Journal of Biotechnology 161 (2012) 294–301

Contents lists available at SciVerse ScienceDirect

Journal of Biotechnology

journal homepage: www.elsevier.com/locate/jbiotec

Bioconversion of major ginsenosides Rg_1 to minor ginsenoside F_1 using novel recombinant ginsenoside hydrolyzing glycosidase cloned from *Sanguibacter keddieii* and enzyme characterization

Jin-Kwang Kim[a,b], Chang-Hao Cui[c], Min-Ho Yoon[b], Sun-Chang Kim[a,c], Wan-Taek Im[a,*]

[a] *KAIST Institute for Biocentury, Korea Advanced Institute of Science and Technology, 291 Daehak-ro, Yuseong-gu, Daejeon 305-701, Republic of Korea*
[b] *Department of Bio-Environmental Chemistry, College of Agriculture and Life Sciences, Chungnam National University, 99 Daehak-ro, Yuseong-gu, Daejeon 305-764, Republic of Korea*
[c] *Department of Biological Sciences, Korea Advanced Institute of Science and Technology, 291 Daehak-ro, Yuseong-gu, Daejeon 305-701, Republic of Korea*

ARTICLE INFO

Article history:
Received 28 March 2012
Received in revised form 18 June 2012
Accepted 19 June 2012
Available online 2 July 2012

Keywords:
Ginsenoside
Bioconversion
β-Glucosidase
Sanguibacter keddieii
Rg_1
F_1

ABSTRACT

This study focuses on the cloning, expression, and characterization of recombinant ginsenoside hydrolyzing glycosidase from *Sanguibacter keddieii* in order to biotransform ginsenosides efficiently. The gene, termed *bglSk*, consists of 1857 bp and revealed significant homology to that of glycoside hydrolase family 3. The enzyme was over-expressed in *Escherichia coli* BL21 (DE3) using a GST-fused pGEX 4T-1 vector system. The over-expressed recombinant enzymes could convert six major ginsenosides Rb_1, Rb_2, Rc, Rd, Re and Rg_1 into more pharmacologically active rare ginsenosides such as C-Y, C-Mc, C-K, Rg_2(S), and F_1. Especially, BglSk could completely convert the Rg_1 into F_1. The GST-fused BglSk was purified with GST·bind agarose resin and then characterized. The kinetic parameters for β-glucosidase had apparent K_m values of 0.456 ± 0.009 and 0.167 ± 0.003 mM and V_{max} values of 30.2 ± 0.7 and 4.1 ± 0.1 μmol min^{-1} mg of protein^{-1} against p-nitrophenyl-β-D-glucopyranoside and Rb_1, respectively.

1. Introduction

Ginseng is one of the most famous medicinal plants in the world (Attele et al., 1999). In particular, in Asian countries, ginseng has been used for thousands of years to heal disease and stay healthy (Qi et al., 2011). Ginsenosides are the major active components in the biological and pharmacological effects of ginseng (Park et al., 2005). In general, the whole ginsenoside family exhibits a variety of pharmaceutical functions: immunomodulatory, anti-tumor, anti-carcinogenic, anti-inflammatory, anti-allergic, anti-atherosclerotic, anti-stress, anti-diabetic, anti-proliferation, anti-genotoxic, and anti-hypertensive functions as well as neurotransmission modulation effects (Choi et al., 2011; Jia and Zhao, 2009; Sun, 2004). To date, over 150 types of naturally occurring ginsenosides have been isolated (Christensen, 2009); however, six types of major ginsenoside (Rb_1, Rb_2, Rc, Rd, Re, and Rg_1) constitute the main portion of Korean and American ginseng (Qu et al., 2009; Shi et al., 2007).

The absorption of the major ginsenosides in the gastrointestinal tract is quite poor (Tawab et al., 2003). Due to their size, low solubility, and poor permeability across the cell membrane, it is difficult for the human body to directly absorb large ginsenosides (Xu et al., 2003), although these components constitute the major portion of the total ginsenosides in raw ginseng (Park et al., 2005). Therefore, the transformation of these major ginsenosides (Rb_1, Rb_2, Rc, Rd, Re, and Rg_1) into smaller deglycosylated rare ginsenosides (C-K, C-Y, C-Mc, F_2, and F_1), which are more effective for in vivo physiological actions, is required (Akao et al., 1998; Quan et al., 2011; Tawab et al., 2003). Thus, enzymatic methods have been researched

Abbreviations: Ginsenoside Rb_1, 3-*O*-[β-D-glucopyranosyl-(1-2)-β-D-glucopyranosyl]-20-*O*-[β-D-glucopyranosyl-(1-6)-β-D-glucopyranosyl]-20(*S*)-protopanaxadiol; Ginsenoside Rb_2, 3-*O*-[β-D-glucopyranosyl-(1-2)-β-D-glucopyranosyl]-20-*O*-[α-L-arabinopyranosyl-(1-6)-β-D-glucopyranosyl]-20(*S*)-protopanaxadiol; Ginsenoside Rc, 3-*O*-[β-D-glucopyranosyl-(1-2)-β-D-glucopyranosyl]-20-*O*-[α-L-arabinofuranosyl-(1-6)-β-D-glucopyranosyl]-20(*S*)-protopanaxadiol; Ginsenoside Rd, 3-*O*-[β-D-glucopyranosyl-(1-2)-β-D-glucopyranosyl]-20-*O*-β-D-glucopyranosyl-20(*S*)-protopanaxadiol; Gypenoside XVII, 3-*O*-β-D-glucopyranosyl-20-*O*-[β-D-glucopyranosyl-(1-6)-β-D-glucopyranosyl]-20(*S*)-protopanaxadiol; Ginsenoside C-O, 3-*O*-β-D-glucopyranosyl-20-*O*-[α-L-arabinopyranosyl-(1-6)-β-D-glucopyranosyl]-20(*S*)-protopanaxadiol; Ginsenoside C-Mc_1, 3-*O*-β-D-glucopyranosyl-20-*O*-[α-L-arabinofuranosyl-(1-6)-β-D-glucopyranosyl]-20(*S*)-protopanaxadiol; Ginsenoside F_2, 3-*O*-β-D-glucopyranosyl-20-*O*-β-D-glucopyranosyl-20(*S*)-protopanaxadiol; Ginsenoside C-Y, 20-*O*-[α-L-arabinopyranosyl-(1-6)-β-D-glucopyranosyl]-20(*S*)-protopanaxadiol; Ginsenoside C-Mc, 20-*O*-[α-L-arabinofuranosyl-(1-6)-β-D-glucopyranosyl]-20(*S*)-protopanaxadiol; Ginsenoside Rg_3(S), 3-*O*-[β-D-glucopyranosyl-(1-2)-β-D-glucopyranosyl]-20(*S*)-protopanaxadiol; Gypenoside LXXV, 20-*O*-[β-D-glucopyranosyl-(1-6)-β-D-glucopyranosyl]-20(*S*)-protopanaxadiol; Ginsenoside Rh_2(S), 3-*O*-β-D-glucopyranosyl-20(*S*)-protopanaxadiol; Ginsenoside C-K, 20-*O*-β-D-glucopyranosyl-20(*S*)-protopanaxadiol.

* Corresponding author. Tel.: +82 42 3504450; fax: +82 42 3504451.
E-mail address: wandra@kaist.ac.kr (W.-T. Im).

0168-1656/$ – see front matter © 2012 Elsevier B.V. All rights reserved.
http://dx.doi.org/10.1016/j.jbiotec.2012.06.021

as a method of converting the major ginsenosides into more pharmacologically active rare ginsenosides in a more specific manner (Kim et al., 2006; Ko et al., 2000). Many ginsenoside-transforming glycoside hydrolases (GHs) have been recently purified and cloned from various sources (Park et al., 2010).

A classification method has been provided to sort these ginsenoside-hydrolyzing enzymes with transformation-pathways. Purified enzymes from *Aspergillus* spp. and *Terrabacter ginsenosidimutans* (An et al., 2010) were characterized and classified into type I, II, III and IV ginsenosidases (Jin et al., 2012; Wang et al., 2011, 2012; Yu et al., 2007, 2009). This classification helped researchers to compare the ginsenoside-transforming activities clearly by transforming-pathways, but it could not cover the all kinds of pathways because of diversity of enzyme activity. Neither has it showed the correlations between protein structure and the transforming pathways because of lack of their sequence information. Thus, the sequenced and recombinant ginsenoside transforming GHs is highly required to reveal the diverse ginsenoside-transformation pathways and have more potential to be used for production for minor ginsenosides specifically.

Ginsenoside F_1 has profound pharmaceutical activities that function as anti-aging and anti-oxidant agents, protect human HaCaT keratinocytes from UVB-induced apoptosis (Lee et al., 2003), and suppress platelet aggregation (Wang et al., 2008). Although F_1 was identified in 1976 (Yahara, 1979), its pharmacology activity was only reported recently (Lee et al., 2003; Zhang et al., 2001a,b) due to the difficulties of obtaining sufficient amounts of F_1 for bioactivity tests, because it is present in ginseng leaf in relatively low concentrations. F_1 production is possible to employ the fermentation or enzymatic method converting ginsenoside Rg_1 or Re, which have glucose residue at the C6 position into F_1 by removing the glucose moiety. For example, ginsenoside F_1 was produced from ginsenoside Rg_1 by *Leuconostoc paramesenterioides* strain PR (Chi and Ji, 2005), naringinase from *Penicillium decumbens* (Ko et al., 2003), and purified β-glucosidase from *Fusarium moniliforme* var. *subglutinans* (Kim et al., 2011).

Recently, the construction of several recombinant enzymes was reported for the large-scale industrial production of particular ginsenosides (An et al., 2010; Wang et al., 2011; Quan et al., 2012). Regarding the process of locating the ginsenoside converting enzyme that has a different pathway as reported previously, a novel gene encoding ginsenoside hydrolyzing glycosidase (BglSk) from *Sanguibacter keddieii* was cloned in this study. Furthermore, the enzymatic properties and substrate specificities of the recombinant enzymes were thoroughly investigated.

2. Materials and methods

2.1. Chemicals

Ginsenosides Rb_1, Rc, Rb_2, Rd, Rg_3(S), Rh_2(S), Rh_2(R), F_2, compound K, protopanaxadiol (PPD), Rg_1, Re, Rg_2(S), Rh_1(S), F_1, protopanaxatriol (PPT) were purchased from Nanjing Zelang Medical Technology Co., Ltd. (China). Ginsenosides gypenoside XVII, gypenoside LXXV, compound O, compound Y, compound Mc_1, and compound Mc were prepared as described by An et al. (2010) and Wang et al. (2011). 5-Bromo-4-chloro-3-indolyl β-D-glucopyranoside (X-Glc), PNP-β-D-glucopyranoside (pNPGlc), PNP-β-D-galactopyranoside, PNP-β-D-fucopyranoside, PNP-N-acetyl-β-D-glucosaminide, PNP-β-L-arabinopyranoside, PNP-β-D-mannopyranoside, PNP-β-D-xylopyranoside, PNP-α-D-glucopyranoside, PNP-α-L-arabinofuranoside, PNP-α-L-arabinopyranoside, PNP-α-L-rhamnopyranoside, PNP-α-D-mannopyranoside, PNP-α-D-xylopyranoside, ONP-β-D-glucopyranoside, ONP-β-D-galactopyranoside, ONP-β-D-fucopyranoside and ONP-α-D-galactopyranoside were obtained from Sigma. The other chemicals used in this study were at least of analytical reagent grade, and the sources are noted individually in Section 2.

2.2. Bacterial strains, vectors and media

The genomic DNA from *Sanguibacter keddieii* KACC 14479^T, *Escherichia coli* BL21 (DE3), and pGEX 4T-1 plasmid (GE Healthcare, USA) were used as a source of ginsenoside hydrolyzing glycosidase gene, a host, and a vector for expression, respectively. *S. keddieii* KACC 14479^T was grown aerobic conditions at 30 °C on R2A agar (BD, USA). The recombinant *E. coli* for protein expression was cultivated in a Luria–Bertani (LB) medium supplemented with ampicillin (100 mg/l).

2.3. Analytical methods of ginsenosides

The TLC analysis was performed using $60F_{254}$ silica gel plates (Merck, Germany) with $CHCl_3$–CH_3OH–H_2O (65:35:10, v/v, lower phase) as the developing solvent. The spots on the TLC plates were detected by spraying with 10% (v/v) H_2SO_4 followed by heating at 110 °C for 5 min. The HPLC analysis of the ginsenosides was performed using an HPLC system (Younglin Co. Ltd., Korea), with a quaternary pump, automatic injector, single wavelength UV detector (model 730D), and Younglin's AutoChro 3000 software for peak identification and integration. The separation was carried out on a Prodigy ODS(2) C_{18} column (5 μm, 150 × 4.6 mm i.d.) (Phenomenex, USA) with a guard column (Eclipse XDB C_{18}, 5 μm, 12.5 × 4.6 mm i.d.). The mobile phase was A (acetonitrile) and B (water). Gradient elution started with 17% solvent A and 83% solvent B changed to: A from 17 to 25%; 12–20 min, A from 25 to 32%; 20–30 min, A from 32 to 55%; 30–35 min, A from 55 to 60%; 35–40 min, A from 60 to 80%; 40–45 min, A from 80 to 100%; 45–50 min, A 100%; 50–54 min, A from 100 to 17%; 54.0–54.1 min, A 17%; 54.1–65 min. The flow rate was 1.0 ml/min, and detection was performed by monitoring absorbance at 203 nm and an inject volume of 25 μl. Electrospray ionization mass spectra (ESI-MS) were measured for eight kinds of biotransformed ginsenosides (GypLXXV, C-K, C-O, C-Y, C-Mc_1, C-Mc, F_1, F_2) after purification steps on a triple-quadrupole tandem mass spectrometer (API-2000, Applied Biosystems, Foster City, USA) with negative ion mode. The ESI parameters were as follows: ionspray voltage, −4200 V; ion source gas 1 (GS1), 20; curtain gas (CUR), 20; collision gas (CAD), 2. The declustering potential (DP), focusing potential (FP), entrance potential (EP), collision cell exit potential (CXP) and collision energy (CE) were variant with regard to measured ginsenosides. For full-scan MS analysis, the spectra were recorded in the m/z range from 400 to 1000.

2.4. Molecular cloning, expression, and purification of recombinant BglSk

The genomic DNA from *S. keddieii* KACC 14479^T was extracted using a genomic DNA extraction kit (Solgent, Korea). The gene encoding ginsenoside hydrolyzing glycosidase was amplified from the genomic DNA as a template via a polymerase chain reaction (PCR) using *Pfu* DNA polymerase (Solgent, Korea). The sequence of the oligonucleotide primers used for the gene cloning was based on the DNA sequence of *S. keddieii* β-glucosidase (GenBank accession number, ACZ20402). Forward (5′-GGT TCC GCG TGG ATC CCC CAC TCC CCT GAC CAC CCT GAC C-3′) and reverse (5′-GAT GCG GCC GCT CGA GTC AGA TGC TCA GCC CGT GCC CCA C-3′) primers were designed as primers to introduce the BamHI and XhoI restriction sites (underlined), respectively, and were synthesized by Macrogen Co. Ltd. (Korea). The amplified DNA fragment obtained from the PCR was purified and then inserted into the pGEX 4T-1 GST