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A complete specific cleavage of glucosyl and ester linkages of stevioside for preparing steviol with a β-galactosidase from *Sulfolobus solfataricus*

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ABSTRACT

β-Galactosidases from *Sulfolobus solfataricus* have been used to synthesize galactooligosaccharide and lactulose. In this work, a β-galactosidase from *S. solfataricus* with weak β-glucosidase activity but high lipase activity was employed as catalyst to assist hydrolysis of stevioside to obtain steviol, an important starting reagent of synthetic bioactive materials and the main metablite of stevioside in human digistion. The β-galactosidase presented a strict substrate specifity on converting stevioside to steviol in a stoichiometric yield. The β-galactosidase favors the cleavage of glycoside linkages prior to cleavage of glycosyl ester linkage. The hydrolysis is external diffusion controlled and hence has to bear low substrate concentration in regular process, but this can be solved with product removal or enzyme immobilization. The immobilization of the β-galactosidase onto cross-linked chitosan microspheres did not enhance the enzyme's thermal or pH stability but eliminated the external diffusion, and therefore speeded the hydrolysis in 3 folds. The relative reaction activity dropped only 1.75% after 6 runs of using the immobilized β-galactosidase.

1. Introduction

Steviol, (5β,8α,9β,10α,13α)-13-hydroxykaur-16-en-18-oic acid, is an important diterpene first isolated from *stevia rebaudiana* in 1931 [1], and was identified with its structure and stereochemistry in 1960 [2]. The intensive notice on steviol has been paid since the approval of using the extract from *Stevia rebaudiana* by FDA in 2008, not only because of its existing in the extract as a bitter component but also as a main metabolite of stevioside and rebaudioside A, those are the glycosides of steviol and two main sweeteners in the extract [3]. In addition, steviol is an important starting material of synthetic bioactive chemicals [4]. Yet itself was fund bioactive as suppressing LPS-mediated TNF-α, IL-1β and IL-6 release, and attenuating LPS-induced pro-inflammatory cytokine productions by affecting cytokine gene expression via IκBα/NF-κB signalling pathway [5]. Steviol is secreted by renal tubular epithelium, causing diuresis, natriuresis, kaliuresis and a fall in renal tubular reabsorption of glucose [6].

Unfortunately, steviol is rare in nature and there are hardly reports about its synthetic methodology. Attempts have been devoted to its synthesis route even since it was discovered [1], but moved slowly. The chemical synthesis revolves classic rearrangement reaction with expensive starting materials [7,8]. The complete acidic hydrolysis of stevioside produces steviol together with isosteviol due to rearrangement [1]. The enzymatic processes include natural pathway such as hydroxylation of ent-kaurenoic acid [9,10] and hydrolysis of its analogue, stevioside with various glycosidases.

Among these processes, enzymatic hydrolysis from stevioside is a promising path for plant scale-up, but manipulating the regioselectivity of the glycosidases is a real challenge; because stevioside has two glucosidic linkages and one ester linkage to be cleaved for preparation of steviol, and β-glucosidases have been disclosed in different regioselective hydrolysis of glucosidic linkages but hardly presented hydrolysis activity to all the glucosidic linkages and ester linkages. For example, steviol was produced from hydrolysis of stevioside using either pancreatin at pH 7.0 (20.2% of yield) and glucosidase from *A. niger* at pH 7.0 (20.8% of yield) [11]; while a β-glucosidase from *Clavibacter michiganense* was reported to hydrolyze glucosyl ester linkages at site C19 of stevioside but did not cleave 13-*O*-linked glucosyl residue of rubusoside or steviol monoside. A β-glucosidase from *Flavobacterium johnsonae* [12] was also used to catalyze hydrolyzing glucosyl ester linkages at site C19 of rebaudioside A, stevioside, and rubusoside, but was not

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active on beta-glucosidic linkages at site C13 of rebaudioside B or steviolbioside. Ko et al. tried 9 commercially available glycosidases, a 64% yield of steviol was the best, which was obtained with a β-glucosidase purified 74-fold from *Penicillium decumbens* naringinase [13]. In previous work, we found a β-galactosidase from *Aspergillus* sp. accelerated converting stevioside to rubusoside in a yield of 91.4%, accompanying with a steviosides conversion of 98.3%; the galactosidase actually presented specific hydrolysis activity on β-1,2 glucosidic linkage of stevioside, the hydrolysis activity was weak on the natural stevioside analogs [14].

β-Galactosidases from *Sulfolobus solfataricus* have been studied well [15–20] for synthesis of galactooligosaccharide and lactulose [18,21–24], and found other hydrolysis functions like hydrolysis of gibberellin A4 glucosyl ester [25]. A β-galactosidase [23] from *S. solfataricus* was found active in hydrolyzing both glucosidic and ester linkages of stevioside [26]; while we did not have any luck in using classic lipases such as Novo435, Lipozyme TL IM and Amano AY 30G (data not published) to cleave the ester linkage in the presence of glycosidase to accomplish the full hydrolysis of stevioside. Therefore, in this experiment, we attempt to use the β-galactosidase from *S. solfataricus* for an applicable preparation of steviol from stevioside, which would also help to enrich the knowledge of mechanism in catalytic hydrolysis with β-galactosidase.

2. Materials and methods

2.1. Enzyme and chemicals

o-Nitrophenyl β-D-galactopyranoside (oNPG) and 4-nitrophenyl-D-glucopyranoside (pNPG) were purchased from Sigma Chemical Co. β-galactosidases [23] from *S. solfataricus* (ATCC no 35092) were provided by Dr Wu Jing from the State Key Laboratory of Food Science and Technology at Jiangnan University (770 U/mL of oNPG hydrolysis activity, strain F441Y purified to 3.1 fold with heat precipitation, DEAE-sepharose and phenyl HP-sepharose Fast Flow. Detailed information is refereed in the supporting material of reference 23). Stevioside was recrystallized from the raw stevioside (90% of HPLC purity, from Niutang chemicals, China). Rebaudioside A was from Niutang chemicals, China (98.7% of HPLC purity). Rubosuside was made in our laboratory. Na_2CO_3, CH_3COONa, CH_3COOH, $Na_2HPO_4{\cdot}12H_2O$, $NaH_2PO_4{\cdot}2H_2O$, glutaraldehyde (25%, aqueous, BR), glyoxal (40%, aqueous, AR), chitosan (80–95% of deacetylation degree, 50–800 mPa s) were purchased from Sinopharm Chemical Reagent Co., Ltd, China. All other reagents were of analytical grade and used as received unless otherwise stated.

2.2. β-Galactosidase, β-glucosidase and lipase assay

The oNPG hydrolysis activity of the β-galactosidase was determined using *o*-nitrophenyl β-D-galactopyranoside (oNPG) as substrate [18]. The increase in the absorbance at 420 nm (T6 New-Century, Beijing Purkinje General Instrument Co., Ltd) caused by the release of *o*-nitrophenol was measured to calculate the hydrolysis activity. The reaction mixture containing 1.8 mL acetate buffer (50 mM, pH 4.5), 100 μL galactosidase solution (dissolved and diluted using the aforementioned buffer) and 100 μL *o*-nitrophenyl β-D-galactopyranoside (20 mM) was shaken at 60 °C for 10 min, and then quenched by 1 mL Na_2CO_3 (1 M). One unit (U) of hydrolysis activity is defined as the amount of enzyme required to release 1 μmol *o*-nitrophenol per min under the above reaction conditions.

The pNPG hydrolysis activity of the β-glucosidase was assayed by using 20 mmol/L 4-nitrophenyl-D-glucopyranoside (pNPG) as substrate in 50 mmol/L sodium acetate buffer (pH 6.0) at 80 °C for 10 min, modified from the literature with conditions offering higher activity [27]. One unit (U) of hydrolysis activity is defined as the amount of enzyme required to release 1 μmol *p*-nitrophenol per min under the above reaction conditions.

The lipase activity of the enzyme was standard olive oil hydrolysis activity, detected at 40 °C.

The enzyme possesses a hydrolysis activity of 770 U/mL as a β-galactosidase on oNPG, 27.97 U/mL as a β-glucosidase on pNPG, and an olive oil hydrolysis activity of 2078 U/mL as a lipase.

2.3. Enzymatic hydrolysis of stevioside with the β-galactosidase from Sulfolobus solfataricus

In a typical reaction, 20 mL stevioside solution (20 g/L) in a 50 mL Erlenmeyer flask was kept at 80 °C in a water bath for 30 min, mixed with β-galactosidase (200 U/g stevioside) and then shaken at 80 °C for 24 h. The reaction mixture was boiled for 3 min to deactivate the galactosidase and terminated the reaction. The precipitated product was filtered and washed with warm DI water until no stevioside was detectable from the washing solution, and then lyophilized to obtain the final product.

The products were characterized with NMR and LC–MS. NMR analysis was recorded at AVANCE III 400 MHz Digital NMR Spectrometer (Bruker, USA). LC–MS–MS profile was taken from Waters Acquity UPLC system (BEH HILIC C18 column; mobile phase: acetonitrile and water (75:25, v/v), 0.3 mL/min; column temperature: 30 °C; collision energy: 20–55 eV; polarity: ES^-). Conversion of stevioside (St) and yield of steviol or other hydrolysis product were calculated based on HPLC analysis with calibration of standard solution of stevioside, described as following:

The reaction mixture and the final product were analyzed with a HPLC system (Waters 2996, United States) equipped a C18 column (Lichrospher C18, 5 μm, Hanbang, China) and a UV detector (detection wavelength was 210 nm), respectively. A mixture of acetonitrile and water was used as eluent, gradient from 75:25 (v/v) to 50:50 (v/v) at 1 mL/min.

The conversion of stevioside (St) was calculated as following:

$$\text{St conversion} = \frac{C_0 - C_t}{C_0} \times 100\%$$

here C_0 is the initial St concentration (g/L), C_t is the real time St concentration in the reaction mixture (g/L). The St concentration was determined with a standard calibration curve. Product yields were calculated according to the percentages of chromatographic areas and calibrated with that of St.

The yield of steviol was production yield, calculated based on the whole analysis of precipitated solid with mass measurement. The yields of steviol monoside (Sm) and rubausoside (Ru) were calculated based on their HPLC chromatograph peak area with stevioside concentration as thereference, calculated as following:

$$\text{Ru or Sm Yield} = \frac{C_t \times A_x / A_{St}}{C_0} \times 100\%$$

The concentration of glucose in the reaction mixture was determined with a SBA-50 Glucose Biosensor (Biology Institute of Shandong Academy of Sciences, Shangdong, China). All tests were performed in triplicate at least; all data presented were with standard deviations less than 5%.

2.4. Immobilization of the β-galactosidase onto cross-linked chitosan microspheres

The optimum parameters for the immobilization were decided by single factor test. The immobilization was applied as following after preparing the cross-linked chitosan microspheres. In a typical cross-linking, 0.5 g chitosan dissolved in 20 mL of 2% acetic