 위해 조 인삼 추출물을 발효시켜 진세노사이드를 형질전환 시키는데 사용되었다. 발효삼 추출물의 TLC (Thin-layer chromatography) 분석 결과, 5일간의 발효 후 주요 사포닌인 진세노사이드 Rg3, Rh1 및 Rh2로 변환되는 것으로 나타났다. HPLC 분석을 수행하여 주요 및 미량 진 세노사이

드를 정량화하였다. 3일째에는 Rg3가 나타나고, 5일째에는 Rh2 및 Rh1이 나타난다. *L. helveticus* KII13과 *P. pentosaceus* KID7의 공동 배양은 주요 진세노사이드(Rb1과 Rg1)를 미량 진세노사이드(Rg3, Rh2, Rh1)로 전환시키는 것을 확인하였다.

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