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Bioconversion of Ginsenoside Rb_1 to Compound K using *Leuconostoc lactis* DC201

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Abstract - Ginseng (*Panax ginseng*) is frequently used in Asian countries as a traditional medicine. The major components of ginseng are ginsenosides. Among these, ginsenoside compound K has been reported to prevent the formation of malignancy and metastasis of cancer by blocking the formation of tumor and suppressing the invasion of cancer cells. In this study, ginsenoside Rb_1 was converted into compound K, via secreted β-glucosidase enzyme from the *Leuconostoc lactis* DC201 isolated, which was extracted from Kimchi. The strain DC201 was suspended and cultured in MRS broth at 37℃. Subsequently, the residue from the cultured broth supernatant was precipitated with EtOH and then dissolved in 20 mM sodium phosphate buffer (pH 6.0) to obtain an enzyme liquid. Meanwhile, the crude enzyme solution was mixed with ginsenoside Rb_1 at a ratio of 1:4 (v/v).The reaction was carried out at 30℃ and 190 rpm for 72 hours, and then analyzed by TLC and HPLC. The result showed that ginsenoside Rb_1 was transformed into compound K after 72 hours post reaction.

Key words - Bioconversion, Ginsenoside Rb_1, Compound K, *Leuconostoc lactis* DC201

Introduction

Panax ginseng is semi-shady and perennial herb, and belong to the family *Araliaceae*. The Genus *Panax* is originated from Greek words, *Pan* means all and *ax* means curing. It has been cultivated and its highly valued root used for medicinal purposes.

The amphipathic molecule is composed of a glycone and an aglycone. The triterpenoids in ginseng saponins include oleanane with 5 rings, and dammarane with 4 rings. Especially a dammarane triterpenoid is well known as major pharmaceutical activity.

If the ginseng saponins are taken orally, the nonpolar alkaloid is absorbed, but most of saponins are not absorbed effectively. These remained and non-digested saponins are degraded by intestinal bacteria such as *Lactobacillus* and *Leuconostoc*. The sugar moiety is used by bacteria and the remained aglycone is dissolved in blood. The dissolved minor saponins are, show highly pharmaceutical effects.

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Based on the intestinal microbial community, the absorption pattern of major saponins is quite different and specific to individuals (Karikura *et al*., 1991; Takino *et al*., 1994; Hasegawa *et al*., 1995). Therefore the conversion of the major saponins to minor saponins which is more absorbable and has higher biological activity, is meaningful.

Odashima (1985) reported that ginsenoside Rh_2 inhibits the growth of lung cancer, carcinogenic B-16, and HELA cells *in vitro*. Lee (1999) showed *in vitro* that IH-901 (compound K) suppresses the metastatic character of HL-60 leukaemia, PC-14 lung cancer, MKN-45 gastric cancer, and Hep-G2 liver cancer cells and that compound K induces cell death in malignant bone marrow cells. Compound K, ginsenoside Rh_2, and Rh_1 are also reported to exert anti-allergic effects (Shin *et al*., 2003; Zhou *et al*., 2006). The method of conversion for major saponins to minor saponins includes acid treatment, base treatment, heat treatment, and enzyme treatment. Especially the conversion by microbes is processed in moderated condition such as neutral, room temperature, pressure of the atmosphere, and the reaction is specific to the substrate (Chi *et al*., 2005a; Chi *et al*., 2005b; Park *et al*., 2001).

Kimchi is a kind of fermented food and good source to lactic acid bacteria (LAB). LAB contributes a singular quality to the taste of Kimchi, as they do in dairy products, such as cheese and fermented milk. A number of type strains have been isolated from Kimchi. In this study, the strain *Leuconostoc lactis* DC201 was isolated from cabbage Kimchi and characterized by a polyphasic approach, which included a physiological analysis and a phylogenetic tree based on 16S rRNA gene sequence.

We used a crude extract of this β-glucosidase-producing lactic acid strain to convert ginsenoside Rb_1 to compound K and to determine optimal conditions for this bioconversion. Our finding may be applied in the large scale production of compound K, a relatively potent ginseng derivative.

Materials and Methods

Sample preparation

Kimchi samples were collected from Kyung-gi province in Korea, and saponin standards (Rb_1, Rd, F_2, compound K and Rh_2) were donated by the Ginseng Genetic Resource Bank, in Korea. Ginsenoside Rb_1 was purified and analyzed with NMR (400 MHz Varian Inova AS 400, Varian USA) in this laboratory. Saponins were detected using thin-layer chromatography on silica gel (60 F_{254} TLC plate, Merck) and analyzed by HPLC (HPLC system NS 3000i, Futecs Co., Ltd.).

Taxonomy of the strain *Leuconostoc lactis* DC201

Lactic acid bacteria were isolated from various types of Kimchi. The Kimchi suspension was spread on MRS agar plates (Difco) after serial dilution with saline solution. Single colonies were selected and transferred to new plates for isolation. After incubation at 37℃ for 2-3 days, the isolated strains were identified by partial 16S rRNA gene sequence, and either cultured further on MRS agar (BD) for immediate study or stored as a suspension in 25% (w/v) glycerol at -70℃. The isolates were tested for β-glucosidase activity on MRS agar containing esculin. The 16S rRNA gene was amplified from the chromosomal DNA using the universal bacterial primer set fD1 and rP1 (Weisburg *et al*., 1991), and the purified PCR product was sequenced by Genotech, in Daejeon, Korea (Kim *et al*., 2005). The partial sequence of the 16S rRNA gene was compiled with SeqMan software (DNASTAR Inc.). The 16S rRNA gene sequences of the related taxa were obtained from GenBank and edited using the BioEdit program (Hall, 1999). Multiple alignments were performed with CLUSTAL X (Thompson *et al*., 1997). Evolutionary distances were calculated using the Kimura two-parameter model (Kimura, 1983). The phylogenetic tree was constructed by the neighbor-joining method (Saitou *et al*., 1987). Bootstrap analysis with 1,000 replicates was also conducted to obtain confidence levels for the branches.

Growth profiles on different culture media

Various media, including de Man, Rogosa and Sharpe (MRS), Luria-Bertani (LB), nutrient broth (NB), and tryptic soy broth (TSB), were used to culture cells and test enzyme activity. For a seed culture, a single colony was inoculated into MRS broth and incubated at 37℃ in shaking incubator (190 rpm) until the optical density at 600 nm reached 1.0. From this suspension, a 500 μl aliquot was inoculated into 50 ml liquid medium, such as MRS, LB, NB, or TSB. The strain was then incubated at 37℃ for 48 hrs in shaking incubator (190 rpm). A sample was withdrawn every 2 hrs to read the OD_{600}.

Effect of temperature on growth

Leuconostoc lactis DC201 was incubated in MRS until the OD_{600} reached 1.0, then diluted 100-fold into fresh MRS broth and subdivided for culture at increasing growth temperatures (25℃, 30℃, 37℃, and 45℃). The cultures were incubated for 24 hrs with mixing at 190 rpm. Growth was monitored at OD_{600}.

Effect of pH on growth

Culture vessels containing MRS broth were individually adjusted with 0.5 N HCl and 0.5 N NaOH to pH 5.0, 5.5, 6.0, 6.5, 7.0, 7.5, and 8.0, respectively. The strain DC201 was preincubated in MRS broth to OD_{600} = 1.0, then diluted 100 fold into MRS broth at a pre-adjusted pH. The cultures were incubated at 37℃ for 24 hrs with 190 rpm. Growth was monitored at OD_{600}.

Preparation of crude enzyme

Growth and appearance on esculin-MRS agar confirmed

β-glucosidase activity in a single colony isolated from the Kimchi. This isolate was thereafter referred to *Leuconostoc lactis* DC201. For crude enzyme preparation, DC201 was grown in MRS broth at 37℃ with mixing (190 rpm) until the OD_{600} reached 1.0. The culture broth was centrifuged at 15,000 g and 4℃ for 10 min. Cell-free supernatant was mixed with 4 volumes cold ethanol and placed on ice for 40 min to precipitate protein. After centrifugation under 4℃ within 40 minutes, the precipitate was dissolved in 20 mM sodium phosphate buffer (pH 6.0), and used as crude enzyme solution.

Conversion of ginsenoside Rb_1

Crude enzyme prepared from strain DC201 was mixed with 0.2 mM ginsenoside Rb_1 (1:4, v/v) and reacted for 72 hrs at 30℃ with stirring (190 rpm). A 1.5 ml sample was removed from the reaction tube every 24 hrs and extracted with water-saturated butanol. The extract was dissolved and applied to a TLC plate (Silica gel, Merck), and developed with the solvent system $CHCl_3/CH_3OH/H_2O$ (65:35:10, v/v, lower phase).

HPLC analysis

A 1.5 ml sample was removed from the enzymatic reaction tube and extracted with water-saturated butanol. The extract was dissolved in methanol, filtered through a 0.45 μm membrane, and then analyzed by HPLC using an RP-18 column (250 × 4.6 mm, ID 5 μm) under UV detector at 203 nm. The injection volume was 25 μl. The mobile phase consisted of acetonitrile (solvent A) and water (solvent B) applied by gradient at a flow rate of 1.6 ml/min. Gradient conditions were as follows: solvent A/solvent B (15/85, 21/79, 58/42, 90/10, 90/10, 15/85, 15/85) with run times (0-5, 5-25, 25-70, 70-72, 72-82, 82-84, and 84-100 min), respectively.

Results and Discussion

Identification of the strain *Leuconostoc lactis* DC201

The 16S rRNA gene sequences of the strain and related taxa were obtained from GenBank, edited by BioEdit, and multi-aligned by CLUSTAL X software. The phylogenetic tree was constructed for strain DC201 with the neighbor-joining method, and bootstrap analysis was conducted with 1,000 replicates to obtain confidence levels for the branches. This tree shows that the strain DC201 is closely related to *Leuconostoc lactis* (99%) (Fig. 1).

Growth profiles of DC201 in different media

A 24-hr culture grown from one colony in MRS broth was diluted 100-fold into LB, NB, TSB, and MRS, and incubated at 37℃ with stirring. Based on a plot of OD_{600} versus time, MRS broth supported optimal growth, followed by TSB> LB>NB. Cultures in MRS broth reached log phase at 4-6 hr after inoculation. After late log phase, the accumulative cell mass in MRS was greater than those in TSB (Data not shown).

Effect of temperature on growth

A stationary phase MRS culture broth (OD_{600} = 1.0) of *Leuconostoc lactis* DC201 was diluted 100-fold into MRS broth and subdivided for incubation at various temperatures (25℃, 30℃, 37℃, 45℃). Growth was monitored at 600 nm for 24 hrs with stirring. DC201 showed optimal growth at

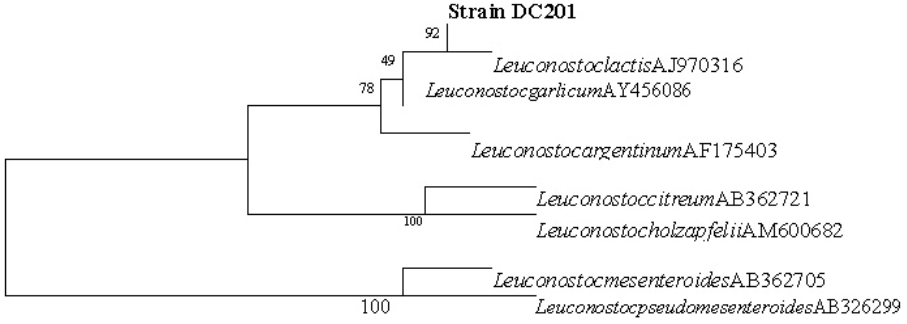

Fig. 1. Phylogenetic tree based on 16S rRNA gene sequences, showing phylogenetic relationships among *Leuconostoc lactis* DC201 and other related type strains.

37℃. The strain grew in MRS broth at 25-37℃, but not above temperatures 45℃ (data not shown).

Effect of pH on growth

A stationary phase culture (OD_{600} = 1.0) of the strain DC201 in MRS broth was diluted 100-fold into MRS media that were pre-adjusted to pH 5.0, 5.5, 6.0, 6.5, 7.0, 7.5, and 8.0. Growth was monitored at 600 nm for 24 hrs at 37℃, with stirring. *Leuconostoc lactis* DC201 grew at pH 6.5-8.0 (data not shown). For further work, MRS broth was not adjusted because its pH is about pH 6.5.

Effect of temperature on the enzyme reaction

The ginsenoside Rb_1 was mixed with crude enzyme solution and placed at different temperatures (25℃, 30℃, 37℃, 50℃, and 60℃) for 60 hrs (Fig. 2). Based on the TLC analysis, the reaction temperature dramatically affected on the production of compound K (Fig. 2 A). The rate was highest at 30℃, and lower at 25℃ and at 37-60℃. HPLC analysis showed a similar pattern to TLC result (Fig. 2 B). Enzymes prepared from *Candida peltata*, *Rhizopus japonicas*, and *Paecilomyces thermophila* show optimal reaction temperatures of 50℃ (Saha and Bothast, 1996), 45℃ (Kim, 1989), and 75℃ (Yang *et al*., 2008), respectively. Enzymes from strain *Lactobacillus brevis* LH8 show an optimal temperature of 30℃ (Quan *et al*., 2008), similar to that of DC201.

Effect of pH on the enzyme reaction

The optimal pH for the reaction of *Leuconostoc lactis* DC201 crude enzyme with ginsenoside Rb_1 was pH 6.0-8.0, the enzyme has no activity below pH 4.0. The final product was analyzed by HPLC (Fig. 3 B). Corresponding enzyme

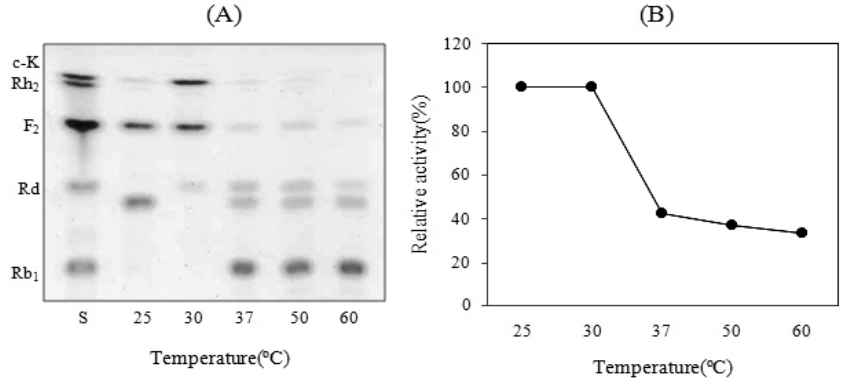

Fig. 2. TLC analysis according to temperature (25-60℃) on the enzymatic conversion of ginsenoside Rb_1 to compound K by *Leuconostoc lactis* DC201 (A), and corresponding effects on β-glucosidase activity (B).

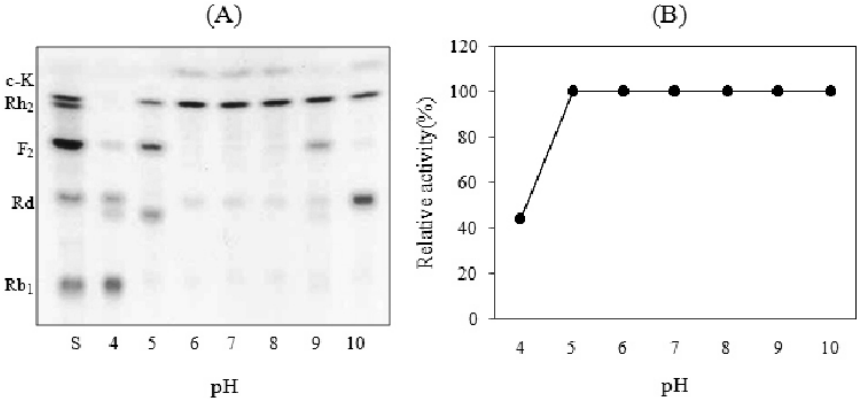

Fig. 3. TLC analysis according to pH (4.0-10.0) on the conversion of ginsenoside Rb_1 to compound K by *Leuconostoc lactis* DC201 (A), and corresponding effects on β-glucosidase activity (B).

activities in other species showed pH optimum for conversion at pH 6.2 (*Paecilomyces thermophila*) (Yang *et al*., 2008), pH4.8-5.0 (*Rhizopus japonicus*) (Kim, 1989), pH 6.0-11.0 (*Lactobacillus brevis* LH8) (Quan *et al*., 2008), and pH 5.0 (*Candida peltata*) (Saha *et al*., 1996).

The time course of ginsenoside bioconversion

During the reaction of strain DC201 crude enzyme with ginsenoside Rb_1, each sample was retrieved for TLC analysis every 24 hrs for 3 days. The level of ginsenoside Rb_1 decreased, and levels of gypenoside XVII, ginsenoside Rd, and F_2 increased. By 72 hrs, all ginsenoside Rb_1 was converted to compound K (Fig. 4).

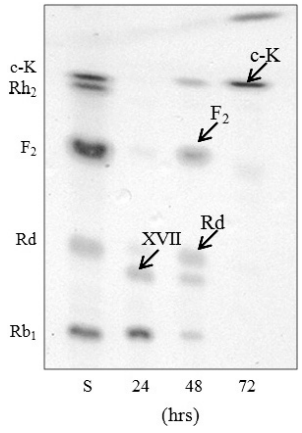

Fig. 4. Time course of ginsenoside Rb_1 bioconversion by *Leuconostoc lactis* DC201 crude enzyme at 30℃ and pH6.0. The TLC analysis was developed in $CHCl_3$/MeOH/H_2O (65:35:10, v/v, lower phase). S: saponin standards.

Analysis of Biotransformation pathway by HPLC

The optimum temperature and pH for the enzyme reaction of *Leuconostoc lactis* DC201 with ginsenoside Rb_1 was 25°-30℃, pH 6.0-8.0, respectively. With time, the level of ginsenoside Rb_1 decreased, and levels of gypenoside XVII, ginsenoside Rd, and F_2 increased. By 72 hrs, all ginsenoside Rb_1 was converted to compound K (Fig. 4). The reaction of ginsenoside Rb_1 with crude enzyme was sampled at 24 hrs

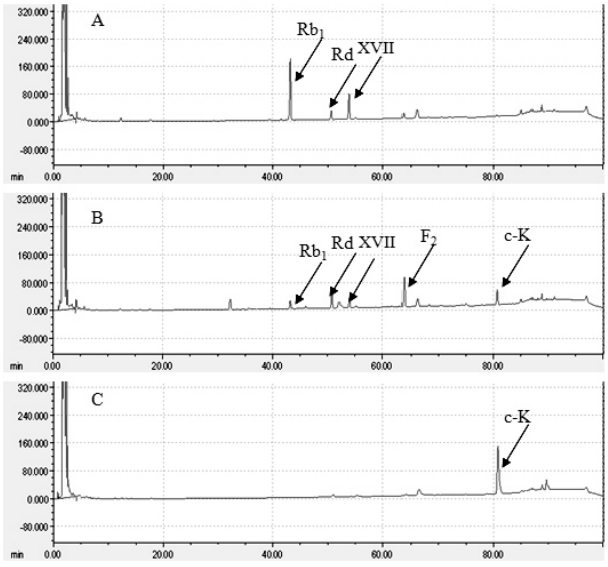

Fig. 5. HPLC profile of ginsenoside Rb_1 metabolites produced by *Leuconostoc lactis* DC201 crude enzyme. The crude enzyme was reacted with 0.2 mM ginsenoside Rb_1 for 24 hrs (A), 48 hrs (B), and 72 hrs (C), extracted with n-butanol, evaporated *in vacuo*, dissolved in methanol, and analyzed by HPLC.

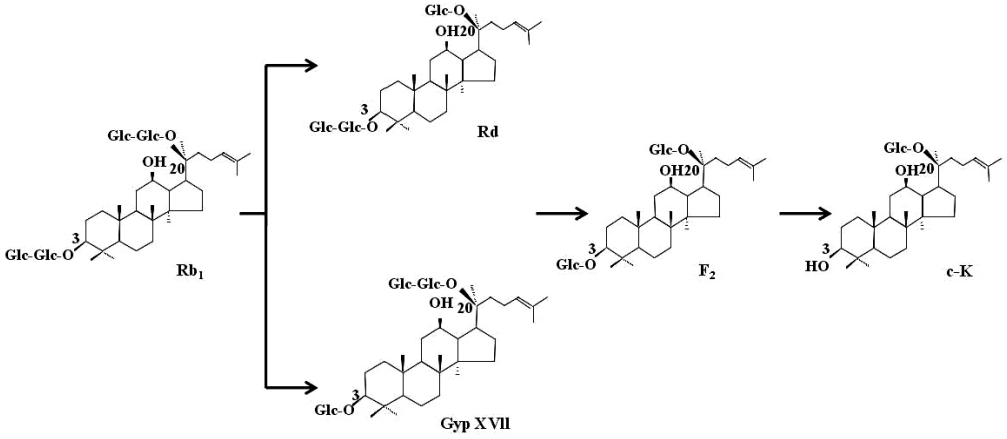

Fig. 6. Proposed pathway of ginsenoside Rb_1 transformation to compound K by the *Leuconostoc lactis* DC201 crude enzyme.

intervals for analysis by HPLC (Fig. 5). The ginsenoside Rb_1 peak (retention time, 44 min) decreased, and the ginsenoside Rd (retention time, 51 min) and gypenoside XVII (retention time, 54 min) peaks increased. The Rb_1 conversion proceeded from $Rb_1 \rightarrow$ Rd, GypXVII $\rightarrow F_2 \rightarrow$ compound K (Fig. 6). The HPLC analysis showed that, in 72 min of reaction time, all of the F_2 was converted to compound K. The proposed pathway is as follows: GypXVII $\rightarrow F_2 \rightarrow$ compound , which indicate that enzymes hydrolyze one glucose molecules at C-3 and two glucose molecules at C-10 of Gypenoside XVll. The major component include protopanaxadiol ginsenoside, such as Rb_1 and Rb_2, and have been shown to be metabolized by human intestinal bacterial to their final derivative, such as compound K. Recently, ginsenoside compound K has been reported to induce tumor cell apoptosis, inhibit tumor metastasis, and restrain tumor invasion.

In this study, we isolated a lactic acid bacteria producing β-glucosidase traditional food Kimchi, and the isolated strain was identified by 16S rRNA sequences. We confirmed that a crude enzyme preparation from *Leuconostoc lactis* DC201 can convert ginsenoside Rb_1 to compound K.

Acknowledgements

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