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Enzymatic preparation of a natural sweetener rubusoside from specific hydrolysis of stevioside with β-galactosidase from *Aspergillus* sp.

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ABSTRACT

Rubusoside is a precious bioactive sweetener which mainly exists in Chinese sweet tea plant (*Rubus suavissimus* S. Lee), while stevioside is an abundant natural sweetener with bitter aftertaste. In this work, a β-galactosidase from *Aspergillus* sp. presented specific hydrolytic activity on β-1,2 glucosidic linkage of stevioside, and converting stevioside to rubusoside. The hydrolytic activity was weak on the natural stevioside analogs, which makes the process a promising approach to produce rubusoside and utilize stevioside. The highest steviosides conversion was 98.3%, accompanying with a rubusoside yield of 91.4%.

1. Introduction

Rubusoside (Ru, 13-O-β-glucosyl-19-O-β-D-glucosyl-steviol) is a rare natural sweetener mainly existed in extract of Chinese sweet tea plant [1,2] (*Rubus suavissimus* S. Lee; known in Japan as Tenryocha or Tencha [3]). The tea plant grows only in southern China with variable yearly yield depending on local climate. Rubusoside is also a minor component in extract of *Stevia rebaudiana* leaf. However, there are few reports focused on synthetic rubusoside. For example, rubusoside was found as an intermediate in a chemo-enzymatic preparation of rebaudioside A, another sweetener that also exists in *Stevia rebaudiana* leaf [4]. Jiang et al. [5] reported that a bacterium (*Chryseobacterium* sp.) fermentation liquid or the β-glucosidase produced from the fermentation stimulated the hydrolysis of stevioside (St, 13-O-β-sophorosyl-19-O-β-D-glucosyl-steviol) into rubusoside, but the results lack enough structure characterization to exclude the isomers.

As an analog of rubusoside, stevioside exists in *Stevia rebaudiana* leaf as an abundant component, which is much less valuable than rubusoside because of its bitter aftertaste [6–8]. Selective cleavage of β-1,2-glucosidic linkage of sophorosyl moiety at C13 of stevioside can produce rubusoside. Whereas, the hydrolysis of stevioside may produce steviol, isosteviol, steviolmonoside, steviolbioside or their mixtures depending on the catalyst and reaction conditions (Scheme 1), because that stevioside possesses three glycosidic bonds (β-linked sophorose, β-1,2-D-glucopyranosyl on C13 and an ester β-glucosidic linkage on the C19 carboxyl group) [9–13].

In fact, most of the hydrolysis products of stevioside exist naturally and exhibit potential pharmaceutical properties, such as anti-inflammatory and anti-tumor activity [12,14–16]. However, few researchers applied hydrolysis of stevioside to improve its taste. A possible reason is that people always focus on the fact that the mono- and di-glycosylation at the 13-O-β-sophorosyl moiety can effectively improve the sweetness character [17].

Among the hydrolysis enzymes, β-galactosidases (β-D-galactoside galactohydrolase, EC 3.2.1.23) are known to catalyze both hydrolysis and transgalactosylation [18,19]. For example, Danieli et al. [20] demonstrated that bovine β-galactosidase (1,4-galactosyltransferase) could catalyze the galactosylation of stevioside and steviolbioside with UDP-galactose, affording corresponding galactosyl derivatives with improved sweetness.

An interesting and valuable hydrolysis using β-galactosidase from *Aspergillus* sp. was noticed when the galactosylation of stevioside was expected, in which rubusoside was obtained as the main product. Therefore, in this work, a β-galactosidase from *Aspergillus* sp. was employed to catalyze the hydrolysis of stevioside, its substrate specificity and regioselectivity as well as the hydrolysis conditions were investigated.

2. Materials and methods

2.1. Enzyme and chemicals

β-Galactosidases from *Aspergillus* sp. (CICIM F0620, from CCTCC) and *Sulfolobus* sp. (ATCC No35092) were provided by Dr Wu

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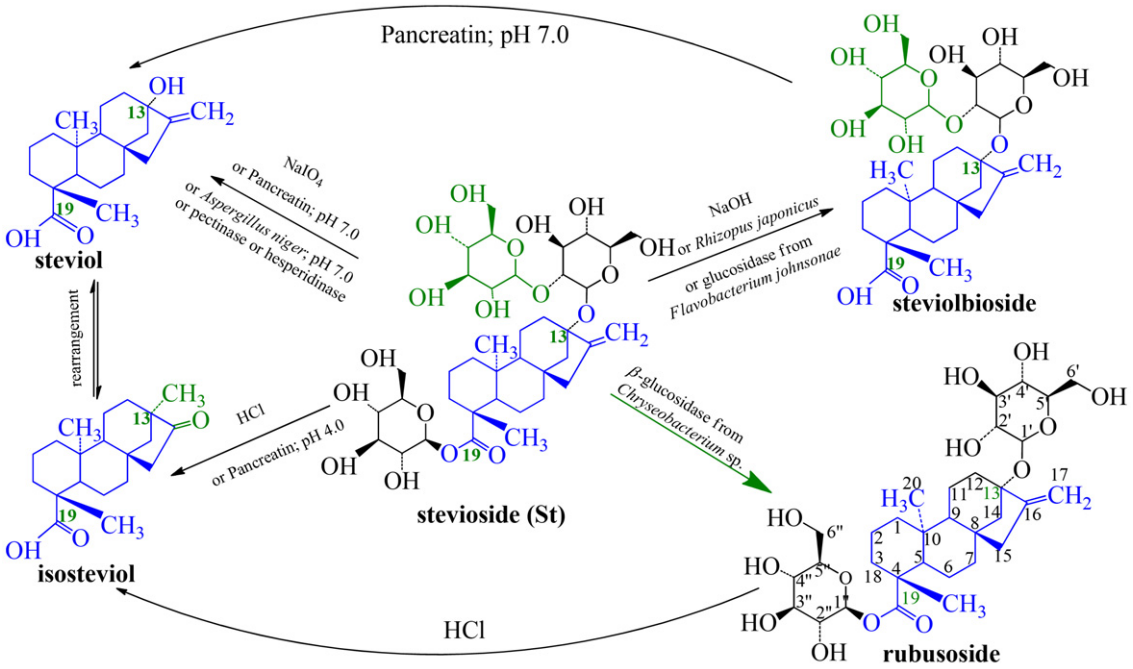

Scheme 1. Hydrolysis of stevioside using different catalysts.

Jing from the State Key Laboratory of Food Science and Technology at Jiangnan University. β-Galactosidase from *Kluyveromyces lactis* (Maxilact® LG 2000, 2000 NLU/g) was provided by Royal DSM N.V. β-Galactosidase from *Kluyveromyces lactis* (Lactozym 3000L HP-G, 3000LAU/mL) was presented by Novozymes (China). α-Galactosidase from *Asperillus niger* was provided by Amano Pharmaceutical Co., Ltd. (China). Stevioside (≥97%, HPLC) was purchased from GLG Life Tech. Co., Ltd. (China). Natural rubusoside (98.6%, HPLC) was provided by Professor Chen Quan-bin at Guangxi Normal University China. Steviol glycosides extracted from *Stevia rebaudiana* leaf (30.0% stevioside, 35.9% rebuside A, 11.2% rebaudioside C, 22.9% others, HPLC) and rebaudioside A (≥99%, HPLC) were provided by Changzhou Niutang Chemical Plant Co., Ltd. *o*-Nitrophenyl β-D-galactopyranoside was purchased from Sigma Chemical Co. Na_2CO_3, CH_3COONa, CH_3COOH, $Na_2HPO_4{\cdot}12H_2O$ and $NaH_2PO_4{\cdot}2H_2O$ were purchased from Sinopharm Chemical Reagent Co., Ltd., China. All other reagents were of analytical grade unless otherwise stated.

2.2. Galactosidase hydrolytic activity assay

The hydrolytic activity was determined using *o*-nitrophenyl β-D-galactopyranoside as substrate [21]. 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 hydrolytic activity. The reaction mixture containing 1.8 mL acetate buffer (50 mM, pH 4.5), 100 μL enzyme 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 hydrolytic activity is defined as the amount of enzyme required to release 1 μmol *o*-nitrophenol per min under the above reaction conditions.

2.3. Enzymatic hydrolysis of stevioside by β-galactosidase

In a typical reaction, 20 mL stevioside solution (10 g/L) and β-galactosidase (0.8 kU/g stevioside) were mixed in a 50 mL Erlenmeyer flask, shaken at 60 °C for 24 h. The reaction mixture was then boiled for 3 min to deactivate the enzyme and the precipitated enzyme was removed by centrifugation. The supernatant was purified with column chromatography over silica gel (200–300 mesh) to obtain the products eluted by CH_2Cl_2:CH_3CH_2OH:H_2O (5:4:1, v/v/v).

The product was analyzed with the HPLC system (Waters 2996, United States) equipped a NH_2 column (APS-2 HYPERSIL, Thermo, United States) and a photodiode array detector. 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. LC–MS-MS profile was taken from Waters Acquity UPLC system (BEH HILIC column, acetonitrile and water (80:20, v/v), 0.3 mL/min, column temperature: 30 °C, collision energy: 20–55 eV, polarity: ES^-). NMR spectra were recorded at AVANCE III 400 MHz Digital NMR Spectrometer (Bruker, USA). The conversion of stevioside was calculated as following:

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

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

The concentration of glucose in the reaction mixture was determined with SBA-50 Glucose Biosensor (Biology Institute of Shandong Academy of Sciences, Shangdong, China). All tests were performed in triplicate.

3. Results and discussion

3.1. Enzyme screen

Initially, five galactosidases were assayed for their hydrolytic activity on stevioside (Fig. 1, a: 40 °C; b: 70 °C).