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Mass Production of Rubusoside Using a Novel Stevioside-Specific β-Glucosidase from *Aspergillus aculeatus*

Jin-A Ko,[†,∥] Young-Min Kim,[†,∥] Young Bae Ryu,[†] Hyung Jae Jeong,[†] Tae-Soon Park,[†] Su-Jin Park,[†] Young-Jung Wee,[‡] Joong-Su Kim,[†] Doman Kim,[§] and Woo Song Lee*,[†]

[†]Infection Control Material Research Center, Korea Research Institute of Bioscience and Biotechnology, Jeongeup 580-185, Republic of Korea

[‡]Department of Food Science and Technology, Yeungnam University, Gyeongsan, Gyeongbuk 712-749, Republic of Korea

[§]School of Biological Sciences and Technology and the Research Institute for Catalysis, Chonnam National University, Gwang-ju 500-757, Republic of Korea

ABSTRACT: Rubusoside (R) is a natural sweetener and a solubilizing agent with antiangiogenic and antiallergic properties. However, currently, its production is quite expensive, and therefore, we have investigated nine commercially available glycosidases to optimize an economically viable R-production method. A stevioside (ST)-specific β-glucosidase (SSGase) was selected and purified 7-fold from *Aspergillus aculeatus* Viscozyme L by a two-step column chromatography procedure. The 79 kDa protein was stable from pH 3.0 to pH 7.0 at 50−60 °C. Hydrolysis of ST by SSGase produced R and steviol monoglucosyl ester as determined by ^{1}H and ^{13}C nuclear magnetic resonance (NMR). Importantly, SSGase showed higher activity toward ST than other β-linked glucobioses. The optimal conditions for R production were 280 mM ST and 16.6 μL of SSGase at pH 5.1 and 63 °C. This is the first discussion detailing the production of R by enzymatic hydrolysis of ST and is useful for the food additive and pharmaceutical industries.

KEYWORDS: *stevioside, rubusoside, β-glucosidase, Aspergillus aculeatus, natural solubilizer*

INTRODUCTION

Stevioside (ST) and rubusoside (R) are sweet steviol glycosides isolated from the leaves of the Compositae *Stevia rebaudiana* Bertoni, which grows in South America, and the Rosaceae *Rubus suavissimus* S. Lee, which grows in South China. ST and R are natural sweeteners that are about 143-fold and 114-fold, respectively, sweeter than sucrose at a concentration of 0.025%. However, they have a slightly bitter taste and aftertaste.[1] Therefore, many researchers have attempted to improve the quality of sweetness by transglycosylation of glycosyl residues by using enzymes such as cyclodextrin glucanotransferase,[2] β-fructofuranosidase,[3] and β-galactosidase.[4] Large amounts of ST have been commercially produced since the 1970s; by 1996, the ST market in Japan was approximately 2000 tons. ST is used primarily as a sweetener for seasonings, pickles, and salted foods in Japan. Comparatively, R is relatively more expensive and is used to make an herbal tea which is different from the well-known tea *Camellia sinesis*. This herbal blend is known as thian-cha or sweet tea in China because of its natural sweetness.[5] In addition to being consumed as a component of herbal tea, R has also been incorporated into a health drink, because it has been recently revealed that R possesses significant bioactivities such as antiangiogenic[6] and antiallergic[7,8] properties. In another use, amphipathic R is a superior natural solubilizer that enhances the solubility of various pharmaceutically important compounds with very low water solubility, such as paclitaxel, curcumin, capsaicin, cyclosporine, nystatin, and erythromycin.[9] However, it is currently not economically viable to purify large quantities of R. The production of R has typically relied on the traditional purification method involving complex components such as gallic acid, rutin, ellagic acid, R, and steviol monoglucoside (SMG) extracted from *R. suavissimus* S. Lee[10] obtained using boiling water or decoction. Another suggested pathway to produce R involves the hydrolysis of the glucose moiety from the relatively cheap ST. Indeed, R is approximately 10-fold more expensive than ST. The mass production of high-purity R increases its potential for use as a natural solubilizer in pharmacology and as a natural sweetener in the food industry.

β-Glucosidase (β-D-glucoside glucohydrolase, EC 3.2.1.21) catalyzes the hydrolysis of β-glucosidic linkages of various oligosaccharides and aryl glycosides. Many β-glucosidases have been isolated from microorganisms, plants, and animals, and as such their substrate specificities have been characterized in detail. Among the reported β-glucosidases, only two enzymes hydrolyze the glucose moiety in ST. β-Glucosidases from *Clavibacter michiganense*[11] and *Flavobacterium johnsoniae*[12] hydrolyze the β-glucosidic linkage of the 19-carboxyl group of steviol glycosides; however, they do not degrade β-glucosidic linkages of the 13-hydroxyl group of rebaudioside A (RebA) or ST. To our knowledge, there is no β-glucosidic enzyme capable of hydrolyzing the linkage of the 13-hydroxyl group of RebA or ST. In our studies of commercially available β-linkage-hydrolyzing enzymes, we identified an enzyme capable of producing R from ST. We optimized the process production of R by using a combination of factorial design and response

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surface methodology (RSM). RSM is an effective statistical tool that is widely used in the optimization of fermentation processes. It has several advantages, including lower experimental numbers, suitability for experiments with multiple factors, the ability to search for relationships between factors, and the ability to find the most suitable conditions. These benefits allow the user to forecast responses with minimal errors with respect to parameter effects. RSM contains many reported designs, such as the Box−Behnken design, the Graeco−Latin design, and the central composite design (CCD).[13]

In this study, the novel ST-specific β-glucosidase, SSGase, was selected to produce R from ST. In this paper we detail the purification, substrate specificities, and kinetic parameters of the enzyme. Moreover, we describe the parameters required for the maximum production of R from ST by RSM.

■ MATERIALS AND METHODS

Materials and Enzymes. Steviol, ST, RebA, *Aspergillus niger* α-glucosidase, hemicellulase, hesperidinase, and β-glucanase, *Trichoderma longibrachiatum* β-glucanase, *Aspergillus aculeatus* Viscozyme L, *Trichoderma reesei* ATCC 26921 β-glucanase, *Clostridium thermocellum* thermostable β-glucanase, almond β-glucosidase, and all other chemicals were purchased from Sigma-Aldrich (St. Louis, MO).

Purification of ST-Specific β-Glucosidase from *A. aculeatus* Viscozyme L. Purification of ST-hydrolyzing enzymes from *A. aculeatus* Viscozyme L was carried out using a phenyl-Sepharose fast flow column (GE Healthcare, Piscataway, NJ) and a Sephacryl S-200 gel filtration (GE Healthcare) column chromatograph according to the manufacturer's protocol. Ammonium sulfate (1 M) was added to the enzyme solution (23 mg/mL) and loaded onto a phenyl-Sepharose fast flow column (1.6 × 10 cm) equilibrated with 40 mM sodium acetate buffer (NAB; pH 5.0) containing 1 M ammonium sulfate. The absorbed proteins were eluted with a 1−0 M ammonium sulfate linear gradient at a flow rate of 1 mL/min. The active fractions were concentrated and loaded onto a Sephacryl S-200 column (2.6 × 60 cm) equilibrated with 40 mM NAB (pH 5.0) in 50 mM sodium chloride. The active fractions were dialyzed against 40 mM NAB (pH 5.0), and the final purified protein was concentrated using an Amicon Ultra 10 000 MWCO (Millipore, Billerica, MA).

Enzyme Assay. β-Glucosidase activity was determined by measuring the release of glucose from *p*-nitrophenyl β-glucoside (*p*NPG-β). When *p*NPG-β (0.80−5 mM) was used as a substrate, the reaction was stopped by the addition of a sodium carbonate solution (final concentration 0.67 M) to the enzyme reaction mixture. The amount of 4-nitrophenol liberated from *p*NPG-β was measured as the absorption at 400 nm in a cuvette (length 1 cm) with a molar extinction coefficient of 55 560 M^{-1} cm^{-1}. The activity of the ST-specific β-glucosidase was determined by the release of glucose from ST, R, steviol monoglycosyl ester (SME), RebA, and glucobioses. One unit of enzyme activity was defined as the amount of enzyme hydrolyzing 1 μmol of glucose/min. The reaction mixture consisted of the substrate (50 mM), the enzyme solution, and 40 mM NAB (pH 5.0). The liberated glucose was analyzed by the Tris−glucose oxidase−peroxidase assay with our modification by the glucose AR-II test (Wako Pure Chemical Industries, Osaka, Japan). The protein concentration was measured by the Bradford method using bovine serum albumin as the standard protein.[14]

Effects of pH and Temperature on Enzyme Activity. The enzyme (46 μg/mL) was incubated at 55 °C in 32 mM Britton−Robinson buffer (pH 2−12) with 50 mM ST to determine the optimum pH. In addition, the enzyme was kept at 4 °C for 20 h in 30 mM Britton−Robinson buffer (pH 2−12), and the residual enzyme activity was examined to determine the pH stability. The optimal temperature was determined by incubating the enzyme at 30−80 °C for 30 min in 40 mM NAB (pH 5.0). The thermal stability was ascertained by incubating the enzyme at 30−80 °C for 12 h in 40 mM NAB (pH 5.0), and the residual enzyme activity was examined using 50 mM ST at 55 °C.

Synthesis, Purification, and Product Analysis. The hydrolysis products of stevioside by various glycosidases were analyzed by thin-layer chromatography (TLC) and high-performance liquid chromatography (HPLC). Nine commercially available glycosidases (3.7−4 U) from *A. niger*, *T. longibrachiatum*, *A. aculeatus*, *T. reesei*, *C. thermocellum*, and almond were incubated for 1 week with 50 mM ST at the pH and temperature described in Figure 1. The reaction mixture was placed in a water bath for 5 min to halt the enzyme activity. At designated time intervals, aliquots (10 μL) were removed, and the reaction products were analyzed by TLC using precoated silica gel 60 F_{254} plates (Merck, Darmstadt, Germany) developed in a solvent system consisting of acetonitrile/water [85:15 (v/v)] with steviol glucosides as the standard compounds. The plates were dipped into a solution containing 0.03 g of *N*-(1-naphthyl)ethylenediamine and 5 mL of concentrated sulfuric acid prepared in 95 mL of methanol, heated at 100 °C,[15] and then visualized.

Chromatographic separation for quantitative analysis was achieved using a 1200 series quaternary HPLC system (Agilent Technologies, Palo Alto, CA) consisting of a G1311A quaternary pump with a G1322A vacuum degasser, a G1329A thermostated autosampler, a G1316A column oven set at 36 °C, a ZORBAX SB-C_{18} column (5 μm, 150 × 4.6 mm), and a G1314B VW detector set at 210 nm. After desalting with Amberlite MB-3 (Organo, Tokyo, Japan), the resultant digests were separated on a C_{18} column with a stepwise ethanol gradient (0%−100%). The fractions containing the reaction products were collected and desalted again with Amberlite MB-3, followed by lyophilization. Finally, two products (P1 and P2 in Figure 1) were isolated by preparative HPLC using an LC-6AD instrument (Shimadzu, Kyoto, Japan) under the following conditions: YMC-Pack Pro C_{18} RS column (250 mm × 20 mm i.d., 5 μm; YMC Co., Ltd., Kyoto, Japan); mobile phase 82% (v/v) acetonitrile; flow rate 0.25 mL/min; room temperature; detection with an SPD-20A UV/vis detector (Shimadzu). High-performance liquid chromatography−mass spectrometry (HPLC−MS)-based analysis for isolated compounds was performed using an HCT ultra PTM Discovery Ion Trap system (Bruker Daltonik, GmbH, Bremen, Germany) in the negative electrospray ionization mode.

For structural elucidation of the hydrolysis products, approximately 50 mg of the isolated R and SME was dissolved in 250 μL of deuterated water (D_2O) and placed into 3 mm nuclear magnetic resonance (NMR) tubes. NMR spectra were acquired on a Unity INOVA 500 spectrometer (Varian, Palo Alto, CA) operating at 500 MHz for ^1H and 125 MHz for ^{13}C at 25 °C. Linkages between the steviol and glucose were evaluated using the spectra obtained via homonuclear correlation spectroscopy (COSY), heteronuclear single quantum coherence (HSQC), and heteronuclear multiple bond correlation (HMBC).

Optimization Procedure and Experimental Design. A five-level central composite design with four factors was applied to produce the R optimization procedure using Design Expert 8.01 software, including the six replicates at the central point, which were utilized in the fitting of a second-order response surface. The ST concentration (x_1), enzyme (3.73 U/mL) concentration (x_2), reaction pH (x_3), and reaction temperature (x_4) were utilized to prepare each of the 30 cultivation conditions summarized in Table 3. Optimization was conducted using a desirability function to determine the effects of x_1, x_2, x_3, and x_4 on R production. A total of 30 experiments composed of 16 factorial points, 8 axial points, and 6 center points were conducted to determine the 14 coefficients of the model as follows:

$$y = \beta_0 + \beta_1 x_1 + \beta_2 x_2 + \beta_3 x_3 + \beta_3 x_4 + \beta_{12} x_1 x_2 + \beta_{13} x_1 x_3 + \beta_{14} x_1 x_4 + \beta_{23} x_2 x_3 + \beta_{24} x_2 x_4 + \beta_{34} x_3 x_4 + \beta_{11} x_1^2 + \beta_{22} x_2^2 + \beta_{33} x_3^2 + \beta_{33} x_4^2 \quad (1)$$

where y is the predicted response, β_0 is the intercept, β_1, β_2, β_3, and β_4 are linear coefficients, β_{11}, β_{22}, β_{33}, and β_{44} are squared coefficients, and β_{12}, β_{13}, β_{14}, β_{23}, β_{24}, and β_{34} are interaction coefficients. Once an appropriate model was obtained, it was used to determine the predicted optimum conditions for the process.

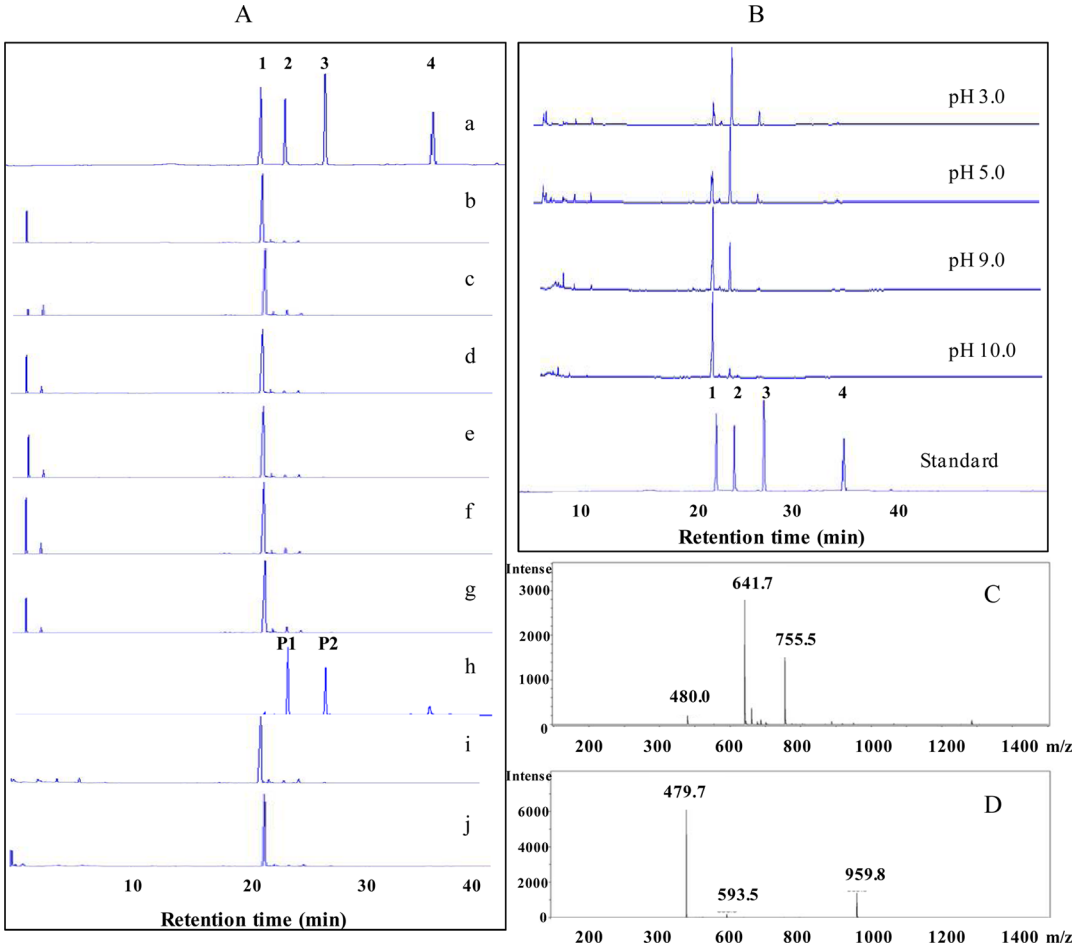

Figure 1. HPLC profiles of the stevioside hydrolysis products and HPLC/MS analysis of two isolated products. (A) HPLC profiles of stevioside hydrolysis products by commercially available glycosidases: lane a, standard steviol glucosides in order of retention time, stevioside (1), rubusoside (2), steviol monoglucosyl ester (3), and steviol (4), respectively; lane b, almond β-glucosidase (pH 5.0, 35 °C); lane c, *A. niger* β-glucanase (pH 5.0, 35 °C); lane d, *A. niger* hesperidinase (pH 4.0, 40 °C); lane e, *A. niger* α-glucosidase (pH 4.0, 35 °C); lane f, *A. niger* hemicellulase (pH 4.5, 40 °C); lane g, *T. longibrachiatum* β-glucanase (pH 5.0, 35 °C); lane h, *A. aculeatus* Viscozyme L (pH 4.5, 60 °C); lane i, *T. reesei* β-glucanase (pH 5.0, 35 °C); lane j, *C. thermocellum* β-glucanase (pH 5.8, 70 °C). (B) HPLC profiles of product patterns by *A. aculeatus* Viscozyme L depend on the pH (3.0−10.0). Viscozyme L (55 μg/mL) was reacted with 50 mM stevioside in Britton−Robinson buffer (pH 3.0−10.0) at 55 °C for 20 h, and then the reaction mixture was analyzed by HPLC under the same conditions as described in the Materials and Methods. (C, D) HPLC−MS analysis of isolated P1 and P2 as unknown compounds from the reaction mixtures (h, *A. aculeatus* Viscozyme L).

RESULTS

Screening for an ST-Hydrolyzing Enzyme from Commercially Available Glycosidases. We screened nine commercially available glycosidases from *A. niger*, *T. longibrachiatum*, *A. aculeatus*, *T. reesei*, *C. thermocellum*, and almonds to identify an enzyme capable of converting ST into R. Each enzyme was incubated with 50 mM ST at the optimal pH for 12 h, and the hydrolysis products were analyzed by TLC or HPLC. Most enzymes did not hydrolyze or exhibited very low hydrolysis activity (<1%) toward ST. Only *A. aculeatus* Viscozyme L hydrolyzed the glucosidic linkage at the sophoroside of ST. As shown in Figure 1, *A. aculeatus* Viscozyme L produced two products from ST as demonstrated by peak 1 (P1; retention time 23.3 min) and peak 2 (P2; retention time 26.5 min). The molecular weights of the constituents identified in P1 and P2 (Figure 1) were determined to be 641.7 and 479.7 by HPLC−MS analysis, and their structures were elucidated as R and SME by ^{1}H and ^{13}C NMR spectroscopy, respectively. Viscozyme L produced two products at a range of acidic pH values (3.0−5.0) and produced the single product R at pH 9.0−10.0 (Figure 1). These data suggest the enzyme could produce pure R at basic pH conditions. *A. aculeatus* Viscozyme L was further purified, characterized, and finally used to produce R from ST.

Purification and Characterization of ST-Hydrolyzing Enzyme from *A. aculeatus*. We purified the ST-hydrolyzing enzyme from Viscozyme L followed by two-step column chromatography utilizing phenyl-Sepharose and Sephacryl S-200 gel filtration (Table 1). The final protein (1.23 U/mg) was purified approximately 10.3-fold from the crude enzyme,

Table 1. Purification of a Stevioside-Hydrolyzing Enzyme from *A. aculeatus* Viscozyme L[a]

step	total activity (U)	total protein amt (mg)	specific activity (U/mg)	yield (%)
crude enzyme	112.44	916.96	0.12	100
phenyl-Sepharose	50.33	62.27	0.81	44.72
Sephacryl S-200	2.02	1.65	1.23	1.81

[a]The enzyme activity was measured by the release of glucose from stevioside.

resulting in a low recovery of activity (1.81%). The purified protein showed a single band on a 12% sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS−PAGE) gel with a molecular mass of 79 kDa (Figure 2). The enzyme

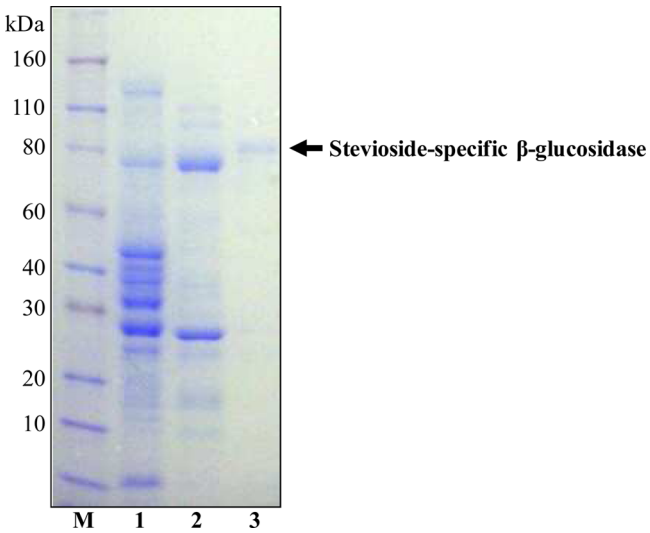

Figure 2. SDS−PAGE of the purified β-glucosidase from *A. aculeatus* Viscozyme L: lane M, molecular mass marker; lane 1, crude enzyme; lane 2, phenyl-Sepharose column chromatography; lane 3, purified stevioside-specific β-glucosidase from Sephacryl S-200 column chromatography.

showed the highest activity at pH 5.0 and maintained more than 95% of its maximal activity in the pH range of 3.0−7.0 (Figure 3). The enzyme showed the highest activity at 60 °C in a 30 min reaction and was stable up to 50−60 °C for 30 min. In addition, the effect of metal ions on the enzyme was studied. Ca^{2+}, Mg^{2+}, Zn^{2+}, and Mn^{2+} ions at 1 mM had no significant effect on the enzyme activity (data not shown).

Substrate Specificities of the ST-Hydrolyzing Enzyme from *A. aculeatus*. The substrate specificities of the ST-hydrolyzing enzyme and almond β-glucosidase were investigated with different β-linked glucobioses and steviol glucosides as summarized in Table 2. The enzymes had similar activities with respect to *p*NPG-β, and the ST-hydrolyzing enzyme exhibited higher specificities toward ST and R than the β-glucobioses. However, it rarely hydrolyzed RebA, which has a β (1 → 3) glucosyl derivative of the 13-hydroxyl group of ST (Figure 4B) and glucosyl linkages at the 19-carboxyl group of the steviol in SME (Figure 4D). In addition, the enzyme did not show hydrolysis activity toward laminaribiose. These results are thought to be due to the steric hindrance, with respect to enzyme catalysis, of the complex glucosidic linkage of RebA. The enzyme kinetic parameters demonstrated that ST was the best substrate, and this enzyme specifically hydrolyzes catalysis between the glucosidic moiety and steviol such as ST and R. These results indicate that the enzyme hydrolyzes only the glucose moiety of the sophoroside at the 13-hydroxyl group in ST. Finally, we named the enzyme ST-specific β-glucosidase (SSGase).

Determination of the Polynomial Equation Coefficients for Optimal Production of R. RSM was used to study the interaction of these variables in relation to R production. From the preliminary experiments, four important factors were selected to optimize the R production: enzyme amount

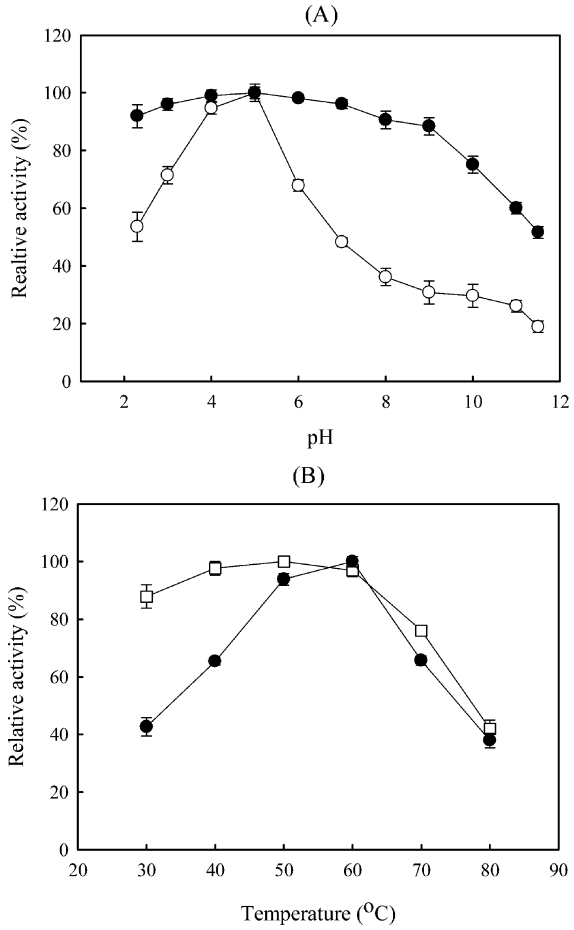

Figure 3. Effects of pH (A) and temperature (B) on the activity and stability of the purified enzyme. (A) Activity profile at various pH values using 46 μg/mL enzyme and 50 mM stevioside in Britton−Robinson buffer at 55 °C for 30 min (○). pH stability profile using 46 μg/mL enzyme in 40 mM Britton−Robinson buffer at 4 °C for 20 h (●). (B) Activity profile at various temperatures using 55 μg/mL enzyme in 40 mM NAB (pH 5.0) for 30 min at various temperatures (●). Thermal stability using 46 μg/mL enzyme in 40 mM NAB (pH 5.0) at 30−80 °C after 12 h of incubation (□). After the reaction mixture was sampled at designated times, the remaining enzyme activity was assayed by a standard method. All data represent the mean value of three independent experiments.

(5−25 μL), ST concentration (10−370 mM), temperature (30−90 °C), and reaction pH (1.5−7.5). The CCD of R production is listed in Table 3. As ascertained from the central points of the corresponding contour plots (Figure 5), the optimal variable values for R production were an ST concentration of 280 mM and an SSGase amount of 16.6 μL at pH (5.1) and 63 °C. R production was measured to be 185 mM under these conditions. The results indicate that R production can be expressed in terms of the following regression equation:

$$y = 184.99 + 22.79x_1 + 0.68x_2 + 17.70x_3 + 4.66x_4 + 3.28x_1x_2 + 9.06x_1x_3 + 3.16x_1x_4 + 1.95x_2x_3 + 1.12x_2x_4 + 14.01x_3x_4 - 29.89x_1^2 - 7.85x_2^2 - 37.39x_3^2 - 33.61x_4^2$$

where x_1, x_2, x_3, and x_4 are coded values of ST concentration (mM), enzyme amount (μL), reaction pH, and reaction

Table 2. Characteristic Parameters of Stevioside-Hydrolyzing Enzyme from *A. aculeatus* Viscozyme L

substrate	almond β-glucosidase activity (U)	SSGase[a] activity (U)	SSGase[a] K_m (mM)	SSGase[a] k_{cat} (s^{-1})	SSGase[a] k_{cat}/K_m ($mM^{-1}\cdot s^{-1}$)	SSGase[a]/ almond β-glucosidase
*p*NPG (β)	20.5	18.7	0.05	4.78	95.6	0.91
stevioside	0.67	14.5	3.57	30.4	8.52	21.6
rubusoside	0.44	4.2	13.64	18.4	1.33	9.55
steviol monoglucosyl ester	–[b]	–				–
rebaudioside A	–	–				–
sophorose	0.45	0.05				0.11
laminaribiose	–	–				–
cellobiose	0.25	0.03				0.12
gentiobiose	0.24	0.08				0.33

[a]SSGase = stevioside-specific β-glucosidase. [b]The dash indicates that the substrate did not hydrolyze.

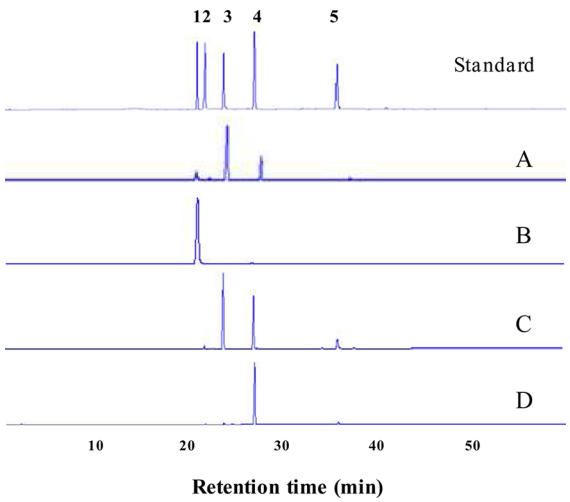

Figure 4. HPLC profiles of the hydrolysis product by the stevioside-specific β-glucosidase with various substrates. A reaction mixture containing stevioside-specific β-glucosidase (55 μg/mL) and 50 mM substrates such as stevioside (A), rebaudioside A (B), rubusoside (C), and steviol monoglucosyl ester (D), respectively, was reacted in 40 mM NAB (pH 5.0) at 55 °C for 12 h. Standard steviol glucosides: 1, rebaudioside A; 2, stevioside; 3, rubusoside; 4, steviol monoglucosyl ester; 5, steviol, respectively.

temperature (°C). The regression equation gave a high R^2 value (0.9742) using an analysis of variance (ANOVA) test. This is an estimate of the fraction of overall variation in the data accounted for by the model, and thus, the model is capable of explaining 97% of the variation in response. On the basis of the model, the predicted response for R production was 185 mM, and the observed experimental value was 193 mM at 16.6 μL of SSGase with 280 mM ST at 63 °C and pH 5.1, demonstrating almost identical results for predicted and observed R production.

DISCUSSION

In the present study, a novel ST-specific β-glucosidase, SSGase, isolated from *A. aculeatus* Viscozymes L was screened, purified, and characterized. In addition, the conditions of production of R were optimized by RSM using four important factors, namely, ST concentration, enzyme activity, temperature, and pH.

β-Glucosidases are widely found in molds, yeasts, bacteria, and higher plants, and many have been purified and characterized. Thus far, only two enzymes, namely, *F. johnsoniae* β-glucosidase (FJGase)[12] and *C. michigaense* β-glucosidase (CMGase),[11] have been reported capable of hydrolyzing the β-glucosyl ester linkages of steviol glycosides. However, the presently discovered SSGase was very different in its properties and substrate specificities from these two enzymes. For example, the novel enzyme has a slightly larger molecular weight (79 000) than FJGase (72 000), and CMGase (65 000). The optimal pH and pH stability (pH 5.0 and 3.0–7.0, respectively) were more acidic than those of FJGase (pH 7.0 and 3.0–9.0, respectively) and CMGase (pH 7.5 and 6.0–10, respectively). In addition, a large difference was found with respect to specificities toward steviol glycosides. FJGase and CMGase exhibited high specificities toward the β-glucosidic linkage at the 19-carboxyl group of ST, whereas SSGase could not hydrolyze the β-glucosidic linkage at the 19-carboxyl group of RebA, ST, and R. The SSGase produced SME from ST via R, indicating the ability of the enzyme to hydrolyze the saccharide chains at the 13-hydroxyl group of ST and R. FJGase degraded R to form steviols via SMG and SME. CMGase could not hydrolyze the β-glucosidic linkage at the 13-hydroxyl group of the steviols. Both SSGase and FJGase could not hydrolyze RebA, whereas CMGase hydrolyzed RebA and ST to form SMG via rebaudioside B (RebB) and steviol bioside (STB). FJGase did not degrade RebB and STB. Finally, FJGase preferred smaller glucosidic linkages at the 19-carboxyl group, such as R, SMG, and SME, whereas CMGase preferred to degrade glucose at the 19-carboxyl group of RebA, which has bulky side chains at the 13-hydroxyl group. In addition, SSGase preferred to hydrolyze glucosidic linkages at the 13-hydroxyl group of ST and R, namely, RebA, as they have bulky side chains at site 13 that may inhibit binding with the enzyme. The three enzymes displayed similar low activities toward the β-glucobioses. This is consistent with the idea that the three enzymes have much higher specificities to steviol glucosides than β-glucooligosaccharides. The natural substrates for the enzymes seem to be steviol glucosides and not the oligosaccharides derived from the naturally abundant glucose polymers, such as cellulose and laminaran. To our knowledge, this is the first study characterizing a β-glucosidase capable of producing R from ST.

Utilizing the optimized conditions, 193 mM R could be produced from ST and SSGase. This optimized condition may be applicable for the economical production of R from the relatively cheap ST for use in Chinese sweet tea and as a natural solubilizer to enhance the water solubility of bioactive materials in the food and pharmaceutical industries. This study indicates the beginning of detailed investigations into SSGase from

Table 3. Central Composite Design Matrix for the Experimental Design and Predicted Responses for Rubusoside Production

	coded value				actual value				rubusoside concn (mM)	
run	x_1	x_2	x_3	x_4	stevioside concn (mM)	enzyme vol (μL)	pH	temp (°C)	exptl	predicted
1	−1	−1	−1	−1	100	10	3	45	46.76	63.015
2	1	−1	−1	−1	280	10	3	45	78.70	77.575
3	−1	1	−1	−1	100	20	3	45	47.14	51.652
4	1	1	−1	−1	280	20	3	45	70.46	79.346
5	−1	−1	1	−1	100	10	6	45	40.16	48.384
6	1	−1	1	−1	280	10	6	45	93.74	99.180
7	−1	1	1	−1	100	20	6	45	42.26	44.837
8	1	1	1	−1	280	20	6	45	100.70	108.767
9	−1	−1	−1	1	100	10	3	75	25.20	35.752
10	1	−1	−1	1	280	10	3	75	65.20	62.965
11	−1	1	−1	1	100	20	3	75	33.97	28.883
12	1	1	−1	1	280	20	3	75	58.83	69.229
13	−1	−1	1	1	100	10	6	75	85.68	77.144
14	1	−1	1	1	280	10	6	75	126.48	140.592
15	−1	1	1	1	100	20	6	75	58.35	78.090
16	1	1	1	1	280	20	6	75	170.58	154.673
17	−2	0	0	0	10	15	4.5	60	34.50	19.869
18	2	0	0	0	370	15	4.5	60	115.34	111.012
19	0	−2	0	0	190	5	4.5	60	164.09	152.233
20	0	2	0	0	190	25	4.5	60	162.06	154.951
21	0	0	−2	0	190	15	1.5	60	11.63	0.036
22	0	0	2	0	190	15	7.5	60	78.22	70.849
23	0	0	0	−2	190	15	4.5	30	58.15	41.225
24	0	0	0	2	190	15	4.5	90	61.90	59.868
25	0	0	0	0	190	15	4.5	60	186.32	184.990
26	0	0	0	0	190	15	4.5	60	183.73	184.990
27	0	0	0	0	190	15	4.5	60	186.59	184.990
28	0	0	0	0	190	15	4.5	60	182.04	184.990
29	0	0	0	0	190	15	4.5	60	184.27	184.990
30	0	0	0	0	190	15	4.5	60	186.99	184.990

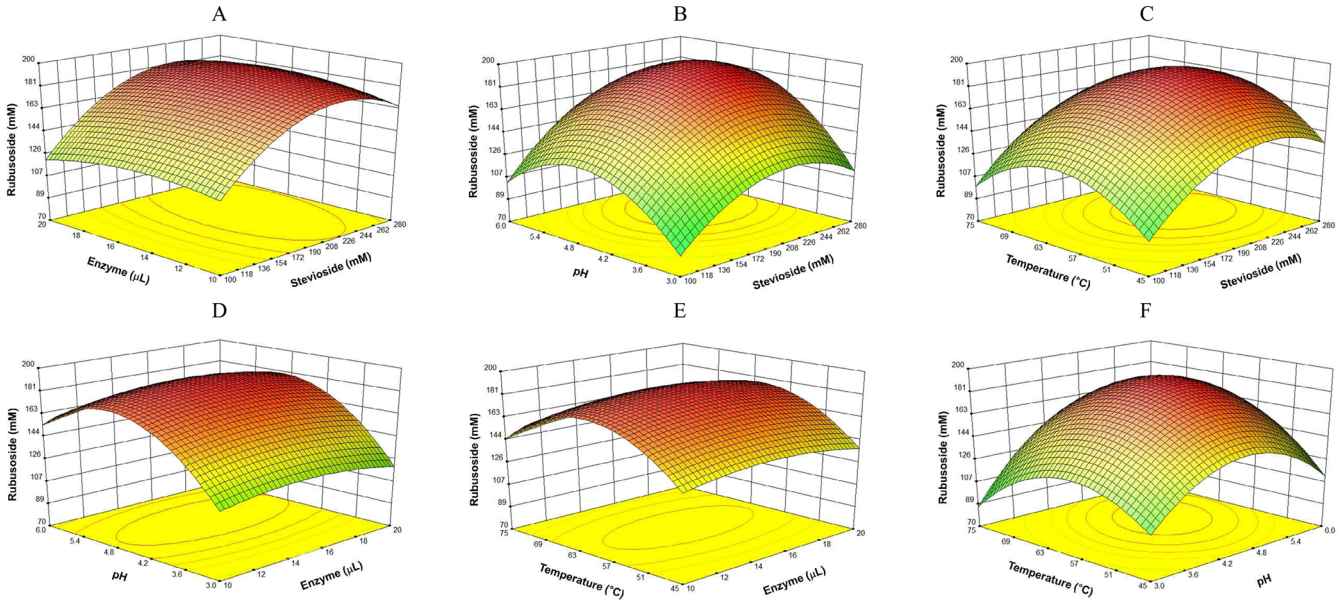

Figure 5. Response surface and contour plots of rubusoside production. Shown are the mutual interactions between stevioside concentration and enzyme amount (A), stevioside concentration and pH (B), stevioside concentration and temperature (C), enzyme amount and pH (D), enzyme amount and temperature (E), and pH and temperature (F). Other parameters, except two parameters in each figure, are zero level in coded units.

A. aculeatus. Future studies should entail cloning the full-length gene as well as protein engineering experiments. The present results demonstrate the potential for industrial production of R by using a novel β-glucosidase from *A. aculeatus*.

AUTHOR INFORMATION

Corresponding Author

*Phone: (8263) 570-5001. Fax: (8263) 570-5239. E-mail: wslee@kribb.re.kr.

Author Contributions

[‖]J.-A.K. and Y.-M.K. contributed equally to this work.

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Notes

The authors declare no competing financial interest.

ABBREVIATIONS USED

CCD, central composite design; CMGase, *Clavibacter michiganense* β-glucosidase; FJGase, *Flavobacterium johnsoniae* β-glucosidase; HPLC, high-performance liquid chromatography; HPLC−MS, high-performance liquid chromatography−mass spectrometry; NAB, sodium acetate buffer; NMR, nuclear magnetic resonance; *p*NPG-β, *p*-nitrophenyl β-glucoside; R, rubusoside; RebA, rebaudioside A; RebB, rebaudioside B; RSM, response surface methodology; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; SME, steviol monoglucosyl ester; SMG, steviol monoglucoside; ST, stevioside; STB, steviol bioside; SSGase, stevioside-specific β-glucosidase; TLC, thin-layer chromatography

REFERENCES

(1) Darise, M.; Mizutani, K.; Kasai, R.; Tanaka, O.; Kitahata, S.; Okada, S.; Ogawa, S.; Murakami, F.; Chen, F. H. Enzymic transglucosylation of rubusoside and the structure-sweetness relationship of steviol-bisglycosides. *Agric. Biol. Chem.* **1984**, *48*, 2483−2488.

(2) Ohtani, K.; Aikawa, Y.; Ishikawa, H.; Kasai, R.; Kitahata, S.; Mizutani, K.; Doi, S.; Nakaura, M.; Tanaka, O. Further study on the 1,4-α-transglucosylation of rubusoside, a sweet steviol bis glucosides from *Rubus suavissimus*. *Agric. Biol. Chem.* **1991**, *55*, 449−453.

(3) Ishikawa, H.; Kitahata, S.; Ohtani, K.; Ikuhara, C.; Tanaka, O. Production of stevioside and rubusoside derivatives by transfructosylation of β-fructofuranosidase. *Agric. Biol. Chem.* **1990**, *54*, 3137−3143.

(4) Kitahata, S.; Ishikawa, H.; Miyata, T.; Tanaka, O. Production of rubusoside derivatives by transgalactosylation of various α-galactosidases. *Agric. Biol. Chem.* **1989**, *53*, 2929−2934.

(5) Tanaka, O. Improvement of taste of natural sweeteners. *Pure Appl. Chem.* **1997**, *69*, 675−683.

(6) Liu, Z.; Schwimer, J.; Liu, D.; Lewis, J.; Greenway, F. L.; York, D. A.; Woltering, E. A. Gallic acid is partially responsible for the antiangiogenic activities of *Rubus* leaf extract. *Phytother. Res.* **2006**, *20*, 806−813.

(7) Ono, Y. Anti-inflammatory and anti-allergic effects of Tiencha (*Rubus suavissimus* S. Lee). *Allergy Pract.* **2004**, *317*, 380−385.

(8) Feng, J.; Xin, N. Advance in studies on chemical constituents and pharmacological action of *Rubus suavissimus* S. Lee and *Lithocarpus polystachyus* Rehd. *Lishizhen Med. Mater. Med. Res.* **2007**, *18*, 1089−1090.

(9) Zhijun, L. Diterpene glycosides as natural solubilizers. PCT/US2009/040324, 2010; pp 10−15.

(10) Tanaka, T.; Kohda, H.; Tanaka, O.; Chen, F. H.; Chou, W. H.; Leu, J. L. Rubusoside (β-D-glucosyl ester of 13-O-β-D-glucosyl-steviol), a sweet principle of *Rubus chingii* Hu (Rosaceae). *Agric. Biol. Chem.* **1981**, *45*, 2165−2166.

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

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

(13) Ashipala, O. K.; He, Q. Optimization of fibrinolytic enzyme production by *Bacillus subtilis* DC-2 in aqueous two-phase system (poly-ethylene glycol 4000 and sodium sulfate). *Bioresour. Technol.* **2008**, *99*, 4112−4119.

(14) Bradford, M. M. Rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* **1976**, *72*, 248−254.

(15) Su, D.; Robyt, J. F. Control the synthesis of dextran and acceptor-products by *Leuconostoc mesenteroides* B-512FM dextransucrase. *Carbohydr. Res.* **1993**, *248*, 471−476.

NOTE ADDED AFTER ASAP PUBLICATION

The original ASAP posting of June 1, 2012, contained an error in the spelling of the fourth author's surname. This has been corrected in the posting of June 6, 2012.