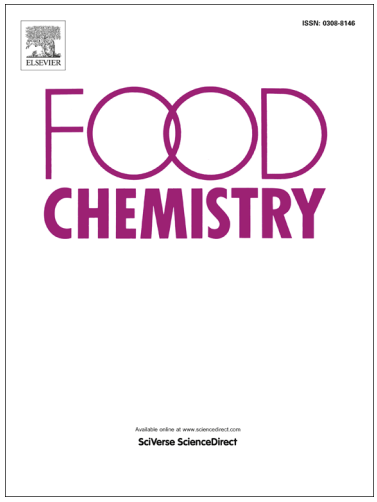

Highly specific sophorose β-glucosidase from *Sphingomonas elodea* ATCC 31461 for the efficient conversion of stevioside to rubusoside

Qing Lan, Tingting Tang, Yu Yin, XiaoYi Qu, Zilong Wang, Hao Pang, Ribo Huang, Liqin Du

PII: S0308-8146(19)30977-X
DOI: https://doi.org/10.1016/j.foodchem.2019.05.164
Reference: FOCH 24890

To appear in: *Food Chemistry*

Received Date: 9 February 2019
Revised Date: 17 May 2019
Accepted Date: 23 May 2019

Please cite this article as: Lan, Q., Tang, T., Yin, Y., Qu, X., Wang, Z., Pang, H., Huang, R., Du, L., Highly specific sophorose β-glucosidase from *Sphingomonas elodea* ATCC 31461 for the efficient conversion of stevioside to rubusoside, *Food Chemistry* (2019), doi: https://doi.org/10.1016/j.foodchem.2019.05.164

Highly specific sophorose β-glucosidase from *Sphingomonas elodea* ATCC 31461 for the efficient conversion of stevioside to rubusoside

Qing Lan[a,1], Tingting Tang[a,1], Yu Yin[a], XiaoYi Qu[a], Zilong Wang[a], Hao Pang[b,*], Ribo Huang[a], Liqin Du[a,*]

[a]State Key Laboratory for Conservation and Utilization of Subtropical Agro-bioresources, Guangxi Research Center for Microbial and Enzymatic Technology, College of Life Science and Technology, Guangxi University, Daxue Road No. 100, Nanning, Guangxi 530005, China

[b]National Engineering Research Center for Non-Food Biorefinery, State Key Laboratory of Non-Food Biomass and Enzyme Technology, Guangxi Key Laboratory of Bio-refinery, Guangxi Academy of Sciences, Daling Road No. 98, Nanning, Guangxi 530007, China

[1]These authors contributed equally to this work.

*Corresponding authors:

Email: haopang@gxas.cn (Hao Pang), duliqin@gxu.edu.cn (Liqin Du)

Phone: +86-771-2503987(Hao Pang), +86-771-3235706(Liqin Du)

Abstract

Enzyme specificity and particularity is needed not only in enzymatic separation methods, but also in enzymatic determination methods for plant compound extraction. Stevioside, rubusoside, and rebaudioside A are natural sweet compounds from plants. These compounds have the same skeleton and only contain different side-chain

glucosyl groups, making them difficult to separate. However, enzymes that target diterpenoid compounds and show specific activity for side-chain glucosyl groups are rare. Herein, we report the identification and characterization of an enzyme that can target both diterpenoid compounds and sophorose, namely, β-glucosidase SPBGL1 from *Sphingomonas elodea* ATCC 31461. SPBGL1 displayed high specificity toward sophorose, and activity toward stevioside, but not rebaudioside A. The stevioside conversion rate was 98%. SPBGL1 also operated at high substrate concentrations, such as in 50% crude steviol glycoside extract. Glucose liberated from stevioside was easy to quantify using the glucose oxidase method, allowing the stevioside content to be determined.

Keywords

β-glucosidase; stevioside; plant content measurement; enzymatic determination method

1. Introduction

Steviol glycosides are natural sweeteners isolated from the leaves of *Stevia rebaudiana*. Steviol glycosides are a class of tetracyclic diterpenoids consisting of at least nine sweet ingredients, including stevioside, rebaudiosides A–D and F, rubusoside, dulcoside, and steviolbioside (Supplementary Fig. S1, Table S1) (Carakostas, Curry, Boilea, & Brusick, 2008). These substances differ in the type and number of glycosyl groups linked to positions C-13 and C-19 (Lemus-Mondaca,

Vega-Gálvez, Zura-Bravo, & Ah-Hen, 2012). Steviol glycosides show various properties and different sweetness levels (Lemus-Mondaca, et al., 2012; Xu, Li, Wang, Yang, & Ning, 2009), with higher sweetness (200–350-fold) and fewer calories (300-fold) compared with sucrose (Carakostas, et al., 2008; Singhania, Patel, Sukumaran, Larroche, & Pandey, 2013). Since 2008, steviol glycoside has been successively approved by the Food and Agriculture Organization of the United Nations, the World Health Organization Joint Expert Committee on Food Additives, the U. S. Food and Drug Administration, and other official organizations as a food and drug additive (Tada, et al., 2013). Therefore, the use of steviol glycoside as a natural sugar substitute is increasing.

Among steviol glycosides extracted from *S. rebaudiana*, rebaudioside A and stevioside have the highest contents. Rebaudioside A produces a clean sweet taste with no significant undesirable taste characteristics, while stevioside has a slightly bitter taste and aftertaste (Prakash, DuBois, Clos, Wilkens, & Fosdick, 2008). Therefore, the separation and purification of steviol glycoside components from the extraction mixture is required to produce high-quality products (Ba, Zhang, Yao, Ma, & Wang, 2014; Li, Chen, & Di, 2012).

Such separation processes and methods have been developed, as well as methods for the rapid detection and quantitation of components from the mixture (Formigoni, et al., 2018; Logue, Dowey, Strain, Verhagen, McClean, & Gallagher, 2017; Pavlíček & Tůma, 2017). Common methods involve measuring the steviol glycoside content in stevia samples or extraction mixtures using standard chemical analyses, such as high-

performance liquid chromatography (HPLC) (Aranda-González, Moguel-Ordoñez, & Betancur-Ancona, 2015; Lorenzo, Serrano-Díaz, Plaza, Quintanilla, & Alonso, 2014). However, these methods are time consuming and expensive. Earlier, Mizukami et al. reported an enzymatic method for the quantitative analysis of stevioside in stevia extract (Mizukami, Shiiba, & Ohashi, 1982). As the stevioside molecule contains three molecules of glucose, glucose liberated from stevioside can be used to quantify the stevioside content using crude hesperidinase. Therefore, this method combined the use of a stevioside-specific enzyme and a standard glucose detection method. Furthermore, Udompaisarn et al. showed that recombinant β-glucosidase from *Bacteroides thetaiotaomicron* HB-13 can selectively hydrolyze stevioside to produce rubusoside and glucose (Udompaisarn, Arthan, & Somana, 2017). Glucose liberation during enzymatic hydrolysis of stevioside can be used to quantify the stevioside content by coupling with a glucose oxidase method. Using this glucosidase, which liberates only one glucose molecule, stevioside determination is more accurate and can be completed in a shorter time. Compared with HPLC-based methods, enzymatic methods are cheaper and faster.

Some enzymes for specific reactions with steviol glycosides have been identified. For example, lactase from *Thermus thermophilus* and β-galactosidase from *Aspergillus* sp. can produce rubusoside from stevioside (Nguyen, et al., 2014; Wan, Guan-junTao, Kim, & Xia, 2012). β-Glucosidase from *Streptomyces* sp. GXT6 can also convert stevioside to rubusoside (Wang, et al., 2015). β-Glucosidase from *Penicillium decumbens* can convert stevioside to steviol (Ko, et al., 2013), while a β-

galactosidase from *Kluyveromyces lactis* was found to specifically catalyze hydrolysis of the glycosyl ester linkage in stevioside to yield steviolbioside (Chen, Ding, Sui, Xia, Wan, & Lu, 2016).

In the present study, we show that a β-glucosidase from *Sphingomonas elodea* ATCC 31461 has high activity in the conversion of stevioside to rubusoside with no activity toward rebaudioside. Therefore, this enzyme can be used for the hydrolysis of stevioside to release glucose, allowing fast and sensitive stevioside determination.

2. Materials and methods

2.1 Strains and materials

S. elodea ATCC 31461 was purchased from the American Type Culture Collection. Vector pSE380 was purchased from Invitrogen Inc. (San Diego, CA). All restriction endonucleases, ligases, and DNA polymerases were from Takara (Dalian, China). *p*-Nitrophenyl-β-D-glucopyranoside (*p*NPG), 4-nitrophenyl-β-D-cellobioside (*p*NPC), 4-nitrophenyl-β-D-galactopyranoside (*p*NPGal), 4-methylumbelliferyl-β-D-glucuronide (MU-Glc), 4-nitrophenyl-2-acetylamino-2-deoxy-β-D-glucopyranoside (*p*NPNAG), *o*-nitrobenzene-β-D-galactopyranoside (*o*NPG), 4-nitrophenyl-β-D-xylopyranoside (*p*NP-Xyl), 4-nitrophenyl-β-D-rhamnoside (*p*NPR), 4-nitrophenyl-β-L-arabinoside (*p*NPA), and *p*-nitrophenyl-α-D-glucopyranoside (α-*p*NPG) were purchased from Sigma (St. Louis, MO). Stevioside (98%, HPLC), rubusoside, rebaudioside, and steviol were purchased from Sichuan Weikeqi Biological Technology Co., Ltd. (Chengdu, China). Steviolbioside was purchased from Chengdu

Herbpurify Co., Ltd. (Chengdu, China). Crude steviol glycoside was obtained from Shandong Huaxian Stevia Co., Ltd. (Shandong, China).

2.2 Expression and purification of recombinant SPBGL1

First, the protein was analyzed using the Simple Modular Architecture Research Tool on the SMART server (http://smart.embl-heidelberg.de/). Signal peptides were predicted using the SignalP 4.1 server (http://www.cbs.dtu.dk/services/SignalP/).

The gene *spbgl1* was amplified from genomic DNA of *S. elodea* ATCC 31461 using primers *spbgl1*-F, 5′-ATTACCATGGATATGCTGCTCCAGGCGGGTGCGCGG-3′ (containing a *Nco*I site at the 5′- end) and *spbgl1*-R, 5′-ATTAAGCTTTCAGTGGTGGTGATGATGATGGGCCAGTTCGAAGCTGCCGGACAC-3′ (containing a *Hin*dIII site and a His_6 tag at the 5′- end). The PCR fragment was digested with *Nco*I and *Hin*dIII and ligated into pSE380. The recombinant plasmid was transformed into *Escherichia coli* XL1-Blue. Transformants were grown in Luria–Bertani (LB) medium until the OD_{600} reached 0.4–0.6. Then, 0.5 mM IPTG was added (final concentration) and incubation was continued at 20°C for 20 h to induce protein expression. The protein was purified by nickel–nitrilotriacetic acid (Ni-NTA) chromatography (Qiagen, Hilden, Germany) according to manufacturer instructions. The purified protein was desalted into 100 mM NaH_2PO_4–Na_2HPO_4 buffer (pH 7.0) using a PD MiniTrap G-25 column (GE Healthcare, Freiburg, Germany). The molecular mass of the recombinant protein was determined by SDS-

PAGE. The purified protein concentration was measured using the Bradford method.

The protein sequence accession number of SPBGL1 is WP_029622673.1 at Genbank.

2.3 Enzymatic activity and kinetic parameters using *p*NPG as substrate

The activity of β-glucosidase SPBGL1 was assessed using *p*NPG as substrate. A standard reaction comprised McIlvaine buffer (170 μL, 0.2 M Na_2HPO_4–0.1 M citric acid, pH 5.5), 20 mM *p*NPG (20 μL), and appropriately diluted enzyme (10 μL) at 45°C for 20 min. The reaction was terminated by adding 2 M Na_2CO_3 (50 μL). The amount of *p*-nitrophenol (*p*NP) released was measured in a 96-well plate at OD_{405} in a Sunrise microplate reader (Tecan Group Ltd., Männedorf, Switzerland). A reaction system without any enzyme was used as a blank control. All reactions were determined simultaneously in three parallel experiments. One enzyme unit was defined as the amount of enzyme that produced 1 μmol of *p*NP per min under the above conditions.

The effect of different pH levels on the enzyme activity (pH 4.5–8.0 at intervals of 0.5) was determined at 37°C in McIlvaine buffer. The optimum reaction temperature for SPBGL1 was assessed at the optimum pH over the temperature range of 25–55°C (5°C intervals). Under the optimum reaction conditions, the enzyme activity was determined at a series of substrate concentrations (0.35–10 mM) by nonlinear regression analysis and double reciprocal mapping using GraphPad Prism 5 software (GraphPad Software, San Diego, CA). The SPBGL1 activity at glucose

concentrations of 0, 20, and 40 mM was determined using different concentrations of *p*NPG (0.35–10 mM) under the optimum reaction conditions. The K_i value of glucose (inhibitor constant) was measured by simultaneous nonlinear regression and calculated using GraphPad Prism 5 software.

2.4 Substrate specificity of recombinant SPBGL1

The substrate specificity of SPBGL1 was determined by incubating purified enzyme with 2 mM α-*p*NPG, *p*NPNAG, *o*NPG, *p*NPGal, *p*NP-Xyl, *p*NPC, *p*NPA, *p*NPR, MU-Glc, and 1% (w/v) cellobiose, gentiobiose, lactose, sophorose, sucrose, trehalose, maltose, and isomaltose, respectively, in McIlvaine buffer (pH 5.5) at 45°C for 20 min. The amounts of *p*NP or *o*NP released were detected using the methods referred to in section 2.3. MU-Glc hydrolysis was visualized under ultraviolet light due to the release of fluorescent methyl umbelliferone. Glucose release was determined using a Glucose Assay Kit (Applygen Technology Inc., Beijing, China). The rates of hydrolysis of different substrates were calculated as relative values compared with the rate of hydrolysis of *p*NPG.

2.5 Hydrolysis activity toward steviol glycosides

To determine the hydrolysis activity of SPBGL1 toward stevioside substrates, the reaction system consisted of 1% (w/v) substrate (stevioside, rubusoside, steviolbioside, and rebaudioside, respectively), buffer (pH 5.5) and appropriately purified enzyme (total system was 50 μL), incubated at 45°C for 20 min. The reaction

was terminated by boiling for 5 min. The products were detected using the Glucose Assay Kit or HPLC. For HPLC, a G1311C 1260 Quat Pump VL, G1329B 1260 ALS automatic sampler, G1314F 1260 VWD detector, and CO-1000 column oven from Agilent Technologies Inc. (Santa Clara, CA, United States) were used. The stationary phase was a C18 column (Alltima, 250×4.6 mm, 5 μm). The column temperature was 40 °C, with a flow rate of 1 mL/min, and detection at 210 nm. The mobile phase consisted of 10 mM NaH_2PO_4 solution in water (adjusted to pH 2.6 with phosphoric acid) and acetonitrile. The gradient elution program was as follows: 32% acetonitrile (1–10 min), 32–80% acetonitrile (10–20 min), 80% acetonitrile (20–21 min), 80–32% acetonitrile (21–28 min), and 32% acetonitrile (28–30 min).

2.6 Enzymatic properties with stevioside as substrate

The effect of different pH levels on the hydrolysis of stevioside by SPBGL1 was determined at 37°C for 20 min. The pH range was 4.0 to 8.0 (intervals of 0.5). The effect of temperature on the hydrolysis of stevioside by SPBGL1 was determined at the optimum reaction pH (pH 5.5) for 20 min at 25–60°C (5°C intervals). Under the optimal reaction conditions, the enzyme activity at a series of substrate concentrations (0.5–25 mM) was determined by nonlinear regression analysis and double reciprocal mapping using GraphPad Prism 5 software. A Glucose Assay Kit was used to measure glucose released by the reaction. Stevioside (1% and 24%, w/v) was incubated with different concentrations of purified enzyme in McIlvaine buffer (pH 6.5) at 45°C for 6 h, and the products were determined by HPLC. The optimum

reaction time with 1% (w/v) stevioside was determined using the optimum concentration of SPBGL1 in McIlvaine buffer (pH 6.5) incubated at 45°C for 0, 5, 10, 20, 40, 60, 120, 240, and 360 min. These products were also determined by HPLC.

2.7 Hydrolysis of crude steviol glycoside and NMR product analysis

The optimum enzyme amounts and reaction time for the hydrolysis of crude 50% (w/v) steviol glycoside were determined using the method described above for SPBGL1. For control enzyme BGL1, the reaction conditions were 50% stevioside (50 μL, 0.1 M Na_2HPO_4–citric acid solution, pH 6.5) at 45°C for 6 h.

Rubusoside and rebaudioside A were isolated and purified from the hydrolysates of crude steviol glycoside by preparative HPLC (Waters Corporation, Milford, MA). A Waters 2535 quaternary gradient module, a 2707 automatic sampler, and a 2489 UV/visible detector were used for preparative HPLC. The stationary phase was a C18 column (SunFire, 250×10 mm, 5 μm). The flow rate was 2 mL/min and detection was at 210 nm. The mobile phase consisted of acetonitrile and H_2O. The gradient elution program was as follows: 32% acetonitrile (0–13 min), 32–80% acetonitrile (13–25 min), 80% acetonitrile (25–30 min), 80–32% acetonitrile (30–40 min), and 32% acetonitrile (40–55 min).

Purified rubusoside and rebaudioside A were lyophilized for NMR determination as follows: Rubusoside (10 mg) dissolved in deuterated water (500 μL) and rebaudioside A (10 mg) dissolved in deuterated pyridine (500 μL) were placed in separate 5-mm tubes for NMR structural elucidation. Spectra were recorded using a

Varian VNMRS 600 MHz spectrometer (Varian, Palo Alto, CA) operating at 600 MHz for ^{1}H and 150 MHz for ^{13}C NMR at 25°C for rubusoside and 40°C for rebaudioside A.

The stevioside conversion rate and rubusoside yield were calculated using the following equations:

Stevioside conversion rate = (initial stevioside amount in reaction – amount of stevioside remaining at the end)/initial stevioside amount in reaction × 100.

Rubusoside yield = amount of rubusoside produced/theoretical maximum amount of rubusoside produced × 100.

3. Results and discussion

3.1 Enzymatic properties of SPBGL1

A β-glucosidase of glycoside hydrolase family 3 was cloned from *S. elodea* ATCC 31461. The open reading frame, designated *spbgl1*, was 2289-bp long, which encoded 763 amino acids. The purified protein was detected by SDS-PAGE, and the molecular weight of SPBGL1 was about 85 kDa, which was consistent with the theoretical molecular weight (Supplementary Fig. S2, lane 3).

The enzymatic properties of SPBGL1 were initially detected using *p*NPG as substrate. The optimum reaction pH of SPBGL1 was 5.5. The enzyme showed high activity (>80%) in the range pH 5.0–6.5 (Supplementary Fig. S3a). The optimum reaction temperature of SPBGL1 was 45°C. Between 35 and 45°C, the activity of SPBGL1 remained over 50% (Supplementary Fig. S3b). The K_m, v_{max}, and K_i

(glucose) values were 1.364±0.11 mM, 11.37±0.28 μmol/min/mg and 16.74±1.29 mM, respectively (Supplementary Figs. S3 and S4).

3.2 Substrate specificity of recombinant SPBGL1

The specificity of SPBGL1 toward various substrates was determined (Supplementary Table S2). In addition to the hydrolysis of β-*p*NPG, SPBGL1 hydrolyzed MU-Glc and had weak hydrolytic activity toward cellobiose, gentiobiose, salicin, and amygdalin. Notably, SPBGL1 had very high activity toward sophorose, about 12.8 times that toward *p*NPG.

SPBGL1 also hydrolyzed daidzin and genistin, showing higher hydrolysis activity toward daidzin than that toward genistin. The conversion rates of daidzin and genistin were 76.6% in 15 min and 22.4% in 3 h, respectively (Fig. S5), meaning that SPBGL1, even as a glucosidase, targeted large substrates, such as diterpenoid compounds.

Compared with other β-glucosidases (SSGase, BGL1, and SPGase) reported to hydrolyze stevioside, SPBGL1 showed higher activity toward cellobiose, gentiobiose, salicin, and amygdalin (Ko, et al., 2012; Ko, et al., 2013; Wang, et al., 2015). SPBGL1 showed 12.8 times the activity toward sophorose that that toward *p*NPG, while BGL1 showed the same activity toward sophorose and *p*NPG. This indicated that sophorose was the optimum substrate for SPBGL1. As SPBGL1 showed the highest activity toward sophorose and also targeted compounds with tetracyclic diterpenoid skeletons, SPBGL1 might be very useful for the hydrolysis of stevioside

266 at the C13 sophorose residue.

267

268 3.3 Hydrolysis of stevioside by SPBGL1

269 The hydrolyses of stevioside, rubusoside, steviolbioside, and rebaudioside A by
270 SPBGL1 were determined at 45°C and pH 5.5 (Fig. 1). SPBGL1 hydrolyzed
271 stevioside to produce rubusoside (Fig. 1c) and converted steviolbioside to
272 steviolmonoside (Fig. 1e). However, SPBGL1 could not hydrolyze rebaudioside A or
273 rubusoside. Therefore, SPBGL1 exhibited highly specific hydrolytic activity toward
274 the C-13 sophorosyl group of stevioside.

275 As SPBGL1 showed the ability to hydrolyze stevioside to produce rubusoside,
276 this activity was studied further. The optimum conditions for stevioside as substrate
277 were different from those for *p*NPG as substrate. For stevioside, the optimum pH was
278 6.5, with SPBGL1 retaining >80% activity between pH 6.0 and 7.0 (Fig. S6a), while
279 the optimum reaction temperature was 45°C, with SPBGL1 retaining >80% activity
280 between 35 and 45°C (Fig. S6b). Using stevioside as substrate, the values of K_m, v_{max},
281 k_{cat}, and k_{cat}/K_m for SPBGL1 were 3.097±0.1939 mM, 85.08±1.854 μmol/min/mg
282 protein, 121.3 s^{-1}, and 39.1 $s^{-1}\cdot mM^{-1}$, respectively (Fig. S7a).

283 When the amount of enzyme was 20 μg/mL at 6 h, the stevioside conversion rate
284 was 95%. When the amount of enzyme was increased to 30 μg/mL, the stevioside
285 conversion rate reached 98% for 1 h, and the final stevioside conversion rate was
286 98.2% when the reaction time was extended to 6 h (Fig. 2).

287

3.4 Activity toward crude steviol glycoside extract

We also determined the conversion rate when the stevioside concentration was increased to 24%. The conversion rate increased to 98.6%, and the rubusoside yield was 99% when using 600 μg/mL of SPBGL1 for 6 h (Fig. 3a). Therefore, SPBGL1 retained high activity even at a high substrate concentration.

In further experiments, we used crude steviol glycoside extract, which contained about 50% stevioside, as substrate instead of purified stevioside. Most stevioside was converted to rubusoside at 4 h. The highest conversion rate of stevioside was >98% and the highest rubusoside yield was >99% in the reaction with 600 μg/mL of SPBGL1 for 6 h (Fig. 3b). The ability of enzyme BGL to hydrolyze the crude steviol glycoside extract was compared with that of SPBGL1, which resulted in stevioside still being present, even when the amount of enzyme was increased to 800 μg/mL (Fig S8e).

The products from the reaction of steviol glycoside were separated by preparative HPLC, and the separated rebaudioside A and rubusoside were lyophilized for NMR analysis. ^{1}H and ^{13}C spectral analyses were performed simultaneously with purchased standards of rebaudioside A and rubusoside, with the results shown in Supplementary Table S3. By comparison with the reported NMR data for rubusoside (Wang, et al., 2015) and rebaudioside A (Steinmetz & Lin, 2009), we concluded that the products obtained by preparative HPLC in this study were indeed rubusoside and rebaudioside A.

Only a few enzymes have been identified to hydrolyze stevioside substrates,

including lactase (Nguyen, et al., 2014), β-galactosidase (Chen, et al., 2016; Chen, Xia, Wan, Wang, & Liu, 2014; Wan, et al., 2012; Wan & Xia, 2015), and β-glucosidase (Katsuyuki, Hirofumi, Tsunneya, Taro, & Sumio, 2000; Ko, et al., 2012; Ko, et al., 2013; Nakano, Okamoto, Yatake, Kiso, & Kitahata, 1998; Wang, et al., 2015; Xu, et al., 2009). However, not all produce rubusoside. The stevioside conversion rate and rubusoside yield were both higher for SPBGL1 than for other reported enzymes (Table 1). In particular, we compared the K_m, k_{cat}, and k_{cat}/K_m values of SPBGL1 with those of three reported β-glucosidases that can hydrolyze stevioside to produce either rubusoside or steviol (Table 2). Among them, SPBGL1 showed the lowest K_m and highest k_{cat}/K_m values. With a high concentration of ST (24% w/v), SPBGL1 (600 μg/mL) still maintained a >98% stevioside conversion rate and 99% rubusoside yield. A small amount of SPBGL1 (30 μg/mL) also rapidly (1 h), and almost completely, converted stevioside to rubusoside. Furthermore, SPBGL1 could not hydrolyze rebaudioside A, with only specific hydrolysis of the glycosidic bond at the C-13 position of stevioside observed. Therefore, SPBGL1 would be suitable for use in an enzymatic stevioside determination method, and may be applicable to the high-throughput analysis of stevioside content. Furthermore, as this enzyme works on crude steviol glycoside extracts, it might be used in method development for the direct measurement of stevioside content in stevia crude extracts.

Acknowledgments

We thank the Natural Science Foundation of China (31360369), the Natural Science Foundation of Guangxi (2014GXNSFAA118078), and the Guangxi Chairman

Technology Fund (17290-03).

References

Aranda‐González, I., Moguel‐Ordoñez, Y., & Betancur‐Ancona, D. (2015). Validation of HPLC-UV method for determination of minor glycosides contained in *Stevia rebaudiana* Bertoni leaves. *Biomedical Chromatography, 29*(5), 733-738.

Ba, J., Zhang, N., Yao, L., Ma, N., & Wang, C. (2014). Separation of Rebaudiana A from Steviol glycoside using a polymeric adsorbent with multi-hydrogen bonding in a non-aqueous system. *Journal of Chromatography B, 971*, 141-149.

Carakostas, M. C., Curry, L. L., Boilea, A. C., & Brusick, D. (2008). Overview: The history, technical function and safety of rebaudioside A, a naturally occurring steviol glycoside, for use in food and beverages. *Food and Chemical Toxicology, 46*(7), S1-S10.

Chen, J.-m., Ding, L., Sui, X.-c., Xia, Y.-m., Wan, H.-d., & Lu, T. (2016). Production of a bioactive sweetener steviolbioside via specific hydrolyzing ester linkage of stevioside with a β -galactosidase. *Food Chemistry, 196*, 155-160.

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*. *Journal of Molecular Catalysis B: Enzymatic, 105*, 126-131.

Formigoni, M., Milani, P. G., Avíncola, A. S., Santos, V. J., Benossi, L., Dacome, A. S., Pilau, E. J., & Costa, S. C. (2018). Pretreatment with ethanol as an alternative to improve steviol glycosides extraction and purification from a new variety of stevia. *Food Chemistry, 241*, 452-459.

Katsuyuki, O., Hirofumi, N., Tsunneya, Y., Taro, K., & Sumio, K. (2000). Purification and some properties of a β-glucosidase from *Flavobacterium johnsonae*. *Bioscience, Biotechnology, and Biochemistry, 64*(2), 333-340.

Ko, J.-A., Kim, Y.-M., Ryu, Y. B., Jeong, H. J., Park, T.-S., Park, S.-J., Wee, Y.-J., Kim, J.-S., Kim, D., & Lee, W. S. (2012). Mass production of rubusoside using a novel stevioside-specific beta-glucosidase from *Aspergillus aculeatus*. *Journal of Agricultural and Food Chemistry, 60*(24), 6210-6216.

Ko, J.-A., Ryu, Y. B., Kwon, H.-J., Jeong, H. J., Park, S.-J., Kim, C. Y., Wee, Y.-J., Kim, D., Lee, W. S., & Kim, Y.-M. (2013). Characterization of a novel steviol-producing β-glucosidase from *Penicillium decumbens* and optimal production of the steviol. *Applied Microbiology and Biotechnology, 97*(18), 8151-8161.

Lemus-Mondaca, R., Vega-Gálvez, A., Zura-Bravo, L., & Ah-Hen, K. (2012). *Stevia rebaudiana* Bertoni, source of a high-potency natural sweetener: A comprehensive review on the biochemical, nutritional and functional aspects. *Food Chemistry, 132*(3), 1121-1132.

Li, J., Chen, Z., & Di, D. (2012). Preparative separation and purification of Rebaudioside A from *Stevia rebaudiana* Bertoni crude extracts by mixed bed of macroporous adsorption resins. *Food Chemistry, 132*(1), 268-276.

Logue, C., Dowey, L. R. C., Strain, J. J., Verhagen, H., McClean, S., & Gallagher, A. M. (2017). Application of Liquid Chromatography–Tandem Mass Spectrometry To Determine Urinary

Concentrations of Five Commonly Used Low-Calorie Sweeteners: A Novel Biomarker Approach for Assessing Recent Intakes? *Journal of Agricultural and Food Chemistry, 65*(22), 4516-4525.

Lorenzo, C. n., Serrano-Díaz, J. s., Plaza, M., Quintanilla, C., & Alonso, G. L. (2014). Fast methodology of analysing major steviol glycosides from *Stevia rebaudiana* leaves. *Food Chemistry, 157*, 518-523.

Mizukami, H., Shiiba, K., & Ohashi, H. (1982). Enzymatic determination of stevioside in *Stevia rebaudiana*. *Phytochemistry, 21*(8), 1927-1930.

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. *Journal of Fermentation and Bioengineering, 85*(2), 162-168.

Nguyen, T. T. H., Jung, S.-J., Kang, H.-K., Kim, Y.-M., Moon, Y.-H., 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 and Microbial Technology, 64-65*, 38-43.

Pavlíček, V., & Tůma, P. (2017). The use of capillary electrophoresis with contactless conductivity detection for sensitive determination of stevioside and rebaudioside A in foods and beverages. *Food Chemistry, 219*, 193-198.

Prakash, I., DuBois, G. E., Clos, J. F., Wilkens, K. L., & Fosdick, L. E. (2008). Development of rebiana, a natural, non-caloric sweetener. *Food and Chemical Toxicology, 46 Suppl 7*, S75-82.

Singhania, R. R., Patel, A. K., Sukumaran, R. K., Larroche, C., & Pandey, A. (2013). Role and significance of beta-glucosidases in the hydrolysis of cellulose for bioethanol production. *Bioresource technology, 127*, 500-507.

Steinmetz, W. E., & Lin, A. (2009). NMR studies of the conformation of the natural sweetener rebaudioside A. *Carbohydrate research, 344*(18), 2533-2538.

Tada, A., Takahashi, K., Ishizuki, K., Sugimoto, N., Suematsu, T., Arifuku, K., Tahara, M., Akiyama, T., Ito, Y., Yamazaki, T., Akiyama, H., & Kawamura, Y. (2013). Absolute Quantitation of Stevioside and Rebaudioside A in Commercial Standards by Quantitative NMR. *Chemical and Pharmaceutical Bulletin, 61*(1), 33-38.

Udompaisarn, S., Arthan, D., & Somana, J. (2017). Development and Validation of an Enzymatic Method To Determine Stevioside Content from *Stevia rebaudiana*. *Journal of Agricultural and Food Chemistry, 65*(15), 3223-3229.

Wan, H.-d., Guan-junTao, Kim, D., & Xia, Y.-m. (2012). Enzymatic preparation of a natural sweetener rubusoside from specific hydrolysis of stevioside with β-galactosidase from *Aspergillus* sp. *Journal of Molecular Catalysis B: Enzymatic, 82*, 12-17.

Wan, H.-d., & Xia, Y.-m. (2015). Enzymatic transformation of stevioside using a β-galactosidase from *Sulfolobus* sp. *Food Function, 6*(10), 3291-3295.

Wang, Z., Wang, J., Jiang, M., Wei, Y., Pang, H., Wei, H., Huang, R., & Du, L. (2015). Selective production of rubusoside from stevioside by using the sophorose activity of beta-glucosidase from *Streptomyces* sp. GXT6. *Applied Microbiology and Biotechnology, 99*(22), 9663-9674.

Xu, Z., Li, Y., Wang, Y., Yang, B., & Ning, Z. (2009). Production of β-fructofuranosidase by *Arthrobacter* sp. and its application in the modification of stevioside and rebaudioside A. *Food Technology and Biotechnology, 47*(2), 137-143.