Identification of novel glycoside hydrolases via whole genome sequencing of *Niabella ginsenosidivorans* for production of various minor ginsenosides

Muhammad Zubair Siddiqi^{1,2} · Majid S. Hashmi³ · Jung-Mi Oh⁴ · Sungkun Chun⁴ · Wan-Taek Im^{1,2}

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Abstract

In this study, many bacterial strains were screened for the production of minor ginsenosides, but based on conversion competence among the strains, the strain *Niabella ginsenosidivorans* BS26^T has the good ginsenoside-transforming ability. Therefore, the strain BS26^T was selected for complete genome sequence analysis to determine the target (glycoside hydrolase) functional genes. Whole genome analysis of strain BS26^T showed 43 glycoside hydrolase genes in total. To determine the target functional gene, 12 sets of six different glycoside hydrolases (3 set of β -glucosidase; 3 set of trehalase; 3 set of arabinofuranosidase; 2 set of xylosidase; and one set of each α -galactosidase and α -fucosidase, respectively) were selected and cloned in *E. coli* BL21 (DE3) using the pGEX4T-1 vector and were characterized. Among these 12 sets of clones, only one, β -glucosidase (BglNg-767), showed ginsenoside conversion ability. The BglNg-767 comprised 767 amino acids and belonged to glycoside hydrolase family 3 (GH3). The recombinant GST-BglNg-767 was capable of altering the ginsenosides Rb₁, Rd, and gypenoside XVII (Gyp-XVII) to F₂; Rb₂ to C-O; Rb₃ to C-Mx1, and Rc to C-Mc1. Besides, complete genome sequence analysis of strain BS26^T also indicates 30 endopeptidase genes, which may be responsible for self-hydrolysis of the proteins. Therefore, using SDS-PAGE analysis, we predict that the difference between the molecular weight of the expressed protein (around 90 kDa) and the predicted amino-acid sequence (102.7 kDa) is due to self-hydrolysis of the proteins.

Keywords *Niabella ginsenosidivorans* · Whole genome sequencing · Bioconversion · Ginsenosides · Recombinant β -glucosidase

Introduction

Ginseng, *Panax Ginseng* was discovered over 2000 years ago in Korea. Taking up to 6 years to mature, this unique root has a plant that grows from 8 to 15 in. in height. The unique feature of this plant is that it grows in cool moist soil that is frequently watered and that the root attains maturity after up to 6 years with the aerial plant growth reaching 8–15 in. in height (Court 2000). Many accounts of ancient Korean history indicate that ginseng was used as a portion of food among farmers and labours for medicinal purposes and was admired for its strength-giving properties and refreshing powers. Ginseng was and still remains a powerful tonic among herbal medicines. Ginseng currently occupies a dominant position in the list of best-selling herbal medicinal plants worldwide (Yun 2001).

Science and technology have proven the therapeutic power of ginseng and revealed the presence of some valuable major compounds commonly known as saponins or

✉ Wan-Taek Im
wandra@hknu.ac.kr

¹ Department of Biotechnology, Hankyong National University, 327 Jungang-ro, Anseong-si, Gyeonggi-do 17579, Republic of Korea

² AceEMzyme Co., Ltd., Room 403, Academic Industry Cooperation, 327 Jungang-ro, Anseong-si, Gyeonggi-do 17579, Republic of Korea

³ Department of Food Science and Technology Faculty of Nutrition Sciences, The University of Agriculture, Peshawar, Khyber Pakhtunkhwa 25120, Pakistan

⁴ Department of Physiology, Medical School, Chonbuk National University, Geonji-ro 20, Jeonju, Jeonbuk 54907, Republic of Korea

major ginsenosides. These major active compounds of *Panax ginseng* are categorized as protopanaxadiol (PPD), protopanaxa triol (PPT), and oleanane-type ginsenosides. The Ginseng root contains more than 90% of PPD- and PPT-type major ginsenosides including Rb₁, Rb₂, Rb₃, Rc, Rd, Re, and Rg₁ (Christensen 2009). Because of the high molecular weight of these major ginsenosides, it is difficult for the human gastrointestinal tract to absorb them into the bloodstream (Tawab et al. 2003; Xu et al. 2003). Therefore, these macro-compounds are transformed into minor ginsenosides for better pharmacological effects. Compared to major ginsenosides, minor ginsenosides [F₂, C–K, Rg₃, Rh₂, and F₁] are present in small amount in the total ginseng extract and also demonstrate high efficacy of anti-oxidative, anti-cancer, anti-aging, anti-osteoporosis, anti-inflammatory, and anti-diabetic effects (Choi et al. 2011a; Kim et al. 2013; Lee et al. 2011; Leung and Wong 2010; Baatar et al. 2018; Siddiqi et al. 2014, 2015), compared to major ginsenosides. To obtain minor ginsenosides from major ginsenosides, three different conversion methods [microbial enzymatic, physical or heat, and chemical (acid or base) treatment] are used to target minor ginsenosides. However, recombinant enzymatic transformation is currently considered a suitable conversion technology for the production of target minor ginsenosides owing to its controlled environmental conditions compared to the physical and chemical conversion methods. Thus, researchers are still trying to determine more suitable and powerful recombinant enzymes for enhanced production (gram unit) of some valuable minor ginsenosides such as Rg₃, Rh₂, F₂, C–K, and others. As a result, our research team succeeded in gram unit production of ginsenoside Rh₂-Mix using a recombinant glucoside hydrolase in 2017 [(BglPm_C) Siddiqi et al. 2017a], but this production was only limited to 20(S)-Rh₂, 20(R)-Rh₂, Rh₃, and Rk₂. Therefore, in this study, we discovered a novel glucoside hydrolase through complete genome sequencing for the production of many kinds of minor ginsenosides. For the target strain selection and functional gene identification, initial biotransformation was carried out as described previously (Siddiqi et al. 2017b).

During the screening of the conversion of ginsenosides, strain BS26^T exhibited efficient conversion of major ginsenosides. The strain was thus subjected to complete genome sequencing to determine the target genes. After complete genome analysis, 12 sets of six (6) different glycoside hydrolases [β , xylosidase, α -galactosidase, α -fucosidase, and β -arabinofuranosidase] were selected for cloning. Thus, among these 12 sets of clones, only one recombinant β -glucosidase (named as BglNg-767) was characterized, which showed the conversion of ginsenosides and belonged to the glycoside hydrolase family 3.

Materials and methods

Materials

The PPD- and PPT-type ginsenoside standards [$\geq 98\%$, Rb₁, Rb₂, Rb₃, Rc, Rd and Gyp-XVII, Re, and Rg₁] were purchased from Republic of Korea (AceEMzyme Co., Ltd.) and were used as the primary substrate in this study. Various types of mono-sugar substrates [with the configuration of *p*-nitrophenyl (*p*NP) and *o*-nitrophenyl (*o*NP)] and other chemicals used in the study were purchased from Sigma.

Screening of β -glucosidase activity of strain BS26^T

To determine the β -glucosidase-positive activity of strain BS26, the strain was transferred to R2A agar plate containing 1.0 g/l esculin and 0.5 g/l ferric citrate. The plates were then incubated at 30 °C for 2 days. The release of esculetin (reddish-brown zone) from esculin was selected and used for the bioconversion of ginsenosides as described previously (Siddiqi et al. 2017b).

Identification and cloning of genes involved in ginsenoside metabolism

Primers designing

Based on the ginsenoside conversion activity, the genomic DNA of the strain BS26^T was extracted by genomic DNA extraction kit and whole genome sequenced by Macrogen Co., Ltd. (Korea). After the whole genome sequencing, the whole genome sequence was accessioned and annotated by NCBI (<https://www.ncbi.nlm.nih.gov>; <https://img.jgi.doe.gov>) under accession number CP015772, as described previously (Lee et al. 2018). Thirty-four (34) target genes for ginsenoside conversion were identified from the WGS by Macrogen Co., Ltd. (Korea) from which six (6) different glycoside hydrolases (trehalase, β -glucosidase, xylosides, α , β -arabinofuranosidase, α -galactosidase, and α -fucosidase) were chosen for cloning and further studies. The oligonucleotide primers designed for cloning of these genes were synthesized with BamHI and XhoI restriction sites by Macrogen Co., Ltd. (Korea) (Table 1). However, in this paper, we focus on the cloning and characterization of a β -glucosidase.

Strain BS26^T is available from the host institute (Department of Biotechnology, Hankyong National University, Anseong-si, South Korea) and from two culture collections KACC 16620^T (Republic of Korea) and JCM 18199^T (Japan).

Table 1 Name of glycoside hydrolases and sets of primers used in this study (sequences 5' → 3')

No of clones	Names	Total amino acids	Ginsenosides conversion activity	Primers sequences (F, R)	Vector
1	Trehalase-643	643	—	GGTTCCGCGTGGATCCAGAAAATGTATGATGGG TTTT_F GATGCGGCCGCTCGAGTTAGTTATAATACCCTT GTTC_R	pGEX 4T-1
2	β -Glucosidase-788	788	—	GGTTCCGCGTGGATCCTTGAAACAGGCGTTTGC GGTGT_F GATGCGGCCGCTCGAGCTATTCCAGCTCGATCT GTTT_R	
3	Trehalase-440	440	—	GGTTCCGCGTGGATCCAAATGGAGGTTTAAAGC GGTA_F GATGCGGCCGCTCGAGTTATTTATTCCTGTCTG CTTC_R	
4	β -Glucosidase-784	784	—	GGTTCCGCGTGGATCCTGTATCTACAATCACCT GATC_F GATGCGGCCGCTCGAGTCAGCGTTGTTTATATT TCAT_R	
5	β -Glucosidase-767	767	+	GGTTCCGCGTGGATCCCGATACAAAAGATTGCT GCTT_F GATGCGGCCGCTCGAGTTACCGTTCCCAATGCA CCGT_R	
6	Arabinofuranosidase-364	364	—	GGTTCCGCGTGGATCCCGAAAAATTTTATTATC AGCC_F GATGCGGCCGCTCGAGTCAGCGCGCCCCGGGCA TCA_R	
7	Arabinofuranosidase-352	352	—	GGTTCCGCGTGGATCCAACCGTTTTATCTTATG GGCT_F GATGCGGCCGCTCGAGCTATGGCCTGTACTCCT GTTT_R	
8	β -Arabinofuranosidase-589	589	—	GGTTCCGCGTGGATCCCAGGTTGCCGTAGACGG GCTT_F GATGCGGCCGCTCGAGTTAATCAATCTGATTTT GAAA_R	
9	Xylosidase-433	433	—	GGTTCCGCGTGGATCCAAAAGGATTGTTTTAAT AGCA_F GATGCGGCCGCTCGAGTCAAACCTTTATCCAATC AATA_R	
10	Xylosidase-349	349	—	GGTTCCGCGTGGATCCCCTGAAGACAGTATTGA ACAT_F GATGCGGCCGCTCGAGCTATTCCAAAACGTTTA ACCC_R	
11	α -Galactosidase-352	352	—	GGTTCCGCGTGGATCCAAAAAACGATTGTACT ATTCT_F GATGCGGCCGCTCGAGTTATGGGATCACCTGG CCGG_R	
12	α -Fucosidase-481	481	—	GGTTCCGCGTGGATCCATTAAGAAATTATTGCT GAGC_F GATGCGGCCGCTCGAGCTAATACACTTCAAAAT TTGA_R	

Cloning of glycosidases, and characterization of novel recombinant BglNg-767

In total, 34 sets of glycoside hydrolases [belonging to different glycoside hydrolase families (GHF 1, 3, 43, and others)] were identified by WGS analysis. Amongst these 34 sets of

6 different glycoside hydrolases, only 12 sets of glycoside hydrolases [containing 3 sets of β -glucosidase (Bgl), 2 sets of trehalase (Thl), 3 sets of arabinofuranosidase (Abf with α and β configuration), 2 sets of xylosidase (Xyl), and 1 set each of galactosidase (Glac) and fucosidase (Fuc)] were nominated for cloning. The selected functional genes were

cloned in *E. coli* DH5- α using the pGEX 4T-1GST fusion vector system. The gene encoding was identified by analyzing the complete genome sequence, and the oligonucleotide primers used for cloning were designed as forward and reverse (as shown in Table 1) by Macrogen Co., Ltd., Korea with BamHI and XhoI restriction sites inserted, respectively. After PCR amplification, the PCR-purified products were inserted into the pGEX 4T-1GST fusion vector using an EzCloning Kit (Enzymomics Co., Ltd., Korea). The resultant recombinants (pGEX-Bgl, pGEX-Thl, pGEX-Abf, pGEX-Xyl, pGEX-Glac, and pGEX-Fuc) were transformed to *E. coli* BL21 (DE3) and were grown in LB-ampicillin broth at 37 °C, until the OD₆₀₀ reached 0.4–0.6. After OD₆₀₀ reached 0.4, the protein was induced with 0.1 mM isopropyl- β -D-thiogalactopyranoside (IPTG), and further incubated for 12–18 h at 28 °C. After 12–18 h incubation, the cells (as mentioned above) were harvested through centrifugation and the pellets were suspended in 100 mM sodium phosphate (pH 7.0) for sonication. After 5–10 min sonication (Branson Digital Sonifier 450, CT, Mexico), the ginsenoside conversion activity of each recombinant enzyme was determined by mixing with the PPD-mix type (Rb₁, Rb₂, Rb₃, Rc, and Rd) and incubation at 37 °C for 24 h. Among these 12 sets, only one recombinant enzyme (named as BglNg-767) showed the conversion of PPD-mix-type major ginsenosides to minor ginsenosides and was characterized in this study. The recombinant enzyme (BglNg-767) was purified using a GST-bound agarose resin column (Elpisbiotech Co., Ltd, Korea) and protein uniformity was evaluated by 10% SDS-PAGE. SDS-PAGE analysis showed a difference between the molecular weights of the well-localized proteins in the gel (around 90 kDa) and the full-length sequences (102.7 kDa). After purification of BglNg-767, the molecular weight of the recombinant enzyme was confirmed to be around 95 kDa (Miyake et al. 1978; Ahmad et al. 2005; Rath et al. 2009; Siddiqi et al. 2017b).

Effect of pH, temperature, and metal ions on enzyme activity

To measure the specific activity of BglNg-767, the recombinant protein was mixed with *p*-nitrophenyl- β -D-glucopyranoside [*p*NPG, (2 mM as final concentration)] and incubated at 37 °C for 10 min. After 10 min, the reaction was stopped with Na₂CO₃ (0.5 M, final concentration), and the amount of *p*-nitrophenol released was measured using a microplate reader at 405 nm (Bio-Rad model 680; Bio-Rad, Hercules, CA). The amount of enzyme required to produce 1 μ mol of *p*-nitrophenol per min is defined as one unit of enzyme activity. The specific activity of the enzyme was expressed as units per milligram of an enzyme. The protein concentration was determined using the Bio-Rad protein

assay (Catalogue Number 500-0006) kit. All assays were performed in triplicate.

The effect of pH (pH 2–10) and temperature (4–65 °C) with pH and temperature stability was determined as described previously (Siddiqi et al. 2017b) with slight modifications of substrate concentration. Metals and heavy metals are high atomic weight and high-density elements that occur naturally. Therefore, the availability of heavy metals at various concentrations of disturbs enzyme activity; as a result, high concentrations of heavy metals could inhibit enzyme activity due to toxic effects. Thus, the effects of metals and other chemicals on BglNg-767 activity were determined in the presence of 1 and 10 mM (final concentration) of NaCl, CaCl₂, KCl, MgCl₂, MnSO₄, CoCl₂, β -mercaptoethanol, MgSO₄, and EDTA for 10 min at 37 °C. The retained activity was determined using *p*NPG as a substrate and was expressed as a percentage of the activity in the absence of the compound.

In this study, substrate affinity (as shown in Table 2) of BglNg-767 was confirmed using 2.0 mM *o*-nitrophenyl (*o*NP) and *p*-nitrophenyl (*p*NP) as substrates. The samples were incubated at 37 °C for 5 min, and enzyme activity was determined. The amount of enzyme required to generate 1 μ mol of *o*NP or *p*NP per min is defined as one unit of enzyme activity (U).

Bioconversion of major ginsenosides by recombinant BglNg-767

Primarily, we confirmed the effect of fused GST on the enzyme activity of BglNg-767. Therefore, our analysis shows that BglNg-767 activity was not affected by the fused GST for the biotransformation of PPD-type ginsenosides. Therefore, the specificity and selectivity of the recombinant enzyme (GST-BglNg-767) were used to determine the glucose moieties attached to the C3 and C20 positions in six PPD and two PPT-type [(PPD-type, Rb₁, Rb₂, Rb₃, Rc, Rd, Gyp-XVII) (PPT-type Re and Rg₁)] ginsenosides. The crude recombinant enzyme [at a concentration of 0.62 mg/ml in 0.1 M sod-phos buffer (pH 7.0)] was mixed with an equal volume of major ginsenosides Rb₁, Rb₂, Rb₃, Rc, Rd, Gyp-XVII Re, and Rg₁ (final concentration of 1000 ppm) at 37 °C for different time intervals. After each specific interval, the samples were collected and the bioconversion of ginsenosides was confirmed by TLC or HPLC analysis.

TLC analysis

Thin layer chromatography (TLC) analysis was performed with CHCl₃–CH₃OH–H₂O (65:35:10, lower phase) as a solvent in 60 F₂₅₄ silica gel plates (Merck, Germany) and the spots were visualized by spraying 10% (v/v) H₂SO₄ after

Table 2 Substrates and ginsenoside conversion activities of 12 sets of clones of strain BS26

Substrates	Clones of <i>Niabella ginsenosidivorans</i> strain BS26 ^T											
	1	2	3	4	5	6	7	8	9	10	11	12
<i>p</i> NP-β-D-glucopyranoside	–	+	–	–	+	–	–	–	–	–	–	–
<i>p</i> NP-β-D-galactopyranoside	–	–	–	–	–	w	–	–	–	–	–	–
<i>p</i> NP-β-D-fucopyranoside	–	–	–	–	–	–	–	–	–	–	–	–
<i>p</i> NP-β-D-glucosaminide	w	+	w	w	+	w	w	–	–	–	–	–
<i>p</i> NP-β-L-arabinofuranoside	–	–	–	–	–	–	–	–	–	–	–	–
<i>p</i> NP-β-β-mannopyranoside	–	–	–	–	–	–	–	–	–	–	–	–
<i>p</i> NP-β-D-xylopyranoside	–	–	–	–	–	–	–	–	–	–	–	–
<i>p</i> NP-α-D-glucopyranoside	–	w	w	w	–	–	w	–	–	–	–	–
<i>p</i> NP-α-L-arabinofuranoside	–	–	–	–	–	–	–	–	–	–	–	–
<i>p</i> NP-α-D-fucopyranoside	–	–	–	–	–	–	–	–	–	–	–	–
<i>p</i> NP-α-L-rhamnopyranoside	–	–	–	–	–	–	–	–	–	–	–	–
<i>p</i> NP-α-D-mannopyranoside	–	–	–	–	–	–	–	–	–	–	–	–
<i>o</i> NP-β-D-glucopyranoside	–	–	–	–	–	–	–	–	–	–	–	–
<i>o</i> NP-β-D-fucopyranoside	–	–	–	–	–	–	–	–	–	–	–	–
<i>o</i> NP-β-D-galactopyranoside	–	–	–	–	–	–	–	–	–	–	–	–
Bioconversion of ginsenosides	–	–	–	–	+	–	–	–	–	–	–	–

Clone sets. **1**, TrehNg-643; **2**, BglNg-788; **3**, TrehNg; **4**, BglNg-784; **5**, BglNg-767; **6**, AbfNg-364; **7**, AbfNg-352; **8**, β-AbfNg-589; **9**, XylNg-433; **10**, XylNg-349; **11**, α-GalNg-352; **12**, α-FucNg-481. +, positive; w, weak positive; –, negative

Bgl, β-glucosidase; Treh, trehalase; Abf, arabinofuranosidase; Xyl, xylosidase; Gal, galactosidase; Fuc, fucosidase

heating at 110 °C for 5–10 min. The results were then compared with ginsenoside standards on a TLC plate.

Results and discussion

Genome properties of strain BS26^T

The complete genome sequence of strain BS26^T shows in total, 43 sets of glycoside hydrolases (14 β-glucosidases; 11 α-glucosidases; 8 β-xylosidase; 8 α-arabinofuranosidase, 1 galactosidase, and 1 fucosidase (<https://img.jgi.doe.gov>). Interestingly, besides glycoside hydrolases, we also found 30 endopeptidase genes (as shown in Table 3) that may be responsible for the self-hydrolysis of these proteins. Thus, from our SDS-PAGE analysis, we predict that some genes cause self-hydrolysis of the protein. The strain BS26^T had a circular chromosome with 5,627,734 bp and the genome sequence has been deposited in GenBank database under the accession number CP015772.

Molecular cloning of β-glucosidase gene (BglNg-767) from strain BS26^T

After target gene cloning, only two clones (BglNg-788, and BglNg-767) of strain BS26^T showed positive activities for *p*NP-β-D-glucopyranoside. The clones in columns 1–7 were

weak positive and negative for *p*NP-β-D-glucosaminide and *p*NP-α-D-glucopyranoside, respectively. Similarly, the clones in columns 8–12 [β-AbfNg-589; XylNg-433; XylNg-349; α-GalNg-352; α-FucNg-481] did not show any activity for the 16 types of substrates (as shown in Table 2). Regarding the bioconversion of ginsenosides, only one clone (BglNg-767) was active for the conversion of ginsenosides (Table 2) and was characterized. Amino-acid sequence analysis indicated the predicted molecular weight of BglNg-767 (GenBank accession number: CP015772) as 102.7 kDa with a theoretical pI value of 4.92 (http://web.expasy.org/compute_pi/). The amino-acid BLAST sequence analysis indicated that BglNg-767 was 66.4% identical to the β-glucosidase from *Bacteroides thetaiotaomicron* VPI-5482 (PUBMED ID: 29131329), which also belongs to glycoside hydrolase family 3 (GH3) and was characterized by Ishiguro et al. 2017. Based on the amino-acid sequence similarity, the glycoside hydrolase families are divided into various groups, which show common structural features of enzymes and substrate specificity (http://www.cazy.org/fam/acc_GH.html) (Henrissat and Davies 1997).

Enzyme expression and bioconversion of ginsenoside by BglNg-767

The recombinant pGEX-BglNg-767 was transformed into *E. coli* BL21 (DE3) and the protein was induced by 0.1 mM

Table 3 Supplementary information of gene annotated for the BS26^T genome

S. nos	Gene_oid	Locus tag	Source	Cluster/gene Information	Total gene count
1	2778109959	Ga0213703_1170	EC:3.4.24; COG3590	Hydrolases. Acting on peptide bonds (peptidases). Metalloendopeptidases; Predicted metalloendopeptidase	8
	2778110699	Ga0213703_11810			
	2778110818	Ga0213703_11929			
	2778111318	Ga0213703_111430			
	2778114241	Ga0213703_114357			
	2778114241	Ga0213703_114357			
	2778113477	Ga0213703_113591			
	2778114612	Ga0213703_114729			
2	2778110061	Ga0213703_11172	EC:3.4.21.-	Hydrolases. Acting on peptide bonds (peptidases). Serine endopeptidases.	1
3	2778110061	Ga0213703_11172	KO:K07259	D-Alanyl-D-alanine-endopeptidase (penicillin-binding protein 4)	2
4	2778110272	Ga0213703_11383	EC:3.4.21.53	Endopeptidase La.	2
5	2778110699, 2778114241	Ga0213703_11810, Ga0213703_114357	KO:K07386	Putative endopeptidase [EC:3.4.24]	3
6	2778110818	Ga0213703_11929	COG1164; KO:K08602	Oligoendopeptidase F	3
			TIGR02289	Oligoendopeptidase, M3 family	1
7	2778113264	Ga0213703_113378	TIGR00382	Endopeptidase Clp ATP-binding regulatory subunit (clpX)	1
	2778113265	Ga0213703_113379	EC:3.4.21.92	Endopeptidase Clp.	3
	2778113266	Ga0213703_113380			
	2778113857	Ga0213703_113971			
8	2778113231	Ga0213703_113345	KO:K17733 Product_name	Peptidoglycan L-alanyl-D-glutamate endopeptidase CwlK [EC:3.4]	2
9	2778113265	Ga0213703_113379	TIGR00493	ATP-dependent Clp endopeptidase, proteolytic subunit ClpP	2
	2778113857	Ga0213703_113971			
10	2778114373	Ga0213703_114490	ITERM:02952	O-Sialoglycoprotein endopeptidase (EC 3.4.24.57)	2

IPTG. To maximize the recombinant enzyme yield, the samples [recombinant *E. coli* BL21 (DE3) in LB + Amp broth] were incubated at various temperatures (18, 22, 25, and 28 °C) with different concentrations of IPTG [0.1, 0.5, and 1.0 mM IPTG (data not shown)]. The maximum level of soluble protein was produced at 28 °C for 18 h with 0.5 mM IPTG induction, as shown in Fig. 1. The bioconversion of PPD- and PPT- type major ginsenosides (Rb₁, Rb₂, Rb₃, Rc, Rd, Gyp-XVII, Re, and Rg₁) by BglNg-767 was confirmed by TLC analysis at regular intervals. Based on the *R_f* values (Fig. 2a–c), BglNg-767 was found to convert only PPD-type major ginsenosides and there was no significant conversion activity for ginsenosides Re and Rg₁. Thus, BglNg-767 could efficiently remove the glucose attached at the C3 and C20 positions of PPD-type ginsenosides. The proposed pathways for the bioconversion of PPD-type major ginsenosides (1 mg/ml) are given as; Rb₁ → Gyp-XVII → F₂; Rb₂ → C–O; Rb₃ → C–Mx₁; Rc → C–Mc₁ → C–Mc; Rd → F₂; and Gyp-XVII → F₂. The biotransformation occurs through stepwise hydrolysis of the outer and inner glucose moieties at the C3 and C20 positions of the aglycon (Fig. 2). However, there

was no further hydrolyzing activity for the minor ginsenosides F₂, C–O, C–Mx₁, and C–Mc₁ after a long incubation at 37 °C. The ginsenoside Rd and Gyp-XVII (1 mg/ml) were transformed into F₂ within 30 min, indicating that the recombinant BglNg-767 had a preference for the outer glucose attached at the C3 or C20 position. Moreover, the conversion rate was much faster than that of a previously described recombinant enzyme (Cheng et al. 2006 and Chi and Ji 2005), which converted 1 mg/ml of Rb₁ to Rd in 24 h.

Purification and characterization of recombinant BglNg-767

The recombinant protein of GST-BglNg-767 was purified using GST•bind agarose resin and the results were confirmed by 10% SDS-PAGE analysis (Fig. 1). From the SDS-PAGE analysis, the difference between the molecular weights of well-localized proteins (around 90 kDa) and the full-length amino-acid sequence (102 kDa) was identified. Therefore, after purifying BglNg-767, the molecular weight of the recombinant enzyme was confirmed to be

Fig. 1 SDS-PAGE analysis of recombinant BglNg-767. Lanes: M, molecular weight standard; 1, un-induced pGEX-BglNg-767; 2, induced pGEX-BglNg-767; 3, GST-BglNg-767 enzyme fraction after purification

around 90 kDa, as shown in Fig. 1. The difference between the molecular weight of the recombinant BglNg-767 protein calculated from amino-acid sequence analysis and that calculated after protein purification might be due to self-proteolysis of the bacterial strain protein (Miyake et al. 1978; Ahmad et al. 2005; Rath et al. 2009; Siddiqi et al. 2017b).

Using purified GST-BglNg-767, the optimum pH and temperature for the bioconversion of ginsenosides were determined. BglNg-767 showed optimal enzyme activity at pH 7.5 (0.1 M sod-phos buffer), and was stable at pH 5.0–7.5 (Fig. 3a). However, at pH less than 5.0 or more than 7.5, the enzyme stability was decreased sharply, as shown in Fig. 3a. The optimal temperature was found to be 40 °C, whereas thermostability was significantly decreased above 37 °C, and no activity was detected when the enzyme was incubated at 60 °C for 10 min (Fig. 3b). BglNg-767 was stable below 37 °C, and about 40% of the activity was lost after incubation at 45 °C for 10–15 min. Thus, the optimal temperature and pH for the activity of BglNg-762 is 37 °C and pH 7.5,

respectively. Therefore, the mild optimal temperature and the near-neutral optimal pH of BglNg-767 is similar to that of other described members of the ginsenoside-converting glycoside hydrolase family 3 (GH3) (Siddiqi et al. 2017b; Choi et al. 2011b). Even though the optimum temperature of BglNg-767 for substrate hydrolysis (*p*NPG) was 40 °C, during a prolonged and constant bioconversion process, the reaction was maintained at 37 °C with an optimum pH of 7–7.5.

As described in the previous section, different concentrations of heavy metals may affect enzyme activity. Therefore, the effects of metal ions and some chemicals (β -mercaptoethanol and EDTA) were examined on the enzyme activity of BglNg-767 and the results were expressed as a percentage of the activity in the absence of the test compound (Table 4). The analysis showed that enzyme activity of BglNg-767 was not affected by 1 mM of metal ions, but was enhanced as compared to that of the control. In addition, BglNg-767 enzyme activity was

Fig. 2 a–c TLC analyses of time course of ginsenoside bioconversion by BglNg-767. **a** Bioconversion of ginsenosides Rb₁, Rb₂, and Rb₃. **b** Transformation of ginsenosides Rc and Rd. **c** Bioconversion of Re,

Rg₁, and Gyp-XVII. Lane S, standards (Rb₁, Rb₂, Rc, Rd, Rg₃, F₂, and C-K). Developing solvent: CHCl₃–CH₃OH–H₂O (65:35:10, v/v, lower phase)

decreased by 10 mM of all the tested metal ions, EDTA, and β-mercaptoethanol, as shown in Table 4.

To determine the substrate specificity of BglNg-767, the recombinant enzyme was reacted with α- and β-configurations of 2.0 mM *p*NP- and *o*NP-glycosides. The results, summarized in Table 2, showed that BglNg-767 was

most active against *p*NPglc, followed by *o*NPglc, but had no effect on any of the other *p*NP- and *o*NP-glycosides.

In this study, we faced a problem with protein BLAST (blastp) in the NCBI for sequence similarity. Using the data of the complete genome sequence of the BS26^T strain, we succeeded in cloning 12 sets of six (6) different glycoside

Fig. 3 a, b Effect of pH on the enzyme activity of BglNG-676. The enzyme activities were measured under standard assay conditions. Enzyme solutions containing 2.0 mM pNPGlc were incubated with various buffers at pH 2–10 for 12 h at 4 °C, and for stability, the enzymes were incubated for 10 min at 40 °C in various buffers at pH 2–10 and the residual activities were measured (**a**). The effect of temperature on the activity and stability of recombinant BglNG-767 was

measured under standard assay conditions. The thermo-dependence of BglNG-767 was assayed in 50 mM potassium phosphate buffer (pH 7.5) at varying temperatures ranging from 4 to 60 °C. Thermostability was tested by incubating aliquots of the enzyme in 0.1 M sodium phosphate buffer (pH 7.5) for different periods at various temperatures. After cooling the sample, residual activity was determined (**b**)

hydrolases in *E. coli* DH5 α . However, when we analyzed the sequence similarity for our target functional gene using blastp, the results did not reveal interesting aspects of the conservation of our target functional gene. The NCBI blast analysis showed that our target gene had a sequence

similarity of more than 50–60% with different glycoside hydrolases (hypothetical proteins), but after enzyme expression, most of the enzymes did not show specific activity against any substrate, as shown in Table 2. Therefore, the corresponding results for our protein in NCBI blastp were

Table 4 Effects of 1 mM and 10 mM metal ions and other chemicals on the activity of recombinant BglNg-767

Metal ions or reagents	Relative activity $\pm$ SD (%) at	
	1 mM	10 mM
NaCl	126.6 $\pm$ 1.3	116.1 $\pm$ 0.7
KCl	118.7 $\pm$ 2.7	105.1 $\pm$ 0.6
CaCl ₂	121.9 $\pm$ 0.9	80.3 $\pm$ 0.6
MgCl ₂	119.1 $\pm$ 2.7	104.1 $\pm$ 2.4
CoCl ₂	131.9 $\pm$ 2.0	60.9 $\pm$ 2.7
MnSO ₄	107.7 $\pm$ 2.0	69.4 $\pm$ 1.3
MgSO ₄	103.3 $\pm$ 2.7	89.8 $\pm$ 1.0
β -Mercaptoethanol	108.4 $\pm$ 2.0	112.7 $\pm$ 0.3
EDTA	109.7 $\pm$ 2.7	88.7 $\pm$ 1.3
Control	100.0 $\pm$ 2.0	100.0 $\pm$ 5.1

not accurate and the NCBI hypothetical protein will be considered as a putative, uncharacterized protein for strain BS26^T.

Ginsenoside transformation and characteristics of recombinant BglNg-767

To confirm the enzymatic transformation pathway, the PPD-type ginsenosides (Rb₁, Rb₂, Rb₃, Rc, Rd, and Gyp-XVII) were treated with BglNg-767 and TLC analyses were performed at regular intervals. GST-BglNg-767 was found to transform six ginsenosides as shown by *R_f* values from the TLC analysis (Fig. 2a–c). During the biotransformation of Rb₁, two types of metabolites (Gyp-XVII and F₂) were detected (data not shown). Thus, BglNg-767 successively hydrolyzed the inner and outer glucose moieties at position C3 and C20. It also transformed all PPD-type major ginsenosides into minor ginsenosides within 30 min. During the conversion of all PPD-type ginsenosides, it was confirmed

through transformation pathways that BglNg-767 preferred to hydrolyze glucose moieties at C3, rather than those at C20. From TLC analysis, it was observed that BglNg-767 could not hydrolyze the ginsenoside Re and Rg₁, indicating that BglNg-767 could not hydrolyze single-glucose molecules attached to C6 and C20 positions.

The suggested pathways for the conversion of ginsenosides by BglNg-767 are given as Rb₁ $\rightarrow$ Gyp-XVII $\rightarrow$ F₂; Rb₂ $\rightarrow$ C–O; Rb₃ $\rightarrow$ C–Mx1; Rc $\rightarrow$ C–Mc1; Rd $\rightarrow$ F₂, and Gyp-XVII $\rightarrow$ F₂. The biotransformation occurs through stepwise hydrolysis of glucose moieties at the C3 and C20 positions of the aglycon (Fig. 4).

Conclusion

Complete genome sequencing (GenBank Accession Number: CP015772) and optical mapping of strain BS26^T were performed to identify the genes encoding glycoside hydrolases responsible for ginsenoside transformation. Through complete genome analysis, a novel ginsenoside-transforming β -glucosidase termed as BglNg-767 was identified, which belongs to the glycoside hydrolase superfamily 3 (GH3). The optimum reaction condition for BglNg-767 was 40 °C at pH 7.5. BglNg-767 could convert various types of major ginsenosides such as Rb₁, Rb₂, Rb₃, Rc, Rd, and Gyp-XVII by selective hydrolysis of glucose moieties. During the conversion of PPD-Mix-type ginsenosides, BglNg-767 efficiently hydrolyzed the glucose attached to the C3 and C20 positions.

Besides the 43 glycoside hydrolases, we also found 30 endopeptidase genes. From our SDS-PAGE analysis, we predicted that some genes among these 30 endopeptidases may cause self-hydrolysis of protein.

In this study, we characterized a novel ginsenoside-transforming β -glucosidase (BglNg-767) isolated from *Niabella ginsenosidivorans* BS26^T. Furthermore, due to its quick and

Fig. 4 Proposed pathway for enzymatic biotransformation of PPD-Mix type ginsenosides by recombinant BglNg-767

efficient reaction with the major ginsenosides Rc, Rd, and Gyp-XVII, BglNg-767 might be useful for mass production of the minor ginsenosides C-Mc1 and F₂.

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Compliance with ethical standards

Conflict of interest The authors declare that there are no conflict of interest.

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