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Complete conversion of major protopanaxadiol ginsenosides to compound K by the combined use of α-L-arabinofuranosidase and β-galactosidase from *Caldicellulosiruptor saccharolyticus* and β-glucosidase from *Sulfolobus acidocaldarius*

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ABSTRACT

The ginsenoside compound K has pharmaceutical activities, including anti-tumor, anti-inflammatory, anti-allergic, and hepatoprotective effects. To increase the production of compound K, the α-L-arabinofuranoside-hydrolyzing α-L-arabinofuranosidase (CS-abf) and/or the α-L-arabinopyranoside-hydrolyzing β-galactosidase from *Caldicellulosiruptor saccharolyticus* (CS-bgal) were mixed with the β-D-glucopyranoside-hydrolyzing β-glucosidase from *Sulfolobus acidocaldarius* (SA-bglu). The optimum conditions for the production of ginsenoside compound K from ginsenoside Rc or Rb_2, or from major protopanaxadiol ginsenosides in ginseng root extract were determined to be pH 6.0 and 75 °C with 8 mg ml^{-1} ginsenoside Rc, 8 mg ml^{-1} Rb_2, or 10% (w/v) ginseng root extract; and 10.5 U ml^{-1} CS-abf or CS-bgal supplemented with 4.5 U ml^{-1} SA-bglu, or 10.5 U ml^{-1} CS-abf and 10.5 U ml^{-1} CS-bgal supplemented with 4.5 U ml^{-1} SA-bglu, respectively. Under optimum conditions, ginsenosides Rc and Rb_2, and major protopanaxadiol ginsenosides in ginseng root extract were completely converted to compound K after 12, 14, and 20 h, respectively, with the respective productivities of 388, 328, and 144 mg l^{-1} h^{-1}. This is the first report of the complete conversion of major protopanaxadiol ginsenosides to compound K.

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1. Introduction

Ginseng (*Panax ginseng* C. A. Meyer) has been used as a traditional medicine in Asian countries, to strengthen immunity, supply nutrition, and decrease fatigue. These beneficial functions are attributed mainly to the ginsenosides in ginseng. The minor ginsenosides (F_2, Rg_3, Rh_1, Rh_2, and compound K) are more pharmaceutically active than the major ginsenosides (Rb_1, Rb_2, Rc, Rd, Re, and Rg_1) (Takino, 1994), which comprise more than 80% of the total ginsenosides (Son et al., 2008). Therefore, many studies have focused on the production of minor ginsenosides by hydrolyzing the sugar moieties of major ginsenosides (Cheng et al., 2008; Quan et al., 2012b; Wang et al., 2011; Ye et al., 2010).

The ginsenoside compound K [20-*O*-β-D-glucopyranosyl-20(*S*)-protopanaxadiol] has attracted attention in recent years because of its pharmaceutical activities, including anti-tumor, anti-inflammatory, anti-allergic, and hepatoprotective effects (Choi et al., 2007; Choo et al., 2003; Lee et al., 2005; Park et al., 2009). Compound K is absent from ginseng root extracts (Yan et al., 2008; Yu et al., 2007), but has been produced from such extracts by fermentations using fungi (Chi and Ji, 2005; Han et al., 2007; Zhou et al., 2008) and enzymatic reactions using recombinant β-glucosidases (Kim et al., 2006; Ko et al., 2007; Yu et al., 2007). The fungus *Aspergillus usamii* produced 310 mg l^{-1} compound K from 998 mg l^{-1} Rb_1 for 48 h, with a molar conversion yield of 55% and a productivity of 6.5 mg l^{-1} h^{-1} (Chi and Ji, 2005), and *Pacilomyces bainier* produced 37.5 mg l^{-1} compound K from the saponin of *Panax notoginseng* leaves for 144 h, with a molar conversion yield of 83% and a productivity of 0.26 mg l^{-1} h^{-1} (Zhou et al., 2008). β-Glucosidase from *Aspergillus niger* produced 1300 mg l^{-1} compound K from 10% root extract of *Panax ginseng* for 24 h, with a molar conversion yield of 80% and a productivity of 54 mg l^{-1} h^{-1} (Kim et al., 2006), and β-glucosidase from *Sulfolobus solfataricus* produced 1630 mg l^{-1} compound K from 10% root extract for 12 h, with a molar conversion yield of 80% and a productivity of 136 mg l^{-1} h^{-1} (Noh et al., 2009). The production of compound K by enzymatic reactions using β-glucosidases showed higher concentration and productivity than that by fermentations using fungi. However, the complete conversion of ginsenoside Rc or Rb_2, or major protopanaxadiol ginsenosides in ginseng root extract to compound K using microorganisms and enzymes has not been reported.

In this study, the complete conversion of ginsenosides Rc and Rb_2, and major protopanaxadiol ginsenosides in ginseng root extract to compound K was achieved by the combined use of

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the α-L-arabinofuranoside-hydrolyzing α-L-arabinofuranosidase from *Caldicellulosiruptor saccharolyticus* (CS-abf) and/or the α-L-arabinopyranoside-hydrolyzing β-galactosidase from *C. saccharolyticus* (CS-bgal) with the β-D-glucopyranoside-hydrolyzing *Sulfolobus acidocaldarius* β-glucosidase (SA-bglu).

2. Materials and methods

2.1. Materials

The ginsenoside standards Rb_1, Rb_2, Rc, Rd, compound Y, compound Mc, and compound K were purchased from BTGin (Daejeon, Korea). To prepare ginseng root extract, 1 l of methanol/water mixture (4:1, v/v) was added to 100 g of dry ginseng root powder and held at 80 °C for 1 h. After cooling, the mixture was filtered, the filtrate was evaporated to remove the methanol, and the residue was dissolved in 1 l of distilled water. The solution was then adsorbed onto a Diaion HP-20 resin, which was rinsed with distilled water to elute the free sugars, leaving the ginsenosides attached to the resin. The adsorbed ginsenosides were successively eluted with methanol, the eluant was evaporated to remove the methanol, and the residue was dissolved in the same volume of distilled water as the original loading volume. Sugar-free ginseng root extract was used because Maillard reactions between free sugars and the enzyme occurred at temperatures above 70 °C.

2.2. Bacterial strains, plasmids, and culture conditions

C. saccharolyticus DSM 8903 and *S. acidocaldarius* DSM 639, *Escherichia coli* ER2566, and plasmid pTrc99A were used as the sources of α-L-arabinofuranosidase, β-galactosidase, and β-glucosidase genes, host cells, and expression vectors, respectively. *C. saccharolyticus* and *S. acidocaldarius* were grown at 70 °C under anaerobic conditions using 100% N_2 gas for 5 days on *Caldicellulosiruptor* medium (DSM Media Formulation No. 640) and *Sulfolobus* medium (DSM Media Formulation No. 88), respectively. The recombinant *E. coli* for protein expression was cultivated with shaking at 200 rpm in a 2-l flask containing 500 ml of Luria-Bertani (LB) medium at 37 °C with 20 μg ml^{-1} of kanamycin. When the optical density of the bacteria reached 0.8 at a wavelength of 600 nm, isopropyl-β-D-thiogalactopyranoside was added to a final concentration of 0.1 mM to induce enzyme expression, after which the culture was incubated at 16 °C with shaking at 150 rpm for 16 h.

2.3. Gene cloning and expression

The genomic DNAs from *C. saccharolyticus* and *S. acidocaldarius* were extracted using the genomic DNA extraction kit (Qiagen, Hilden, Germany). The genes encoding CS-abf, CS-bgal, and SA-bglu were amplified from the each genomic DNA as templates by polymerase chain reaction (PCR) using *Pfu* DNA polymerase (Solgent, Daejeon, Korea). The sequences of the oligonucleotide primers used for gene cloning were based on the DNA sequences of CS-abf (GenBank accession number, CP000679), CS-bgal (GenBank accession number, YP 001179823), and SA-bglu (GenBank accession number, YP 256448). Forward (5′-TTTGGATCCATGAAAAAAGCAAAAGTCATCTA-3′) and reverse primers (5′-TTTCTGCAGTTAATTTTCTCTCTTCTTCAATCTG-3′) were designed to introduce the *BamH*I and *Pst*I restriction sites (underlined) for CS-abf gene insertion, forward (5′-CACTGGATCCATGGCTAAAATCAAACTGAAA-3′) and reverse primers (5′-CGCCGTCGACTTTTCTTAATATCATAACTTCTTTT-3′) were designed to introduce the *BamH*I and *Sal*I restriction sites for CS-bgal gene insertion, and forward (5′-GAATTCATGTTATCATTCCCAAAGGGTTTC-3′) and reverse primers (5′-CTGCAGTTAATGTCTCAAAGGTTTTATTGGTGG-3′) were designed to introduce the *Eco*RI and *Pst*I restriction sites for SA-bglu gene insertion. Each amplified DNA fragment obtained by PCR was purified and digested with the respective two restriction endonucleases (Lim et al., 2010; Park et al., 2010). The digested DNA fragment was extracted from gel using the QIA quick gel extraction kit (Qiagen, Hilden, Germany), and then inserted into the pTrc99A vector digested with the same restriction enzymes. *E. coli* ER2566 strain was transformed with the ligation mixture and plated on LB agar containing 50 μg ml^{-1} of ampicillin. Ampicillin resistant colony was selected, and plasmid DNA from the transformant was isolated using a plasmid purification kit (Promega, Madison, WI). DNA sequencing was performed at the Macrogen facility (Seoul, Korea). The expression of CS-abf, CS-bgal, and SA-bglu genes was determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and by enzyme activity assay.

2.4. Enzyme purification

The harvested *E. coli* cells were resuspended in 50 mM Tris–HCl buffer (pH 7.0) containing 0.1 mM phenylmethylsulfonyl fluoride as protease inhibitor. The resuspended cells were disrupted by sonication using Sonic Dismembrator (Fisher Scientific Model 100, Pittsburgh, PA, USA) on ice for 2 min. The unbroken cells and cell debris were removed by centrifugation at 13,000 *g* for 20 min at 4 °C, and the supernatant obtained was used as a crude extract. The crude extract was subsequently heated at 75 °C for 10 min, the suspension was centrifuged at 13,000 *g* for 20 min to remove insoluble denatured proteins. The supernatants were used as the soluble enzyme.

2.5. Hydrolytic activity

One unit (U) of CS-abf, CS-bgal, or SA-bgl activity was defined as the amount of enzyme required to liberate 1 nmol of ginsenoside Rd, Rd, or compound K as a product, respectively, from ginsenoside Rc, Rb_2, or Rd as a substrate, respectively, per min at 80 °C and pH 6.0. Unless otherwise indicated, the hydrolytic reactions were performed at 80 °C for 1 h in 50 mM citrate/phosphate buffer (pH 6.0) containing 0.4 mg ml^{-1} ginsenoside Rc or Rb_2, 1.4 U ml^{-1} CS-abf or CS-bgal supplemented with 0.6 U ml^{-1} SA-bgl, respectively.

2.6. Optimization of reaction conditions for compound K production

The effect of the ratio of CS-abf to SA-bglu or CS-bgal to SA-bglu on compound K production was investigated by the two-enzyme system which was obtained by mixing CS-abf or CS-bgal with SA-bglu, at ratios of 0:2 to 2:0 U ml^{-1}. The effects of pH and temperature on compound K production were investigated by varying the pH from 5.0 to 7.0, and the temperature from 70 to 90 °C. The effect of temperature on the stability for the mixture of two enzymes was monitored as a function of incubation time by maintaining the solution of enzymes at five different temperatures (65, 70, 75, 80, and 85 °C). The effects of the concentrations of the two enzymes on compound K production were evaluated with 4 mg ml^{-1} ginsenoside Rc or Rb_2 after 2 h by varying the concentration of CS-abf or CS-bgal relative to that of SA-bglu, ranging from 3.5 and 1.5 U ml^{-1} to 14 and 6 U ml^{-1}.

2.7. Conversions of ginsenoside Rc, ginsenoside Rb_2, and ginseng root extract to compound K

The conversion of ginsenoside Rc or Rb_2 to compound K was performed at 75 °C in 50 mM citrate/phosphate buffer (pH 6.0) containing 8 mg ml^{-1} ginsenoside Rc or Rb_2, 10.5 U ml^{-1} CS-abf or

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