Biotransformation of ginsenoside Rb1 for ginsenoside Rd preparation by *Lysinibacillus massiliensis* No. 24

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Bacterium *Lysinibacillus massiliensis* No. 24 was tested for its ability to transform the major ginsenosides Rb1. The transformation product was identified by thin layer chromatography (TLC) and high performance liquid chromatography (HPLC), and its structure was assigned by $^{13}$C-NMR. The bacterium was found to specifically transform major ginsenoside Rb1 and ginsenoside Rd was the sole product. The optimal conditions for transforming Rb1 by *L. massiliensis* No. 24 were determined to be: substrate concentration, 3 mg/mL; temperature, 34°C; pH 10; the time of substrate addition, 48 h; cell concentration, 0.30×10⁹ cells/mL; and biotransformation period, 3 d. Under the optimum conditions, the maximum yield of ginsenoside Rd reached 97%. Further, a preparative scale transformation with *L. massiliensis* No. 24 was performed with 150 mg Rb1 to give a yield of 79%. This bacterium would be potentially applied in the preparation of Rd.

Keywords: Biotransformation, ginsenoside Rb1, ginsenoside Rd, β-glucosidase, *Lysinibacillus massiliensis*

Introduction

Biotransformation is a strong tool in the pharmaceutical industry. Many natural products were converted to pharmaceutically active compounds by biotransformation. Ginseng, the root of *Panax ginseng* C A Meyer, has been used as traditional natural medicine in Asian countries for thousands of years. Ginsenosides are regarded as the principal components responsible for the pharmacological properties. Until now, more than 40 kinds of ginsenosides have been isolated and characterized from ginseng roots. In recent decades, many studies have focused on the pharmacological activities of the minor ginsenosides as their activities were found to be superior to those of the major ginsenosides. It has been reported that the protopanaxadiol-type ginsenoside Rd has the protection ability against the renal dysfunction caused by ischemia (local anemia) and recirculation. It enhances the differentiation of neural stem cells and protects the kidney from apoptosis and DNA fragmentation caused by chemical drugs and cancer. Ginsenoside Rd has also been known to arrest the aging process of the suppressing anti-oxidative defense system and lipid peroxidation by elevating the GSH/GSSG ratio of glutathione and increasing the activities of glutathione peroxidase and glutathione reductase, which are both significantly lower in old organisms.

It is noticed that the separation of ginsenoside Rd from ginseng was quite difficult because of its low concentration. A possible pathway for preparation of Rd is through transforming structurally related compounds to it. The amount of the major ginsenoside Rb1 is high in ginseng, and it has the same aglycone (protopanaxadiol) as Rd (Fig. 1). Theoretically, Rd can be obtained by hydrolysis of Rb1 with removing the outer glucosidic residue at position C-20. Chemical transformation usually has poor selectivity and generates more environmental pollution. Biotransformation has more potential because of its high specificity and environmental compatibility. Some studies have looked for suitable microbes or enzymes that can transform Rb1 to Rd.

However, most of them showed poor specificity and can further transform Rd to other metabolites, such as ginsenoside F2, compound K, Rg3 and Rh2, which resulted in a low yield of Rd. We now report a high specificity transformation of Rb1 to Rd by *Lysinibacillus massiliensis* No. 24. This biotransformation has great potential to be applied...
Materials and Methods

Materials

Standard ginsenoside Rd was purchased from Mansite Biotechnology Ltd. (Chengdu, China). Ginsenosides Rb1 was prepared from Chinese white ginseng roots (5-yr-old, cultivated in Fusong, Jilin Province of China), and identified by $^{13}$C-NMR spectrometry. Bacterium No. 24 was isolated from ginseng cultivation soil in Yanji (Jilin Province, China) by our laboratory.

Analytical Methods

TLC was carried out using a silica gel G60 plate and a developing solvent consisting of chloroform:methanol:distilled water (65:35:10, v/v/v, lower phase). The product developed on the silica gel plate was stained with 5% sulphuric acid (in ethanol, v/v), then heating at 120°C for 2 min. Under above conditions, the relative mobility rates (Rf) of Rb1 and Rd were determined to be 0.2 and 0.34, respectively.

HPLC was performed using a Shimadzu HPLC system with Shim-pack PREP-ODS (H) column (4.6 mm×250 mm, 5 μm), using acetonitrile/water (23:77, v/v) as elution buffer. Each sample was tested for 30 min. The flow rate was 0.8 mL/min and the elution process was monitored via absorbance at 203 nm.

Cultivation and Biotransformation

The testing bacterium was cultivated in LB agar medium (per liter: 10 g tryptone, 5 g yeast extract, 10 g NaCl, 15 g agar) at 37°C for 12 h, then transferred to LB liquid medium for 24h shaking at 170 rpm. After that ginsenoside Rb1 was added to the cells (final cells concentration 0.58×10⁸ cells/mL). The mixture was incubated at 37°C with a 3 d shaking. During transformation, samples were withdrawn at different time points and extracted with n-butanol. The n-butanol phase containing transformed product was dried under vacuum, resuspended in 23% acetonitrile (in water) and then analyzed by TLC and HPLC. Transformation yield was further determined based on the peak area ratio in HPLC analysis.

Condition Optimization for Ginsenoside Rd Preparation

The transformation conditions for ginsenoside Rd preparation by L. massiliensis No. 24 were optimized. Substrate concentration (1, 2, 3, 4, 5 mg/mL), time of substrate addition (0, 4, 12, 24, 48 h), pH (2, 4, 6, 8, 10), temperature (25, 28, 31, 34, 37°C) and cell concentration (0.58×10⁹, 0.42×10⁹, 0.35×10⁹, 0.30×10⁹, 0.25×10⁹ cells/mL) were optimized. The transformed product was extracted with equal volume of n-butanol; n-butanol phase was dried, resuspended in 23% acetonitrile in water (v/v) and subjected to HPLC analysis.

Preparative Scale Biotransformation

Preparative-scale biotransformation of ginsenoside Rb1 by L. massiliensis No. 24 was carried out in 250 mL Erlenmeyer flasks containing 50 mL of cell suspension (cells concentration of 0.3×10⁹ cell/mL) with shaking (170 rpm at 34°C). Ginsenoside Rb1 (150 mg) was dissolved in ddH₂O, filtered with 0.22 μm filter and added. After 3 d of incubation, the biotransformation products were extracted with equivalent volume of n-butanol, which gave a crude extract.

Purification and Identification of Transformed Product

The resulting crude extract was separated by column chromatography using silica gel (60-200 μm) with the compound:sorbent ratio of 1:50 and with chloroform:methanol (8:2) as the eluent. The $^{13}$C-NMR spectrum of the solution in MeOD was recorded on a Bruker Av-600 NMR spectrometer with TMS as internal standard.

Identification of Isolated Bacterium No. 24

Bacterium No. 24 was identified by 16S rDNA sequencing. The genomic DNA of the ginsenoside Rb1-hydrolyzing bacterium was extracted using the TianAMP Bacteria DNA Kit (Tiangen, China).
The 16S rDNA gene was amplified using the universal bacterial primers 27F and 1492R\textsuperscript{15}, the PCR product was recovered by TIANgel Midi purification kit (Tiangen, China) and sequenced by Sangon Biotech (Shanghai, China). The 16S rDNA gene sequence was blasted in NCBI database, and the phylogenetic tree was constructed with MEGA 4.0 software.

**Assay of Glycosidase Activity**

During biotransformation, the \(\beta\)-glucosidase activity was monitored with p-nitrophenyl-\(\beta\)-D-glucopyranoside (pNPG) as substrate\textsuperscript{16}. Briefly, 50 \(\mu\)L of cell-free extract was incubated with 20 \(\mu\)L of 5 mM pNPG and 100 \(\mu\)L of 25 mM Tris-HCl (pH 7.5) at 37°C for 1 h. The released p-nitrophenol (pNP) was measured at 405 nm. One unit of enzyme activity was defined as the amount of enzyme that released 1 \(\mu\)mol pNP per min at above conditions.

**Results and discussion**

**Screening of Ginsenoside Rb1 Hydrolyzing Strains and Identification of Strain No.24**

With ginsenoside Rb1 as the sole carbon source, the bacteria isolated from ginseng-cultivated soil in Changbai Mountain, China were tested for their abilities to transform ginsenoside Rb1. For efficiency, TLC method was used to monitor the biotransformation process. Among the isolated bacteria, one bacterium (isolate no. 24) transformed ginsenoside Rb1 with high efficiency. Isolate no. 24 was identified by 16S rDNA sequencing. The obtained sequence was blasted in NCBI database and the closest type strain was identified (Fig. 2). The nucleotide sequence of 16S rDNA gene was deposited in NCBI under accession number KJ011902. The strain was classified as *Lysinibacillus massiliensis* No. 24. This strain had potential to transform major ginsenoside Rb1 to ginsenoside Rd and, therefore, chosen for further study.

**Preparation of Ginsenoside Rd from Rb1 by L. massiliensis No.24**

Transformation of ginsenoside Rb1 by *L. massiliensis* NO. 24 was performed in LB broth. The transformation process was monitored by TLC and HPLC. As shown in TLC results after 24 h incubation, most of ginsenoside Rb1 was transferred to a product having Rf value identical with that of the standard Rd (Fig. 3). After 36 h incubation, the conversion of Rb1 to Rd was almost completed. Further, ginsenoside Rd did not convert to any other metabolites. The HPLC results also confirmed the TLC results (Fig. 4). After incubation with *L. massiliensis* No. 24, the amount of ginsenoside Rb1 decreased, while a new peak appeared with retention time identical with that of standard Rd, which indicated that *L. massiliensis* No. 24 had high selectivity to cleave the glucosyl residue at position C-20 of Rb1, without attacking on other glycosidic linkages. The high selectivity is eminently suitable for the production of Rd.
Identification of Transformed Product

The transformed product was prepared and further identified by $^{13}$C-NMR spectroscopy. The sugars attached to C-20 position in ginsenoside Rb1 are $\beta$-D-glucopyranosyl-(1→6)-$\beta$-D-glucopyranose, while $\beta$-D-glucopyranosyl-(1→2)-$\beta$-D-glucopyranose attached to C-3 position (Fig. 1). As shown in the spectral signals of ginsenoside Rb1, the signal of $\delta$105.4 and $\delta$98.2 represented the anomeric carbons of 20-O-outer-glucopyranosyl and 20-O-inner-glucopyranosyl residues, and the signal of $\delta$106.1 and $\delta$105.2 represented the anomeric carbon of 3-O-outer glucopyranosyl and 3-O-inner-glucopyranosyl residues in ginsenoside Rb1$^{17}$. Comparing the $^{13}$C-NMR spectral signals of substrate Rb1 and bio-transformed product (Table 1), it is noticed that the signal of $\delta$105.4 disappeared, while the spectral signals of the sugars at C-3 position still existed, which confirms that only the C-20-outer glucopyranosyl residue of ginsenoside Rb1 was removed and the number of sugar residues decreased from four sugar residues in Rb1 to three sugar residues in the metabolite. The above results further confirm that the metabolite of Rb1 biotransformation by L. massiliensis No. 24 was ginsenoside Rd, i.e. 3-O-[$\beta$-D-glucopyranosyl-(1→2)-$\beta$-D-glucopyranosyl]-20-O-$\beta$-D-glucopyranosyl-20(S)-protopanaxadiol.

Optimal Conditions for Ginsenoside Rd Preparation by L. massiliensis No. 24

To optimize the biotransformation of ginsenoside Rb1 in Rd preparation, the effect of time of substrate addition, substrate concentration, pH, temperature and bacterial cells concentration on the yield of transformed product were evaluated. HPLC was used to evaluate the yield by calculating the peak area of the transformed product. As shown in Fig. 5, the yield arrived at the highest level when the time of substrate addition was 48 h. Meanwhile, the optimum substrate concentration was 3 mg/mL; higher or lower substrate concentration caused the lower yields (Fig. 6). pH and temperature are key factors and affect the biotransformation. Generally, ginsenoside-hydrolyzing bacteria and fungi played their optimum activities at acidic pH in the range of pH 5-7$^{18}$. However, L. massiliensis No. 24 exhibited its optimum transformation ability at pH 10 (Fig. 7). Temperature did not affect the transformation significantly. As shown in Fig. 8, the biotransformation yield was slightly higher at 34°C compared to other temperatures. Also, the cells concentration did not affect the biotransformation significantly. The optimum cells concentration was 0.30×10$^9$ cell/mL (Fig. 9). The biotransformation yield reached 97% after 3 d of incubation, which was the maximum yield for the conditions tested in the present study.

The preparative scale biotransformation of ginsenoside Rb1 (150 mg) by L. massiliensis No. 24 was performed under the optimum conditions. The product was confirmed by $^{13}$C-NMR. The yield of Rd by preparative biotransformation was 79%. This indicated that the biotransformation has potential for industrial application.

Glycosidase Activity Assay

Glycosidase activity was conducted during biotransformation at 0, 1, 2, 4, 8, 12, 24 and 36 h. As
Table 1—$^{13}$C-NMR data for ginsenoside Rb1 and Rd (150 MHz, solvent: MeOD)

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Fig. 5—Effects of substrate addition time on biotransformation by *L. massiliensis* No. 24. [A solution of Rb1 in 20 mM NaHPO$_4$/Na$_2$HPO$_4$ buffer (pH 7.0) was added to the tubes containing cells of *L. massiliensis* No. 24 preincubated for 0, 4, 12, 24 and 48 h. The mixtures were incubated with shaking (170 rpm) at 34°C for 3 d. HPLC analysis was performed using acetonitrile/water (23:77, v/v) for 30 min. Data were expressed as mean±SD from three independent experiments.]

Fig. 6—Effects of substrate concentration on biotransformation by *L. massiliensis* No. 24. [A solution of Rb1 in 20 mM NaHPO$_4$/Na$_2$HPO$_4$ buffer (pH 7.0) was added to the tubes containing cells of *L. massiliensis* No. 24, preincubated for 48 h at a final concentration of 1, 2, 3, 4 and 5 mg/mL. The mixtures were incubated with shaking (170 rpm) at 34°C for 3 d. HPLC analysis was performed as described before. Data were expressed as mean±SD from three independent experiments.]
Fig. 7—Effects of pH on biotransformation by *L. massiliensis* No. 24. [A solution of Rb1 in 20 mM Na$_2$HPO$_4$/NaH$_2$PO$_4$ buffer (pH 7.0) was added to the tubes containing cells of *L. massiliensis* No. 24, preincubated for 48 h. The mixtures were incubated at different pH (2, 4, 6, 8 & 10) with shaking (170 rpm) at 34°C for 3 d, then analyzed by HPLC as described before. The maximum yield obtained was defined as 100%. Data were expressed as mean±SD from three independent experiments.]

Fig. 8—Effects of temperature on biotransformation by *L. massiliensis* No. 24. [A solution of Rb1 in 20 mM Na$_2$HPO$_4$/NaH$_2$PO$_4$ buffer (pH 7.0) was added to the tubes containing cells of *L. massiliensis* No. 24, preincubated for 48 h. The mixtures were shaked (170 rpm) at 25, 28, 31, 34 and 37°C for 1-3 d, then analyzed by HPLC as described before. Data were expressed as mean±SD from three independent experiments.]

Fig. 9—Effects of cells concentration on biotransformation by *L. massiliensis* No. 24. [A solution of Rb1 in 20 mM Na$_2$HPO$_4$/NaH$_2$PO$_4$ buffer (pH 7.0) was added to the tubes containing cells of *L. massiliensis* No. 24, preincubated for 48 h at a final concentration of 0.58×10$^9$, 0.42×10$^9$, 0.35×10$^9$, 0.30×10$^9$ and 0.25×10$^9$ cell/mL. The mixtures were shaken (170 rpm) at 34°C for 3 d, then analyzed by HPLC as described before. Data were expressed as mean±SD from three independent experiments.]

Fig. 10—Effect of glycosidase activity on the biotransformation by *L. massiliensis* No. 24, conducted at the different time (0, 1, 2, 4, 8, 12 & 36 h). [The released p-nitrophenol (pNP) was measured at 405 nm. One unit of enzyme activity was defined as the amount that released 1 µmol pNP per min.]

shown in Fig. 10, the enzyme activity was relatively low at 0 to 24 h, after that the activity increased sharply, which was identical with the result of biotransformation. As shown in Fig. 3, transformation between 0 to 24 h was relatively slow, which was caused by the low amount of β-glucosidase in the reaction system. β-Glucosidase is the key enzyme which caused the transformation from ginsenoside Rb1 to Rd. The secreted β-glucosidase by *L. massiliensis* No. 24 catalyzed the hydrolysis of the β-glucosidic linkage in the β-D-glucopyranosyl-(1→6)-β-D-glucopyranose at C-20 position of ginsenoside Rb1, without hydrolyzing other sugar linkage of Rb1, which showed high specificity.

Minor ginsenoside Rd exhibited various activities, which is regarded as a potential drug molecule. Preparation of ginsenoside Rd with simple steps, high yield and easy operation is of great importance for its application. Biotransformation is designed for the preparation of bioactive molecules. Previously, the
production of pharmaceutical active ginsenoside Rd from ginsenoside Rb1 has been performed with several enzymes, such as, β-D-glucosidase from the China white jade snail and Penicillium oxalicum, and Cladosporium flavum. Compared with these enzymes, biotransformation process by L. massiliensis NO. 24 for producing ginsenoside Rd would be more efficient because the enzymes involved in this transformation do not need to be separated. As the present results show that the preparation procedure of Rd would be shorter, the cost would also decrease automatically. This biotransformation is very significant in the utilization of ginseng resources and would be useful for preparing ginsenoside Rd from naturally abundant saponins.

In summary, a β-glucosidase-producing bacterium, identified to be L. massiliensis NO. 24, was able to specifically hydrolyze ginsenoside Rb1 to produce Rd. Under the optimized conditions, the biotransformation showed high selectivity and productivity, which would potentially be used for the Rd production in industry.

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References