Food Chemistry 196 (2016) 155–160

Contents lists available at ScienceDirect

Food Chemistry

journal homepage: www.elsevier.com/locate/foodchem

Production of a bioactive sweetener steviolbioside via specific hydrolyzing ester linkage of stevioside with a β-galactosidase

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ARTICLE INFO

Article history:
Received 2 April 2015
Received in revised form 12 August 2015
Accepted 10 September 2015
Available online 11 September 2015

Keywords:
Steviolbioside
Stevioside
Hydrolysis
β-Galactosidase
Reaction coupling separation
Anti-cancer

ABSTRACT

A β-galactosidase from *Kluyveromyces lactis* was found to specifically catalyze hydrolysis of the glycosyl ester linkage of stevioside to yield steviolbioside, a rare sweetener that also exists in *Stevia rebaudiana* leaves. In a packed bed reactor, a reaction coupling separation was realized and a production yield of steviolbioside reached 90% in 6 h. The hydrolysis product steviolbioside presented higher cytoxicity on human normal cells (hepatocytes cell L02 and intestinal epithelial cell T84) than stevioside did. Comparing to the typical chemotherapy agent, 5-fluorouracil (5-FU), steviolbioside presents much lower cytotoxicity on all assayed human normal cells; it presented notable inhibition on human hepatocarcinoma cell Hep3B, human breast cancer cell MDA-MB-231 and human pancreatic cancer cell BxPC-3. The remarkable inhibition on MDA-MB-231 cells makes steviolbioside a potential remedy for human breast cancer, when steviolbioside is served as a natural sweetener.

1. Introduction

Steviolbioside, 13-[(2-O-β-D-glucopyranosyl-β-D-glucopyranosyl)oxy]kaur-16-en-18-oic acid, is a bioactive nature sweetener (Ibrahim et al., 2014; Kataev et al., 2011; Purkayastha et al., 2014; Sharipova et al., 2011; Well, Frank, & Hofmann, 2013), which is rare in *Stevia rebaudiana* leaves (Kohda, Kasai, Yamasaki, Murakami, & Tanaka, 1976). It is also the main metabolite of the star sweetener rebaudioside A and its analogue stevioside (Nikiforov, Rihner, Eapen, & Thomas, 2013; Roberts & Renwick, 2008; Williams & Burdock, 2009), yet it can be used as an intermediate of some medications, which makes steviolbioside an important reagent in medical science in addition to a food additive.

Synthetically, steviolbioside could be obtained from hydrolysis of stevioside, the cheaper and abundant analogue (see also Supporting Material, Scheme 1s). Hydrolysis of stevioside may yield multiproducts because stevioside has three glucose moieties at the C13 and C19 sites, which could be fully or partially cleaved, yielding steviolbioside, steviol, isosteviol, steviol mono-glucosyl ester, rubusoside, steviol mono-glucoside. There are few reports about the specific synthetic methodology for this minor sweetener, such as alkaline (Chaturvedula & Prakash, 2011; Wood, Allerton, Diehl, & Fletcher, 1955) or enzymatic (Ko et al., 2013) hydrolysis of stevioside. For example, alkaline hydrolysis of stevioside yields both steviolbioside and rebaudioside B (Chaturvedula & Prakash, 2011). Therefore, enzymatic hydrolysis of stevioside is a possible path for plant scale-up, but manipulating the regioselectivity of the glycosidase is still a challenge.

To assist hydrolysis of ester linkages, lipases would be the first choice as they should specifically release the glucose moiety from the C19 site; but neither literature nor our preliminary experiment were successful with some popular commercial lipases, such as Novo435, Lipozyme TL100L, Lipozyme TL IM, Lipozyme RM IM, Lipase AF-15 and Amano AY 30G. Therefore, glycosidase especially glucosidases turned to be the next enzyme candidate, as they have been disclosed in different regioselectivities in hydrolysis of glucosidic linkages. Researchers have still not succeeded with glucosidases in hydrolysis of ester linkage of stevioside. Terai disclosed that stevioside was hydrolyzed with catalysis of *Rhizopus japonicus* culture and afforded steviolbioside in a yield of 30.1% in 5 days at 30 °C (Terai, 1989). β-Glucosidases from *Clavibacter michiganense* and *Flavobacterium johnsonae* were reported to catalyze hydrolysis of the glucosyl ester linkages at the C19 site of stevioside but saved 13-O-linked glucosyl moiety of rubusoside or steviol monoglucoside (Kiso, Kitahata, Okamoto, Miyoshi, & Nakano, 2000; Nakano, Okamoto, Yatake, Kiso, & Kitahata, 1998). With pPNG (4-nitrophenyl-D-glucopyranoside) as control, the relative catalysis activity of the β-glucosidase from *C. michiganense* with stevioside and RA as substrate was 12.3% and 6.92%, respectively (Yore et al., 2014). Yet as a byproduct, steviolbioside was found in the

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http://dx.doi.org/10.1016/j.foodchem.2015.09.035
0308-8146/

production of steviol, with the catalysis of a β-glucosidase purified from a *Penicillium decumbens* naringinase (Ko et al., 2013).

Galactosidases were not the choice in our experiments until some of them were found to be active in yielding steviolbioside, in the experiment of enzymatic preparation of steviol from stevioside (Chen, Xia, Wan, Wang, & Liu, 2014). Considering that hydrolysis is a nucleophilic reaction that can be driven and affected by not only the catalyst but also the reaction microenvironment; solubility differences between steviolbioside and its starting material stevioside, reaction coupling purification is desirable. Yet the packed bed reactor can be counted on for reusing the enzyme. Therefore, in this experiment, aimed at a promising process to produce steviolbioside, the enzymatic production of steviolbioside was studied in detail; also, the cytoxicity of steviolbioside on some human normal cells and carcinoma cells was investigated.

2. Materials and methods

2.1. Enzymes and chemicals

o-Nitrophenyl β-D-galactopyranoside (oNPG) and 4-nitrophenyl-D-glucopyranoside (pNPG) were purchased from Sigma–Aldrich Chemical Co (China). β-Galactosidase from *Kluyveromyces lactis* (Maxilact® LG 2000, 1300 U/ml of oNPG hydrolysis activity) was provided by Royal DSM N.V. β-Galactosidases from *Sulfolobus solfataricus* (ATCC No35092, 770 U/ml of oNPG hydrolysis activity) was provided by Dr. Wu Jing from the State Key Laboratory of Food Science and Technology at Jiangnan University. β-Glucosidase from *Aspergillus niger* (10 U/ml, pNPG hydrolysis activity) was presented by Novozymes (China). α-Glucosidase from *A. niger* (9 U/ml, pNPG hydrolysis activity) was presented by Novozymes (China). β-Glucosidase from *Penicillium multicolor* (Aromase™, 80 U/g-pNPG hydrolysis activity) was provided by Amano Pharmaceutical Co., Ltd. (China). Novozyme 435 (from *Candida antarctica*, 10,000 U/g of hydrolytic activity on olive oil) and Lipozyme RM IM (from *Rhizomucor miehei*, 5800 U/g of hydrolytic activity on olive oil) was provided by Novozymes (China).

Stevioside (97% of HPLC purity) was recrystallized from the raw stevioside (90% of HPLC purity, from Niutang chemicals, China). Rebaudioside A (98.7% of HPLC purity) was from Niutang chemicals, China. Na_2CO_3, CH_3COONa, CH_3COOH, $Na_2HPO_4{\cdot}12H_2O$, $NaH_2PO_4{\cdot}2H_2O$, glutaraldehyde (25%, aqueous, BR), chitosan (80–95% of deacetylation degree, 50–800 mPa s) and 5-fluorouracil (5-FU, BR) 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. Cell culture

Cells involved in the human digestion system were employed in this experiment. The human oral epidermoid carcinoma cell KB, human moderately differentiated gastric cancer cell SGC-7901, human colon adenocarcinoma cell Caco-2, human ileo-caecal adenocarcinoma epithelial cell HCT-8, human colorectal cell HCT 116, human hepatocytes cell L02, human hepatocarcinoma cells (BEL-7404, BEL-7402), human pancreatic cancer cell BxPC-3, human breast cancer cell MDA-MB-231, human epithelioid carcinoma cell A-431and human neuroblastoma cell SH-SY5Y were purchased from Shanghai Institute of Biochemistry and Cell Biology, Chinese Academy of Science. The human gastric mucosal epithelial cell GES-1, human poorly differentiated gastric cancer cell MGC-803, human intestinal epithelial cell T84, three hepatoma cells, Huh-7 (mutated p53), HepG2 (wild-type p53) and Hep3B (p53 deleted), human hepatocellular carcinoma cell with low metastatic potential MHCC97-L, human hepatocellular carcinoma cell with high metastatic potential HCCLM3 and human papillary thyroid carcinoma cell BCPAP were purchased from the American Type Culture Collection. The cells were cultured in DMEM medium containing 10% fetal bovine serum, 1% glutamine (200 mmol/l), penicillin (100 IU/ml), and streptomycin (100 mg/l) in a humidified 5% CO_2 atmosphere at 37 °C before use.

2.3. β-Galactosidase and β-glucosidase assay

The oNPG hydrolysis activity of the β-galactosidase was determined using o-Nitrophenyl β-D-galactopyranoside (oNPG) as substrate (Park, Kim, Lee, Kim, & Oh, 2008). 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 (Chen et al., 2014). One unit (U) of hydrolysis activity is defined as the amount (ml) of enzyme required to release 1 μmol o-nitrophenol per min at 40° in acetate buffer (50 mM, pH 6.5).

The pNPG hydrolysis activity of the β-glucosidase was assayed by using 20 mmol/l pNPG as substrate in 50 mmol/l sodium acetate buffer (pH 6.0) at 40 °C for 10 min, modified from the literature with conditions offering higher activity (Wood & Bhat, 1988). One unit (U) of hydrolysis activity is defined as the amount (ml) of enzyme required to release 1 μmol p-nitrophenol per min under the above reaction conditions.

2.4. Enzymatic hydrolysis of stevioside with the β-galactosidase

In a typical reaction (Chen et al., 2014), 20 ml stevioside solution (20 g/l) in a 50 ml Erlenmeyer flask was kept at 40 °C for 30 min, then mixed with β-galactosidase and shaken in a water bath at 40 °C for 12 h. The reaction mixture was boiled for 3 min to deactivate the galactosidase to terminate the reaction. The precipitated white powder was filtered and washed with warm DI water until no stevioside was detectable from the washing solution. The powder was then recrystallized from 95% aqueous methanol and white needles were obtained as final product. The product was characterized with NMR and LC–MS (Chen et al., 2014). The conversion of stevioside (St) was calculated based on HPLC analysis with calibration of standard solution of stevioside, described as following:

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

There C_0 and C_t is the initial and real time concentration (g/l) of stevioside in the reaction mixture, respectively. The St concentration was determined with a standard calibration curve.

The yield of steviolbioside was production yield, calculated based on the whole analysis of precipitated solid with mass measurement. The yields of other byproducts, if needed, were calculated based on their corresponding HPLC chromatograph peak area (A_x) with chromatograph peak area of stevioside (A_{St}) as the reference, calculated as following:

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

All tests were performed in triplicate at least; all data presented were with standard deviations less than 5%.

2.5. Optimizing of immobilization of the β-galactosidase onto crosslinked chitosan microspheres

The immobilization of the β-galactosidase onto crosslinked chitosan microspheres was conducted as the previous experiment with glutaraldehyde as the cross-linker instead of glyoxal (Chen et al., 2014). Before attempting multivariate statistical design, single fact experiments were carried out to determine the crucial