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

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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.

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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

fusion vector digested with BamHI and XhoI using an EzCloning Kit (Enzynomics Co. Ltd., Korea). The resulting recombinant pGEX-*bglSk* was transformed into *E. coli* BL21(DE3). *E. coli* BL21(DE3) harboring the recombinant plasmid was grown in an LB-ampicillin medium at 37 °C until the culture reached an O.D.$_{600}$ of 0.6, at which point the protein expression was induced through the addition of 0.1 mM isopropyl-β-D-thiogalactopyranoside (IPTG). The bacterial cells were incubated further for 24 h at 18 °C and then harvested via centrifugation at 13,000 rpm for 15 min at 4 °C. The cells were washed twice with a solution consisting of 50 mM sodium phosphate, 5 mM EDTA, and 1% Triton X-100 (pH 7.0), and then they were resuspended in 50 mM sodium phosphate (pH 7.0). The cells were disrupted via ultrasonication (Vibra-cell, Sonics & Materials, CT, USA). The intact cells and debris were removed via centrifugation at 13,000 rpm for 15 min at 4 °C in order to obtain a crude cell extract. The GST tag was purified using GST-bind agarose resin. The homogeneity of the protein was assessed using 10% SDS-PAGE and EZ-Gel staining solution (Daeillab Co. Ltd., Korea).

2.5. Enzyme characterization and determination of kinetic parameters

The standard reaction to investigate the specific activity of purified BglSk and the effects of the pH level, temperature, metal ions, and chemical reagents on the enzyme activity were performed as previously described (An et al., 2010). A substrate preference was examined using chromogenic o-nitrophenyl (ONP) and p-nitrophenyl (PNP). The release of o- or p-nitrophenol was measured using a microplate reader at 415 nm (Bio-Rad Model 680, USA). Kinetic studies were performed with freshly purified enzymes using pNPGlc and Rb_1 at concentrations from 0.1 to 2 mM. One unit of activity was defined as the amount of protein required to generate 1 μmol of *p*-nitrophenol or to convert 1 μmol of Rb_1 per minute. All assays were performed in triplicate. The parameters (K_m and V_{max}) were determined using the enzyme kinetics program reported by Cleland. (1979).

2.6. Enzymatic conversion of ginsenosides Rb_1, Rb_2, Rc, Rd, Gyp XVII, Rg_3, Re, and Rg_1

The initial biotransformation experiments using the major ginsenosides Rb_1 and Rg_1 as substrates revealed that the GST-fused enzyme does not affect the activities of BglSk. Therefore, the fusion protein (GST-BglSK) was used to determine the specificity and selectivity of the enzymes for the hydrolysis of the glucose moieties attached at the C3 and C20 sites in the six PPD type ginsenosides and two PPT type ginsenosides. The enzyme solutions at concentrations of 0.1 mg/ml in 50 mM sodium phosphate buffer (pH 8.0) were reacted with an equal volume of Rb_1, Rb_2, Rc, Rd, Gyp XVII, Rg_3(S), Re, and Rg_1 solution at a concentration of 0.1% (wt/vol) in 50 mM of sodium phosphate buffer (pH 8.0) at 25 °C. Samples were withdrawn at regular intervals. For the TLC analyses, an equal volume of water-saturated *n*-butanol was added to stop the reaction, and the reactant present in the *n*-butanol fraction was analyzed after pretreatment. For the HPLC analyses, an equal volume of methanol was added to stop the reaction. After centrifugation at 15,000 × *g* for 10 min, the supernatant was used for the sample analyses.

3. Results

3.1. Cloning, expression, and purification of recombinant BglSk

The ginsenoside hydrolyzing glycosidase gene consisting of 1857 bp encoding 618 amino acids, which have homology to the protein domain of the glycoside hydrolase family 3, was amplified via PCR and then inserted into the pGEX 4T-1 vector. In order to maximize the yield of the fusion protein in a soluble form, different induction conditions were tested and it was found that an induction with 0.1 mM IPTG at 18 °C for 24 h cultivation after induction produced the maximum level of soluble active fusion enzyme (data not shown). The GST-BglSk fusion protein was purified using the GST·bind agarose resin and the GST tag was removed using thrombin at room temperature for 12 h incubation. The calculated molecular mass of the native BglSk by amino acid sequence was 66.6 KDa but, BglSk ran slower than expected migration in SDS-PAGE (Fig. 1).

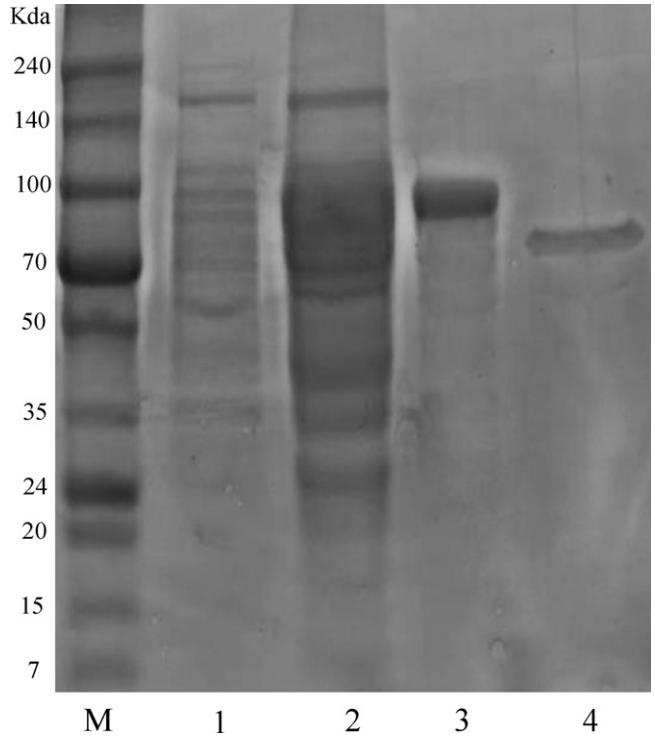

Fig. 1. SDS-PAGE analysis of recombinant BglSk after purification using GST-bind agarose resin. Lanes: (1) precipitated fraction of crude extract of induced recombinant BL 21 (DE3) cells; (2) soluble fraction of crude extract of induced recombinant BL 21 (DE3) cells; (3) GST-BglSk after purification with GST-bind agarose resin; (4) purified recombinant BglSk after cleavage by thrombin.

3.2. Enzyme characterization

BglSk had optimal pH activity and stability at pH 8.0 in a sodium phosphate buffer at 37 °C, and from pH 9.0 the enzyme activity decreased, while at pH 6.0 the enzyme activity decreased 70% (Fig. 2A). The optimal temperature activity was 25 °C, and at 30 °C and 37 °C the enzyme lost 15% and 25% activity, respectively, while thermostability was not detected at 65 °C (Fig. 2B). The enzyme activity appeared to be strongly inhibited in the presence of Cu^{2+}, Hg^{2+}, and Zn^{2+}. Na^{2+} and K^{2+} had very little positive effect on the enzyme activity at the low concentration; however, dramatic positive effects on the BglSk activity were not found for the tested metal ions (Table 1). The substrate specificity of BglSk was tested using 2.0 mM of p-nitrophenyl (pNP) and o-nitrophenyl (ONP)-glycosides with α and β configurations. The results, summarized in Table 2, show that BglSk hydrolyzed both pNP-β-D-glucopyranoside and oNP-β-D-glucopyranoside. The *K*m and *V*max for the hydrolysis of pNPGlc by GST-BglSK were determined to be 0.456 ± 0.009 mM and 30.2 ± 0.7 μmol min^{-1} mg of protein^{-1}, respectively. The Km and Vmax for the transformation of ginsenoside Rb1 by GST-BglSK were determined to be 0.167 ± 0.003 mM and 4.1 ± 0.1 μmol min^{-1} mg of protein^{-1}.

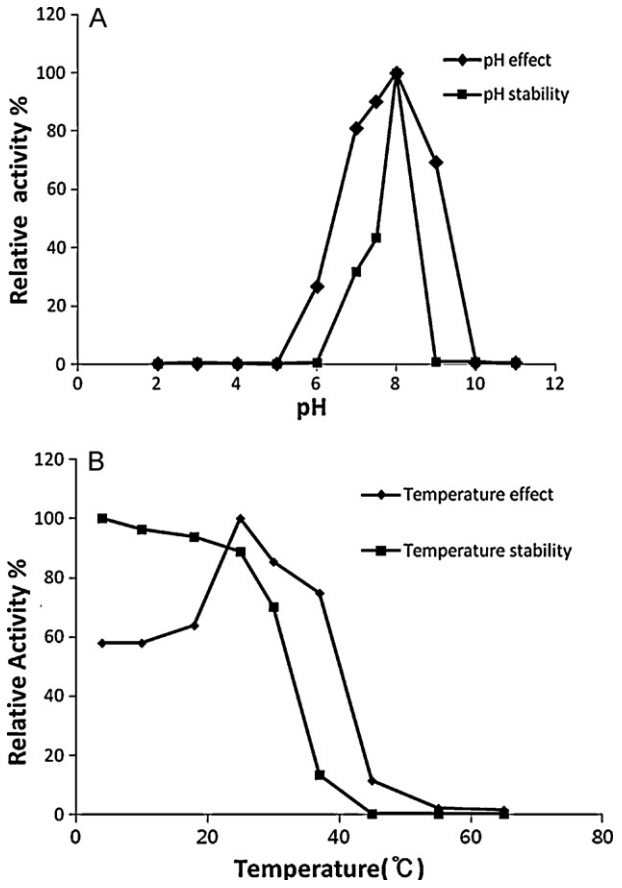

Fig. 2. Effects of pH (A) and temperature (B) on the activity and stability of recombinant BglSk.

Table 1
Effects of metal ions and chemical agents on the activity of purified recombinant BglSk.

Metal ions or regents	Relative activity ± SD (%)	
	1 mM	10 mM
NaCl	104.4 ± 1.21	82.8 ± 0.43
KCl	103.2 ± 1.90	83.0 ± 1.05
$MgCl_2$	84.5 ± 4.15	76.2 ± 0.75
$MnCl_2$	85.9 ± 2.37	61.9 ± 0.57
$CoCl_2$	84.2 ± 3.00	62.2 ± 0.50
$ZnCl_2$	52.2 ± 1.02	24.8 ± 0.53
$CaCl_2$	99.2 ± 2.63	62.2 ± 1.59
$CuCl_2$	1.5 ± 0.05	6.4 ± 1.25
$HgCl_2$	2.2 ± 0.07	2.1 ± 0.02
SDS	83.6 ± 1.75	63.9 ± 2.09
EDTA	97.7 ± 1.53	75.8 ± 0.87
β-Mercaptoethanol	86.0 ± 2.63	77.0 ± 1.22
DTT	99.3 ± 4.26	74.1 ± 2.03
Control	100 ± 1.28	100 ± 1.28

Table 2
Relative activity of the purified recombinant BglSk on various chromogenic substrates as measured by ONP or PNP release at 37 °C.

Substrate	Relative activity ± SD (%)
pNP-α-D-glucopyranoside	0
pNP-α-D-mannopyranoside	0
pNP-α-D-xylopyranoside	0
pNP-α-L-arabinofuranoside	0
pNP-α-L-arabinopyranoside	0
pNP-α-L-rhamnopyranoside	0
pNP-β-D-fucopyranoside	0
pNP-β-D-galactopyranoside	0
pNP-β-D-glucopyranoside	100 ± 3.70
pNP-β-D-glucosaminide	0
pNP-β-D-mannopyranoside	0
pNP-β-D-xylopyranoside	0
pNP-β-L-arabinopyranoside	0
oNP-α-D-galactopyranoside	0
oNP-β-D-fucopyranoside	0
oNP-β-D-glucopyranoside	112 ± 4.52

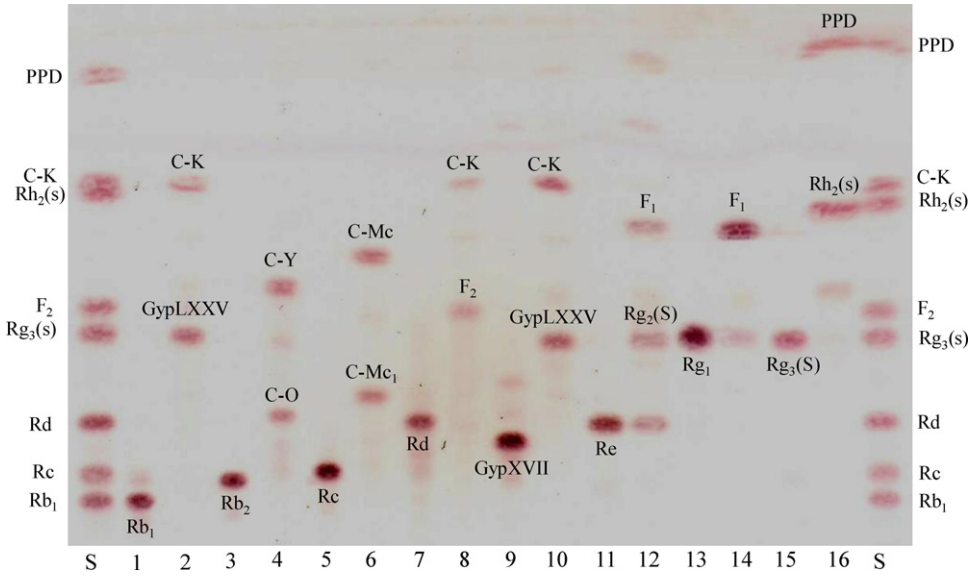

Fig. 3. Thin layer chromatography (TLC) analyses of biotransformation of Rb_1, Rb_2, Rc, Rd, Gyp XVII, Rg_3(S), Re, and Rg_1 by recombinant BglSk derived from *S. keddieii* KACC 14479^T. The reaction time was 1 h. Developing solvent: $CHCl_3$–CH_3OH–H_2O (65:35:10, v/v, lower phase). Standards: 1, 3, 5, 7, 9, 11, 13, and 15; reaction mixture: 2, 4, 6, 8, 10, 12, 14, and 16; S, ginsenoside standards (PPD type ginsenoside mixtures); 1, Rb_1; 2, reaction mixture of Rb_1; 3, Rb_2; 4, reaction mixture of Rb_2; 5, Rc; 6, reaction mixture of Rc; 7, Rd; 8, reaction mixture of Rd; 9, GypXVII; 10, reaction mixture of GypXVII; 11, Re; 12, reaction mixture of Re; 13, Rg_1; 14, reaction mixture of Rg_1; 15, Rg_3(S); 16, reaction mixture of Rg_3(S). Abbreviations: C-K, compound K; PPD, protopanaxadiol.

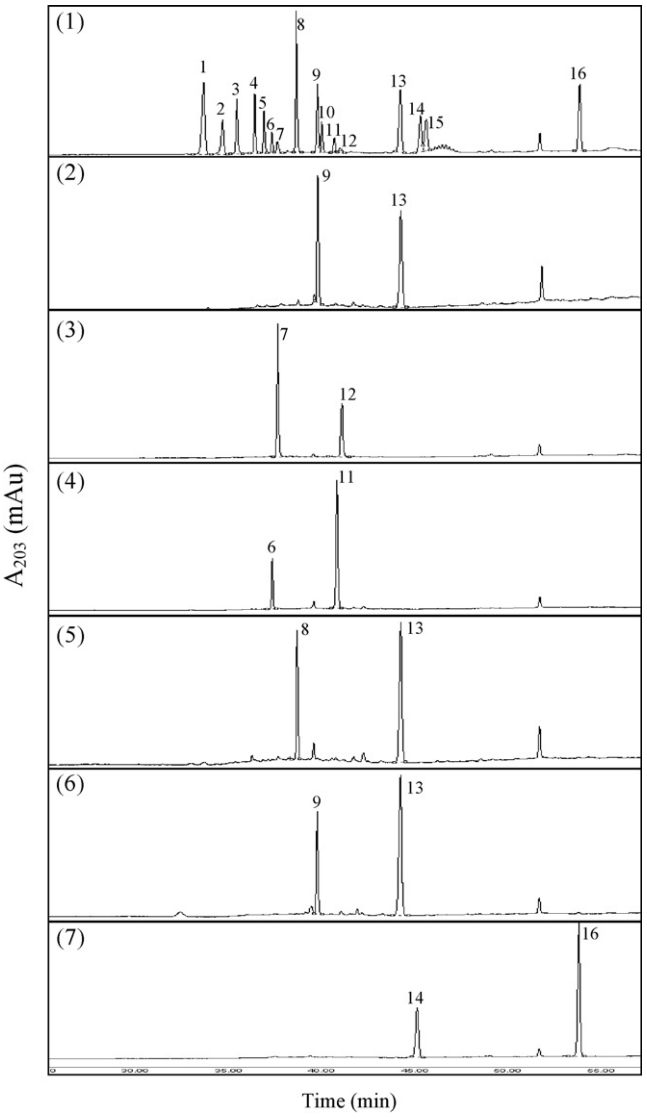

Fig. 4. HPLC results of transformation of PPD types of ginsenosides Rb_1, Rb_2, Rc, Rd, GypXVII, and $Rg_3(S)$ by recombinant BglSk after 2 h incubation. (1) Ginsenoside standard peaks: 1, Rb_1; 2, Rc; 3, Rb_2; 4, Rd; 5, GypXVII; 6, C-Mc_1; 7, C-O; 8, F_2; 9, $Rg_3(S)$ + Gyp LXXV; 10, $Rg_3(R)$; 11, C-Mc; 12, C-Y; 13, C-K; 14, $Rh_2(S)$; 15, $Rh_2(R)$; 16, PPD. (2) Reaction mixture of Rb_1. (3) Reaction mixture of Rb_2. (4) Reaction mixture of Rc. (5) Reaction mixture of Rd. (6) Reaction mixture of GypXVII. (7) Reaction mixture of $Rg_3(S)$. Abbreviations: C-K, compound K; GypXVII, gypenoside XVII; Gyp LXXV, gypenoside LXXV; C-Mc, compound Mc; C-Mc_1, compound Mc_1; C-Y, compound Y; C-O, compound O.

3.3. Biotransformation of ginsenosides Rb_1, Rb_2, Rc, Rd, Gyp XVII, $Rg_3(S)$, Re and Rg_1

For the verification of the bioconversion pathway of the major ginsenosides by GST-BglSk, TLC and HPLC analyses were performed at regular intervals. As shown in Fig. 3, it is clear that GST-BglSk could transform the eight ginsenosides Rb_1, Rb_2, Rc, Rd, Gyp XVII, $Rg_3(S)$, Re, and Rg_1 based on the R_f values. The HPLC profiles of the reaction mixture of the eight ginsenosides using GST-BglSk after 2 h incubation are shown in Figs. 4 and 5. The transformed ginsenosides were determined by their retention times. The GST-BglSk demonstrated the variety of ginsenosides hydrolyzing activity. The proposed biotransformation pathways for the PPD ginsenosides are as follows: $Rb_1 \rightarrow$ GypXVII $\rightarrow$ Gyp LXXV $\rightarrow$ compound K (C-K), $Rb_2 \rightarrow$ C-O $\rightarrow$ C-Y, Rc $\rightarrow$ C-Mc_1 $\rightarrow$ C-Mc, Rd $\rightarrow F_2 \rightarrow$ C-K, Gyp XVII $\rightarrow$ Gyp LXXV $\rightarrow$ C-K, and $Rg_3(S) \rightarrow Rh_2(S) \rightarrow$ Protopanaxadiol

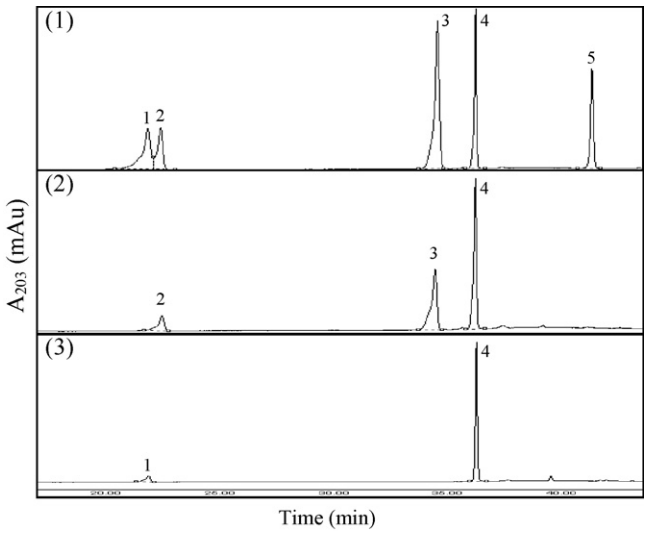

Fig. 5. HPLC results of transformation of PPT types of ginsenosides Re, and Rg_1 by GST-BglSk. (1) Ginsenoside standard peaks: 1, Rg_1; 2, Re; 3, $Rg_2(S) + Rh_1(S)$; 4, F_1; 5, PPT. (2) Reaction mixture of Re. (3) Reaction mixture of Rg_1. Abbreviation: PPT, protopanaxatriol.

via stepwise hydrolysis of the outer and inner glucose in the C3 position, followed by hydrolysis of the outer glucose in the C20 position. For the PPT ginsenosides, the proposed biotransformation pathways are Re $\rightarrow Rg_2(S)$ and F_1, $Rg_1 \rightarrow F_1$ through hydrolysis of the inner glucose in the C3 position. Almost all eight ginsenosides used as substrates at a concentration of 1.0 mg/ml were biotransformed within 2 h. Negative ion ESI MS-MS spectra of the intermediate metabolites (C-O, C-Mc_1, GypLXXV, F_2) and final products (C-Y, C-Mc, C-K, F_1) were obtained on API 2000 to confirm their identity (Supplementary data is available in online system).

4. Discussion

The constructed recombinant ginsenoside hydrolyzing glycosidase, termed BglSk, is homologous to the glycoside hydrolase family 3, based on the amino acid sequence similarities to the β-glucosidase in *Actinosynnema mirum* DSM 43827^T (GenBank accession number: YP_003100744, 66%), *Microbacterium testaceum* StLB037 (YP_004225291, 65%), and *Terrabacter ginsenosidimutans* Gsoil 3082^T (ACZ66247, 63%). The last sequence characterized by An et al. (2010) led the investigation of the BglSk function. The characterized ginsenoside hydrolyzing glycosidase (GHG) have been primarily in glycosyl hydrolase families 1 and 3. The enzymes in family 3 commonly form in a closely related subfamily with a wide range of activities, although they have high sequence similarities. In addition, many enzymes in this group were able to hydrolyze various types of glycosides; for example, isoflavone glycosides could be hydrolyzed by isoflavonoid β-glucosidases (Kaya et al., 2008). Until now, only three GHG belonging to the glycoside hydrolase family 3 with catalytic activity for ginsenosides biotransformation have been reported (An et al., 2010; Quan et al., 2012; Ruan et al., 2009).

Theoretically, four types of glucose moieties attached to ginsenoside Rb_1 and two types of glucose moieties attached to the Re and Rg_1 ginsenosides were available for hydrolysis by BglSk, namely the outer and inner glucose moieties attached at positions C3, C6, and C20. Based on an analysis of the hydrolysis products of the Rb_1 ginsenoside, BglSk hydrolyzed three of the four possible types of glucose moieties: the outer and inner glucose moieties at position C3 and the outer glucose at position C20. The preferential reactivity of BglSk toward these four glucose moieties of Rb_1 could be deduced from the Rb_1 biotransformation pathway, i.e.

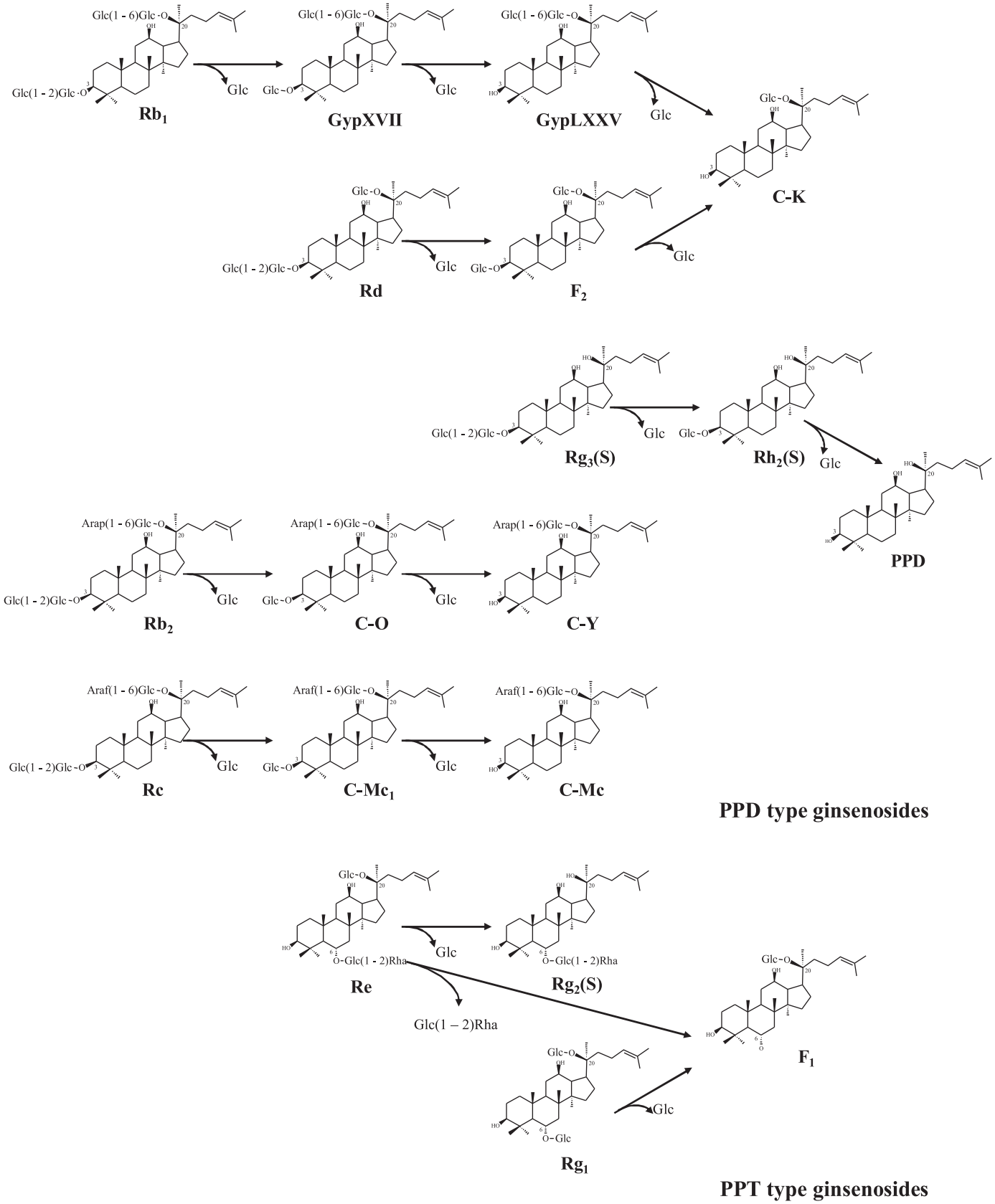

Fig. 6. Transformation pathways of ginsenosides Rb_1, Rb_2, Rc, Rd, Gyp XVII, $Rg_3(S)$, Re, and Rg_1 by recombinant BglSk, respectively.

$Rb_1 \rightarrow$ GypXVII $\rightarrow$ GypLXXV $\rightarrow$ C-K. BglSk preferred the outer glucose at position C3, followed by the inner glucose at position C3, and finally the outer glucose at position C20. In order to investigate whether BglSk showed the same specificity and selectivity for glycone residues attached at the C3 and C20 positions of other PPD ginsenosides, the Rb_2, Rc, Rd, Gyp XVII, and Rg_3(S) ginsenosides were also used as substrates. The Rb_2 and Rc ginsenosides have L-arabinopyranoside and L-arabinofuranoside as their outer glycone residue at the C20 position, respectively (Fig. 6). BglSk transformed Rb_2 and Rc to compound Y (C-Y) and compound Mc (C-Mc), respectively, through cleavage of the terminal glucose at the C3 position. However, BglSk could not further hydrolyze C-Y and C-Mc due to the L-arabinopyranoside and L-arabinofuranoside moiety attached at the C20 site. This activity was caused by very specific reactivity for the substrate preferences of BglSk as shown in Table 2. Interestingly, this conversion pathway is similar to that of the thermostable β-glycosidase belonging to glycoside family 1 (Noh et al., 2009), except BglSk could not hydrolyze C-Y further to C-K. The Rd and GypXVII ginsenosides were converted to C-K via F_2 and GypLXXV, respectively, via cleavage of the outer glucose and inner glucose at the C3 position. When the Rg_3(S) ginsenoside was used as a substrate, BglSk hydrolyzed the outer glucose and inner glucose at the C3 position of Rg_3(S) to form PPD via Rh_2(S). These results indicate that BglSk demonstrates substrate specificity for PPD ginsenosides that have one or two glucose moieties at the C3 or C20 position, and prefers the outer glucose at position C3, followed by the inner glucose at position C3, and finally the outer glucose at position C20. This conversion pathway for Rb_1, Rb_2, Rc, Rd, and Rg_3 is the same as that of BgpA from *Terrabacter ginsenosidimutans* Gsoil 3082^T (An et al., 2010; Jin et al., 2012), which was named as ginsenosidases type III. This similar characteristic is attributed to the high amino acid sequence similarity (63%).

Furthermore, in order to investigate whether BglSk demonstrated the specificity and selectivity for glycone residues attached at the C6 and C20 positions of the PPT ginsenosides, the Re and Rg_1 ginsenosides were also used as substrates. It was interesting to note that the ginsenoside Re was converted into both Rg_2(S) and F_1 cleavage of the inner glucose at the C6 position irrespective of the existence of outer rhamnose moiety at the C6 position. When the Rg_1 ginsenoside was used as a substrate, BglSk hydrolyzed one glucose moiety at the C6 position to produce F_1, without further reaction. This bioconversion pathway is a novel finding that has not been reported in the GHG group of the glycoside hydrolase family 3. Bgp1, belonging to glycoside hydrolase family 3 and derived from *Microbacterium esteraromaticum*, preferentially hydrolyzed both outer and inner glucose moieties at the C20 position of the Rb_1 ginsenoside until the Rg_3(S) ginsenoside was produced (Quan et al., 2012), and it could hydrolyze the one glucose moiety attached to the C20 position of the Re and Rg_1 ginsenosides in order to produce the Rg_2(S) and Rh_1(S) (Quan et al., 2012). Until now, the F_1 production using the Rg_1 ginsenoside as substrate was reported by purified β-glucosidase from *Fusarium moniliforme* var. *subglutinans* (Kim et al., 2011) and fungal strains (Wu et al., 2008). However, a report on the conversion of the Re ginsenoside into F_1 has not yet been published. Furthermore, these productions do not have the advantages of the recombinant enzyme, which could be produced for the production of the required ginsenoside using the Re and Rg_1 mixture. Overall, BglSk has a very diverse biotransformation ability for ginsenosides as shown in Fig. 6. Furthermore, BglSk could be a practical tool for the preparation of large amounts of C-K and F_1 ginsenosides with high yields if the purified PPD mixture (Rb_1, Rd) or PPT mixture (Re, Rg_1) were used as the substrates.

In conclusion, a recombinant ginsenoside hydrolyzing glycosidase (BglSk) belonging to glycoside hydrolase family 3 was constructed from *S. keddieii* KACC 14479^T for the biotransformation of major ginsenosides. This enzyme was expressed in *E. coli* BL21(DE3) in a soluble form and was characterized; it had optimum reaction conditions at 25 °C and pH 8.0. BglSk could convert the Rb_1, Rb_2, Rc, Rd, Gyp XVII, Rg_3(S), Re, and Rg_1 ginsenosides into the minor ginsenosides C-K, C-Y, C-Mc, PPD, and F_1 through selective hydrolysis of the glucose moieties. This is the first report of the cloning of an enzyme that selectively produces ginsenoside F_1 through the biotransformation of ginsenoside Rg_1.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.jbiotec.2012.06.021.

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