Biotransformation of Ginsenoside Rb₁ to Prosapogenins, Gypenoside XVII, Ginsenoside Rd, Ginsenoside F₂, and Compound K by Leuconostoc mesenteroides DC102

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Ginsenoside Rb₁ is the main component in ginsenosides. It is a protopanaxadiol-type ginsenoside that has a dammarane-type triterpenoid as an aglycone. In this study, ginsenoside Rb₁ was transformed into gypenoside XVII, ginsenoside Rd, ginsenoside F₂, and compound K by glycosidase from Leuconostoc mesenteroides DC102. The optimum time for the conversion was about 72 h at a constant pH of 6.0 to 8.0 and the optimum temperature was about 30°C. Under optimal conditions, ginsenoside Rb₁ was decomposed and converted into compound K by 72 h post-reaction (99%). The enzymatic reaction was analyzed by high-performance liquid chromatography, suggesting the transformation pathway: ginsenoside Rb₁→ gypenoside XVII and ginsenoside Rd→ginsenoside F₂→compound K.

Keywords: Biotransformation, Gypenoside XVII, Ginsenoside Rd, Compound K, Leuconostoc mesenteroides DC102

INTRODUCTION

Ginseng, the root of Panax ginseng Meyer, belongs to family Araliaceae. It has been used as a medicine to treat various diseases in the Orient for several thousand. The major active components of ginseng are ginsenosides, having bioactive and pharmacological activities, including anti-cancer activities [1], anti-inflammatory [2], anti-aging activities [3]. However, these naturally occurring ginsenosides are observed to be poorly absorbed along the 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 to their final derivative, 20-O-β-D-glucopyranosyl-20(S)-protopanaxadiol, also called compound K [5,6].

Compound K, reported to be easily absorbed by the human body, is the genuine active form of protopanaxadiol-type saponins [7], and it has recently attracted increasing interest because of its intriguing biological actions. compound K was demonstrated to anti-genotoxic activity, anti-allergic effect and the prevention of tumor invasion and metastasis, have shown to be mediated by this metabolite [8-12]. Since compound K does not exist in natural products and the natural transforming ability of human intestinal bacteria is rather limited, much attention has been paid to the preparation of compound K. Several chemical approaches have been tried to deglycosylate ginsenosides [13]. However, none of them was suitable for preparation.
of compound K, because both glycone moieties at C-20 could be cleaved nonspecifically under chemical hydrolysis conditions. Hence, enzymatic transformation, which is considered to be highly region-specific, might be a promising method.

Ginsenoside Rb₁ is the main component in ginsenosides. It is a protopanaxadiol-type ginsenoside that has a structure similar to that of compound K. By enzymatically hydrolyzing the two glucose molecules at C-3 and one of the glucose molecule at C-20, compound K can easily be transformed from ginsenosides Rb₁. In the present study, we characterized the transformation of the major protopanaxadiol ginsenoside Rb₁ into minor compound K by using cell-free extracts of the Leuconostoc mesenteroides DC102 strain isolated from kimchi, a traditional Korean fermented food.

MATERIALS AND METHODS

Materials

The L. mesenteroides DC102 strain was isolated from kimchi. Difco MRS broth was purchased from Becton Dickinson and Co. (Franklin Lakes, NJ, USA). Ginsenoside Rb₁ was obtained from Panax quinquefolius, and standard ginsenosides including 20(S)-Rb₁, 20(S)-Rd, 20(S)-Rg₁, 20(S)-Rh₂, and compound-K were obtained from GGRB at Kyung-Hee University (Yongin, Korea). A 60 F-254 silica gel plate (Merck, Darmstadt, Germany) was used for TLC, and silica gel 60 column (70–230 mesh, Merck) was used for column chromatography. A HPLC (NS 3000i system; Futecs, Daejeon, Korea) equipped with a UV/Vis detector and gradient pump was also used for analysis.

Screening of lactic acid bacteria producing β-glucosidase

Esculin-MRS agar was used to isolate β-glucosidase-producing lactic acid bacteria [14]. This growth medium contains (per 1 L) 3 g esculin and 0.2 g ferric citrate in MRS agar (Becton Dickinson and Co.). The lactic acid bacteria which produced the β-glucosidase that hydrolyzes esculin appeared on the esculin-MRS agar as colonies surrounded by a reddish-brown to dark brown zone. Subsequently, single colonies from those plates were subjected to an additional two-day incubation at 37°C.

Phylogenetic analysis

The complete sequence of the 16S rRNA gene from the L. mesenteroides DC102 strain was compiled through SeqMan program in the DNASTAR package, and edited using the BioEdit program [15]. 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 4 program [16]. 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. mesenteroides DC102 strain was grown in the MRS broth at a temperature of 37°C until absorbance at 600 nm reached 1.0. After culture broth centrifugation (15,000 ×g for 10 min at 4°C) using a MICRO 17R microcentrifuge (Hanil Science Industrial, Seoul, Korea), four volumes of ethanol were added to the supernatant. This solution was mixed sufficiently and placed in an ice chamber for 20 min to foster further reactions. Meanwhile, the protein pellet was collected via centrifugation (10,000×g for 40 min at 4°C) and was dissolved in a 20 mM sodium phosphate buffer (pH 7.0).

Parameter optimization for the biotransformation of ginsenoside Rb₁

The effects of temperature on the transformation of ginsenoside Rb₁, were investigated at temperatures in the range of 25°C to 60°C. Reactions were performed in sodium phosphate buffer (pH 7.0) containing ginsenoside Rb₁ (1 µmol) and the crude enzyme (250 µL) for 48 h.

The optimum reaction pH for the conversion of ginsenoside Rb₁ varied between 3.0 and 13.0. Reactions were performed in various buffers (pH 3.0–13.0) containing ginsenoside Rb₁ (1 µmol) and the crude enzyme (250 µL) at 30°C for 48 h, followed by extraction with water-saturated n-butanol. Subsequently, the n-butanol fraction was evaporated to dryness, and the methanolic extract was analyzed using HPLC.

Biotransformation of ginsenoside Rb₁ by crude enzymes

The L. mesenteroides DC102 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 1 mM ginsenoside Rb₁ dissolved in distilled water at a 1:4 ratio (v/v). Subsequently, the mixture was incubated at 30°C and stirred at 190 rpm for 72 h. During the reaction period, a 1.25-mL aliquot was collected every 12 h, extracted in water-saturated n-
butanol and then analyzed using both TLC and HPLC.

Analysis of ginsenosides by thin-layer chromatography

TLC was performed with silica gel plates (60F254, Merck), and CHCl3-CH3OH-H2O (65:35:10, v/v, lower phase) was used as its developing solvent. The spots on the TLC plates were detected through spraying with 10% H2SO4, followed by heating at 110°C for 10 min [17].

Analysis of ginsenosides by high-performance liquid chromatography

The HPLC-grade acetonitrile and water were purified by passing through a 0.45 μm filter, and the mobile phases were mixed appropriately. The flow rate was 1.6 mL/min and the sample was detected at UV 203 nm.

Structural identification

Reaction products 1, 2, 3 and 4, from the ginsenoside Rb1 bacterial biotransformation by the L. mesenteroides DC102 strain, were separated on an ODS flash column using acetonitrile (solvent A) and distilled water (solvent B) mobile phases at A/B ratios of 15:85, 21:79, 58:42, 90:10, and 15:85; with run times of 0-5, 5-25, 25-80, 70-82, and 82-100 min, respectively. The flow rate was 1.6 mL/min and the sample was detected at UV 203 nm.

Table 1. 13C-NMR chemical shifts of material Rb, and metabolites 1 and 2 (100 MHz, solvent: pyridine-d5)

| Carbon site | Gypenoside XVII | Ginsenoside Rd |
|-------------|----------------|---------------|
| Aglycone moiety | Reference | Metabolite 1 | Reference | Metabolite 2 |
| C-1 | 107.0 | 106.8 | 105.0 | 105.0 |
| C-2 | 75.8 | 75.6 | 83.3 | 83.1 |
| C-3 | 78.8 | 78.6 | 78.1 | 77.9 |
| C-4 | 72.0 | 71.8 | 71.6 | 71.6 |
| C-5 | 78.4 | 78.2 | 78.1 | 78.2 |
| C-6 | 63.2 | 63.0 | 62.7 | 62.6 |
| Sugar moiety | | | | |
| 3-O-inner-Glc | | | | |
| C-1 | 98.2 | 98.0 | 98.2 | 98.2 |
| C-2 | 75.8 | 75.5 | 75.0 | 75.1 |
| C-3 | 78.8 | 78.7 | 78.1 | 78.2 |
| C-4 | 72.0 | 71.9 | 71.6 | 71.6 |
| C-5 | 78.4 | 78.3 | 78.1 | 78.2 |
| C-6 | 63.2 | 62.9 | 62.7 | 62.6 |
| 20-O-inner-Glc | | | | |
| C-1 | 105.4 | 105.2 | – | – |
| C-2 | 75.3 | 75.1 | – | – |
| C-3 | 78.4 | 78.2 | – | – |
| C-4 | 71.8 | 71.6 | – | – |
| C-5 | 78.4 | 78.2 | – | – |
| C-6 | 62.9 | 62.6 | – | – |
| 20-O-outer-Glc | | | | |
| C-1 | 105.4 | 105.2 | – | – |
| C-2 | 75.3 | 75.1 | – | – |
| C-3 | 78.4 | 78.2 | – | – |
| C-4 | 71.8 | 71.6 | – | – |
| C-5 | 78.4 | 78.2 | – | – |
| C-6 | 62.9 | 62.6 | – | – |

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RESULTS AND DISCUSSION

Phylogenetic study

The 16S rRNA gene sequence of DC102 was aligned with other neighboring strains, confirming the taxonomic relationships. As shown in Fig. 1, the phylogenetic tree revealed that DC102 was actually grouped with the Leuconostoc species. Moreover, the highest degree of 16S rRNA gene sequence identities were with L. mesenteroides FJ655776.1 (99%). Hence, based on the phylogenetic tree and homology analysis, the DC102 strain can be designated as L. mesenteroides DC102.

Biotranformation of ginsenoside Rb1

After L. mesenteroides DC102 was cultured until A600 reached 1.0, the crude enzyme solution of the strain was used to transform ginsenoside Rb1 to both metabolite 1, metabolite 2, metabolite 3 and 4, as shown in Fig. 2. Evidently, this indicated that metabolite 1, metabolite 2 and metabolite 3 was an intermediate metabolite, and that metabolite 4 was the final product. The Rf values to metabolite product 1, 2, 3 and 4 on TLC were similar to those of gypenoside XVII, ginsenoside Rd, ginsenoside F2 and compound K. This result suggested that Rb1 was converted by crude enzyme from L. mesenteroides DC102. Reaction products 1, 2, 3 and 4, were separated on a ODS flash column chromatography structure.

The structure of metabolite 3 (=ginsenoside F2) and metabolite 4 (=compound K) has already been identified and confirmed in our previous study [18].

Structure of metabolites 1 and 2

In the 1H-NMR spectrum of metabolite 1, the anomeric proton signals for the H-1 of the 3-O-innerglucopyranosyl moiety, 3-O-outerglucopyranosyl moiety, and 20-glucopyranosyl moiety appeared at δ 4.89 ppm (1H, d, J=7.6 Hz, H-3-glc(inner)-1'), 5.03 ppm (1H, d, J=7.6 Hz, H-20-glc(inner)-1''), and δ 5.08 ppm (1H, d, J=7.6 Hz, H-20-glc(terminal)-1''') respectively, showing that the aglycon of metabolite (1) harbored three β-D-glucoses. The anomeric proton signals of metabolite (1) showed that there was a loss of one terminal glucose at C-3 compared with that of ginsenoside Rb1, whose anomeric proton signals appeared at 4.88 ppm [1H, d, J=7.6 Hz, H-3-glc(inner)-1'], 5.06 ppm [1H, d, J=7.7 Hz, H-20-glc(inner)-1''], 5.09 ppm [1H, d, J=7.7 Hz, H-20-glc(terminal)-1'''], and 5.34 ppm [1H, d, J=7.6 Hz, H-3-glc(out)-1''] . 13C-NMR (pyridine-d5, 100 MHz) data are shown Table 1. A comparison of the 13C-NMR spectrum of metabolite (1) with that of ginsenoside Rb1 showed that the signal for the C-2' of the 3-inner-glucose was shifted upfield, from 83.5 ppm to 75.6 ppm, but the other signals were similar to those of ginsenoside Rb1 [19]. It is believed that the terminal glucose on C-3 of the aglycon of ginsenosides Rb1 was hydrolyzed by the from L. mesenteroides DC102, and this hypothesis is consistent with the 1HNMR data. Therefore, the metabolite (1) produced by from L. mesenteroides DC102 from...
ginsenoside Rb₁ is 3-O-[β-D-glucopyranosyl]-20-O-[β-D-glucopyranosyl-(6→1)-β-D-glucopyranosyl]-20(S)-protopanaxadiol, identical to gypenoside XVII.

The ¹H-NMR spectrum of metabolite 2, the proton signals for the H-1 of the 3-O-inner-glucopyranosyl moiety, 3-O-outer-glucopyranosyl moiety, and 20-glucopyranosyl moiety appeared respectively, at δ 4.87 ppm [1H, d, J=7.6 Hz, 3-O-Glc (inner) H-1], δ 5.34 ppm [1H, d, J=7.6 Hz, 3-O-Glc (outer) H-1], and δ 5.14 ppm [1H, d, J=7.6 Hz, 20-O-Glc H-1]. Metabolite 2 was shown to harbor three β-D-glucoses. According to the results of comparisons of the ¹³C-NMR spectrum of metabolite 2 with the material ginsenosides Rb₁, the signal for the C-6 of the 20-innerglucopyranosyl moiety was shifted upfield, from δ 71.6 ppm to δ 62.8 ppm, and the signal for the C-5 of the 20-inner-glucopyranosyl moiety was shifted downfield, from δ 77.1 ppm to δ 78.2 ppm. However, the other signals were similar to those of ginsenoside Rb₁. It is believed that the terminal glucose combining C-20 of aglycon of ginsenoside Rb₁ was hydrolyzed by the from *L. mesenteroides* DC102. Therefore, metabolite 2 was identified as 3-O-[β-D-glucopyranosyl-(1→2)-β-D-glucopyranosyl]-20-O-[β-D-glucopyranosyl]-20(S)-protopanaxadiol, identical to ginsenoside Rd.

**Biotransformation by pathway**

The process through which ginsenoside Rb₁ was decomposed by the strain DC102 was analyzed using HPLC, as were any changes in reaction time. As is shown in Fig. 3, the concentrations of ginsenosides Rb₁ and those of the decomposition products gypenoside XVII, ginsenosides Rd, ginsenosides F₂ and compound K exhibited regular changes with reaction time. After 24 h of reaction, ginsenoside Rb₁ was simultaneously transformed into gypenoside XVII (metabolite 1) and ginsenoside Rd (metabolite 2). And the majority of ginsenoside Rb₁ had been decomposed into gypenoside XVII (metabolite 1), ginsenoside Rd (metabolite 2) and ginsenosides F₂ (metabolite 3) after 48 h of reaction. From 48 to 72 h, all the intermediates were continuously transformed into compound K (metabolite 4). Generally, there are two pathways for the transformation of ginsenoside Rb₁ into compound K, one is ginsenosides Rb₁→ginsenosides Rd→ginsenosides F₂→compound K, the other is
ginsenosides $R_b_1 \rightarrow$ gypenoside XVII $\rightarrow$ ginsenosides $F_2 \rightarrow$ compound K. However, these findings indicated that gypenoside XVII, ginsenosides Rd and ginsenosides $F_2$ were intermediate metabolites, and that compound K was the final product after 72 h of reaction time. This result suggested that strain DC102 exerts potent $\beta$-glucosidase activity, and that ginsenoside $R_b_1$ was converted in the following sequence: ginsenosides $R_b_1 \rightarrow$ gypenoside XVII and ginsenosides Rd $\rightarrow$ ginsenosides $F_2 \rightarrow$ compound K (Fig. 4) by the enzymes produced from strain DC102, consecutively hydrolyzing 3-C $\beta$-(1→2) glucoside, 20-C $\beta$-(1→6) glucoside and 3-C $\beta$-glucose of ginsenoside $R_b_1$.

The effect of temperature on enzyme activity

This experiment was performed at various temperatures (25°C, 30°C, 37°C, 50°C, 60°C) in order to determine the crude enzyme’s optimum temperature. The results shown in Fig. 5 revealed that both the L. mesenteroides DC102 and ginsenosides $R_b_1$ strains’ crude enzyme reactions were greatly influenced by temperature. In addition, the TLC analysis showed that the crude enzyme activity of L. mesenteroides DC102 was the highest at 30°C. The optimum temperature was further confirmed by quantitative HPLC analysis. It was also observed that ginsenoside $R_b_1$ started degrading at 25°C, reached its maximum concentration at 30°C and 37°C, and suddenly decreased after 37°C. Hence, the enzyme could not perform catalytic reactions under temperatures lower than the optimum, because the energy released was insufficient to power the reaction. Under high temperatures, enzymes lose their activities due to denaturalization.

The effect of pH on enzyme activity

The ginsenoside conversion activity of the crude enzyme was tested at various pH (4.0-10.0) levels. TLC analysis revealed that the optimum pH range was pH 6.0 to 8.0. Similarly, quantitative HPLC analysis also explained that ginsenoside $R_b_1$ started degrading at pH 5.0. Moreover, it sustained maximum enzyme activities at pH 6.0 to 8.0 and suddenly decreased at pH 8.0 (Fig. 6).

Ginsenoside $R_b_1$ can be converted to gypenoside XVII and ginsenoside Rd by loss of a glucose moiety at the C-3 and C-20 position of the ginsenoside aglycon, respectively. Compound K can be produced from ginsenoside $F_2$, which was transformed from gypenoside XVII and ginsenoside Rd, by hydrolysis of additional single glucose moiety at the same positions described above (Fig. 4).

The L. mesenteroides DC102 strain is an aerobic and edible lactic acid bacteria. The enzyme activity of this strain was found to be greatest at 30°C and decreased at temperatures higher than 37°C. According to these results, the optimum temperature determined in this study was slightly lower than those reported for other species, such as 45°C for Rhizopus japonicas derivative $\beta$-glucosidase [20], 40°C for Aspergillus niger 48 g and A. niger 848 g derivative $\beta$-glucosidase [21], and 40°C to 50°C for general $\beta$-glucosidase [22,23]. In addition, the crude enzyme showed a high activity rate at pH 5.0 to 9.0, while the maximum hydrolysis activity was found at pH 6.0. These pH values were slightly higher than those reported for other species, such as pH 5.0 for A. niger 48 g and A. niger 848 g derivative $\beta$-glucosidase [21], pH 4.8-5.0 for R. japonicas derivative $\beta$-glucosidase [20], pH 6.0 for Fusobacterium K-60 derivative $\beta$-glucosidase [24].
and pH 5.0 for ginseng derivative β-glucosidase [25].

This study is the first report on the aerobic bacteria, which is capable of converting ginsenoside Rb₁ into compound K via simultaneous bioconversion of gypenoside XVII and ginsenoside Rd. There are reports on microbial sources able to convert the major ginsenoside Rb₁ to minor ginsenosides. As such, enzymes secreted by microbes such as A. niger and A. usamii, and Caulobacter leidyia GP45 transformed ginsenoside Rb₁ into compound K via ginsenoside Rd and ginsenoside F₂ [26,27]. Additionally, Intrasporangium sp. GS603 transformed ginsenoside Rb₁ into ginsenosides F₂ via gypenoside XVII [28]. The ginsenoside Rb₁-converting enzymes from the strain DC102 can be applied in industry for the production of gypenoside XVII, ginsenoside Rd, ginsenoside F₂ and compound K after the target enzymes for the conversion have been purified and characterized, a goal that should be included in future studies.

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