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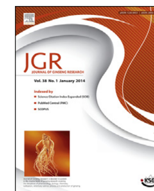

Research article

Evaluation of glucosidases of *Aspergillus niger* strain comparing with other glucosidases in transformation of ginsenoside Rb1 to ginsenosides Rg3

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ABSTRACT

The transformation of ginsenoside Rb1 into a specific minor ginsenoside using *Aspergillus niger* KCCM 11239, as well as the identification of the transformed products and the pathway via thin layer chromatography and high performance liquid chromatography were evaluated to develop a new biologically active material. The conversion of ginsenoside Rb1 generated Rd, Rg3, Rh2, and compound K although the reaction rates were low due to the low concentration. In enzymatic conversion, all of the ginsenoside Rb1 was converted to ginsenoside Rd and ginsenoside Rg3 after 24 h of incubation. The crude enzyme (β-glucosidase) from *A. niger* KCCM 11239 hydrolyzed the β-(1→6)-glucosidic linkage at the C-20 of ginsenoside Rb1 to generate ginsenoside Rd and ginsenoside Rg3. Our experimental demonstration showing that *A. niger* KCCM 11239 produces the ginsenoside-hydrolyzing β-glucosidase reflects the feasibility of developing a specific bioconversion process to obtain active minor ginsenosides.
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1. Introduction

Ginseng, the root of *Panax ginseng* Meyer, is one of the most popular traditional herbal medicines. It has been used for thousands of years in Asian countries, and has also recently become popular in Western countries. Ginseng harbors a variety of bioactive compounds, including ginsenosides (ginseng saponins), acidic polysaccharides, phenolics, and polyacetylenes [1]. Among these, the ginsenosides have been well characterized for their functionality, and are thus regarded as the principal components responsible for the pharmacological and biological activities of ginseng [2].

Ginsenosides are composed of a dammarane backbone with several side chains, including glucose, arabinose, xylose, and rhamnose side chains [3]. Thus far, more than 50 types of ginsenosides have been isolated and identified from *Panax ginseng* Meyer [4]. Based on the differences in their chemical constitutions, the ginsenosides are generally classified into three types: protopanaxadiol (PPD), protopanaxatriol, and olenolic acid. Among those thus far identified, six major ginsenosides (Rb1, Rb2, Rc, Rd, Re, and Rg1) have been determined to account for 90% of the total ginsenoside content of *P. ginseng* Meyer [5]. In particular, ginsenoside Rb1 is present in greater abundance (usually >20% of total ginsenosides) than any other ginsenosides in *P. ginseng, Panax quinquefolius, Panax japonicum* and *Panax notoginseng* [6]. Earlier reports have shown that the major PPD-type ginsenosides (Rb1, Rb2, Rc, Rd) are metabolized by intestinal bacteria after oral administration to minor ginsenosides such as Rg3, Rh2, F2, and compound K (CK) [7]. In recent years, it has been demonstrated that the minor ginsenosides possess remarkable pharmaceutical activity and can be readily absorbed by the human body [8]. For example, ginsenoside Rg3 induces tumor cell apoptosis, inhibits tumor cell proliferation and attenuates tumor invasion and metastasis [9,10]. In addition, Rg3 serves as a natural cytoprotective agent against environmental carcinogens [11]. Therefore, a variety of studies have focused on the conversion of major ginsenosides to the more active minor ginsenosides via methods such as heating [12], acid treatment [13], alkali treatment [14], and enzymatic conversion [15,16]. Chemical transformation induces side reactions including epimerization, hydroxylation, and hydration, and also generates more environmental pollution [17]. By contrast, microbial or enzymatic approaches have arisen as the predominant conversion modalities, owing to their marked selectivity, mild reaction conditions, and environmental compatibility.

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Some studies have involved attempts to find suitable microbes or enzymes that can transform Rb1 into minor ginsenosides such as Rd, F2, Rg3, and compound K [4,17–20]. However, the majority of the microorganisms employed in these experiments are not of food-grade.

Aspergillus niger strain has been known to be one of the most popular fungi in fermentation of the crops such as soybean and in brewing industry due to its production of various hydrolyzing exoenzymes [21]. In particular, production of glucosidase by using *A. niger* as a good producer has been recently studied by many researchers [22]. Therefore, in this study, we focused narrowly on the transformation of ginsenoside Rb1 into specific minor ginsenosides using *A. niger*, which has been generally regarded as safe by the Food and Drug Administration.

2. Materials and methods

2.1. Materials

Ginsenosides Rb1, Rd, 20(S)-Rg3, 20(R)-Rg3, Rh2, and CK were purchased from Vitrosys, Inc. (Yeongju, Korea). Ginsenoside Rb1, Rd, 20(S)-Rg3, and 20(R)-Rg3. *ρ*-Nitrophenyl-β-D-glucopyranoside (PNPG), *ρ*-nitrophenol (PNP), and β-glucosidase from almond were purchased from Sigma-Aldrich (St Louis, MO, USA). Potato dextrose broth was purchased from Difco (Miller, Becton Dickinson, and Co., Sparks, MD, USA). Celluclast 1.5L and Cellulase 12T were purchased from Novozymes (Bagsværd, Denmark) and Bioland Co. (Chungnam, Korea), respectively. High performance liquid chromatography (HPLC; Agilent 1100 series; Agilent Technologies, Palo Alto, CA, USA) was conducted using a UV/vis detector and a gradient pump. All solvents used in chromatography were of HPLC grade and all other chemicals were of analytical reagent grade.

2.2. Growth and crude enzyme production

A. niger KCCM 11239 was purchased from the Korean Culture Center of Microorganisms (KCCM, Seoul, Korea). The fungus was cultured on potato dextrose agar at 30°C for 4 d, and the stock cultures were maintained at 4°C. Erlenmeyer flasks were filled to 20% of their volume with potato dextrose broth, and subsequently inoculated with 5-d cultures. The cultures were grown for 16 d under shaking conditions at 200 rpm at 30°C. During the shake flask culturing, a few glass beads were added to prevent mycelial clumping and thus to achieve homogeneous growth. After incubation, the culture broth was centrifuged at 9,000 × g at 4°C for 10 min, and a crude enzyme was obtained by precipitation with 70% of $(NH_4)_2SO_4$ of the supernatant. The specific activity of crude enzyme was detected to 91 U/mg.

2.3. Assay of enzyme activity

Beta-glucosidase activity was evaluated via a colorimetric method using PNPG as a substrate. The reaction mixture, which contained 1 mL of 5 mM PNPG and 100 μL of enzyme solution, was incubated at 50°C for 10 min. The reaction was subsequently terminated via the addition of 1 mL of 0.5 M NaOH, and the absorption of the released PNP was measured at 400 nm. One unit of β-glucosidase activity was defined as the quantity of enzyme required to liberate 1 μM of PNP/min under standard conditions [23].

2.4. Biotransformation of ginsenoside Rb1

Microbial transformation was conducted via a modified Cheng's method [24]. In brief, suspensions of the 5 d-old cultures were mixed with an equal volume of 1 mM ginsenoside Rb1 dissolved in 0.5 M sodium phosphate buffer (pH 5.5) and were shaken for 16 d, 200 rpm, at 30°C. Enzymatic transformation was conducted with 200 μL of a 16-d culture supernatant (centrifuged at 14,400 × *g* for 30 min at 4°C) and the same volume of 1 mM ginsenoside Rb1 was reacted for 48 h at 30°C and 50°C. Aliquots were withdrawn at suitable time intervals (0.5 h, 1 h, 2 h, 4 h, 8 h, 12 h, and 24 h). To compare of ginsenosides produced by commercial β-glucosidase, a 200 μL of each enzyme solution (approximately 200 U) was added into a reaction mixture and samples were picked for thin layer chromatography (TLC) after 48 h.

2.5. Extraction of crude saponin

The reaction mixtures were extracted twice with 200 μL of water-saturated *n*-butanol. The *n*-butanol fraction was evaporated to generate the crude saponin fraction with a rotary vacuum evaporator (N-1000V, EYELA, Tokyo, Japan). Crude saponin was dissolved in 50 μL of methanol, which was subjected to TLC and HPLC determination. The samples were then passed through a 0.45 μm PTFE syringe filter (Whatman, Brentford, Middlesex, UK) prior to injection.

2.6. TLC and HPLC analysis for determination of ginsenosides

TLC was conducted on silica gel $60F_{254}$ plates. A solvent mixture of chloroform:methanol:water (65:35:10 v/v/v, lower phase) was used as the developing solvent. The spots were detected by spraying with 10% sulfuric acid followed by heating under a lamp flame until the spots became clearly visible. Ginsenosides and transformed ginsenosides were identified and assayed via comparison with known ginsenoside standards.

HPLC was conducted using an Agilent 1100 system (Agilent Technologies) at a detection wavelength of 203 nm. The column used was a reverse-phase column (C_{18}, 4.6 mm × 150 mm, 5 μm) and an injection volume of sample was 20 μL. The mobile phase utilized gradient conditions with solvents A ($CH_3CN:H_2O$ = 100:0) and B ($CH_3CN:H_2O$ = 14:86). The solvent A and B ratios were as follows: [20% A (0 min)]; 20% A (5 min); 30% A (10 min); 30% A (15 min); 60% A (20 min); 60% A (23 min); 0% A (25 min)] with a 1.2 mL/min flow rate.

2.7. Statistical analysis

Each experiment was individually repeated three times. All data were assigned for purposes of comparison and an analysis of variance (ANOVA) was carried out by using SPSS version 8.0 (SPSS Inc., Chicago, IL, USA). A *p*-value of < 0.05 was considered significant.

3. Results

3.1. Microbial conversion of ginsenoside Rb1 during culturing

Aspergillus species are known as a useful source of β-glucosidase and *A. niger* is by far the most efficient β-glucosidase producer among the microorganisms investigated thus far. Changes in the growth and β-glucosidase activity of *A. niger* KCCM 11239 on potato dextrose broth medium at 30°C were evaluated under aerobic conditions (data not shown). Very little β-glucosidase was detected in the culture broth until 12 d, but then the activity dramatically increased and reached to a maximum level (197.7 U/mL) after approximately 16 d. After that time, it appeared that the activity was slightly decreased by protease existing in culture broth. From the results, it is presumed that a production pattern of β-glucosidase is nongrowth associated type.

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