Bioconversion of Ginsenoside Rd into Compound K by *Lactobacillus pentosus* DC101 Isolated from Kimchi

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Ginsenosides are the principal components responsible for the pharmacological and biological activities of ginseng. Ginsenoside Rd was transformed into compound K using cell-free extracts of food microorganisms, with *Lactobacillus pentosus* DC101 isolated from kimchi (traditional Korean fermented food) used for this conversion. The optimum time for the conversion was about 72 h at a constant pH of 7.0 and an optimum temperature of about 30°C. The transformation products were identified by thin-layer chromatography and high-performance liquid chromatography, and their structures were assigned using nuclear magnetic resonance analysis. Generally, ginsenoside Rd was converted into ginsenoside F2 by 36 h post-reaction. Consequently, over 97% of ginsenoside Rd was decomposed and converted into compound K by 72 h post-reaction. The bioconversion pathway to produce compound K is as follows: ginsenoside Rd→ginsenoside F2→compound K.

Keywords: Bioconversion, Compound K, Ginsenoside modification, Kimchi, *Lactobacillus pentosus* DC101

INTRODUCTION

In Asian countries, ginseng (from the roots of *Panax ginseng* C. A. Meyer) is frequently used orally in traditional medicine. The major active components of ginseng are ginsenosides, which are glycosides containing an aglycone (protopanaxadiol or protopanaxatriol) with a dammarane skeleton. These ginsenosides were previously reported to exhibit various biological activities, including anti-inflammatory activity [1] and antitumor effects (inhibition of tumor-induced angiogenesis and prevention of tumor invasion and metastasis) [2,3], but these naturally occurring ginsenosides are poorly absorbed along human intestinal tracts [4]. Previous studies demonstrated that protopanaxadiol-type ginsenosides such as Rb1, Rb2, and Rc are metabolized by intestinal bacteria after oral administration of their final derivative,

20-*O*-β-D-glucopyranosyl-20(*S*)-protopanaxadiol, also called compound K. Compound K, reported to be easily absorbed by the human body, is the genuine active form of protopanaxadiol-type saponins [5], and it has recently attracted increasing interest because of its intriguing biological actions. Compound K was demonstrated to induce tumor cell apoptosis, inhibit tumor metastasis, and restrain tumor invasion [6–10]. Additionally, it has exhibited hepatoprotective and anti-inflammatory activities, which are more therapeutic effects than those of currently used drugs such as dimethyl diphenyl bicarboxylate and indomethacin [11,12].

Several studies have shown that transformation of ginsenosides into deglycosylated ginsenosides is required for more effective *in vivo* physiological activities

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of ginsenosides [13]. Various transformation methods have been used, including mild acid hydrolysis [14], enzymatic conversion [15], and microbial conversion [16], although chemical methods produce side reactions, such as epimerization, hydration, and hydroxylation. Most of the microorganisms that have been used for ginsenoside transformation are not of food-grade standard.

In the present study, we characterized the transformation of the major protopanaxadiol ginsenoside Rd using cell-free extracts of the *Lactobacillus pentosus* (*L. pentosus*) DC101 strain isolated from kimchi, a traditional Korean fermented food.

MATERIALS AND METHODS

Materials

The *L. pentosus* DC101 strain was isolated from kimchi and collected from 10 different areas in Gyeonggi-do, Korea, on MRS agar. Difco MRS broth was purchased from Becton Dickinson and Co. (Franklin Lakes, NJ, USA), while crude ginseng leaf saponin was acquired from Fusong, China. The standard ginsenosides, 20(*S*)-Rb1, 20(*S*)-Rd, 20(*S*)-Rg3, 20(*S*)-Rh2, 20(*S*)-F2, and compound K, were obtained from KT&G in Daejeon, Korea.

Separation of ginsenoside Rd

Ginsenoside Rd was separated from crude ginseng leaf saponin using both C₁₈ and silica gel 60 columns (70-230 mesh; Merck KGaA, Darmstadt, Germany). Thus, 1 g of crude ginseng leaf saponin was dissolved in methanol (MeOH), absorbed onto RP-18 (10 g), and applied to a flash chromatography system (C₁₈ column-discovery; Φ 4.6 mm $\times$ 25 cm; Supelco, Bellefonte, PA, USA). Next, acetonitrile (solvent A) and water (solvent B) were used at an A/B ratio with the following temporal profiles: 0-20 min, from 10:90 to 30:70; 20-30 min, maintained at 30:70; 30-45 min, from 30:70 to 45:55; 45-50 min, maintained at 45:55; and 50-60 min, from 45:55 to 52:48. The eluate fraction was collected every 10 mL and was subsequently analyzed by thin-layer chromatography (TLC). The fraction containing ginsenoside Rd was concentrated *in vacuo*. The residue was reabsorbed with a silica gel 60 column (Φ 5 mm $\times$ 50 cm) and eluted with chloroform:MeOH (CHCl₃:MeOH; 7:3, v/v). Finally, the pure separated ginsenoside Rd fraction was concentrated *in vacuo* and subsequently used in reactions.

Screening of lactic acid bacteria producing β -glucosidase

Esculin-MRS agar [17] was used to isolate β -glu-

cosidase-producing lactic acid bacteria. This growth medium contains (per 1 L) 3 g esculin and 0.2 g ferric citrate with MRS agar. The lactic acid bacteria producing β -glucosidase that hydrolyzes esculin appeared as colonies, which were surrounded by a reddish-brown to dark brown zone on the esculin-MRS agar. Subsequently, single colonies from those plates were subjected to an additional incubation for 2 days at 37°C.

Phylogenetic analysis

The complete sequence of the 16S rRNA gene from the *L. pentosus* DC101 strain was compiled through SeqMan software and edited using the BioEdit program. The 16S rRNA gene sequences of related taxa were obtained from GenBank (National Center for Biotechnology Information; Bethesda, MD, USA), and the phylogenetic tree was constructed using the neighbor-joining method of the MEGA 3.1 program. A bootstrap analysis with 1,000 replicates was similarly performed to obtain confidence levels for the branches. Finally, the closest type strains were included in the phylogenetic tree.

Preparation of crude microbial enzymes

The *L. pentosus* DC101 strain was incubated in MRS broth at 37°C for 8 h with shaking. Following culture broth centrifugation (15,000 $\times$ g for 10 min at 4°C), four volumes of ethanol were added to the supernatant. This was mixed sufficiently and placed in an ice chamber for 20 min until further reactions. Meanwhile, the protein pellet was collected by centrifugation (10,000 $\times$ g for 40 min at 4°C) and then was dissolved in 20 mM sodium phosphate buffer (pH 7.0).

Parameter optimization for the conversion of ginsenoside Rd

The effects of temperature on the conversion of ginsenoside Rd were investigated at temperatures in the range of 25-60°C. Reactions were performed in sodium phosphate buffer (pH 7.0) containing ginsenoside Rd (0.2 μ mol) and crude enzyme (250 μ L) for 48 h.

The optimum reaction pH for the conversion of ginsenoside Rd varied between 3.0 and 13.0. Reactions were performed in various buffers (pH 3.0-13.0) containing ginsenoside Rd (0.2 μ mol) and crude enzyme (250 μ L) at 30°C for 60 h, followed by extraction with water-saturated *n*-butanol. Subsequently, the *n*-butanol fraction was evaporated to dryness and the methanolic extract was analyzed by TLC and high-performance liquid chromatography (HPLC).

Conversion of ginsenoside Rd by crude enzymes

The *L. pentosus* DC101 strain was grown in MRS broth at 37°C, until its absorbance at 600 nm reached 1.0. The crude enzymes from each culture broth were dissolved in 20 mM sodium phosphate buffer (pH 7.0) and then mixed with 0.2 mM ginsenoside Rd that was dissolved in distilled water at a 1:4 ratio (v/v). Subsequently, the mixture was incubated at 30°C and 190 rpm for 72 h. During the reaction period, a 1.25-mL aliquot was taken every 12 h. This was extracted via water-saturated *n*-butanol and then analyzed by both TLC and HPLC.

Analysis of ginsenosides by thin-layer chromatography

TLC was performed using silica gel plates (60F₂₅₄; Merck, Darmstadt, Germany), and the mixture CHCl₃:CH₃OH:H₂O (65:35:10; v/v; lower phase) was used as its developing solvent. The spots on TLC plates were detected by spraying 10% sulfuric acid, followed by heating the plate at 110°C for 10 min.

Analysis of ginsenosides by high-performance liquid chromatography

The HPLC-grade acetonitrile and water were purchased from SK Chemicals (Ulsan, Korea). Next, the reaction mixture was extracted using water-saturated *n*-butanol and evaporated *in vacuo*. Additionally, the residue was dissolved in CH₃OH and injected for HPLC analysis. This experiment also used a C₁₈ (250×4.6 mm; ID 5 µm) column containing acetonitrile (solvent A) and distilled water (solvent B) at A:B ratios of 15:85, 21:79, 58:42, 90:10, 90:10, 15:85, and 15:85 and with run times of 0-5, 5-25, 25-70, 70-72, 72-82, 82-84, and 84-100 min, respectively. Finally, the flow rate was 1.6 mL/min and the sample was detected at a UV absorbance of 203 nm.

Structural identification

Reaction products 1 and 2, from the ginsenoside Rd bacterial bioconversion by the *L. pentosus* DC101 strain, were separated on a silica gel column using the solvent mixture CHCl₃:CH₃OH:H₂O (8:3:1, by vol., lower phase) (Fig. 1). Their structures were assessed using the proton and carbon nuclear magnetic resonance (NMR; ¹H-NMR, ¹³C-NMR) spectrum method with a Fourier transform NMR spectrometer (Inova AS 400 MHz NMR system; Varian, Vernon Hills, IL, USA) and pyridine-*d*₅ as the solvent.

Metabolite 1: white powder; melting point (mp): 184-186°C; ¹H-NMR (pyridine-*d*₅, 400 MHz): δ0.82 parts per million (ppm; 3H, s, H-19), δ0.96 ppm (3H,

Fig. 1. Time course thin-layer chromatography analysis of metabolites of ginsenoside Rd bioconverted by *Lactobacillus pentosus* DC101. Ginsenoside Rd was used as the substrate to yield ginsenoside F2 (1) and compound K (C-K, 2).

s, H-30), δ0.97 ppm (3H, s, H-18), δ1.00 ppm (3H, s, H-29), δ1.31 ppm (3H, s, H-28), δ1.60 ppm (3H, s, H-26, H-27), δ1.63 ppm (3H, s, H-21), δ4.95 ppm (1H, d, J=7.2Hz, H-20-glc-1), δ5.21 ppm (1H, d, J=7.2Hz, H-3-glc-1); for ¹³C-NMR (pyridine-*d*₅, 100 MHz) data, see Table 1.

Metabolite 2: white powder; mp: 220-222°C; ¹H-NMR (pyridine-*d*₅, 400 MHz): δ0.83 ppm (3H, s, H-19), δ0.90 ppm (3H, s, H-18), δ0.94 ppm (3H, s, H-30), δ1.00 ppm (3H, s, H-29), δ1.19 ppm (3H, s, H-28), δ1.57 ppm (6H, s, H-26, H-27), δ1.59 ppm (3H, s, H-21), δ5.16 ppm (1H, d, J=7.2Hz, H-20-glc-1); for ¹³C-NMR (pyridine-*d*₅, 100 MHz) data, see Table 1.

RESULTS AND DISCUSSION

Phylogenetic study

The 16S rRNA gene sequence of the *L. pentosus* DC101 strain was aligned with other neighboring type strains, and the taxonomic relationships were confirmed. The phylogenetic tree revealed that the *L. pentosus* DC101 strain was grouped with the *Lactobacillus* species (Fig. 2). Additionally, the highest degrees of 16S rRNA gene sequence identities were listed, including *L. plantarum* subsp. *argentoratensis* CIP 108320^T (accession number, AJ640078; 98.9%), *L. paraplantarum* DSM 10667^T (accession number, AJ306297; 99.3%), *L. plantarum* subsp. *plantarum* NCDO 1752^T (accession number, X52653; 99.4%), and *L. pentosus* JCM 1558^T (accession number, D79211; 99.8%). Hence, based on the phylogenetic tree and homology analysis, the

Table 1. ^{13}C -NMR chemical shift of material Rd and metabolites 1 and 2 (100 MHz, solvent: pyridine- d_5)

Carbon site	Ginsenoside Rd	Ginsenoside F2 (ppm)		Compound K (ppm)	
		Reference metabolite 1		Reference metabolite 2	
Aglycone moiety					
C-1	39.1	39.2	39.2	39.5	39.6
C-2	26.6	26.8	26.8	28.3	28.4
C-3	88.9	88.8	88.8	78.1	78.2
C-4	39.7	39.7	39.9	39.6	39.7
C-5	56.3	56.4	56.4	56.5	56.5
C-6	18.4	18.4	18.5	18.8	18.9
C-7	35.1	35.1	35.2	35.3	35.3
C-8	40.0	40.1	40.1	40.2	40.2
C-9	50.1	50.2	50.2	50.4	50.4
C-10	36.8	36.9	37.0	37.5	37.5
C-11	30.8	30.8	30.8	30.9	30.9
C-12	70.2	70.2	70.1	70.2	70.3
C-13	49.3	49.5	49.5	49.6	49.7
C-14	51.4	51.4	51.5	51.5	51.6
C-15	30.8	30.9	31.0	31.1	31.2
C-16	26.7	26.6	26.7	26.7	26.8
C-17	51.7	51.7	51.7	51.7	51.8
C-18	16.3	16.3	16.4	16.3	16.4
C-19	15.9	16.0	16.0	16.1	16.2
C-20	83.3	83.3	83.3	83.4	83.4
C-21	22.5	22.4	22.5	22.4	22.3
C-22	36.0	36.1	36.2	36.3	36.4
C-23	23.3	23.2	23.3	23.3	23.6
C-24	125.8	126.0	126.6	126.0	126.0
C-25	130.8	130.9	131.5	131.0	130.9
C-26	25.8	25.8	25.8	25.8	25.9
C-27	17.8	17.8	17.8	17.8	17.6
C-28	28.1	28.2	28.2	28.7	28.8
C-29	16.6	16.8	16.9	16.4	16.5
C-30	17.3	17.4	17.4	17.5	17.3
Sugar moiety					
3-O-inner-Glc					
C-1	105.0	106.9	106.8		
C-2	83.1	75.8	75.9		
C-3	77.9	79.3	78.7		
C-4	71.6	71.7	71.6		
C-5	78.2	78.3	78.3		
C-6	62.6	62.9	62.8		
3-O-outer-Glc					
C-1	105.8				
C-2	77.0				
C-3	79.1				
C-4	71.4				
C-5	78.0				
C-6	62.6				
20-O-inner-Glc					
C-1	98.2	98.3	98.3	98.3	98.3
C-2	75.1	75.1	75.1	75.2	75.2
C-3	78.2	78.8	79.2	79.3	79.3
C-4	71.6	71.9	71.8	71.9	71.9
C-5	78.2	78.3	78.4	78.1	78.2
C-6	62.8	63.1	63.0	63.1	63.1

DC1010 strain could be *L. pentosus*.

The effect of temperature on enzyme activity

Generally, the present study focused on finding the crude enzyme's optimum temperature, and experiments were performed at various temperatures (25, 30, 35, 40, 50, and 60°C) to determine that aforementioned variable. Our results shown revealed that the crude enzyme reaction of both the *L. pentosus* DC101 and ginsenoside Rd strains was greatly influenced by temperature (Fig. 3A). Additionally, TLC analysis showed that the crude

enzyme activity of the *L. pentosus* DC101 strain was highest at 30°C. The optimum temperature was further confirmed by quantitative HPLC analysis. At 25°C, ginsenoside Rd began degrading, reached its maximum activity at 30°C, and suddenly decreased its activity above 35°C. Thus, the enzyme could not perform catalytic reactions at temperatures lower than the optimum because the energy released was insufficient for the reaction. At high temperatures, enzymes lose their activities because of denaturation.

Fig. 2. Phylogenetic tree based on the 16S rRNA gene sequence showing the phylogenetic relationships between the *Lactobacillus pentosus* DC101 strain and related *Lactobacillus* species.

The effect of pH on enzyme activity

The ginsenoside conversion activity of the crude enzyme was tested at various pH (3.0-13.0) levels. Indeed, TLC analysis revealed that the optimum pH range was pH 7.0 to pH 9.0. Similarly, quantitative HPLC analysis also clarified that ginsenoside Rd began degrading at pH 4.0 and then gradually increased its activity up to pH 5.0. Additionally, it sustained its maximum enzymatic activity at pH 7.0 and then suddenly decreased at pH 9.0 (Fig. 3B).

Fig. 3. Effects of temperature (A) and pH (B) on the conversion of ginsenoside Rd as substrate to F2 and compound K (C-K) by the crude enzyme of *Lactobacillus pentosus* DC101 using thin-layer chromatography analysis.

Bioconversion of ginsenoside Rd

After *L. pentosus* DC101 was cultured until the absorbance at 600 nm reached 1.0, the crude enzyme solution of the *L. pentosus* DC101 strain was used to convert ginsenoside Rd into both metabolite 1 and metabolite 2 (Fig. 1). This indicated that metabolite 1 was an intermediate metabolite and metabolite 2 was the final product. The retention factor values of metabolite products 1 and 2 on TLC were similar to those of ginsenoside F2 and compound K. This result suggested that ginsenoside Rd was converted by the crude enzyme from *L. pentosus* DC101. Reaction products 1 and 2 were separated on a silica gel column to confirm their chemical structures.

Structure of metabolites 1 and 2

In the $^1\text{H-NMR}$ spectrum of metabolite 1, the proton signals for H-1 of the 3-glucopyranosyl moiety and the 20-glucopyranosyl moiety appeared at $\delta 5.21$ (1H, d, $J=7.2\text{Hz}$, H-3-glc-1) and $\delta 4.95$ ppm (1H, d, $J=7.2\text{Hz}$, H-20-glc-1), respectively. Metabolite 1 was demonstrated to harbor two β -D-glucoses. According to the comparison of the $^{13}\text{C-NMR}$ spectrum of metabolite 1 with that of ginsenoside Rd, the signal for the C-2 of the 3-inner-glucopyranosyl moiety was shifted upfield, from $\delta 83.1$ to $\delta 75.9$ ppm, and other signals were similar to those of ginsenoside Rd. Additionally, because anomeric carbon

signals at δ 106.8 and δ 98.3 ppm were similar to signals for C-1 of the 3-*O*-inner-glucopyranosyl moiety and 20-*O*-inner-glucopyranosyl moiety of ginsenoside Rd, two glucoses connected with the C-3 and C-20 sites of the aglycone moiety were confirmed. Thus, metabolite 1 was identified as 3-*O*-[β -D-glucopyranosyl]-20-*O*-[β -D-glucopyranosyl]-20(*S*)-protopanaxadiol, identical to ginsenoside F2.

The ^1H -NMR spectrum of metabolite 2 revealed that only one proton signal for H-1 of the 20-glucopyranosyl moiety appeared at δ 5.16 ppm (1H, d, $J=7.2\text{Hz}$, H-20-glc-1); metabolite 2 was shown to harbor only one β -D-glucose. According to the comparison of the ^{13}C -NMR spectrum of metabolite 2 with decomposition product 1, the signal for C-3 of aglycone was shifted upfield, from δ 88.8 to δ 78.2 ppm, and other signals were similar to those of metabolite 1. So the glucose combined the C-3 site of aglycon of metabolite 1 was hydrolyzed. The signal for the C-1 of glucopyranosyl moiety appeared at δ 98.3 ppm, further indicating that the glucopyranosyl moiety of metabolite 2 combined with the C-20 site of aglycone. Therefore, metabolite 2 was identified as 20-*O*-[β -D-glucopyranosyl]-20(*S*)-protopanaxadiol, identical to compound K.

Bioconversion by pathway

The conversion of ginsenosides by *L. pentosus* DC101 was confirmed using quantitative HPLC analysis. Here, the conversion reaction started with ginsenoside Rd (retention time, 44 min) and gradually decreased with increasing time period (36 h post-reaction). Notably, the amount of degraded ginsenoside Rd was about 27% after 36 h, 77% after 48 h, 80% after 60 h, and 97% after 72 h (Table 2, Fig. 4). The degraded amount of ginsenoside Rd was converted into ginsenoside F2 (metabolite 1; retention time, 52 min), followed by conversion into compound K (metabolite 2; retention time, 64 min). The ginsenoside F2 (metabolite 1) peak reached a maximum level (123 μg) by 48 h post-reaction (Fig. 4A), which then disappeared gradually (Figs. 3C and 4B). Note that the compound K (metabolite 2) peak increased continu-

Table 2. The conversion pattern of major ginsenosides (Rd) to minor ginsenosides by *Lactobacillus pentosus* DC101 depending on reaction time

Reaction time (h)	Converted ginsenosides (μg)		
	Rd	F2	Compound K
0	200	0	0
36	146	24	0
48	46	123	3
60	43	57	53
72	5	13	115

Fig. 4. High-performance liquid chromatography analysis of the bioconversion of ginsenosides. The reaction time-course transformation of ginsenoside Rd by the crude enzyme of *Lactobacillus pentosus* DC101. Incubation time; (A) 48 h, (B) 60 h, (C) 72 h. F2, ginsenoside F2; C-K, compound K.

ously after 60 h, finally reaching its maximum at 72 h post-reaction. Consequently, 87% of ginsenoside Rd was converted into compound K (metabolite 2) by 72 h post-reaction with *L. pentosus* DC101 (Table 2). Thus, our results revealed that the transformation pathways of ginsenoside Rd by *L. pentosus* DC101 was ginsenoside Rd→ginsenoside F2→compound K (Fig. 5).

In the present study, ginsenoside Rd was converted into compound K using the enzyme secreted by the β -glucosidase-producing bacteria *L. pentosus* DC101, which was isolated from kimchi. Importantly, the bioconversion pathway to produce compound K followed the sequence: ginsenoside Rd→ginsenoside F2→compound K. As such, enzymes secreted by microbes such as *Bifidobacterium* sp. SJ32 (SJ32) also transformed ginsenoside Rb2 into compound K via ginsenoside Rd and ginsenoside F2 [18]. Additionally, *Aspergillus niger* and *Aspergillus usami*, and *Caulobacter leidyia* GP45 transformed ginsenoside Rb1 into compound K via ginsenoside Rd and ginsenoside F2 [19,20]. Thus, the

Fig. 5. Proposed bioconversion pathway of ginsenoside Rd to ginsenoside F2 and compound K by the crude enzyme of *Lactobacillus pentosus* DC101.

biotransformation of ginsenoside Rd into compound K has potential for applications in industry. Further work is under way to enhance the enzyme's activities to shorten its production cycle and increase its yield.

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