Research article

Highly regioselective biotransformation of ginsenoside Rb2 into compound Y and compound K by β-glycosidase purified from Armillaria mellea mycelia

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Background: The biological activities of ginseng saponins (ginsenosides) are associated with type, number, and position of sugar moieties linked to aglycone skeletons. Deglycosylated minor ginsenosides are known to be more biologically active than major ginsenosides. Accordingly, the deglycosylation of major ginsenosides can provide the multibioactive effects of ginsenosides. The purpose of this study was to transform ginsenoside Rb2, one of the protopanaxadiol-type major ginsenosides, into minor ginsenosides using β-glycosidase (BG-1) purified from Armillaria mellea mycelium.

Methods: Ginsenoside Rb2 was hydrolyzed by using BG-1; the hydrolytic properties of Rb2 by BG-1 were also characterized. In addition, the influence of reaction conditions such as reaction time, pH, and temperature, and transformation pathways of Rb2, Rd, F2, compound O (C-O), and C-Y by treatment with BG-1 were investigated.

Results: BG-1 first hydrolyzes 3-O-outer β-D-glucoside of Rb2, then 3-O-β-D-glucoside of C-O into C-Y. C-Y was gradually converted into C-K with a prolonged reaction time, but the pathway of Rb2 → Rd → F2 → C-K was not observed. The optimum reaction conditions for C-Y and C-K formation from Rb2 by BG-1 were pH 4.0–4.5, temperature 45–60°C, and reaction time 72–96 h.

Conclusion: β-Glycosidase purified from A. mellea mycelium can be efficiently used to transform Rb2 into C-Y and C-K. To our best knowledge, this is the first result of transformation from Rb2 into C-Y and C-K by basidiomycete mushroom enzyme.

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1. Introduction

Ginseng, the root of Panax ginseng Meyer, contains various bioactive components, such as ginsenosides, polyacetylenes, phenolic acids, and polysaccharides [1–3]. Among these compound classes, ginsenosides are known as the major active compounds, and dammarane-type ginsenosides in ginseng are divided into two groups according to attached position and number of hydroxyl groups in dammarane-type skeletons, i.e., protopanaxadiol (PPD)-type and protopanaxatriol-type [5,4]. A few ginsenosides including Rd1, Rd2, and Rc constitute nearly 80–90% of the total ginsenosides of ginseng [5]. However, the absorption of these major ginsenosides in human intestine is known to be poor because of large molecular size, low water solubility of hydrophobic aglycones, and poor permeability through the cell membrane [6,7]. Meanwhile, the human intestinal bacterial metabolites, i.e., minor ginsenosides, such as compound O (C-O), C-Y, C-Mc, C-Mc1, and C-K, which are nearly absent in unprocessed ginseng, are more easily absorbed into the bloodstream [8,9]. Previous studies reported an attempt to convert major ginsenosides into minor ginsenosides by physical and chemical techniques [10–12], microbial [13,14] or enzymatic transformations [5,14]. In particular, minor ginsenosides could be prepared by crude microbial preparations or purified β-glycoside hydrolases

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from major ginsenosides, which were easily obtained from un-
processed extract [5,13].

Recently, biological effects of C-K, such as anti-inflammatory,
anticarcinogenic, antiangiogenesis, antiaging, antidiabetic, and
hepatoprotective activities, have been reported [14,15], but rela-
tively little is known about the bioactivity of C-Y [16]. However,
during microbial or enzymatic biotransformation of ginsenosides, it
is difficult to stop the hydrolysis of Rb2 at the stage of C-O or C-Y
formation because C-O or C-Y can be further transformed into C-K.
Furthermore, the application of some ginsenoside-hydrolyzing β-
glycosidases has a serious limitation in that they have poor activ-
ities against Rb2, which possess α-L-arabinopyranosidic linkage
[13]. Therefore, it is very useful to search for Rb2-hydrolyzing β-
glycosidases having a good regioselectivity and a high yield. Our
attention has been focused on the transformation of major ginse-
osides into minor ginsenosides by glycosidase hydrolyses secreted
from basidiomycetes mycelia. In previous studies [17,18], we found
that minor ginsenosides, C-K and C-Mc, could be efficiently pro-
duced from Rb1 and Rc by a crude enzyme extract or purified β-
glycosidase from Armillaria mellea mycelium. Rb2 has α-L-arabi-
nofuranosidic linkage different from Rb1 (∇-D-glucosidic) and Rc
(α-L-arabinofuranosidic) at the C-20 outer position of the aglycone.
Therefore, we report on the biotransformation of Rb2 using β-
glycosidase (BG-1) purified from A. mellea mycelium.

2. Materials and methods

2.1. Materials

Individual ginsenoside standards and the standard mixture
(Rb1, Rb2, Rc, Rd, Rg3, F2, Rh2, C-K) were provided by the Korea
Ginseng Corporation Research Institute (Daejeon, South Korea). C-Y
and C-O were provided by Professor Tae-Hoo Yi (Department of
Oriental Medicinal Materials and Processing College of Life Science,
Kyung-Hee University, Yongin, South Korea). Ginsenoside Rb2 was
also isolated from white ginseng roots [19]. p-Nitrophenol and p-
nitrophenyl-β-D-glucopyranoside were purchased from Sigma-
Aldrich Co. (St. Louis, MO, USA). Silica gel 60 (70–230 mesh) for
column chromatography and silica gel 60 F254 TLC plates were
purchased from Merck Co. (Darmstadt, Germany). HPLC-grade
distilled water and acetonitrile were purchased from J.T. Baker
(Center Valley, PA, USA). Other reagents (analytical grade) were
purchased from Daeyung Chemicals & Metals Co. (Siheung,
Gyonggi-do, South Korea).

![Fig. 1. Two main pathways of the transformation of ginsenoside Rb2 by microbial glycosidases [13]. ara (p), α-L-arabinopyranosyl; C-K, compound K; C-O, compound O; C-H, compound Y; Glc, β-D-glucopyranosyl.](image1)

![Fig. 2. TLC analysis of reaction products of Rb2 and ammonium sulfate (30–80%) precipitates from mushroom mycelia. The reaction mixture contained Rb2 (2.0 mg) and ammonium sulfate (30–80%) precipitate containing 1.5 U as a β-glucosidase in 1.0 mL of 0.2M acetate buffer (pH 4.5), and distilled water to make a final volume of 2.0 mL was incubated for 96 h at 45°C, respectively. After heating for 10 min in boiling water, the reaction mixture was extracted with water-saturated n-butanol (2.0 mL × 2) and concentrated. The residue was dissolved in methanol (1 mL), S, ginsenoside standard mixture; AM, Armillaria mellea; GL, Ganoderma lucidum; PB, Phellinus baumii; GA, Ganoderma aplanta; PO, Pleurotus ostreatus.](image2)
2.2. Production of mushroom mycelia and purification of β-glycosidase from A. mellea mycelium

The strains of five mushrooms—A. mellea (KACC 50013), Ganoderma lucidum (KACC 42231), Phellinus baumii (KACC 53719), Ganoderma applanata (KACC 53688), and Pleurotus ostreatus (KACC 50356)—were provided by the Korean Agricultural Culture Collection (Suwon, Gyeonggi-Do, South Korea). Unless otherwise stated, all procedures for mushroom mycelia production, preparations of crude enzymes (30–80% salt precipitates), and purification of β-glycosidase from A. mellea mycelium were performed according to the methods described in our previous studies [17,18].

2.3. Screening of ginsenoside Rb2-hydrolyzing activity using crude enzyme preparations from mushroom mycelia

A reaction mixture containing Rb2 (2.0 mg) as a substrate and each crude enzyme preparation (1.5 U as a β-glucosidase) containing β-glycosidase activity in 1.0 mL of 0.2M sodium acetate buffer (pH 4.5), and distilled water to make a final volume of 2.0 mL was incubated for 96 h at 45°C, respectively. After heating for 10 min in boiling water, the mixture was extracted with water-saturated n-butanol (2.0 mL x 2). The extract was concentrated under reduced pressure, dissolved in methanol (1.0 mL).

2.4. Influence of reaction condition on transformation of ginsenoside Rb2 by BG-1

For investigation of the time course of transformation of Rb2 by BG-1, the reaction mixture containing Rb2 (20 mg), BG-1 enzyme
(15 U), 10 mL of 0.2M acetate buffer (pH 4.5), and distilled water to make a final volume of 20 mL was incubated for 96 h at 45°C. The mixture (2.0 mL) during reaction was taken at different time intervals and extracted with water-saturated n-butanol (2.0 mL x 2). The extract was concentrated under reduced pressure, dissolved in methanol (1.0 mL), and was subjected to TLC and HPLC analysis. Reaction mixture (2.0 mL) containing individual ginsenoside (Rd, F2, C-O, or C-Y) in methanol (0.2 mL) and BG-1 (1.5 U) in 1.8 mL of 0.1M acetate buffer (pH 4.5) were incubated for 96 h at 45°C. The reaction mixture was extracted with water-saturated n-butanol (2.0 mL x 2). The extract was concentrated to dryness under reduced pressure, and the residue was dissolved in methanol (1.0 mL).

The influence of temperature and pH on transformation of Rb2 by BG-1 was investigated under the following conditions: temperature range 30–70°C and pH range 4.0–9.0. Each reaction was incubated for 96 h at 45°C.

2.5. General analytical methods

β-Glucosidase activity was assayed as described by Mfombep et al. [20]. TLC, HPLC, and ultra-high performance liquid chromatography-quadrupole time-of-flight mass spectrometry (UPLC/Q-TOF-MS) analysis were performed as described in our previous studies [17,18]. The relative ratio of the transformation products in the reaction mixtures was calculated from the peak area percentages in HPLC analysis without considering the detector response factor. HPLC analyses were performed in triplicate, and the results are expressed as the mean ± standard deviation.

3. Results

3.1. Biotransformation of ginsenoside Rb2 by BG-1

Microbial β-glycosidases hydrolyze Rb2 through two different main pathways as shown in Fig. 1 [13]. One pathway is Rb2 → Rd → F2 → C-K and another pathway is Rb2 → C-O → C-Y → C-K. In preliminary experiments, when crude enzyme preparations (30–80% ammonium sulfate precipitate) obtained from five mushrooms mycelia were incubated with Rb2 as a substrate, high Rb2-hydrolyzing activity was only observed with enzyme preparation from A. mellea, whereas other enzyme preparations showed very weak hydrolytic activities against Rb2 (Fig. 2). Therefore, this result demonstrated that A. mellea has the potential to be used to produce minor ginsenosides from Rb2.

The sample was taken from the reaction mixture at different time intervals during a 96-h reaction to investigate the transformation pattern of Rb2 by BG-1, and analyzed by TLC and HPLC. The TLC profile (Fig. 3) showed that Rb2 was gradually transformed into three products having different Rf values with those of the authentic standards. Rb2 was transformed into product 1 in the

![Fig. 5. MS spectra (negative ion mode) of the transformation products of Rb2 by BG-1. The reaction mixture (2.0 mL) contained Rb2 (2.0 mg) and BG1 (1.5 U) in 2.0 mL of 0.1M sodium acetate buffer (pH 4.5) and was incubated for 24 h at 45°C. After heating for 10 min in boiling water, the reaction mixture was analyzed by UPLC/Q-TOF-MS. (A) C-O m/z 961.5005 [M-H+HCOOH]+; (B) C-Y m/z 799.4680 [M-H+HCOOH]+; (C) C-K m/z 667.4415 [M-H+HCOOH]+. C-K, compound K; C-O, compound O; C-Y, compound Y.]
initial stage of the reaction. After 72–96 h reaction, almost all of the Rb2 and product 1 were transformed into products 2 and 3 (Fig. 4). The mixture after 24 h reaction was analyzed by UPLC/Q-TOF-MS to determine the molecular weights of the products (Fig. 5). Product 1 showed a quasi-molecular ion peak at \( m/z \) 961.5005 [M- H + HCOOH] (molecular formula C47H80O17). Product 2 showed a quasi-molecular ion peak at \( m/z \) 799.4680 [M-H + HCOOH] (molecular formula C41H70O12), and product 3 showed a quasi-molecular ion peak at \( m/z \) 667.4415 [M-H + HCOOH] (molecular formula C36H62O18). HPLC retention times and Rf values on TLC of products were also consistent with those of the authentic standards, C-O, C-Y, and C-K. These results suggested that BG-1 hydrolyzed \( \beta \)-sophorosyl moiety at the C-3 position of Rb2 to form C-Y via C-O, followed hydrolysis for C-K formation at the \( \alpha-\(1 \rightarrow 6\) \)-arabinopyranosidic linkage at the C-20 position with a prolonged reaction time, but the transformation pathway of C-K formation via Rd and F2 from Rb2 was not observed.

3.2. Influence of reaction conditions and substrate specificity

The enzymatic transformation of ginsenosides was highly influenced by reaction conditions such as pH and temperature. When the influence of pH on transformation of Rb2 by BG-1 was investigated at broad pH range (pH 4.0–9.0), C-Y and C-K formation reached their maxima in the range of pH 4.0–4.5 (Fig. 6). These results indicate that the optimum pH range for the transformation of Rb2 by BG-1 is between pH 4.0 and 4.5. Then the influence of temperature was investigated in the range of 30–70°C. The transformation of Rb2 was maximized at 40–55°C (Fig. 7). The Rb2 molecule contains two \( \beta \)-D-glucopyranosyl moieties at C-3 position and one \( \alpha \)-L-arabinopyranosyl and one \( \beta \)-D-glucopyranosyl moiety at C-20 position of aglycone skeleton. Therefore, Rb2 can be hydrolyzed by enzymes via more than one pathway. The results from TLC and HPLC analysis showed that the biotransformation of Rb2 by BG-1 followed one pathway as shown in Figs. 3 and 4. BG-1 attacked the outer and inner \( \beta \)-glucosidic linkage attached to the C-3 position of aglycone to form C-O and C-Y. To investigate whether BG-1 showed the same specificity and...
selectivity against glycosyl moieties attached to the C-3 and C-20 positions of other PPD-type ginsenosides, C-O, C-Y, Rd and F2 were also used as substrates. C-O was transformed into C-Y by hydrolysis of the glucosyl moiety at the C-3 position. When C-Y was used as a substrate, BG-1 was also gradually transformed C-Y into C-K by hydrolysis of the \(\alpha\)-L-arabinopyranosidic linkage at the C-20 position (Fig. 8). But Rd was transformed into C-K by way of F-2, formed by hydrolysis of outer \(\beta\)-glucosyl linkage at the C-3 position (Fig. 9).

4. Discussion

By screening for mushrooms that have Rb2-hydrolyzing activity, we found that a crude enzymatic preparation from \(A. mellea\) mycelium can efficiently transform Rb2 into C-Y and C-K, whereas crude enzymes prepared from other mushroom mycelia showed very weak transformation activities against Rb2. These results demonstrate that \(\beta\)-glycosidases from other mushroom mycelia used in this study could hardly hydrolyze the \(\alpha\)-(1 \(\rightarrow\) 6)-L-arabinopyranosidic linkage at the C-20 position of Rb2. Previous studies reported that contents of minor ginsenosides in red ginseng extracts or its by-products could be enhanced by fermentation with some mushrooms [21–23]. However, the enhanced component in fermented products by mushrooms used in previous studies was Rd that was contained in unprocessed ginseng rather than minor ginsenosides, The medicinal fungus \(Codyceps sinensis\) was also shown to be capable of producing C-K from Rb1 [24].

Ginsenoside-hydrolyzing enzymes can be divided into four categories, based on the hydrolyzing characteristics of glycosidic linkages of ginsenoside molecules [25,26]. Interestingly, type III enzyme could selectively hydrolyze 3-\(\beta\)-sophorosyl and the outer 20-\(\alpha\)-L-arabinopyranosidic linkages of Rb2 but not the inner 20-\(\beta\)-D-glucosidic linkage [26]. BG-1 also attacked the \(\beta\)-sophorosyl
linkage bonded to the hydroxyl group at C-3 and the outer 20-α-L-arabinopyranosidic linkage at C-20 to form C-Y via C-O in Rb2. C-Y was also very slowly transformed into C-K via F2. These results indicate that BG-1 has a weak activity in hydrolyzing the outer 20-α-L-arabinopyranosidic linkage of the aglycone [18]. Meanwhile, when Rb2 was used as a substrate, BG-1 transformed it into C-K via F2. These transformation characteristics of BG-1 on Rb2 are similar to those of previously published studies on Rb2 transformation by Rb2. The transformation characteristics of BG-1 on Rb2 are similar to the findings of glucosidic linkage bonded to the hydroxyl group at C-20 position of Rb2. The transformation characteristics of BG-1 on Rb2 are similar to those of previously published studies on Rb2 transformation by β-glucosidases from gut bacteria [9,27], Sulfobolus acidocaldarius [16], Microbacterium esteromartcum [28], Dictyoglomus turigdium [29], Penicillium aculeatum [30], Penicillium oxalicum [31], Sanguibacter keddeii [32], recombinant Sphingomonas sp. [33], and crude enzyme extracts from Aspergillus niger and Aspergillus usamii [34].

The optimum pH range for transformation of Rb2 into C-Y and C-K by BG-1 was between 4.0 and 4.5. β-Glucosidase activities secreted by white rot fungi such as A. mellea exhibited pH optima between 3.5 and 5.0 [20]. The influence of pH on enzymatic transformation of ginsenosides has been extensively investigated using microbial enzymes isolated from various sources. These enzymes have a weak activity in hydrolyzing the outer 20-α-L-arabinopyranosidic linkage at the C-20 position to form C-K given a prolonged reaction time. However, the use of a large quantity of enzyme (BG-1) did not result in the formation of C-Y and C-K pathway.

In conclusion, A. mellea β-glucosidase exhibited potent transformation activity against Rb2. The incubation conditions for optimum transformation of Rb2 into C-Y and C-K were a reaction time of 72–96 h, a pH of 4.0–4.5, and a temperature of 40–55 °C. The pathway for C-Y and C-K formation from Rb2 was Rb2 → C-O → C-Y, but the pathway Rb2 → G-Rd → G-F2 → C-K was not observed. C-Y was also slowly hydrolyzed at α-(1 → 6)-arabinopyranosidic linkage at the C-20 position to form C-K given a prolonged reaction time. The results of the present study suggest that β-glucosidase purified from A. mellea mycelium can be efficiently used to transform Rb2 into C-Y and C-K.

Conflicts of interest

All contributing authors declare that they have no conflicts of interest.

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