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Microbial Conversion of Major Ginsenosides to Minor Pharmacological Ginsenoside Compound-K by *Sphingomonas* sp. ZY-3

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***Abstract*—Ginsenoside is the most important secondary metabolite of ginseng, and the metabolite of Ginsenoside Rb1 has various pharmacological activities, such as anti-tumor properties. Although various chemical and biological methods have been reported of preparation for it, enzymatic production by microbial bioconversion is able to produce engineering range metabolite with specific selectivity and high efficiency. In this study, to achieve minor pharmacological ginsenoside Compound-K from major ginsenoside using microbial biotransformation, many soil bacteria which have strong β–glucosidase activity were isolated. The screening was performed on esculin containing 1/10 TSA agar plate and then observed color development. The selected positive isolates were cultured 1000ppm G-Rb1 and the products were analyzed by TLC (thin-layer-chromatography) and HPLC (high-performance liquid chromatography) method. One novel bacterial strain ZY-3 could hydrolyze ginsenoside Rb1 to the active metabolites Compound-K through F2 and Rd. Based on phylogenetical tree, it was found to belong to genus *Sphingomonas* and was very close to *Sphingomonas jaspsi* TDMA-16^T and *Sphingomonas astaxanthinifaciens* TDMA-17^T (96.8%, 96.5%, similarity based on 16S rRNA sequence). The bioconversion production of Compound-K occurred by consecutive hydrolyses of the terminal sugar moieties at the C-20 carbon and hydrolyses of the terminal and inner C-3 carbon of ginsenoside Rb1 showing the biotransformation pathway: Rb1 → Rd → F2→Compound-K.**

Keywords-ginsenoside; microbial biotransformation; 16S rRNA gene

I. INTRODUCTION

Ginseng, a celebrated traditional herbal products were used in empirical for thousands of years in Asian countries to cure disease and stay healthy. Especially in Korea, China and Japan, ginseng was deemed to have omnipotent function and was cultivated on a large scale in those areas due to its beneficial effects on antitumor, anti inflammatory, blood circulation, properties [1-3], and neuroprotection and immunoprotection [4-5].

The ginseng saponin (ginsenoside), obtained from the seeds, roots, and leaves have been regarded as the primary chemical components responsible for the bioactive and pharmacological effects. Till now, approximately 50 kinds of naturally occurring saponins have been isolated [6-9]. including malonyl Ra1, Ra2, Ra3, Rb1, Rb2, Rc, Rd, Ra1, Ra2, Ra3, Rb1, Rb2, Rb3, Rc, Rd, Re, Rf, Rg1, Rg2 (R,S), Rg3 (R,S), Rg5, Rg6, Rh1 (R,S), Rh2 (R,S), Rh3, Rh4, Rk1, Rk2, Rk3, R1, R2, F2, Rs1, Rs2, Rs3 (R, S), Rs4, Rs5, Rs6, Rs7, Ro, F1, F2, F4,compound K, compound, compound O, notoginsenoside R1, and quinquenoside R1[10]. The major compound structure was shown in figure 1. These chemical compounds name was established by their Rf value on thin layer chromatography plates from low position to high by Shibato etc, 1963. Among these ginsenosides, Compound-K has many pharmacological activities such as tumor-suppressing inhibitory effect on tumor necrosis factor expression [9], on NO and prostaglandin E2 biosynthesis of RAW264.7 cells [10]. In some other reports it also declared to inhibit proliferation of some certain cancer cells such as B16-BL6 mouse melanoma cells and activated rat hepatic stellate cells, and to induce morphological changes at certain concentration so as induction of apoptosis [13-15].

The enzymatic conversion via hydrolyzing glycons on C3 and C20 position of ginsenoside is practical for production of Compound-K manufacture. In this study, we isolated ginsenoside-bioconversion bacteria ZY-3 from soil of ginseng field, investigated the ability of transformation of ginsenoside Compound-K from major ginseng saponins, and identified its intermediate metabolites.

II. MATERIALS AND METHODS

A. Chemicals.

Ginsenoside Rb1 was purchased from Dalian Green Bio Ltd (Dalian, China), standard ginsenoside were kindly provided by professor Jin (Dalian Polytechnic University, China). The other chemicals used in this study were at least of analytical reagent grade, and the sources are noted individually in the methods sections.

B. Thin-Layer Chromatography (TLC) analysis.

TLC was performed using $60F_{254}$ silica gel plates (Merck, Germany) with $CHCl_3$-CH_3OH-H_2O (70:30:5) as the solvent. The spots on the TLC plates were detected by spraying with 10% H_2SO_4, followed by heating at 110ºC for 5 min.

C. *High-Performance Liquid Chromatography (HPLC) analysis.*

HPLC system (Waters, MA) and a C18 (250× 4.6 mm, ID 5 μm) column was performed with acetonitrile (A) and distilled water (B) at ratios of (A:B) 15:85, 21:79, 58:42, 90:10, 90:10, and 15:85 with run time of 0-5, 5-25, 25-70, 70-72, 72-82, and 82-100 min, respectively, at a flow rate of 1.6ml/min. Detection absorbance was 203 nm.

D. *Isolation and phylogenetic analysis of strain ZY-3 for ginsenoside bioconversion.*

Strain ZY-3 was isolated from the soil of ginseng field in China by using screening for β-glucosidase positive strains with escullin hydrolysis activity. The phylogenetic relatedness of ZY-3 strain to other bacteria was determined by analyzing a portion of the 16S rRNA gene which had been amplified by PCR using universal primers 27F and 1492R and sequenced (SolGent).The 16S rRNA gene sequences of related taxa were obtained from the GenBank database. Multiple alignments were performed using the CLUSTAL_X program [16], Gaps were edited in the BioEdit program [17], and evolutionary distances were calculated using the Kimura two-parameter model [18]. A phylogenetic tree was constructed using the neighbor-joining method [19] using the MEGA4 Program [20] with bootstrap values based on 1000 replicates.

Soil samples taken from ginseng root were thoroughly suspended in 50 mM phosphate buffer, serially diluted with the same buffer, and the suspension was spread on one tenth strength TSA agar plates. Isolated colonies were transferred to esculin-TSA plates to screen for strains with high β-glucosidase activity. The strain was cultured on 1/10 tryptic soy broth, the suspension culture of the strain ZY-3 until it reached logarithmic phase then mixed with the same volume of ginsenoside Rb1 and incubated for 24 hr at 30°C shaking incubator. After 24hr culture, 100 μl was extracted with the same volume of water-saturated *n*-butanol and analyzed biotransformation ability of ginsenoside by TLC and HPLC method.

III. Results and Discussion

A. *Partial taxonomic characterization of strain ZY-3.*

One of the isolates, designated ZY-3, was characterized by a polyphasic approach to clarify its taxonomic position. The ZY-3 isolate was Gram-positive, aerobic, non-motile, non-spore-forming, and short-rod-shaped. Phylogenetic analysis based on 16S rRNA gene sequences indicated that the isolate belongs to the genus *Sphingomonas* in the phylum *Proteobacteria* and is most closely related to *Sphingomonas jaspsi* TDMA-16^T (96.8% similarity), followed by *Sphingomonas astaxanthinifaciens* TDMA-17^T (96.5%), as seen in figure 2, in this study we preliminarily hypothesized to be species novel strain.

B. *Bioconversion of ginsenoside Rb1*

In order to check the ability of ginsenoside conversion by the strain ZY-3, we mixed the suspension culture of strain ZY-3 and ginsenoside Rb1 and confirmed at regular intervals by TLC and HPLC analysis. As shown TLC data in figure 3 the amount of ginsenoside Rb1 was gradually decreased from 1hr to 36hr and during the last 72hr reaction the Rb1 was hardly appeared because of transformation to other minor ginsenosides, at the same process, our target production-ginsenoside compound-K was ultimate appeared at 72hr reaction according to comparison of Rf values analyzed by TLC with standard ginsenosides showing a positive result for the bioconversion activity of our work. Moreover, we precisely confirmed the phenomenon from TLC results by HPLC method. Again from the 1hr and 72hr HPLC data, it illustrated that the bacterial enzyme produced three metabolites from ginsenoside Rb1 during the 72hr reaction. In the figure 4, by HPLC chromatogram, the retention time were 4.79, 7.27, 9.88 and 15.04 min correspond to ginsenoside Rb1, Rd, F2 and Compound-K. Based on the retention times of metabolite measured by HPLC analysis with standard ginsenosides, the final production of transforming process by strain ZY-3 was identified as Compound-K accompany with ginsenoside Rd and F2 as intermediate products. The pathway could be listed as ginsenoside Rb1—Rd—F2—Compound K.

IV. Conclusions

Traditional method to prepare for pharmacological mior ginsenosides from major ginsenosides was carried on by chemical or physical process such as heating or acid treatment, however, it was either low output or excess side-production, in the recent decades, the enzymatic conversion process was in a prosperous prospect for the mild reaction conditions and seldom side products than chemical or physical process.

In this study, we isolated alomost 100 β-glucosidase-producing microorganisms from soil of a ginseng field and identified one strain, *Sphingomonas* sp. ZY-3 showing a strong ability to bioconversion of ginsenoside Rb1 into Compound-K confirmed by TLC and HPLC analysis. The biotransformation pathway was from ginsenoside Rb1 to Compound-K via intermediate products ginsenoside Rd and F2 by isopyknic reacted with the suspension culture of the strain ZY-3. The bacteria strain ZY-3 used in this study was partial confirmed by phylogenetical method based on comparative analysis of 16S rRNA gene sequences as species novel bacteria in genus *Sphingomonas* which belongs to the phylum *Proteobacteria.*

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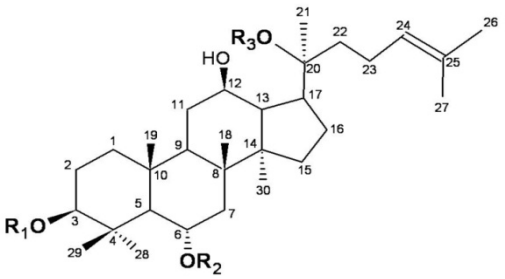

Ginsenoside	R_1	R_2	R_3
Rb1	glc(2→1)glc	H	glc(6→1)glc
Rb2	glc(2→1)glc	H	glc(6→1)arap
Rc	glc(2→1)glc	H	glc(6→1)araf
Rd	glc(2→1)glc	H	glc
F2	glc	H	glc
20-Rg3	glc(2→1)glc	H	H
20-Rh2	glc	H	H
COMPOUND-K	H	H	glc
Re	H	glc(2→1)rha	glc
Rg1	H	glc	glc
Rh1	H	glc	H

Figure 1. Chemical structure of ginsenosides. glc: β-D-glucopyranosyl, arap: α-L-arabinopyranosyl, araf: α-L-arabinofuranosyl

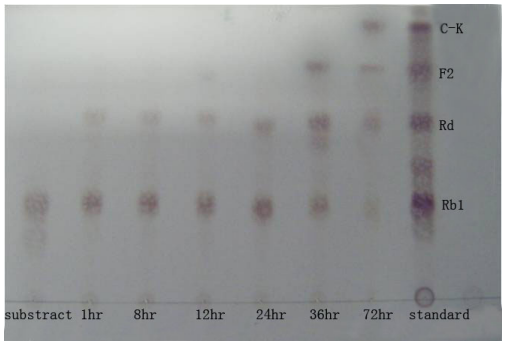

Figure 3. Analysis of the time course ginsenoside Rb1 bioconversion by Sphingomonas sp. ZY-3 using TLC. Lane: Rb1 to COMPOUND-K, Standard; 1 to 72 h, reaction time.

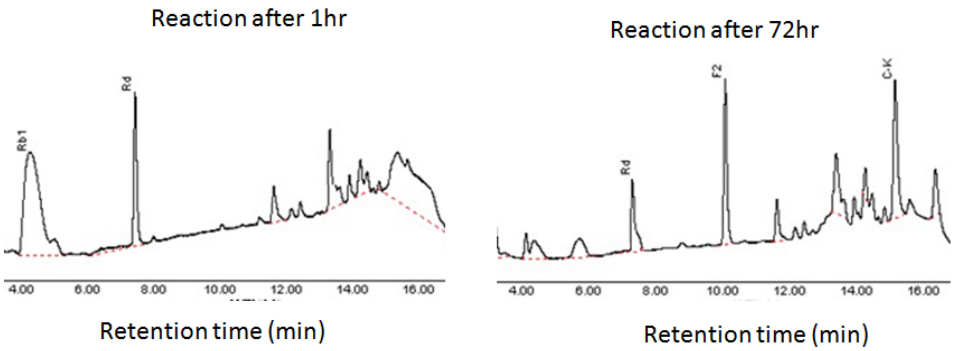

Figure 4. HPLC analysis of the metabolites of ginsenoside Rb1 converted by the strain Sphingomonas sp. ZY-3. Retention time are 1hr and 72hr seperately.

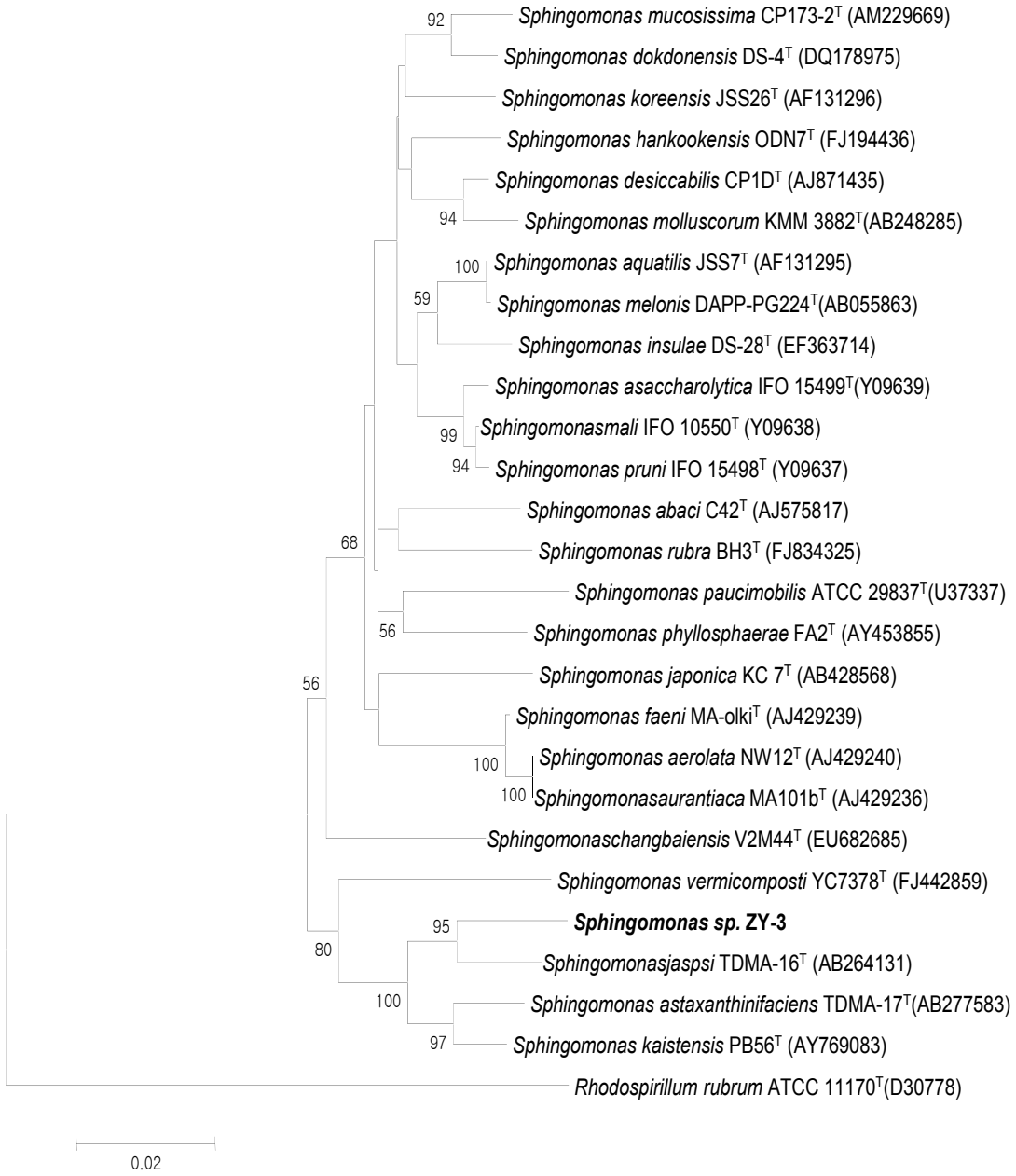

Figure 2. phylogenetical tree a comparative analysis of 16S rRNA gene sequences showing the relationships of Sphingomonas sp. ZY-3 with other related species. Dots indicate generic branches. Bootstrap values greater than 55% are shown at the branch points. Bar, 0.01 substitutions per 1 nucleotide position.