Appl Microbiol Biotechnol
DOI 10.1007/s00253-015-6802-z

Selective production of rubusoside from stevioside by using the sophorose activity of β-glucosidase from *Streptomyces* sp. GXT6

Zilong Wang[1] · Jinpei Wang[1] · Minhua Jiang[1] · Yutuo Wei[1] · Hao Pang[2] · Hang Wei[1] · Ribo Huang[1,2] · Liqin Du[1]

Received: 5 March 2015 / Revised: 5 June 2015 / Accepted: 27 June 2015

Abstract In order to produce rubusoside, enzymes with preferential specificity for the saccharide sophorose were tested for ability to produce rubusoside from stevioside. We identified BGL1, a β-glucosidase from *Streptomyces* sp. GXT6, as an enzyme for rubusoside production. Out of several saccharide substrates, BGL1 showed the most affinity to sophorose. This enzyme only hydrolyzes the glucose moiety of the sophoroside at C-13 in stevioside. Production of rubusoside was determined by ^{1}H and ^{13}C nuclear magnetic resonance (NMR). Thus, rubusoside was produced from stevioside and the stevioside conversion rate was 98.2 %. The production yield of rubusoside was 78.8 % in 6 h.

Keywords *Streptomyces* sp · β-Glucosidase · Rubusoside · Stevioside · Sophorose · *Rubus suavissimus*

Electronic supplementary material The online version of this article (doi:10.1007/s00253-015-6802-z) contains supplementary material, which is available to authorized users.

✉ Hao Pang
haopang@gxas.cn

✉ Liqin Du
duliqin@gxu.edu.cn

[1] State Key Laboratory for Conservation and Utilization of Subtropical Agro-bioresources, College of Life Science and Technology, Guangxi University, 100 Daxue Road, Nanning, Guangxi 530005, China

[2] National Engineering Research Center for Non-Food Biorefinery, Guangxi Academy of Sciences, 98 Daling Road, Nanning, Guangxi 530007, China

Introduction

Rubusoside and stevioside are natural sweeteners. They belong to the steviol glucoside family (chemical structures are shown in Fig. 1) and are about 114- and 143-fold sweeter than sucrose at a concentration of 0.025 %, respectively (Ceunen and Geuns 2013; Ko et al. 2012). In addition to its use as a sweetener, rubusoside is an excellent solubilizing agent. It can enhance the solubility of a number of pharmaceutically important compounds, such as liquiritin, teniposide, curcumin, and etoposide (Nguyen et al. 2014; Zhang et al. 2012). Also, rubusoside possesses significant bioactivities such as antiangiogenic activity and antiallergic activity (Koh et al. 2009; Liu et al. 2006). Indeed, rubusoside is approximately 10-fold more expensive than stevioside (Ko et al. 2012).

Rubusoside is produced primarily by extraction from *Rubus suavissimus* S. Lee (known in China as tiancha or Chinese sweet tea), although it can also be extracted from *Stevia rebaudiana* leaves as a minor component (Koh et al. 2009; Prakash Chaturvedula and Prakash 2011). *R. suavissimus* S. Lee grows in southern China, and it has a variable yield year-round depending on local climate (Chou et al. 2009). It has a history of use as a local herbal tea going back hundreds of years (Koh et al. 2009; Wan et al. 2012). The production of rubusoside is mainly dependent on extraction and purification from *R. suavissimus* S. Lee, which is complicated and economically impractical for large-scale production (Ko et al. 2012). By contrast, stevioside is more abundant. Stevioside can be extracted from the leaves of the composite *Stevia rebaudiana* Bertoni, which has been used as a sweetener in South America, Asia, and some parts of Europe for many years (Geuns 2003).

Stevioside has three glycosidic bonds (β-linked sophorose, β-1,2-D-glucopyranosyl on C-13, and an ester β-glucosidic linkage on the C-19 carboxyl group) (Nguyen et al. 2014).

Published online: 22 July 2015

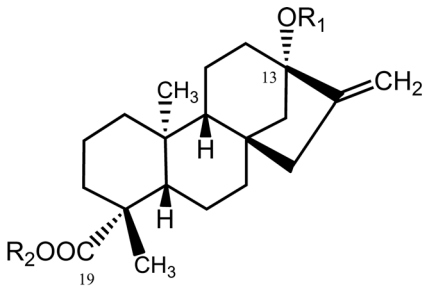


Fig. 1 Chemical structure of some known steviol glycosides. Steviol (R_1=R_2=H) is the aglycone of the steviol glycosides. Glc, Rha, and Xyl represent glucose, rhamnose, and xylose sugar moieties, respectively

Compound name	R_1 (C-13)	R_2 (C-19)	Formula	Mass
Steviol	H	H	$C_{20}H_{30}O_3$	318.22
Steviol mono-glucoside	β-Glc	H	$C_{26}H_{40}O_8$	480.27
Steviol mono-glucosyl ester	H	β-Glc	$C_{26}H_{40}O_8$	480.27
Rubusoside	β-Glc	β-Glc	$C_{32}H_{50}O_{13}$	642.33
Steviolbioside	β-Glc-(2→1)-β-Glc	H	$C_{32}H_{50}O_{13}$	642.33
Stevioside	β-Glc-(2→1)-β-Glc	β-Glc	$C_{38}H_{60}O_{18}$	804.38
Rebaudioside A	β-Glc-(2→1)-β-Glc ＼(3→1)-β-Glc	β-Glc	$C_{44}H_{70}O_{23}$	966.43
Rebaudioside B	β-Glc-(2→1)-β-Glc ＼(3→1)-β-Glc	H	$C_{38}H_{60}O_{18}$	804.38
Rebaudioside C	β-Glc-(2→1)-α-Rha ＼(3→1)-β-Glc	β-Glc	$C_{44}H_{70}O_{22}$	950.44
Rebaudioside D	β-Glc-(2→1)-β-Glc ＼(3→1)-β-Glc	β-Glc-(2→1)-β-Glc	$C_{50}H_{80}O_{28}$	1128.48
Rebaudioside F	β-Glc-(2→1)-β-Xyl ＼(3→1)-β-Glc	β-Glc	$C_{43}H_{68}O_{22}$	936.42
Dulcoside A	β-Glc-(2→1)-α-Rha	β-Glc	$C_{38}H_{60}O_{17}$	788.38

Relative to rubusoside, stevioside has an additional glucosyl group at C-13. Selective cleavage of β-1,2-glucosidic linkage of sophorosyl moiety at C-13 of stevioside produces rubusoside. Enzymatic preparation of stevioside from rubusoside has specific advantages over the chemical preparation, including higher selectivity, milder reaction conditions, convenient workup, and minimal damage to the environment (Ko et al. 2013). However, previous studies have shown that few of the 30 enzymes tested can convert stevioside to rubusoside as a primary product (Nguyen et al. 2014). Many β-linkage-hydrolyzing enzymes were screened for stevioside hydrolysis (Chen et al. 2014; Ko et al. 2012; Ko et al. 2013; Nakano et al. 1998; Nguyen et al. 2014; Okamoto et al. 2000; Wan et al. 2012). However, only three enzymes have been identified that can produce rubusoside (Ko et al. 2012; Nguyen et al. 2014; Wan et al. 2012). Many enzymes that showed specificity for stevioside exhibited nonspecific hydrolysis of sequential glucosyl units of stevioside and produced various by-products, such as steviol, isosteviol, steviolmonoglucoside, steviolbioside, steviol mono-glucosyl ester, or mixtures of these (Wan et al. 2012).

Identification of an enzyme with regiospecific activity for sophorose hydrolysis would be advantageous for large-scale production of rubusoside from stevioside. A β-glucosidase from *Novosphingobium* sp. GX9, *S*BGL, was found to show very high affinity toward compounds that contained heterocyclic aglycone, such as isoflavonoid (Du et al. 2014). In this context, searching for β-glucosidases with high sophorose activity is a promising approach for industrial production of rubusoside from stevioside by enzymatic approach. In this work, a β-glucosidase from *Streptomyces* sp. GXT6 selected for its high sophorose activity was tested for potential use in rubusoside production.

Materials and methods

Materials and microbial strains

Esculin, *p*-nitrophenyl-β-D-glucopyranoside (*p*NPG), cellobiose, and gentiobiose were purchased from Sigma (St. Louis, MO, U.S.). Compounds rebaudioside A, stevioside, rubusoside, and steviol were purchased from Sichuan Weikeqi Biological Technology Co., Ltd. (Chengdu, China). Steviolbioside was purchased from Chengdu Herbpurify Co., Ltd. (Chengdu, China). All restriction endonucleases, ligases,

and DNA polymerases were purchased from Takara (Dalian, China). *Escherichia coli* strain XL1-Blue was used as the host strain for cloning and expression, and pSE380 (Invitrogen, CA, U.S.) was used as the expression vector. *Streptomyces* sp. GXT6 was isolated from soil of cassava gardens and collected in-lab. It is also currently deposited in the China Center for Type Culture Collection [CCTCC] under accession number CGMCC7091. The genome of *Streptomyces* sp. GXT6 has been sequenced and deposited in GenBank under accession number AYRX00000000.

Overexpression and purification of the recombinant protein

The gene *bgl*1 was amplified from genomic DNA from *Streptomyces* sp. GXT6 by using the primers listed below. *Pag*I and *Hin*dIII restriction enzyme sites were introduced for cloning into the pSE380 vector. The following primers were used: bgl1-F (5′-CACTCATGATGCACCACCACCAC CACCACACGCCCCCGTTCG GGTCCGAAC-3′) and bgl1-R (5′-CATAAGCTTCTAACGCGCGGTCGGGG CACCG-3′). The underlined sequences represent the recognition sites of restriction enzymes *Pag*I and *Hin*dIII, respectively. PCR was performed with a total volume of 50 μL. Concentrations of LA DNA polymerase (Takara, Dalian, China), GC buffer II, and other components were taken from the manufacturer's recommendations. The PCR program consisted of a denaturation step at 95 °C for 2 min, followed by 30 cycles of 98 °C for 10 s, 54.5 °C for 15 s, and 72 °C for 1 min and 30 s, and a final extension at 72 °C for 10 min. After isolation from agarose gel, the PCR product was digested with *Pag*I and *Hin*dIII and then ligated into pSE380 expression vector pretreated with *Nco*I and *Hin*dIII. The ligation mixture was transformed into *E. coli* XL1-Blue, and positive transformants were able to form black halos on LB agar plates containing 2 g/L esculin and 5 g/L ferric ammonium citrate after induction by isopropyl-β-D-thiogalactopyranoside (IPTG). The recombinant plasmid pSE-*bgl*1 was verified by DNA sequencing (BGI Tech, Shenzhen, China).

A single colony was inoculated into LB medium containing 100 μg/mL ampicillin and grown overnight at 37 °C with shaking at 200 rpm. Then, it was inoculated into a 500-mL flask containing 200 mL LB broth containing 100 μg/mL ampicillin at a ratio of 1 % (*v*/*v*) and incubated at 37 °C until the cell density (OD_{600}) reached to 0.6. The culture was induced by adding 0.5 mM IPTG at 30 °C for 8 h with shaking at 200 rpm. Cells were then harvested by centrifugation (6000 *g*, 10 min) at 4 °C, and the protein was purified according to the protocol provided in the QIAexpressionist™ kit (Qiagen, Hilden, Germany). Briefly, cell pellets were re-suspended in lysis buffer (50 mM NaH_2PO_4, 300 mM NaCl, 10 mM imidazole, pH 8.0) and then incubated with lysozyme (1 mg/mL) on ice for 15 min. After ultrasonication, the lysate was centrifuged at 12,000*g* for 20 min at 4 °C to remove cell debris. The supernatant was mixed with nickel nitrilotriacetic acid agarose (Ni-NTA Agarose, Qiagen) resin for 1 h at 4 °C with slow shaking and then loaded onto a prechilled column. After washing with 4 mL wash buffer (50 mM NaH_2PO_4, 300 mM NaCl, 20 mM imidazole, pH 8.0), recombinant protein was eluted in elution buffer (50 mM NaH_2PO_4, 300 mM NaCl, 250 mM imidazole, pH 8.0). The target fraction was collected and then the buffer was replaced with 100 mM NaH_2PO_4-Na_2HPO_4 buffer (pH 7.0) using a PD MiniTrap G-25 column (GE Healthcare, Freiburg, Germany). Protein concentrations were determined using the Bradford method with bovine serum albumin (BSA) as a standard protein. The molecular mass and the purity of the recombinant protein were determined using 10 % sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and visualized by staining with Coomassie blue R-250.

Enzymatic activity analysis

The β-glucosidase activity was assessed using *p*-nitrophenyl-β-D-glucopyranoside as substrate (*p*NPG; Sigma). The standard reaction (200 μL) was performed in McIlvaine buffer (100 mM citric acid-200 mM Na_2HPO_4, pH 6.5) containing 2 mM *p*NPG and appropriately diluted enzyme at 45 °C for 20 min. After 2 min of preheating, the reaction was started by adding the diluted enzyme solution and was stopped by adding 50 μL of 1 M Na_2CO_3 to the reaction mixture. The amount of *p*-nitrophenol (*p*NP) released was monitored at 405 nm in 96-well microtiter plates using a microplate reader (μ-Quant, BioTek, VT, USA). A reaction mixture without enzyme was used as a blank control, and all enzyme activities were determined in three independent experiments. A standard curve of *p*NP was used to quantify the product. One unit of β-glucosidase activity (U) was defined as the amount of enzyme that produced 1 μmol of *p*-nitrophenol or glucose per minute under the experimental conditions described above.

pH, temperature, and enzyme activity

To measure the optimum pH of recombinant BGL1, the enzyme activity was assayed at 37 °C for 20 min in the range of pH 4.5–8.0 using McIlvaine buffer supplemented with 2 mM *p*NPG. To characterize the pH stability, the purified enzyme was incubated for 24 h at 4 °C in the absence of substrate in the following buffer systems: McIlvaine buffer (pH 5.0–8.0), $Na_2B_4O_7$-H_3BO_3 buffer (100 mM, pH 7.5–9.0), and glycine-NaOH buffer (100 mM, pH 9.0–10.5). Then, the residual activity was measured under standard assay conditions.

To determine the optimal temperature of recombinant BGL1, enzymatic activity was determined in McIlvaine buffer (pH 6.5) containing 2 mM *p*NPG within a temperature range

of 25–65 °C for 20 min. Thermostability was measured by analyzing residual activity under standard assay conditions after incubation of aliquots of the purified enzyme for varying periods of time at temperatures ranging from 35 to 55 °C. The experimental data for thermal deactivation of enzyme was fitted to a first-order curve, and the half-life of the enzyme was calculated using Graphpad Prism 5.0 software (GraphPad Software, San Diego, CA, U.S.).

Substrate specificity assays

The substrate specificity of the recombinant BGL1 was determined by incubating the purified enzyme in McIlvaine buffer (pH 6.5) containing 2 mM *p*NP-β-D-glucopyranoside, *p*NP-α-D-glucopyranoside, *p*NP-β-D-xylopyranoside, *p*NP-β-D-cellobioside, *p*NP-β-D-galactopyranoside, *p*NP-N-acetyl-β-D-glucosaminide, *o*NP-β-D-galactopyranoside, 4-methylumbelliferyl-β-D-glucopyranoside (MUGlc) (Sigma, St. Lois, MO, USA), or 10 g/L sophorose, laminaribiose, cellobiose, gentiobiose, amygdalin, salicin, sucrose, trehalose, lactose, maltose, isomaltose, carboxymethylcellulose (CMC), acid-swollen cellulose, xylan from oat spelts, xylan from birchwood, and starch at 45 °C for 20 min. The amounts of *p*-nitrophenol and *o*-nitrophenol released were determined under standard enzyme assay conditions. Released glucose concentration was analyzed using a Glucose Assay Kit (Applygen Technologies Inc., Beijing, China). Concentrations of reducing sugars released from polysaccharose were determined using the dinitrosalicylic acid (DNS) method (Miller 1959). BGL1 hydrolysis of MUGlc was visualized under ultraviolet light due to release of fluorescent methyl umbelliferone. The relative rates of hydrolysis on different substrates were determined as relative values of the initial rate of hydrolysis obtained with *p*NPG.

Determination of kinetic parameters

Michaelis-Menten kinetic parameters and the inhibition constant (K_i) for glucose inhibition of recombinant BGL1 were determined by fitting data to nonlinear regression analysis. Competitive inhibition model fitting and plotting were performed using Graphpad Prism 5.0 software (GraphPad Software, San Diego, CA, USA). The initial velocity of the reaction was determined by incubating the enzyme in McIlvaine buffer (pH 6.5) with *p*NPG (0.25 to 16 mM) as a substrate either in the absence or presence of glucose at two fixed concentrations (400 and 600 mM) for 20 min at 45 °C.

Analysis of stevioside hydrolysis

To measure the effect of pH on the enzymatic hydrolysis activity for stevioside, the purified enzyme (5.3 μg/mL) was incubated with 10 g/L stevioside at 37 °C for 20 min in the following buffers: McIlvaine buffer (pH 5.0–8.0), $Na_2B_4O_7$-H_3BO_3 buffer (100 mM, pH 7.5–9.0), and Na_2CO_3-$NaHCO_3$ buffer (100 mM, pH 9.0–10.0). To measure the effect of temperature on enzymatic hydrolytic activity for stevioside, the purified enzyme (3.8 μg/mL) was incubated in $Na_2B_4O_7$-H_3BO_3 buffer (100 mM, pH 8.5) with 10 g/L stevioside for 20 min at temperatures ranging from 25 to 65 °C. To determine the kinetic properties of recombinant BGL1 toward stevioside, the purified enzyme (0.77 μg/mL) was incubated in $Na_2B_4O_7$-H_3BO_3 buffer (100 mM, pH 8.5) for 20 min at 50 °C with stevioside ranging from 0.2 to 2.5 g/L. After 2 min of preheating, the reaction was initiated by addition of enzyme solution and then later stopped by boiling that solution for 5 min. The liberated glucose was analyzed by the Glucose Assay Kit (Applygen Technologies Inc.) according to the manufacturer's recommendations. All enzyme activities were determined in duplicates and a reaction mixture without enzyme served as a blank control.

Analysis of hydrolysis product of steviol glucosides

To identify the product of hydrolysis of stevioside and other steviol glucosides, the purified enzyme (15 μg/mL) was incubated in $Na_2B_4O_7$-H_3BO_3 buffer (100 mM, pH 8.5) with 10 g/L of rebaudioside A, stevioside, rubusoside, and steviolbioside at 50 °C for 12 h. To analyze stevioside hydrolysis by BGL1 over time, the reaction mixture containing the purified enzyme (30 μg/mL) and 10 g/L stevioside was reacted in $Na_2B_4O_7$-H_3BO_3 buffer (100 mM, pH 8.5) at 50 °C for varying periods of time. The reaction mixture was withdrawn at specified time points and boiled for 5 min to inactivate the enzyme. The products of the reaction were analyzed using high-performance liquid chromatography (HPLC) and ultra performance liquid chromatography coupled with quadrupole time-of-flight mass spectrometry (UPLC-MS).

To purify reaction products, preparative scale reactions were set up; 20 mL reaction mixture containing the purified enzyme (50 μg/mL) and 10 g/L stevioside in $Na_2B_4O_7$-H_3BO_3 buffer (100 mM, pH 8.5) was incubated at 50 °C for 6 h. The enzymatic reaction was terminated by boiling for 5 min. A Waters HPLC system, comprised of a Waters 2535 quaternary gradient module, a 2707 autosampler, a 2489 UV/Visible detector, a reverse phase C18 column (SunFire™, 250×10 mm, 5 μm), and a fraction collector III (Waters Corporation, Milford, USA), was used for product preparation. The sample was injected and eluted with a linear gradient of solvent A (acetonitrile) and solvent B (H_2O): 68 % B (0–13 min), 68 to 20 % B (13–25 min), 20 % B (25–30 min), 20 to 68 % B (30–40 min), and 68 % B (40–55 min) at a flow rate of 2 mL/min at room temperature, and the UV absorption was measured at 210 nm. The purified rubusoside fractions were

pooled and freeze-dried for determination by nuclear magnetic resonance (NMR).

A Waters HPLC system, comprised of a Model e2695 separation module system, a reverse phase C18 column (Alltima, 250 × 4.6 mm, 5 μm), and a Model 2998 photodiode array detector (Waters Corporation, Milford, USA), was used in this study for general analysis. The sample was injected and eluted with a linear gradient of solvent A (acetonitrile) and solvent B (10 mM sodium phosphate buffer, pH 2.6): 68 % B (0–10 min), 68 to 20 % B (10–20 min), 20 % B (20–21 min), 20 to 68 % B (21–28 min), and 68 % B (28–30 min) at a flow rate of 1 mL/min. The column temperature was set at 40 °C, and the UV absorption was measured at 210 nm. A Waters ACQUITY UPLC™ system (Waters, Milford, MA, USA) equipped with a photodiode array detection (PDA) system and quadrupole time-of-flight mass spectrometry (QTOF) was used for mass spectrometric analysis of the hydrolysis product. The chromatography was performed using ACQUITY UPLC® BEH C18 column (1.7 μm, 2.1 × 100 mm, Waters, Milford, MA, USA). The mobile phase consisted of solvent A (0.1 % formic acid in water) and solvent B (acetonitrile). A linear gradient elution was performed as follows: 30 to 40 % B (0–4 min), 40 to 80 % B (4–7 min), 80 % B (7–8 min), 80 to 30 % B (8–8.1 min), and 30 % B (8.1–10 min). The flow rate was 0.4 mL/min and the injection volume was 1 μL. The temperature of column was set to 40 °C. The scan range for PDA was 190–400 nm. All samples were diluted appropriately using 50 % acetonitrile and filtered through a 0.22-μm membrane. Mass spectrometry was performed on a Waters Xevo® G2-S QTof Mass Spectrometry (Waters, Milford, MA, USA) equipped with an electrospray ionization (ESI) source. Detection was performed in negative-ion (ESI) mode. The ESI conditions were as follows: capillary voltage 2600 V, cone voltage 40 V, source temperature 100 °C, and desolvation temperature 400 °C. High-purity nitrogen was used as desolvation and cone gas at flow rates of 700 and 20 L/h, respectively. The MS experiment, which records exact mass precursor and fragment ion information in the same run, was performed with the low collision energy set to 6 V and the high collision energy ramped from 30 to 40 V. Data were collected in centroid mode over the m/z range of 100–1500 Da with an acquisition time of 0.1 s. All MS data were acquired using LockSpray™ to ensure mass accuracy and reproducibility. Leucine-enkephalin (m/z 556.2771 or 554.2615 in positive and negative ionization modes, respectively) was used as the lock mass at a concentration of 1 μg/mL at a flow rate of 10 μL/min. The data were processed using Waters MassLynx software version 4.1 (Waters, Milford, MA, USA). The QTOF-MS was calibrated daily according to the manufacturer's recommendations.

For NMR structural elucidation of the hydrolysis products, approximately 10 mg of the purified rubusoside was dissolved in 500 μL of deuterated water and then placed into 5-mm NMR tubes. NMR spectra were acquired on a Varian VNMRS 600 MHz spectrometer (Varian, Palo Alto, CA, USA) operating at 600 MHz for ^{1}H and 150 MHz for ^{13}C at 25 °C.

Nucleotide acid sequence accession number

The GenBank accession numbers of the nucleotide sequences of the *bgl*1 gene and the 16S rRNA gene of *Streptomyces* sp. GXT6 presented in this report were KJ958924 and GU348992, respectively.

Results

Enzyme overexpression and purification

From the genome sequence of *Streptomyces* sp. GXT6, an open reading frame of 1278 bp encoding a glycoside hydrolase family 1 (GH family 1) protein of 425 amino acids was found and named *bgl*1. Protein blast (BLASTP) analysis indicated that BGL1 shares the highest identity (73 %) with a β-glucosidase from *Streptomyces leeuwenhoekii* (GenBank accession number: WP_029385013). The *bgl*1 gene was cloned into pSE380 expression vector and then transformed into *E. coli* XL1-Blue for expression. The cells were harvested after induction with IPTG and disrupted by sonication in an ice-water bath. The cell lysate supernatant displayed hydrolyzing activity for esculin, which suggested that the recombinant enzyme was expressed in soluble form. The recombinant enzyme was purified using Ni-NTA chromatography, and purified protein was separated by SDS-PAGE. The calculated molecular weight of the recombinant BGL1 with an N-terminal histidine tag was≈47.1 kDa. A clear protein band of≈47 kDa as shown in Supplementary Fig. S1 (lane 4) matches the predicted molecular weight of BGL1. Our purified BGL1 has a specific activity of 18.5 U/mg corresponding to a 5.6-fold purification with 16.6 % recovery.

Enzymatic properties of BGL1 on *p*NPG substrate

The enzymatic activity of recombinant BGL1 was measured at various pH values (pH 4.5–8.0) using *p*NPG as substrate. The optimum pH for BGL1 enzymatic activity was 6.5 (Supplementary Fig. S2a) and the enzyme displayed high activity (more than 80 %) at a narrow pH range, between 6.5 and 7.0. The enzyme also showed good stability, retaining over 80 % of its original activity between pH 7.0 and 8.5 after incubation in specified buffers for 24 h at 4 °C (Supplementary Fig. S2c). The optimal temperature of recombinant BGL1 was 45 °C, and the enzyme retained over 80 % of its activity within the temperature range from 40–50 °C (Supplementary Fig. S2b). However, the enzyme showed poor thermostability over 40 °C (Supplementary Fig. S2d).

Table 1 Substrate specificity of the recombinant BGL1

Substrate	Linkage of glycosyl group	Relative activity (%)
Aryl-glycosides		
*p*NP-β-D-glucopyranoside	β-Glc	100±2.2
*p*NP-β-D-galactopyranoside	β-Gal	7.1±0.2
*p*NP-β-D-xylopyranoside	β-Xyl	1.7±0.2
*p*NP-β-D-cellobioside	β-Cel	1.5±0.3
*o*NP-β-D-galactopyranoside	β-Gal	1.5±0.2
*p*NP-N-acetyl-β-D-glucosaminide	β-Glc	0
*p*NP-α-D-glucopyranoside	α-Glc	0
Amygdalin	β-Glc	0.9±0.0
Salicin	β-Glc	[a]
Saccharides		
Cellobiose	Glc β-(1→4) Glc	1.3±0.0
Gentiobiose	Glc β-(1→6) Glc	0.2±0.0
Lactose	Gal β-(1→4) Glc	0.04±0.0
Laminaribiose	Glc β-(1→3) Glc	16.1±0.7
Sophorose	Glc β-(1→2) Glc	99.9±0.7
Sucrose	Glc α-(1→2) Fru	0
Trehalose	Glc α-(1→1) Glc	0
Maltose	Glc α-(1→4) Glc	0
Isomaltose	Glc α-(1→6) Glc	0
CMC	β-Glc	0
Acid-swollen cellulose	β-Glc	0
Xylan from oat spelts	β-Xyl	0
Xylan from birchwood	β-Xyl	0
Starch	α-Glc	0

Data represent the means of three separate experiments with standard deviation. The activity levels measured using *p*NPG as substrate were considered to be 100 %

[a] The substrate can be hydrolyzed by BGL1, but no accurate activity data were available here

Table 2 Kinetic parameters of three β-glucosidases toward steviol glucosides

Substrate	SSGase[a]			SPGase[b]			BGL1[c]		
	K_m (mM)	k_{cat} (1/s)	k_{cat}/K_m (1/s•mM)	K_m (mM)	k_{cat} (1/s)	k_{cat}/K_m (1/s•mM)	K_m (mM)	k_{cat} (1/s)	k_{cat}/K_m (1/s•mM)
*p*NPG	0.05	4.8	95.6	1.3	3.2	2.5	1.4	24.4	17.8
Stevioside	3.6	30.4	8.5	10.5	10.2	1.0	1.5	13.2	9.0
Rubusoside	13.6	18.4	1.3	9.9	10.7	1.1	–	–	–
Steviol Mono-glucosyl ester	[d]	[d]	[d]	14.4	15.4	1.1	[d]	[d]	[d]
Steviol Mono-glucoside	–	–	–	16.6	32.1	2.0	[d]	[d]	[d]
Rebaudioside A	–	–	–	–	–	–	–	–	–

Hyphen (–): No hydrolysis activity was detected

[a] β-glucosidase from *Aspergillus aculeatus* (Ko et al. 2012)

[b] β-glucosidase from *Penicillium decumbens* (Ko et al. 2013)

[c] β-glucosidase from *Streptomyces* sp. GXT6 (this study)

[d] Not reported

BGL1 half-life ($t_{1/2}$) was 1220, 324, 38, 5.6, and 0.9 min at 35, 40, 45, 50, and 55 °C, respectively.

Michaelis-Menten kinetic parameters and the inhibition constant (K_i) for glucose inhibition of recombinant BGL1 were determined by fitting data to a competitive inhibition model by nonlinear regression analysis (Supplementary Fig. S3). The values of K_m, V_{max}, k_{cat}, and k_{cat}/K_m of BGL1 were 1.37±0.07 mM, 31.1±0.38 μmol/min/(mg protein), 24.36 1/s, and 17.78 1/s•mM, respectively. The inhibition constant K_i was 385.0±29.3 mM, which indicated that the BGL1 had strong tolerance for glucose.

Substrate specificity of BGL1

The substrate specificity of BGL1 for different substrates were determined as shown in Table 1. Recombinant BGL1 showed broad substrate specificity but exhibited the highest activity for *p*NPG. BGL1 also hydrolyzed *p*NP-β-D-galactopyranoside, *p*NP-β-D-xylopyranoside, *p*NP-β-D-cellobioside, *o*NP-β-D-galactopyranoside, and MUGlc. No activity was detected for *p*NP-α-D-glucopyranoside or *p*NP-N-acetyl-β-D-glucosaminide. Our BGL1 hydrolyzed sophorose, laminaribiose, cellobiose, amygdalin, gentiobiose, esculin, and salicin. Weak activity for lactose was also observed. However, BGL1 displayed no activity for sucrose, trehalose, maltose, or isomaltose (Supplementary Table S1). No hydrolytic activity was detected for the soluble polysaccharides CMC, acid-swollen cellulose, xylan (from oat spelts or birchwood), or starch.

Analysis of BGL1 hydrolysis of stevioside

The optimal pH and temperature for stevioside hydrolysis were determined using a Glucose Assay Kit and as shown in Supplementary Fig. S4. Recombinant BGL1 showed the highest activity at pH 8.5 and retained more than 80 % of its maximal activity in the pH range 8.0–9.0. The enzyme showed the highest activity at 50 °C and retained over 80 % of its maximal activity from 40–50 °C. However, activity decreased to 49 % of its maximal activity, at 55 °C.

The enzyme kinetic parameters of BGL1 for stevioside were as follows: K_m 1.47±0.13 mM, V_{max} 16.83±0.7 μmol/min/(mg protein), k_{cat} 13.18 1/s, and k_{cat}/K_m 8.97 1/s•mM. Enzyme kinetic parameters for BGL1 and two additional stevioside-specific enzymes are summarized in Table 2.

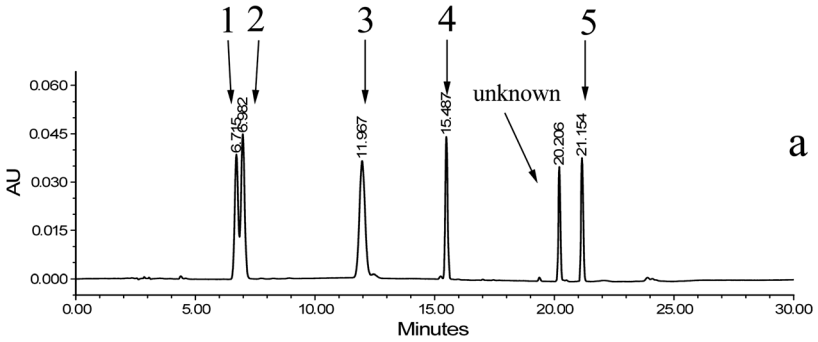
The hydrolytic activity and hydrolysis products of rebaudioside A, stevioside, rubusoside, and steviolbioside were analyzed using HPLC (Figs. 2 and 3). Our results indicated that BLG1 exhibited high hydrolytic activity for stevioside and steviolbioside but did not hydrolyze rebaudioside A or rubusoside. Four products were detected from stevioside hydrolysis and are marked peak1, peak2, and peak3 (all three as by-products with small yields) and peak4 (the major product) after retention times of 4.12, 4.58, 6.15, and 11.91 min in HPLC profiles, respectively (Fig. 2). Each product peak was analyzed using mass spectrometry. Peak1 represented a compound with a 1011.42 *m/z* ratio (Fig. 2c), which is an adduct between peak1 and formic acid as the form of a $[M-H+HCOOH]^-$ species in the negative-ion (ESI^-) mode. The molecular weight of peak1 is 162 Da greater than the basic molecular weight of stevioside and the addition equal to the molecular weight of a glucose moiety. Peak1 is likely a transglycosylation product of stevioside. Both peak2 and peak3 showed a 849.37 *m/z* ratio (Fig. 2d, e), a form of $[M-H+HCOOH]^-$ species, which indicated that peak2 and peak3 shared the same molecular weight with stevioside. According to the retention time of stevioside standards, peak2 and peak3 are likely isomers of stevioside, but the mechanism underlying their formation was not clear. The mass of peak4 had a 687.32 *m/z* ratio (Fig. 2f) and was identified as rubusoside in the form of a $[M-H+HCOOH]^-$ species after comparison to the standards.

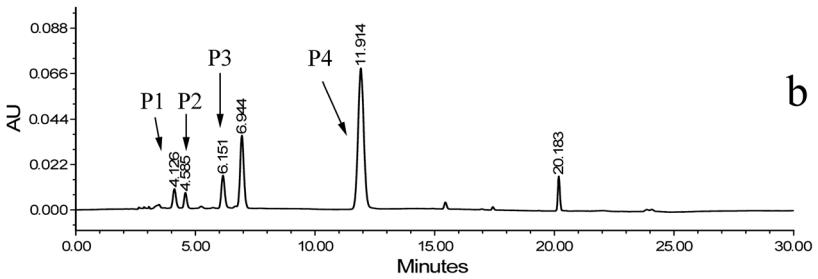
Rebaudioside A was not hydrolyzed by BGL1 (Fig. 3b). Hydrolysis of rubusoside did not produce any detectable product (Fig. 3c), even when the concentration of enzyme was 55 μg/mL and the reaction time reached 24 h (data not shown). BGL1 produced one product from steviolbioside, which was eluted at 17.44 min (Fig. 3d). This product had a 525.27 *m/z* ratio in a form of $[M-H+HCOOH]^-$ species in the negative-ion (ESI^-) mode (data not shown). This product was inferred to be steviolmono-glucoside from the chemical structure of steviolbioside.

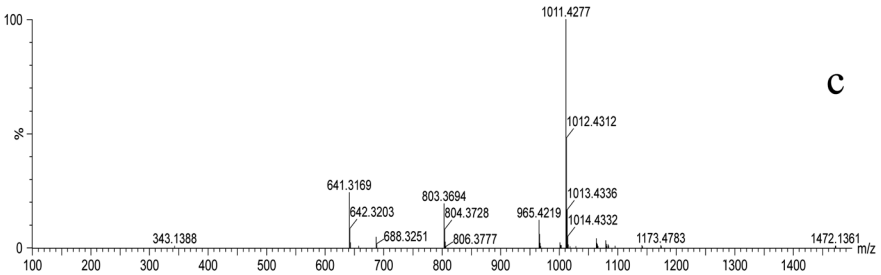
Production of rubusoside from stevioside by BGL1

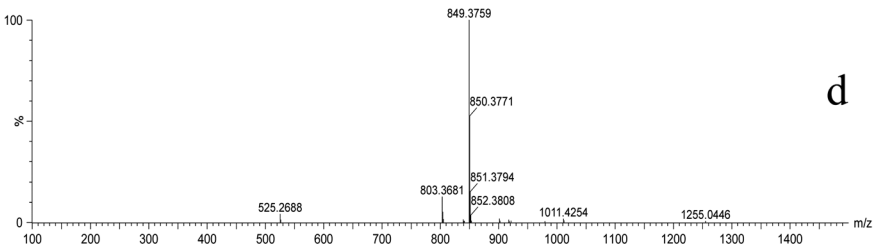
The results of time course analysis showed that the process of stevioside hydrolysis was complete after 6 h (Fig. 4). The same result was obtained using an increased concentration of BGL1 (55 μg/mL) under the same reaction conditions. Almost all stevioside substrate had been converted to product, leaving less than 2 % of substrate. These results indicate that the enzyme hydrolyzes only the glucose moiety of the sophoroside at C-13 in stevioside to produce rubusoside, leaving other glucose moieties untouched. The NMR analysis confirmed that the transfer product was rubusoside. The NMR data of transfer product is shown in Supplementary Table S2.

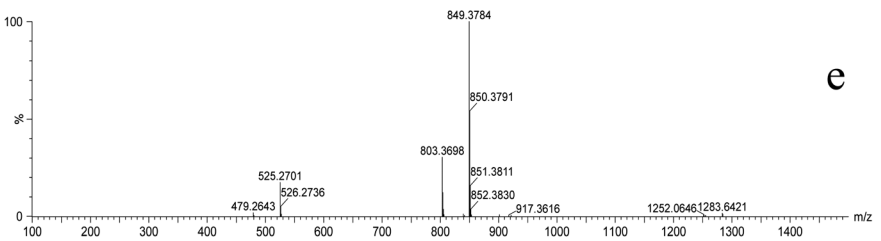
At a preparative scale production of rubusoside, the stevioside conversion rate was 98.2 % and rubusoside yield was 78.8 %. The amount of rubusoside synthesis/1000 U/h was 56.7 g/L.

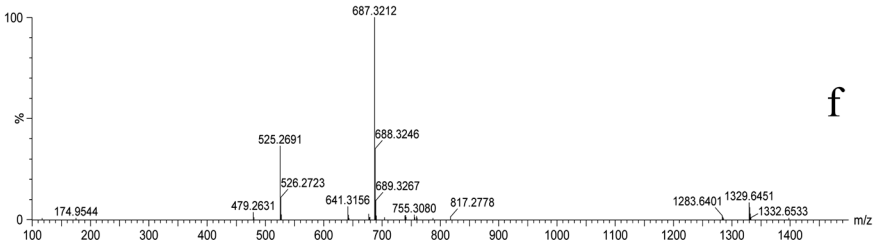

1
2
3
4
5
unknown
6.715
6.982
11.967
15.487
20.206
21.154
AU
Minutes
a


P1
P2
P3
P4
4.126
4.585
6.151
6.944
11.914
20.183
AU
Minutes
b


1011.4277
1012.4312
641.3169
803.3694
642.3203
804.3728
1013.4336
965.4219
1014.4332
343.1388
688.3251
806.3777
1173.4783
1472.1361
m/z
c


849.3759
850.3771
803.3681
851.3794
525.2688
852.3808
1011.4254
1255.0446
m/z
d


849.3784
850.3791
803.3698
525.2701
851.3811
526.2736
479.2643
852.3830
917.3616
1252.0646
1283.6421
m/z
e


687.3212
525.2691
688.3246
526.2723
689.3267
479.2631
641.3156
755.3080
817.2778
1283.6401
1329.6451
1332.6533
174.9544
m/z
f

◀ **Fig. 2** HPLC-MS analysis of stevioside hydrolysis. **a** Standard steviol glucosides in order of retention time: (*1*) rebaudioside A, (*2*) stevioside, (*3*) rubusoside, (*4*) steviolbioside, and (*5*) steviol, respectively; the peak marked "*unknown*" (at retention time 20.2 min) was identified as an impurity, and it was found in every tested commercially available substrate and had no effect on the enzyme assay. **b** The product of hydrolysis of stevioside. The reaction was performed by incubating the purified enzyme (15 μg/mL) in $Na_2B_4O_7$-H_3BO_3 buffer (100 mM, pH 8.5) with 10 g/L of stevioside at 50 °C for 12 h. **c–f** MS analysis of four hydrolysis products from stevioside in the order of P1–P4 (four peaks shown in (**b**))

Discussion

To date, many studies have been performed to search for stevioside hydrolysis enzymes. Previous efforts have identified a total of seven enzymes suitable for hydrolysis of the β-glucosyl linkages of steviol glucosides. Among these, three β-galactosidases from *Aspergillus* sp. (Wan et al. 2012), *Sulfolobus solfataricus* (Chen et al. 2014), and *Thermus thermophilus* (Nguyen et al. 2014) focused on the optimization of steviol or rubusoside production, but they provided limited information about their specificity for stevioside. The remaining four enzymes were all β-glucosidases: *Clavibacter michiganense* β-glucosidase (CMGase) (Nakano et al. 1998), *Flavobacterium johnsoniae* β-glucosidase (FJGase) (Okamoto et al. 2000), *Aspergillus aculeatus* β-glucosidase (SSGase) (Ko et al. 2012), and *Penicillium decumbens* β-glucosidase (SPGase) (Ko et al. 2013). Each has been described in detail with respect to hydrolysis of steviol glucoside substrate. The biggest similarities between these four β-

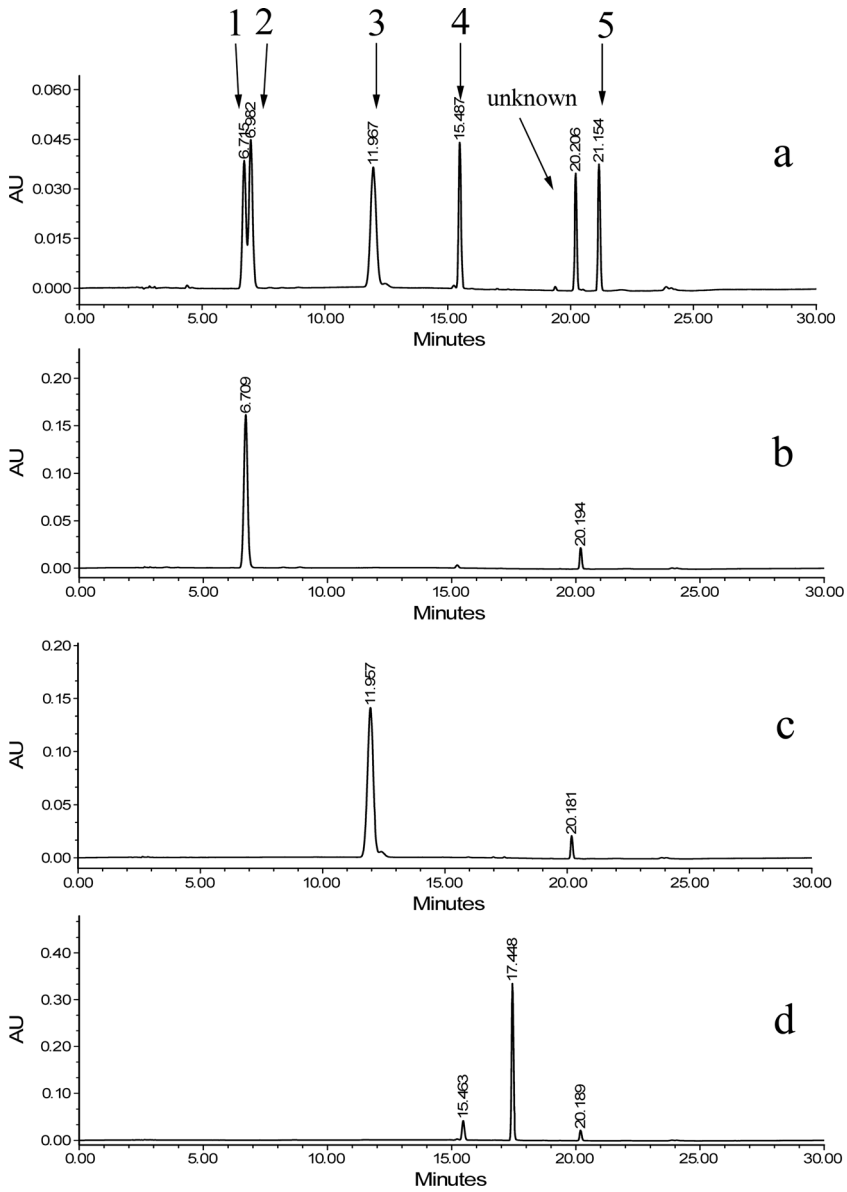


Fig. 3 HPLC profiles of various steviol glucoside hydrolysis products. **a** Standard steviol glucosides in order of retention time referring to that provided in Fig. 2. **b–d** The products of hydrolysis of rebaudioside A, rubusoside, and steviolbioside, respectively. The reaction was performed by incubating the purified enzyme (15 μg/mL) in $Na_2B_4O_7$-H_3BO_3 buffer (100 mM, pH 8.5) with 10 g/L of rebaudioside A, rubusoside, and steviolbioside at 50 °C for 12 h

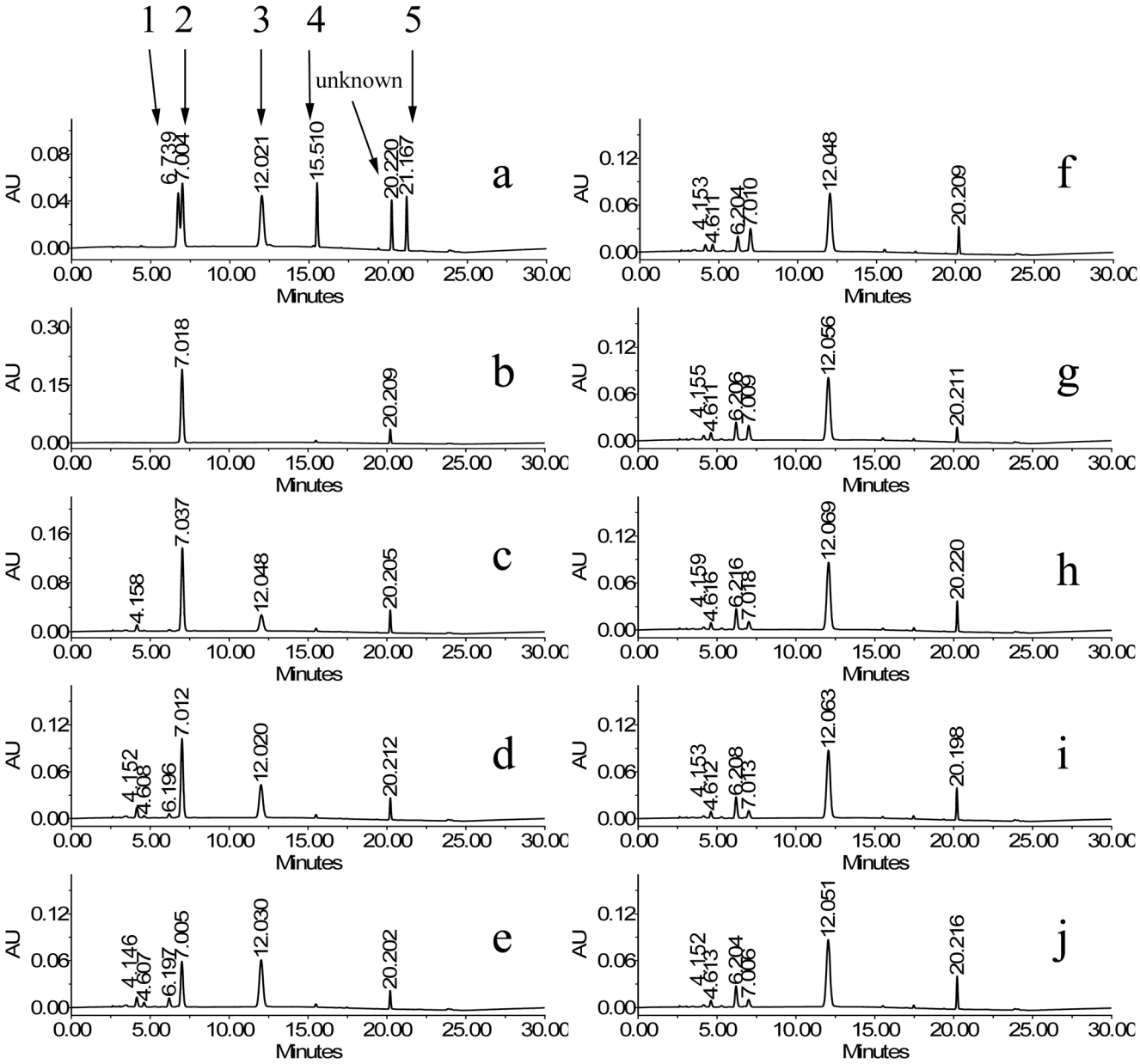


Fig. 4 Time course analysis of stevioside hydrolysis by recombinant BGL1. The reaction was performed by incubating the purified enzyme (30 μg/mL) in $Na_2B_4O_7$-H_3BO_3 buffer (100 mM, pH 8.5) with 10 g/L of stevioside at 50 °C for 0–360 min. **a** Standard steviol glucosides in order of retention time referring to that provided in Fig. 2. **b-j** The reaction mixture was incubated for 0, 5, 10, 20, 40, 60, 120, 240, and 360 min, respectively

glucosidases were that they displayed little activity for cellobiose. The natural substrates for these enzymes appear to be other saccharides rather than the cellobiose.

BGL1 is a β-glucosidase. This enzyme appears to use sophorose as natural substrate. Our study showed that it exhibits activity toward stevioside. BGL1 is very different from previously reported stevioside-hydrolyzing enzymes. For example, BGL1 has a smaller molecular mass (47 kDa) than CMGase (65 kDa), FJGase (72 kDa), SSGase (79 kDa), and SPGase (121 kDa). For stevioside hydrolysis, the optimal pH of BGL1 (pH 8.5) was slightly more alkaline than that of CMGase (pH 7.5) and FJGase (pH 7.0). The optimal pH was also more alkaline than SSGase (pH 5.0) and SPGase (pH 4.0). The optimal temperature (50 °C) of BGL1 was a little higher than that of CMGase and FJGase (both were 45 °C) and slightly lower than SSGase and SPGase (both were 60 °C). A large difference was found with respect to specificities toward steviol glucosides. CMGase was found to hydrolyze glucosyl ester linkages at C-19 of rebaudioside A, stevioside, rubusoside, and steviol mono-glucosyl ester. It was also found to degrade the glucosidic bond in the saccharide chain at C-13 of rebaudioside B and steviolbioside to a lesser extent. However, it did not cleave glucosyl residue of rebaudioside A, stevioside, rubusoside, or steviol mono-glucoside at C-13. Steviol mono-glucoside was

accumulated as a final product for the hydrolysis steviol glucoside by CMGase (Nakano et al. 1998). FJGase specificity was comparable to CMGase, as both enzymes preferentially hydrolyzed glucosyl ester linkages of rebaudioside A, stevioside, rubusoside, and steviol mono-glucosyl ester at site C-19 and did not hydrolyze the glucosidic bond in the saccharide chain at C-13 of rebaudioside A and stevioside. However, this enzyme had a considerable amount of hydrolytic activity for the glucosidic bond when a glucose residue was the only component present at the 13-hydroxyl group of steviol glucosides, such as rubusoside and steviolmono-glucoside, and it showed no activity toward the glucosidic bond in the saccharide chain at C-13 of rebaudioside B and steviolbioside (Okamoto et al. 2000). SPGase hydrolyzed all the glucosidic bonds and glucosyl ester linkages between the glucosidic moiety and steviol, such as in stevioside, rubusoside, steviolmono-glucoside, and steviol mono-glucosyl ester. However, it did not hydrolyze rebaudioside A. SPGase was the only one of these β-glucosidases that could produce steviol from stevioside in a single-enzyme system (Ko et al. 2013). SSGase did not hydrolyze the glucosyl ester linkages at the 19-carboxyl group of rebaudioside A, stevioside, rubusoside, and steviol mono-glucosyl ester or the glucosidic bonds in the saccharide chain at C-13 of rebaudioside A. This enzyme hydrolyzed glucosidic linkages at the 13-hydroxyl group of stevioside and rubusoside. For this reason, it was selected for use as a specific β-glucosidase for producing rubusoside from stevioside (Ko et al. 2012). BGL1 only hydrolyzed stevioside and steviolbioside. It did not hydrolyze rebaudioside A, which may have been due to steric hindrance, as rebaudioside A has a β-glucosyl (1→3) derivative of the C-13-hydroxyl group of stevioside.

BGL1 specifically hydrolyzed the glucose moiety of the sophoroside at C-13 in stevioside or steviolbioside, and there were no further products of hydrolysis from rubusoside or steviol mono-glucoside. Our results indicate that rubusoside was the final product of the stevioside hydrolysis process and suggest that BGL1 is sufficient for the production of rubusoside from stevioside. Unlike other enzymes used for rubusoside production, BGL1 accomplished the converted reaction in less than 6 h. The time for lactase from *T. thermophilus*, SSGase from *A. aculeatus*, and β-galactosidase from *Aspergillus* sp. was 9, 12, and 24 h, respectively (Ko et al. 2012; Nguyen et al. 2014; Wan et al. 2012). In this process, all four enzymes showed a high stevioside conversion rate (95.4–98.3 %) and rubusoside yield (66–91.4 %). Rubusoside synthesis/1000 U/h was also elevated using BGL1, as our enzyme preparation yielded 56.7 g/L rubusoside product compared with 14.4 g/L from *T. thermophilus* lactase and 0.19 g/L from *Aspergillus* sp. β-galactosidase.

In this study, we describe a novel stevioside-specific β-glucosidase from *Streptomyces* sp. GXT6. BGL1 specifically hydrolyzes the glucose moiety of the sophoroside at C-13 in stevioside to produce rubusoside. Thus, this enzyme possesses valuable properties that make it suitable for industrial use, including high tolerance to glucose. The present results indicated that this novel β-glucosidase from *Streptomyces* sp. GXT6 is a promising candidate in the industrial production of rubusoside.

Acknowledgments This work was supported by the National Natural Science Foundation of China (No. 31360369, 21366007), the Innovation Project of Guangxi Graduate Education (No. YCBZ2015005) and the Natural Science Foundation of Guangxi (No. 2014GXNSFAA118097).

Conflict of interest The authors declare that they have no conflicts of interest.

References

Ceunen S, Geuns JM (2013) Steviol glycosides: chemical diversity, metabolism, and function. J Nat Prod 76(6):1201–1228

Chen J-M, Xia Y-M, Wan H-D, Wang H-J, Liu X (2014) A complete specific cleavage of glucosyl and ester linkages of stevioside for preparing steviol with a β-galactosidase from *Sulfolobus solfataricus*. J Mol Catal B-Enzym 105:126–131

Chou G, Xu SJ, Liu D, Koh GY, Zhang J, Liu Z (2009) Quantitative and fingerprint analyses of Chinese sweet tea plant (*Rubus suavissimus* S. Lee). J Agric Food Chem 57(3):1076–1083

Du L, Wang Z, Zhao Y, Huang J, Pang H, Wei Y, Lin L, Huang R (2014) A beta-glucosidase from *Novosphingobium* sp. GX9 with high catalytic efficiency toward isoflavonoid glycoside hydrolysis and (+)-catechin transglycosylation. Appl Microbiol Biotechnol 98(16):7069–7079

Geuns J (2003) Stevioside. Phytochemistry 64(5):913–921

Ko JA, Kim YM, Ryu YB, Jeong HJ, Park TS, Park SJ, Wee YJ, Kim JS, Kim D, Lee WS (2012) Mass production of rubusoside using a novel stevioside-specific beta-glucosidase from *Aspergillus aculeatus*. J Agric Food Chem 60(24):6210–6216

Ko JA, Ryu YB, Kwon HJ, Jeong HJ, Park SJ, Kim CY, Wee YJ, Kim D, Lee WS, Kim YM (2013) Characterization of a novel steviol-producing beta-glucosidase from *Penicillium decumbens* and optimal production of the steviol. Appl Microbiol Biotechnol 97(18):8151–8161

Koh GY, Chou G, Liu Z (2009) Purification of a water extract of Chinese sweet tea plant (*Rubus suavissimus* S. Lee) by alcohol precipitation. J Agric Food Chem 57(11):5000–5006

Liu Z, Schwimer J, Liu D, Lewis J, Greenway FL, York DA, Woltering EA (2006) Gallic acid is partially responsible for the antiangiogenic activities of Rubus leaf extract. Phytother Res 20(9):806–813

Miller GL (1959) Use of dinitrosalicylic acid reagent for determination of reducing sugar. Anal Chem 31(3):426–428

Nakano H, Okamoto K, Yatake T, Kiso T, Kitahata S (1998) Purification and characterization of a novel β-glucosidase from *Clavibacter michiganense* that hydrolyzes glucosyl ester linkage in steviol glycosides. J Ferment Bioeng 85(2):162–168

Nguyen TT, Jung SJ, Kang HK, Kim YM, Moon YH, Kim M, Kim D (2014) Production of rubusoside from stevioside by using a thermostable lactase from *Thermus thermophilus* and solubility enhancement of liquiritin and teniposide. Enzyme Microb Technol 64–65:38–43

Okamoto K, Nakano H, Yatake T, Kiso T, Kitahata S (2000) Purification and some properties of a β-glucosidase from *Flavobacterium johnsonae*. Biosci Biotechnol Biochem 64(2):333–340

Prakash Chaturvedula VS, Prakash I (2011) A New diterpene glycoside from *Stevia rebaudiana*. Molecules 16(4):2937–2943

Wan H-D, Tao G-J, Kim D, Xia Y-M (2012) Enzymatic preparation of a natural sweetener rubusoside from specific hydrolysis of stevioside with β-galactosidase from *Aspergillus* sp. J Mol Catal B-Enzym 82: 12–17

Zhang F, Koh GY, Hollingsworth J, Russo PS, Stout RW, Liu Z (2012) Reformulation of etoposide with solubility-enhancing rubusoside. Int J Pharm 434(1-2):453–459