BIOTRANSFORMATION OF GINSENOSES RB1 BY BACTERIAL CRUDE ENZYME OF *Paenibacillus* spp. strain E3 ISOLATED FROM VIETNAMESE GINSENG SOIL

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ABSTRACT

In ginseng, minor ginsenosides were more effective pharmacological properties than major ginsenosides. Therefore, finding bacteria that can convert major ginsenosides has been paying attention. Ginsenoside Rb1 is one of major ginsenosides of ginseng and its biotransformation produces pharmacologically active compounds such as compound K. In this study, the isolation of bacterial strains from Vietnamese ginseng cultivated soil was carried out with the objective of evaluating their hydrolytic capacity for use in biotransformation of ginsenosides Rb1. In the screening of β -glucosidase producing bacteria, seven isolates exhibited black color zones on R2A agar medium containing esculine. Among seven isolates, strain E3 showed the highest ability biotransformation of ginsenosides Rb1 in Luria-Bertani broth. Therefore, strain E3 was selected for further research. Biotransformation of Rb1 was observed by using thin layer chromatography (TLC) and high performance liquid chromatography (HPLC) with ginsenoside standards for comparison. We determined the optimal conditions for biotransformation of ginsenoside Rb1 into the compound K of crude enzymes of strain E3 were at 30°C, pH 7.0 and 3 days. Ginsenoside Rb1 was converted into compound K via ginsenoside Rd and F2. The analyses of 16S rRNA gene and phylogenetic tree indicated that strain E3 was closely related to *Paenibacillus terrigena* with 99.4% identity and formed a discrete cluster with type strain *Paenibacillus terrigena* A35^T with high bootstrap support (99%), supporting strain E3 belonging to the species *Paenibacillus terrigena*. Physiological characteristics also supported strain E3 belonging to the genus *Paenibacillus*. This is the first report of biotransformation of ginsenosides using bacterial strains isolated from Vietnamese ginseng cultivated soil in Vietnam. Based on our the obtained results, strain E3 could be applied for the preparation of ginsenoside compound K for use in the cosmetic and pharmaceutical industries.

Keywords: *Paenibacillus terrigena*, Biotransformation, Rb1, compound K, strain E3, Vietnamese ginseng soil.

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INTRODUCTION

Panax vietnamensis Ha et Grushv. (Vietnamese ginseng), has been used as an herbal medicine in Vietnam for life-saving and enhancing physical strength. *P. vietnamensis* contains bioactive substances such as ginsenosides, acidic polysaccharides, phenolic compounds, and polyacetylenes. Ginsenosides

are considered as the main active components of ginseng. Ginsenosides in *P. vietnamensis* are classified into four groups including oleanane-type, protopanaxadiol type, protopanaxatriol type and ocotillol type. Ginsenoside Rc, Rb1, Rb2, Rd, vinanosides-R2, majonoside-R, Rc, Rg1+Re are major compositions of *P. vietnamensis* root, whereas ginsenosides F1, Rh1, F2, Rg3, and Rh2, compound K (C-K)

play as minor components or absence (Van Le et al., 2014; Van Le et al., 2015). However, F1, Rh1, F2, Rg3, and Rh2, and compound K (C-K) were pointed out more effective pharmacological properties than major ginsenosides (Choi et al., 2008; Kim et al., 2017; Li et al., 2018). Major ginsenosides can be converted to minor ginsenosides by chemical method (Han et al., 1982; Im et al., 1995), physical method (Park, 2004), and biotransformation (Hoang et al., 2013; Upadhyaya et al., 2016). Despite of mild conditions, selectivity, regiospecificity and stereospecificity, biotransformation of major ginsenosides using microbial enzymes has attracted much attention.

A large of number β -glucosidase producing bacterial strains have been isolated from Korean ginseng soil (Kim et al., 2005; Park et al., 2010) and Chinese ginseng soil (Jin et al., 2012; Fu et al., 2014). However, biotransformation of ginsenosides by bacteria resident in the Vietnamese ginseng soil is lacking. Due to that, in this study, we aimed to find the β -glucosidase producing bacterial strains originating from Vietnamese ginseng soil and used for biotransformation of ginsenosides Rb1. We found a strain E3 converted ginsenoside Rb1 into compound K through the pathway $Rb1 \rightarrow Rd \rightarrow F2 \rightarrow C-K$.

MATERIALS AND METHODS

Materials

The media R2A (Becton Dickinson) and LB (Luria-Bertani) broth were purchased from Difco, Becton Dickinson Co. (MD, USA). The standard ginsenosides Rb1, Rc, Rd, Re, Rg1, Rg2(S), Rh1(S), and compound K were purchased from Dalian Green Bio, Ltd. (Dalian, China). Silica gel-60 used for thin liquid chromatography (TLC) was purchased from Merck (Germany). High performance liquid chromatography (HPLC, Agilent 1260 Infinity, Agilent Technologies, Palo Alto, CA, USA) was conducted by using a UV/Vis detector and a gradient pump system. All chemicals and solvents were of analytical reagent grade.

Screening of β -glucosidase producing bacteria

Ginseng soil samples were collected from Nam Tra My district, Quang Nam province, Vietnam. Esculin-R2A agar was used to

isolate β -glucosidase producing bacteria from Vietnamese ginseng soil. The isolation medium contained 0,3 g/l of esculin and 0,02 g/l of ferric citrate in R2A agar (Becton Dickinson). After 10^{-4} to 10^{-5} dilution with sterile 0,85% NaCl solution, 100 μ l of suspension was spread on an Esculin-R2A agar plate and incubated at 30°C for 48 hours. The bacteria that produced β -glucosidase, which hydrolyzes esculin, appeared as colonies surrounded by a reddish-brown to dark brown zone on esculin-R2A agar. Single colonies from these plates were picked up and transferred to fresh R2A agar plates to purify.

Preparation of a microbial crude enzyme

Cells of bacterial strains were cultured in 100 ml of LB broth by inoculating a single colony from a pure culture from a LB agar medium. The inoculation was incubated at 200 rpm and 30°C for 24 hours. After that, cells were harvested by centrifugation at 8000 rpm for 20 min. The cells were washed with sterile phosphate buffered saline (PBS) twice, then re-suspended in 5 ml of 20 mM sodium phosphate buffer (pH 7,0), and disrupted by cell sonication. The disrupted cell suspension was used as a crude enzyme solution.

Conversion of ginsenoside Rb1 by crude enzyme

One ml of the whole-cell β -glucosidase of bacterial culture was mixed with an equal volume of ginsenosides Rb1 at 1 mg/ml in 20 mM sodium phosphate buffer. The mixture was then incubated at 30°C with shaking at 200 rpm. After 4 days of inoculation, 200 μ L aliquot was taken and the ginsenosides were collected by adding water-saturated n-butanol and analyzed using TLC.

Effects of temperature, pH, and reaction time on the enzyme activity

The effect of temperature on the whole-cell β -glucosidase activity of strain E3 was investigated at pH 7,0 $\pm$ 0,1 and the range of 20 to 60°C. Reactions were performed in 1 ml of sodium phosphate buffer (pH 7,0) containing 1 mg of ginsenoside Rb1 and 1 ml of crude enzyme for 48 hours.

The optimum reaction pH for the conversion of ginsenoside Rb1 varied between 4,0 and 12,0. Reactions were performed in 1 ml of various buffers (pH 4,0–12,0) containing

1 mg of ginsenoside Rb and 1 ml of crude enzyme at 30°C for 48 hours.

The time course of metabolites of ginsenoside Rb1 by strain E3 was checked at pH 7,0 and 30°C. Reactions were performed in 1 ml of sodium phosphate buffer (pH 7,0) containing 1 mg of ginsenoside Rb1 and 1 ml of crude enzyme. During the reaction period, a 200 µL aliquot was taken every 1 day.

The ginsenosides were extracted with water-saturated *n*-butanol. Subsequently, the *n*-butanol fraction was evaporated to dryness and the methanolic extract was analyzed by TLC.

TLC and HPLC analysis of the biotransformation

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

The reaction mixture was extracted using water-saturated *n*-butanol and then dried using a SpeedVac freeze dryer. After drying, the residue was dissolved in methanol and applied to HPLC (Agilent Technology 1260, Infinity) analysis. The separation was carried out on a Poroshell 120 EC-C18 column (2,7 µm, 3 × 50 mm i.d). The mobile phase was A (water) and B (acetonitrile). Gradient elution started with 83% solvent A and 17% solvent B, 0–6 min; then changed to: A from 83 to 77%, 6–9 min; 77 to 76,5%, 9–16,5 min; 76,5 to 69%, 16,5–19 min; 69 to 55%, 19–29 min; 55 to 53%, 29–31 min; 53 to 10%, 31–33 min; 10%, 33–34 min; 10 to 83%, 34–35 min; 83%, 35–37 min. The flow rate was 1,0 ml/min, and detection was performed by monitoring absorbance at 203 nm, with an injection volume of 5 µl. The ginsenoside transformation was monitored by the comparison with the chromatogram of ginsenoside standards (Rb1, Rd, and mixture of Rg1, Re, Rf, Rg2+Rg1, Rb1, Rc, Rb2, Rd, F2, Rg3, Ck, Rh2) individually injected into HPLC system.

Identification of strain E3

The genomic DNA extraction for 16S rRNA gene sequencing was performed by using a

commercial GeneJET genomic DNA purification kit (Thermo Fisher Scientific Inc. (USA)). The 16S rRNA gene was amplified from the chromosomal DNA of strain E3 using the universal bacterial primer sets 27F, and 1492R (Lane et al., 1991). Purified PCR products were sequenced using primers 27F, 518F, 800R and 1492R in a 3500 Genetic Analyzer (Applied Biosystems-Hitachi, Carlsbad, CA, USA) at Institute of Genome Research (Ha Noi, Vietnam). The complete sequence of the 16S rRNA gene from strain E3 (1489 bp) were compiled through SeqMan software and edited using the BioEdit program. The 16S rRNA gene sequences similarity of strain E3 was blast on EzTaxon-e server (Kim et al., 2012). The 16S rRNA gene sequences of related taxa were obtained from GenBank (National Center for Biotechnology Information; Bethesda, MD, USA), and trees were constructed by neighbor joining (Saitou & Nei, 1987) using the MEGA 7 program (Kumar et al., 2016). The resultant tree topologies were evaluated by bootstrap analysis based on 1000 replications (Felsenstein, 1985).

Carbon utilization and enzyme productions of strain E3 were carried out using the API 50CH and API ZYM according to the manufacturer's instruction (bioMérieux).

RESULTS AND DISCUSSION

Screening of β -glucosidase producing bacteria and identification

A total of seven isolates of β -glucosidase producing bacteria were obtained from the soil of Vietnamese ginseng (Table 1). Among these isolates, strain E3 exhibited the strongest ability to convert ginsenosides Rb1 in LB broth (Fig. 1). Therefore, strain E3 was selected for further research.

Table 1. List of β -glucosidase producing bacterial strains isolated from Vietnamese ginseng cultivated soil in Nam Tra My district

No.	Strain	Closest taxonomy	16S rRNA identity (%)
1	E1	<i>Bacillus mycoides</i>	96.64
2	E2	<i>Bacillus bataviensis</i>	99.30
3	E3	<i>Paenibacillus terrigena</i>	99.86
4	E4	<i>Bacillus methylotrophicus</i>	100.00
5	E5	<i>Lysinibacillus xylanilyticus</i>	99.93
6	E6	<i>Bacillus luciferensis</i>	99.16
7	E8	<i>Paenibacillus terrae</i>	99.17

Fig. 1. Biotransformation of ginsenoside Rb1 of seven strains isolated from Vietnamese ginseng cultivated soil. Rb1: ginsenoside Rb1 standard; E1→E8, bacterial strains

The 16S rRNA gene sequence of strain E3 was deposited on NCBI Genbank under accession number KY000528 (*Paenibacillus terrigena* strain E3). The relationship between strain E3 and other members of *Paenibacillus* was also shown in the phylogenetic tree (Fig. 2). Strain E3 formed a discrete cluster with type strain A35^T of the species *Paenibacillus terrigena* with high bootstrap support (99%), indicating that strain E3 is a member of the genus *Paenibacillus*. Strain E3 shared the 99.4% sequence identity to *Paenibacillus terrigena* A35^T, supporting that strain E3 belonging to the species *Paenibacillus terrigena*. Strain E3 was deposited in Vietnam Type Culture Collection (VTCC) as VTCC 91007.

Fig. 2. Phylogenetic tree derived from 16S rRNA gene sequences of strain E3 and their taxonomic neighbours in the genus *Paenibacillus*, constructed with the neighbour-joining method. The scale bar corresponds to 0.02 substitutions per nucleotide position

Effects of temperature, pH, and reaction time on the activity of crude enzyme

Effects of different temperatures, pH, and reaction time on the activity of crude enzyme

were shown in Fig. 3. At 25°C, the crude enzyme started degrading consecutively ginsenoside Rb1 into ginsenoside Rd, F2 and compound K. The crude enzyme reached maximum activity at 30°C, decreased activity above 40°C, and lost activity from 50°C (Fig. 3a). It can be explained that, at low temperatures, enzyme is insufficient because of energy deficiency; at high temperatures, enzyme loses its activities because of denaturation.

Fig. 3. Effects of temperature (a), pH (b), and reaction time (c) on the biotransformation of ginsenoside Rb1 by the crude enzyme of strain E3. S: ginsenoside standards including ginsenoside Rb1, Rd, Rg3, F2, and compound K (C-K); C: ginsenoside Rb1; (a) Temperature at 20°C, 25°C, 30°C, 35°C, 40°C, 50°C, and 60°C; (b) pH at 4, 5, 6, 7, 8, 9, 10, 11, and 12; (c) Time course 1 day, 2 days, and 3 days. C, control; S, ginsenoside standards; C-K, compound K

HPLC analysis of the biotransformation of ginsenosides

After strain E3 was inoculated until the absorbance at 600 nm reached 1.0, the crude enzyme solution strain E3 was used to convert ginsenoside Rb1 into ginsenosides compound K. Conversion of ginsenoside Rb1 by strain E3 was confirmed using quantitative HPLC analysis (Fig. 4). Peaks with retention times of 10.02; 10.34; 14.9; 17.40; 18.28; 19.28; 20.16;

22.10; 27.81; 29.02; 31.14 and 31.47 min corresponded to ginsenosides Rg1, Re, Rf, Rg2, Rg2 mix Rh1, Rb1, Rc, Rb2, Rd, F2, Rg3, C-K and Rh2, respectively (Fig. 4a). Fig. 4b showed the peak of ginsenoside Rb1 as a control. Ginsenoside Rb1 disappeared almost entirely, followed by the appearance of a new peak (Fig. 4c). The retention time was similar to the time of ginsenoside compound K.

Fig. 4. HPLC profiles and proposed biotransformation pathway of ginsenoside Rb1 by the crude enzyme of strain E3. Ginsenoside standards (a); ginsenoside Rb1 control (b); ginsenoside Rb1 converted to C-K by the crude enzyme of strain E3 (c); proposed biotransformation pathway Rb1 → Rd → F2 → C-K (d)

Pathway of biotransformation of ginsenosides

In this study, ginsenoside Rb1 was converted into ginsenoside compound K (C-K) via intermediates ginsenoside Rd and F2 (Fig. 4d). The ginsenoside Rb1 belongs to protopanaxadiol (PPD) type ginsenoside with two glucosyl residues attached to the position C3 and two glucosyl residues attached to the position C20 (Fig. 4d). Strain E3 catalyzed the cleavage of one glucose at C20 of Rb1 to form ginsenoside Rd, then continued hydrolyzing one glucose at C3 of Rd to form ginsenoside F2, and finally removed one glucose at C3 of F2 to produce compound-K (Fig. 4d). The biotransformation of ginsenoside of strain E3 was similar to the pathway by which ginsenoside Rb1 is converted to compound K by *Leuconostoc citreum* LH1 (Quan et al., 2011) and *Armillaria mellea* (Upadhyaya et al., 2016).

C-K is the final metabolite of PPD (Lee et al., 2000), therefore it is easier to absorb from the gut than other PDD (Wakabayashi, 1997). C-K has many pharmacological activities such as anticarcinogenic, anti-inflammation, antiallergic, anti-diabetic, anti-angiogenesis, anti-aging, neuroprotective and hepatoprotective effects (Yang et al., 2015), and antidepressant effects (Song et al., 2018). However, ginsenoside C-K is found in small amounts in ginseng roots. Therefore, it is desirable to produce a greater amount of C-K. One of the methods is utilizing of microbial enzyme. In this study, we demonstrated that strains E3 is capable of producing C-K using its crude enzymes. However, crude enzymes are limited by the complexity of enzymes, long time reactions, and low yield. In the future we need to clone, purify and characterize of a specific β -glucosidase from strain E3 to reduce the time and increase the yield of biotransformation. For example, Quan et al. (2012) cloned and characterized β -glucosidase (bgp-3) from *Microbacterium esteraromaticum* isolated from ginseng field, bgp3 transformed 1,0 mg/ml ginsenoside Rb1 into 0,46 mg/ml C-K within 60 min.

Physiological and biochemical analysis

In the API zym kit, strain E3 was positive for produced enzyme alkaline phosphatase, esterase lipase (C8), leucine arylamidase,

α -chymotrypsin, acid phosphatase, naphthol-AS-BI-phosphohydrolase, α -galactosidase, β -galactosidase, α -glucosidase, β -glucosidase, and *N*-acetyl- β -glucosaminidase; but negative for produced enzyme valine arylamidase, cystine arylamidase, trypsin, esterase (C4), lipase (C14), α -mannosidase, α -fucosidase, and β -glucuronidase. In the API 50 CH test, strain E3 produced acids from glycerol, D-ribose, D-xylose, methyl- β -D-xylopyranoside, D-galactose, D-glucose, D-fructose, D-mannose, methyl- α -D-mannopyranoside, methyl- α -D-glucopyranoside, *N*-acetyl-glucosamine, amygdalin, arbutin, esculin, salicin, D-cellobiose, D-maltose, D-lactose, sucrose, D-melibiose, D-raffinose, starch, xylitol, gentiobiose, D-turanose, L-fucose, and potassium gluconate; but not from erythritol, L-arabinose, D-arabinose, L-xylose, D-adonitol, D-trehalose, L-sorbose, glycogen, L-rhamnose, dulcitol, inositol, D-mannitol, D-sorbitol, inulin, D-melezitose, D-lyxose, D-tagatose, D-fucose, D-arabitol, L-arabitol, potassium 2-ketogluconate, and potassium 5-ketogluconate.

These biochemical characteristics of strain E3 were similar to members of the genus *Paenibacillus* (Xie et al., 2007; Guo et al., 2014; Yao et al., 2014).

CONCLUSION

In conclusion, this is the first report about the conversion of ginsenoside Rb1 into compound K via intermediates ginsenoside Rd and F2 by the crude enzyme of strain *Paenibacillus terrigena* strain E3 isolated from ginseng soil. This crude enzyme was optimum at mild conditions at pH 7,0, 30°C and 3 days. Strain E3 can be used as a potential microbial source for obtaining compound K.

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