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Abstract

β-Glucosidase (EC. 3.2.1.21) is of industrial interest due to its critical role in the utilization of cellulosic biomass to produce high-value chemical compounds and biofuels. Moreover, β-glucosidases can be utilized in the biotransformation of high value plant active materials such as ginsenosides. In this report, we confirmed the biotransformation activity of ginsenosides by β-glucosidases from *Bacillus* sp. 3KP and *Serratia marcescens* LII61 strains isolated from Indonesia. β-Glucosidases from both bacterial strains were cloned and overexpressed in *Escherichia coli* BL21(DE3). Crude cell extract of *E. coli* BL21(DE3) overexpressing the β-glucosidase were used for the biotransformation of ginsenosides Rb1. Results showed that the Rb1 was biotransformed to the more pharmacologically active rare ginsenosides, gypenoside XVII and F2. This work is the first effort to use β-glucosidases from Indonesian bacterial strains for ginsenosides biotransformation and is expected to encourage further exploration of β-glucosidase-producing bacterial strains from Indonesia.

Keywords: β-glucosidase; biotransformation; ginsenosides; *Bacillus* sp.; *Serratia marcescens*

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1. Introduction

β-Glucosidases (β-d-glucopyranoside glucohydrolase) [E.C.3.2.1.21] are diverse group of enzymes which hydrolyse the glycosidic bond of a carbohydrate moiety (Singh et al., 2016; Zang et al., 2018). The enzymes are best-known for their critical role in the degradation of lignocellulosic materials into glucose in chemical and biofuel industries (Srivastava et al., 2019). Recently, the enzymes are also highly sought after due to their ability to transform plant active materials with high economic value such as ginsenosides.

Ginsenosides, a group of triterpenoid saponins, are the major active components of ginseng, roots of *Panax* species plants (Park et al., 2019). For thousands of years ginseng has been used as traditional medicine in East Asian countries, and recently ginseng has been increasingly used in foods and dietary supplements worldwide (Cui et al., 2019). More than 90% of total ginsenosides in ginseng are less pharmacologically active major ginsenosides group such as ginsenoside (G)-Rb1, G-Rb2, G-Rc, and G-Rd (Du et al., 2014). Those major ginsenosides are poorly absorbed by human intestinal track (Upadhyaya et al., 2016). Hydrolysis of sugar moieties of major ginsenosides is needed to obtain more easily absorbed, thus more pharmacologically active minor/rare ginsenosides (Kim et al., 2012; Zhong et al., 2016). Among various hydrolysis methods (heating, acid treatment, enzymatic transformation), the use of enzymes especially β-glucosidase is preferred due to their advantages of higher stereo-specificity, conversion yield, as well as lower negative effects to the environment (Kim et al., 2019).

β-Glucosidases for ginsenoside biotransformation are commonly cloned from microorganisms isolated from the soils of ginseng plantation in East Asia (An et al., 2010; Cui et al., 2017; Yan et al., 2008). A considerable number of those ginsenoside-transforming β-glucosidases belong to glycose hydrolase (GH) family 1, which hydrolyse the sugar moieties at both the C-3 and C-20 positions in PPD-type ginsenosides (Noh et al., 2009; Wang et al., 2011; Yuan et al., 2015). In this study, we report the first effort to clone ginsenoside-transforming β-glucosidase from GH family 1-producing bacterial isolates from Indonesia, *Bacillus* sp. 3KP (Ni'matuzahroh et al., 2017; Triawan et al., 2017) and *Serratia marcescens* LII61 (Fatimah et al., 2019). The efficacy of β-glucosidases from those isolates to catalyse the biotransformation of PPD-type major ginsenosde Rb1 to the more pharmacologically active minor ginsenosides Gypenoside XVII (Gyp-XVII) and F2 was also investigated.

2. Materials and Methods

2.1. *Chemicals*

Ginsenoside Rb1, Rd, and F2 standards (98% purity), were purchased from Zelang Medical Technology Co., Ltd. (China). *p*-Nitrophenyl-*β*-D-glucopyranoside (*p*NP*β*Glc) was purchased from Sigma Aldrich (USA), while 5-Bromo-4-chloro-3-indolyl β-d-glucopyranoside (X-Glc) was purchased from Wako Co. Ltd. (Japan). All the chemicals used in this study were at least analytical or reagent grade, and the sources are noted individually.

2.2. *Molecular cloning of the β-glucosidase genes*

Genomic DNA of *Bacillus* 3KP and *S. marcescens* was extracted by using a genomic DNA extraction kit (Qiagen, USA). The extracted genomic DNA was used as template for the amplification of β-glucosidase encoding genes designated as *bgl3KP* and *bglSM* via polymerase chain reaction (PCR) using Pfu DNA polymerase (Solgent, Korea). The primers used in the PCR reaction were designed based on the sequence glycoside hydrolase family 1 genes of *Bacillus* sp. (GenBank accession no. CP042874.1) and of *Serratia marcescens* (GenBank accession no. CP027300.1), respectively. Primers (*bgl3KP* forward: 5'- GTCTAACCATGGATAAGTTTCCACATGATTTTTTATTCGGA-3'; *bgl3KP* reverse: 5'- CCGTTAGGATCCTTATCATTATAACTCTTCCCCTCTCGTTTCG-3'; *bglSM* forward; 5'-GTCTAACATATGGAATATCAATTTGCCGACGG-3' *bglSM* reverse: 5'-CCGTTACTCGAGTTATCATTAGTCAAATCCGTTGCGTCTGG-3') which include NcoI and BamHI (*bgl3KP*) and NdeI and XhoI (*bglSM*) restriction sites (underlined) were synthetized by Genotech (Korea). The amplified DNA fragments of *bgl3KP* and *bglSM* were purified and inserted into the pRSFDuet-1 vector (Novagen, USA) digested with appropriate enzymes (NEB. USA).

2.3. *Expression of recombinant β-glucosidase*

The resulting recombinant pRSF-bgl3KP and pRSF-bglSM was each transformed into *E. coli* BL21(DE3). The *E. coli* BL21(DE3) harbouring the recombinant plasmid was grown in LB kanamycin medium at 37°C (200 rpm) until the culture reached OD_{600} of 0.6, at which point induction with 0.1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) was conducted. The bacterial cells were grown at 25°C (200 rpm) for a further 8 h and were then harvested by centrifugation at 13,000 rpm for 10 min at 4°C. The cells were resuspended in Phosphate Buffer Saline (pH 7.0), and then disrupted by ultrasonication (Bioruptor; Cosmobio, Korea). Soluble and precipitate (cell debris and inclusion bodies) fractions were

separated by centrifugation at 21,600× g for 15 min at 4 °C. The insoluble pellet was washed twice in 1% Triton X-100 and resuspended in 10 mM TE buffer (pH 7.6) (precipitate fraction). The expression of *bgl3KP* and *bglSM* genes was confirmed by SDS-PAGE (Elpisbio, Korea) and EZ-Gel staining solution (Daeillab, Korea).

2.4. *Activity confirmation of the recombinant β-glucosidase*

The activity of the recombinant β-glucosidase Bgl3KP and BglSM was tested using X-Glc assay (An et al., 2012). *E. coli* BL21(DE3) harbouring pRSF-bgl3KP (BglKP strain) and pRSF-bglSM (BglSM strain) were inoculated on LB agar plate supplemented with 50 mg/ml kanamycin, 0.1 M IPTG, and 50 mg/ml X-Glc and incubated at 37^{0}C for 18 h. The β-glucosidase activity was indicated by the presence of blue coloured colonies. While, the specific activity of the recombinant β-glucosidase was confirmed using *p*-nitrophenyl-*β*-D-glucopyranoside (*p*NP*β*Glc) as surrogate substrate (Cui et al., 2019; Gouripur and Kaliwal, 2017). Twenty-five mg of *E. coli* bglKP and bglSM strains were harvested after 8 h induction at 25^{o}C using 0.1 mM IPTG, resuspended in 500 μl Phosphate Buffer Saline (pH 7.0) and disrupted by ultrasonication (Bioruptor; Cosmobio, Korea). The cell lysate was then mixed with *p*NP*β*Glc and incubated at 37^{o}C for 1 hour until yellow colour appeared, indicating the release of p-nitrophenol.

2.5. *Ginsenosides biotransformation assay*

The biotransformation ability of recombinant bgl3KP and bglSM was evaluated using ginsenoside Rb1 as a substrate. Twenty-five mg of *E. coli* BglKP and BglSM strains were harvested after 8 h induction at 25^{o}C using 0.1 mM IPTG, resuspended in 500 μl Phosphate Buffer Saline (pH 7.0) and disrupted by ultrasonication (Bioruptor; Cosmobio, Korea). One hundred ul of the cell lysate were reacted with an equal volume of 0.1% (wt/vol) in 50 mM of sodium phosphate buffer (pH 6.0) at 37°C for 48 hours. An equal volume of water-saturated n-butanol was added to stop the reaction, and the reactant present in the n-butanol fraction was analysed by thin-layer chromatography (TLC) (Park et al., 2014).

TLC was performed using 60 F254 silica gel plates (Merck, Germany) with $CHCl_3$-CH_3OH-H_2O (65:35:10, vol/vol/vol, lower phase) in the solvent system. Spots detection on the TLC plate was conducted by spraying with 10% (vol/vol) H_2SO_4, followed by heating at 110°C for 5 min.

3. Results and Discussion

3.1. *Cloning, expression, and purification of recombinant Bgl3KP and BglSM*

The β-glucosidase genes *bgl3KP* and *bglSM* both were 1,410 and 1,386 bp in lengths, encoding proteins of 469 and 461 amino acids with molecular masses of 54.7 and 52.8 kDA (Figure 2) and theoretical pI values of 5.67 and 5.95 (http://web.expasy.org/compute_pi/), respectively. Bgl3KP and BglSM both belong to the glycoside hydrolase family 1 (GH1). Analysis of the amino acid sequences of Bgl3KP indicated that it was most similar (99.6%) to the glycoside hydrolase family 1 protein of *Bacillus* sp. (GenBank accession no. WP_000671629.1), while BglSM showed high similarity (99.6%) to the glycoside hydrolase family 1 protein of *Serratia marcescens* (GenBank accession no. WP_110146706.1).

To determine the evolutionary position of Bgl3KP and BglSM within the enzymes in GH family 1 with reported ginsenoside biotransformation activity (Cui et al., 2013; Park et al., 2014), neighbor joining phylogenetic tree was constructed using the MEGA X program (Kumar et al., 2018). Bootstrap value of 1000 was used, and the resulting tree is presented in Figure 1. Bgl3KP and BglSM are clustered with β-glucosidases from *Sphingomonas* sp. 2F2 and *Pyrococcus furiosus* DSM 3638 (Oh et al., 2014; Wang et al., 2011). BglSM especially formed a separate, well-supported
clade (bootstrap of 100) with β-glucosidases of Sphingomonas sp. 2F2, indicating that both enzymes showed high amino acid sequences similarities.

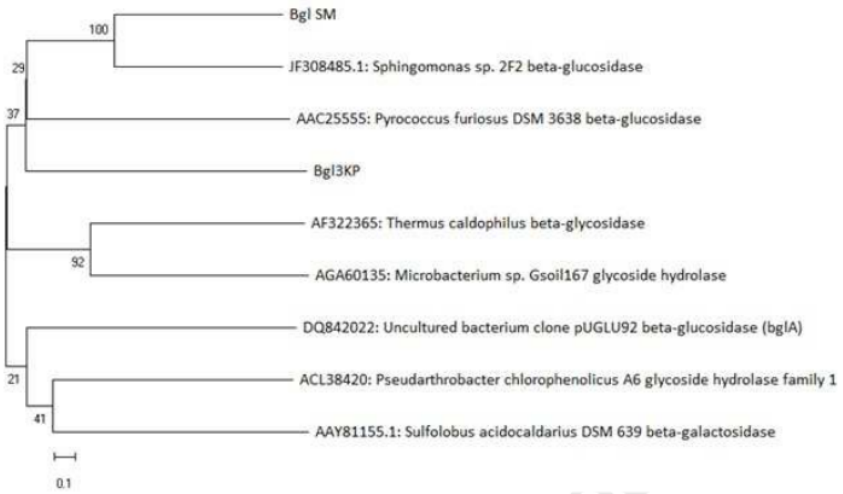

Fig. 1. Phylogenetic analysis of Bgl3KP and BglSM and other ginsenoside-hydrolysing glycoside hydrolase family 1 (GH1) enzymes with reported ginsenoside-biotransforming activity. Amino acid sequences were obtained from the NCBI database with accession numbers as indicated in the tree. This tree was made using the neighbour-joining method with a Poisson model and pairwise deletion (Du et al., 2014). Bootstrap values are expressed as percentages of 1000 replications where greater than 65% values are shown at the branch points. The bar represents 10 amino acid residue substitutions per 100 amino acid residues.

Bgl3KP and BglSM were successfully expressed in *E. coli* BL21(DE3) after 8 hours at 25°C induction using 0.1 M IPTG. Based on the SDS PAGE result, most of the β-glucosidase were in soluble fraction (Figure 2), thus the crude cell extracts were expected to exhibit enzymatic activities.

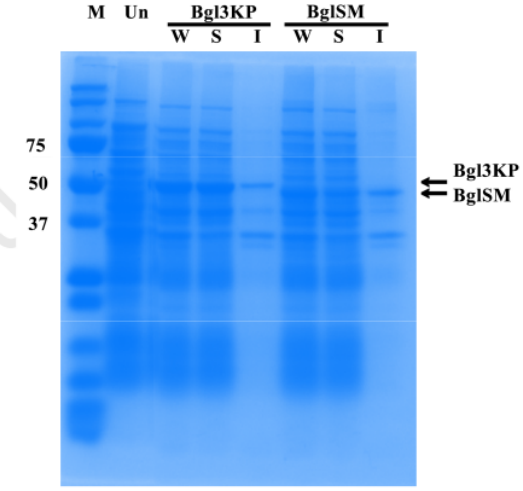

Fig. 2. SDS-PAGE analysis of Bgl3KP and BglSM expression: lane M, protein marker with bands corresponding to proteins with size of 75, 50, and 37 kDa marked (Biorad, USA); lane Un, cell lysate of Bgl3KP strain growth for 8 h at 25°C without IPTG induction; lane Bgl3KP/BglSM W, cell lysate of Bgl3KP / BglSM strain induced with IPTG for 8h at 25°C; S, soluble fraction of cell lysate of Bgl3KP / BglSM strain induced with IPTG for 8h at 25°C; I, precipitated fraction of cell lysate of Bgl3KP / BglSM strain induced with IPTG for 8h at 25°C.

3.2. *Activity of the recombinant β-glucosidases in crude cell extract*

The activity of expressed β-glucosidases was confirmed using X-Glc. The presence of blue colonies indicated that the expressed Bgl3KP and BglSM were active (Figure 3a and 3b). The activity was further confirmed using *p*NP*β*Glc assay. Hydrolysis of *p*NP*β*Glc by crude cells extract of *E. coli* strain Bgl3KP and BglSM induced with IPTG for 8 h at

25°C was observed after 1-hour incubation (Figure 3c). The results suggest that both enzymes show potential to catalyse the biotransformation of major ginsenoside.

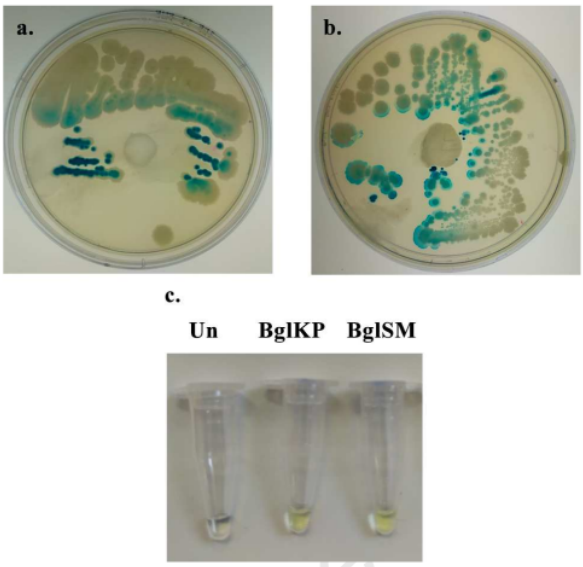

Fig. 3. Blue colonies of *E. coli* strain Bgl3KP (a) and BglSM (b) on LB + IPTG + X-Glc plate after 18 hours incubation at 37°C. *p*NP*β*Glc hydrolysis was observed in the presence of crude cell extract of Bgl3KP and BglSM strains induced with 0.1 M IPTG for 8 hours (c). The cell lysate of uninduced Bgl3KP strain was used as control (Un).

3.3. *Biotransformation of ginsenosides*

Rb1 biotransformation activity of Bgl3KP and BglSM from crude cell extract were confirmed using TLC analysis. After 48 hours incubation, spots corresponding to ginsenosides Rd and another spot below the spot corresponding to ginsenoside Rd, which based on previous reports was predicted to be Gyp-XVII (An et al., 2010; Wang et al., 2011) were observed, indicating that the two β-glucosidases were able to catalyze the hydrolysis of outer glucose moeities at the 3 and 20 position on Rb1 (Figure 4a and 4b). These results are amenable with provious reports where GH family 1 β-glucosidases cloned from *Sulfolobus solfataricus*, *Sulfolobus acidocaldarius*, *Pyrococcus furiosus*, and *Arthrobacter chlorophenolicus*(Noh et al., 2009; Noh and Oh, 2009; Park et al., 2014; Yoo et al., 2011) . However, only BglSM showed significant biotransformation of Rd and Gyp-XVII to F2 (Figure 4b). Interestingly, BglSM showed similar ginsenoside transformation activity with as β-glucosidases from Sphingomonas sp. 2F2 which belong to the same clade in the constructed neighbour joining tree (Wang et al., 2011) (Figure 1).

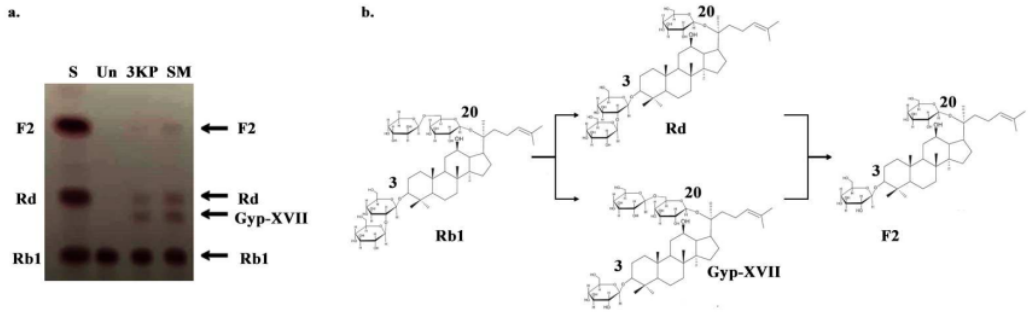

Fig. 4. (a) Thin layer chromatography (TLC) analyses of biotransformation of ginsenosides Rb1. Lane S, Ginsenoside standards (Zelang Medical Technology Co., Ltd., China) ; Lane Un, biotransformation of Rb1 by cell lysate of Bgl3KP strain growth for 8 h at 25°C without IPTG induction;

Lane 3KP / SM, biotransformation of Rb1 by soluble fraction of cell lysate of IPTG induced Bgl3KP/ BglSM strain.
(b) The proposed conversion pathway of ginsenoside Rb1 using crude cell extract of induced Bgl3KP and BglSM strains.

Ginsenoside Gyp-XVII and F2 have been reported to show beneficial health effects. Gyp-XVII has been reported for its cardiovascular protective effect by alleviating atherosclerosis via the ERα-mediated PI3K/Akt pathway and neuroprotective protective effect against Alzheimer's disease by activating transcription factor EB (Meng et al., 2016; Yang et al., 2017), while F2 has been reported for its anti-cancer activity which mediated through inhibition of proliferation judged by Ki67 and apoptosis induced by activation of caspase-3 and -8 in glioblastoma multiforme, as well as anti-inflammatory effects (Mai et al., 2012; Park et al., 2016; Shin et al., 2012). Therefore, Bgl3KP and BglSM has potential application in the pharmaceutical industry.

This report is one of the first endeavors on exploring the potency of bacterial isolates from outside East Asia especially from Indonesia as sources for ginsenoside-biotransforming β-glucosidase. As one of the mega-biodiversity countries with various unique ecosystems such as hot springs, acidic crater lakes, and peatlands, Indonesia could provide the sources for β-glucosidase enzymes with unique characteristic which are useful for efficient ginsenosides biotransformation at industrial scale (i.e. thermostability and tolerance to low pH) (Geraldi et al, 2019). Thus, further exploration of β-glucosidases producing microbes in Indonesia would to be conducted.

4. Conclusion

Genes encoding recombinant ginsenoside-hydrolysing β-glycosidases, Bgl3KP and BglSM, belonging to the GH1 family, were cloned from *Bacillus* sp. 3KP and *Serratia marcescens* LII61, respectively. These enzymes were expressed in *E. coli* BL21(DE3) in a soluble form and could convert major ginsenoside Rb1 into pharmacologically active minor ginsenoside F2 via Rd and Gyp-XVII. To our knowledge this is the first report on using β-glucosidases of bacterial isolates from Indonesia for ginsenoside biotransformation. This report is expected to encourage further exploration of ginsenoside transforming-β-glucosidases, especially those with industrial potential, in Indonesia.

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