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.

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 $\times$ 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 $\times$ 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 $\times$ 150 mm, 5 μm) and an injection volume of sample was 20 μL. The mobile phase utilized gradient conditions with solvents A (CH$_3$CN:H$_2$O = 100:0) and B (CH$_3$CN:H$_2$O = 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.
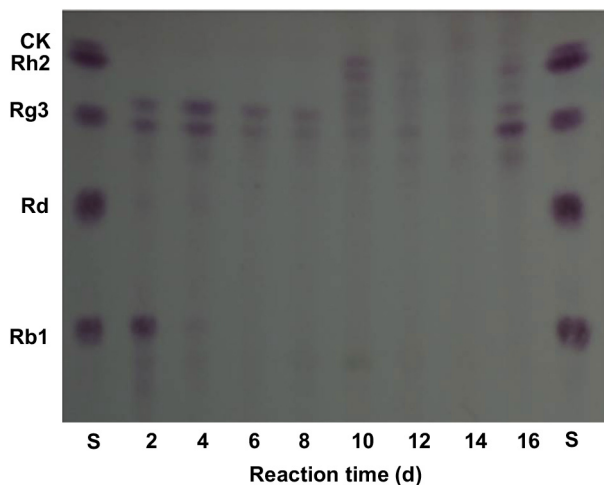
Fig. 1. TLC analysis of the time-course transformation of ginsenoside Rb1 by *Aspergillus niger* KCCM 11239. Suspensions of the 5-d cultures were mixed with an equal volume of 1 mM ginsenoside Rb1. S is the mixture of ginsenoside standards; TLC, thin layer chromatography.

The microbial conversion of ginsenoside Rb1 was prepared by inoculating ginsenoside Rb1 into precultured suspensions, followed by 16 d of incubation of the mixture at 30°C and 200 rpm. The microbial conversion was checked via TLC at 2-d intervals. During the 2-d growth period, much of the enzyme seemed to be produced, as evidenced by the observation of increased ginsenoside Rg3 levels. Fig. 1 shows that most of the ginsenoside Rb1 was converted to ginsenoside Rg3 and generated less polar metabolites after 4 d of incubation. Indeed, after 10 d, *A. niger* produced a less polar metabolite, with an Rf value similar to that of ginsenoside Rh2 and compound K. This result suggests that the microbial conversion of ginsenoside Rb1 using *A. niger* KCCM 11239 induced the production of diverse PPD-type ginsenosides. We estimated that this result was induced by different types of β-glucosidase from *A. niger* KCCM 11239.

Aspergillus species are known to produce different types of β-glucosidase. For example, *Aspergillus* sp. g48p produces two types of ginsenoside-hydrolyzing β-glucosidases: ginsenosidase type II

hydrolyzes 20-*O*-glycosides of PPD-type ginsenosides and ginsenosidase type I hydrolyzes 3-*O* and 20-*O* glycosides of PPD-type ginsenosides [25].

3.2. Enzymatic conversion of ginsenoside Rb1

The enzymatic conversion of ginsenoside Rb1 over a time-course was conducted using a crude enzyme. Ginsenoside Rb1 was reacted with the same volume of crude enzyme for 48 h at 30°C and 50°C; the TLC results are shown in Fig. 2. When ginsenoside Rb1 was reacted at 30°C, the levels of ginsenoside Rd were increased within 30 min and the levels of ginsenoside Rg3 were increased after 4 h. All of the ginsenoside Rb1 was converted to ginsenoside Rd and ginsenoside Rg3 after 24 h of incubation. Ginsenoside Rb1 was reacted with crude enzyme at 50°C to compare the effects of the reaction temperature. The results demonstrate that high reaction temperatures accelerate the reaction to produce ginsenoside Rg3. In addition, the conversion activity of *A. niger* after 48 h of a reaction time were compared by using three commercial enzymes (Fig. 3). When ginsenoside Rb1 was reacted with Celluclast 1.5L and Cellulase 12T, the content of ginsenoside Rb1 was reduced and a productivity of Rd increased after 48 h. However, these enzymes were not converted further into active minor ginsenosides Rg3. In case of β-glucosidase from almond, ginsenoside hydrolyzing activity was not detected.

3.3. HPLC assay of metabolites

Various products of Rb1 transformed by the crude enzyme isolated from *A. niger* KCCM 11239 were confirmed via HPLC analysis. The profile of the reaction mixture of ginsenoside Rb1 at 30°C for 24 h of reaction is shown in Fig. 4. HPLC analysis yielded results similar to the findings of TLC. In addition, the amount of ginsenoside Rb1 was reduced with the extension of the reaction time, whereas other hydrolysis products including ginsenoside Rd and S-Rg3 increased after 24 h of incubation at 30°C. Ginsenoside Rb1 harbors four β-glucosidic linkages, including a 20-C, β-(1→6) and a 3-C, β-(1→2) linkage. Fig. 4 shows that a peak area of Rd in a sample increased after 8 h, but decreased after 24 h. In the meantime, ginsenoside Rg3 was detected in a 24 h-reaction mixture and a ratio of the peak area was approximately 48.5%. By contrast, ginsenoside Rb1 was not detected in a 24-h reaction mixture. These
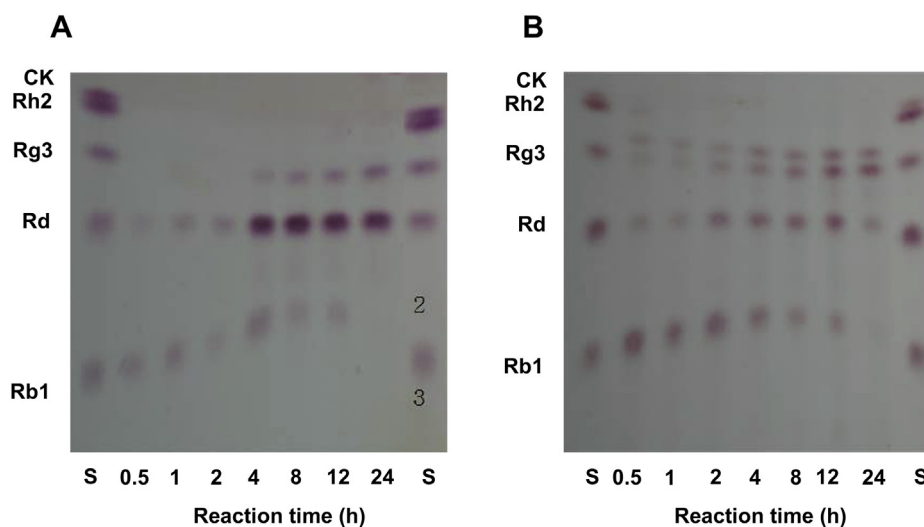

Fig. 2. TLC analysis of time-course transformation of ginsenoside Rb1 by the crude enzyme from *Aspergillus niger* KCCM 11239. Crude enzyme was mixed with an equal volume of 1 mM ginsenoside Rb1 and then reacted for 24 h at (A) 30°C and (B) 50°C. S is the mixture of ginsenoside standards; TLC, thin layer chromatography.
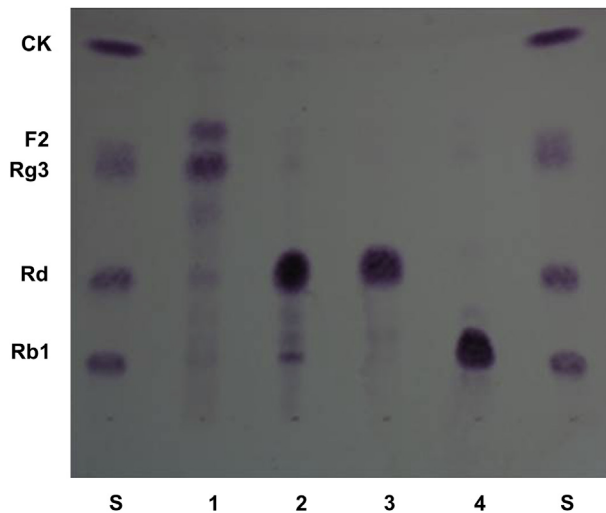
Fig. 3. Comparison of ginsenoside Rb1 transformation activity using commercial enzymes. S, ginsenoside standards; 1, crude enzyme from *Aspergillus niger* KCCM 11239; 2, Celluclast 1.5L; 3, Cellulase 12-T; 4, β-glucosidase from almond.

results show that ginsenoside Rb1 can be converted to ginsenoside Rd by the loss of a glucose moiety at the β-(1→6)-glucosidic linkage of the C-20 position of the ginsenoside aglycon [26]. Finally, ginsenoside Rg3 can also be produced from ginsenoside Rd via the additional hydrolysis of a glucose moiety. In production of ginsenoside Rg3, a peak area ratio of ginsenoside 20(S)-Rg3:20(R)-Rg3 isomer was calculated to be approximately 83:17. There have been

previous reports on microbial sources capable of converting the major ginsenoside Rb1 to ginsenoside Rg3. *Microbacterium* sp. GS514 exhibited a marked ability to convert ginsenoside Rb1 to Rg3 [4]. The enzymes isolated from the strain GS514 hydrolyzed the terminal glucose and then the inner glucose at position C-20. Ginsenosidase type II from *Aspergillus* sp. g48p hydrolyzed PPD ginsenosides such as Rb1, Rb2, Rc, or Rb3 to generate Rd, and also slowly hydrolyzes the 20-O-glucoside of Rd to produce a very small quantity of ginsenoside Rg3 [25].

It was reported that ginsenoside Rd as an intermediate has a variety of pharmaceutical activities, including the prevention of kidney injury by chemical drugs [27], the prevention of the concentration of blood vessels [28], enhancement of the differentiation of neural stem cells [29]. In addition, it has been shown that ginsenoside Rg3, in particular the S form Rg3, prevents endothelial cell apoptosis via the Akt-dependent inhibition of the mitochondria [10], and regulates voltage-dependent Ca^{2+}, Na^+, and K^+ channel activity [30].

In conclusion, it has been shown that ginseng has various biofunctional effects including ginsenosides and their derivatives. Ginsenoside Rb1 is present in greater abundance than any other ginsenosides in the root, but ginsenoside Rg3 has greater biological effects for human health though its content is relatively very low in ginseng. We can transform from Rb1 to Rg3 by using enzymatic hydrolysis for a larger production. The pathway of enzymatic hydrolysis of Rb1 to produce Rb3 has already been shown by Chang et al [31]. A production yield of minor ginsenoside such as Rg3 depends on many kinds of glucosidase. In this study, hydrolysis of Rb1 by β-glucosidase produced from the *A. niger* strain was evaluated comparing with a commercial enzyme such as Celluclast 1.5L, Cellulase 12T, and other β-glucosidase (from almond) for higher
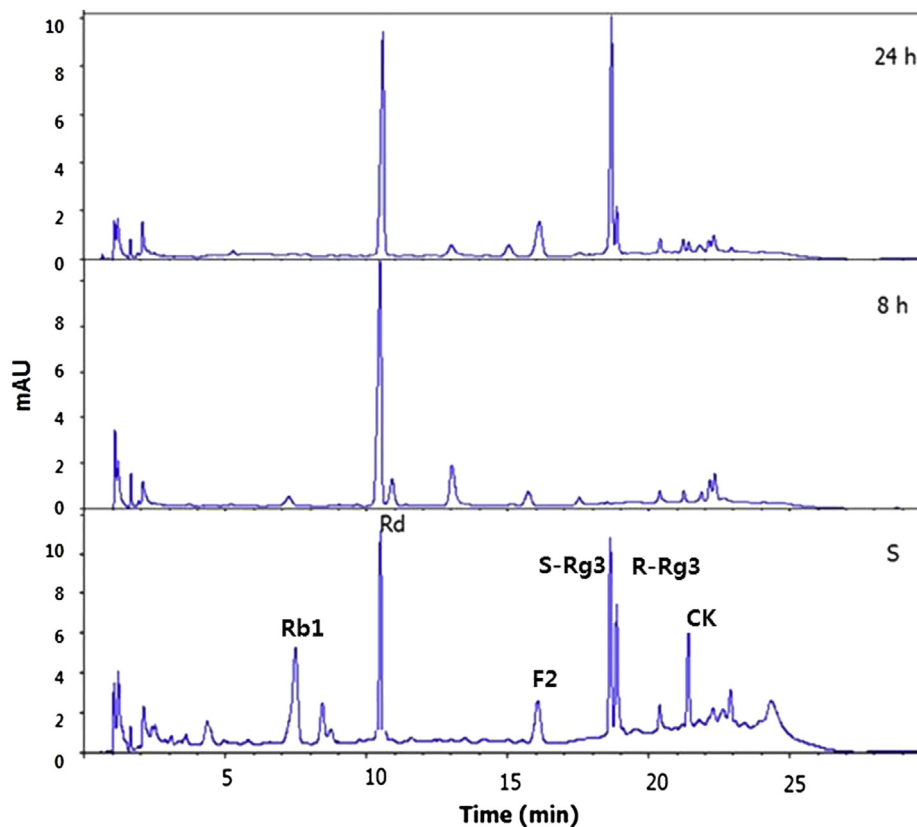

Fig. 4. Time-course HPLC analysis of metabolites of ginsenoside Rb1 converted by the crude enzyme from *Aspergillus niger* KCCM 11239. The crude enzyme was mixed with an equal volume of 1 mM ginsenoside Rb1 and reacted for 24 h at 30°C. HPLC, high performance liquid chromatography; S is the mixture of ginsenoside standards.

yields of Rg3. From these results, it appeared that β-glucosidase produced from *A. niger* strain had a greater hydrolytic activity on Rb1 than any other glucosidase tested in this study. Actually, a crude enzyme of this study has various glucosidase [25], and it is thought that hydrolysis of Rb1 is done by a combination of these glucosidases. As further research, we will examine the mechanism of hydrolysis with combined enzymes of crude samples. These compounds can be used for the development of new pharmaceutical materials such as antiturmeric agents and this is a valuable technique for bioconversion for new compounds in the pharmaceutical industry.

Conflicts of interest

All contributing authors declare no conflicts of interest.

Acknowledgments

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