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Dekkera bruxellensis, a beer yeast that specifically bioconverts mogroside extracts into the intense natural sweetener siamenoside I

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ABSTRACT

In response to growing concerns about the consumption of artificial sweeteners, the demand for natural sweeteners has recently increased. Mogroside V is a common natural sweetener extracted from the fruit of *Siraitia grosvenorii*, but its taste should be improved for marketability. Here, we screened various microbes for the ability to perform selective hydrolysis of glycosidic bonds in mogroside V, converting it to siamenoside I, which has a higher sweetening power and better taste than other mogrosides. *Dekkera bruxellensis* showed the most promising results in the screen, and the *Exg1* gene (coding for a *β*-glucosidase) of *D. bruxellensis* was cloned and purified. We then used HPLC-MS/MS to assess the *β*-glucosidase activity of purified enzymes on *p*-nitrophenyl *β*-glucoside and mogroside V. The results demonstrated that *D. bruxellensis* had a unique enzyme that can selectively hydrolyze mogrol glycosides and promote the conversion of the natural sweetener mogroside V to siamenoside I.

1. Introduction

Global demands for natural sweeteners have significantly increased over past decades (Sylvetsky & Rother, 2016) due to concerns about the long-term consumption of artificial sweeteners (NAS) as sugar substitutes (Suez, Korem, Zilberman-Schapira, Segal, & Elinav, 2015). To date, the U.S. Food and Drug Administration has only approved two types of high-intensity natural sweeteners, including purified rebaudiana A (from the leaves of *Stevia rebaudiana*) and mogroside extracts (from the fruit of *Siraitia grosvenorii*, commonly called Lo Han Kuo: LHK) (FDA, 2008, 2010). Mogrosides consist of a triterpenoid aglycone (mogrol) with different numbers of attached glucose molecules. The sweetness of mogrosides may be determined by the number and locations of the glucose moieties (Matsumoto, Kadai, Ohtani, & Tanaka, 1990). For example, mogroside V contains five glucose moieties in its molecular structure and is 392 times sweeter than 5% sucrose in water (Matsumoto et al., 1990). Moreover, Siamenoside I and mogroside IV have four glucose moieties each, but at different locations, and are respectively 563 and 465 times sweeter than 5% sucrose. On the other hand, mogroside III E, with three glucose moieties, is somehow less sweet (Matsumoto et al., 1990). Indeed, siamenoside I is currently considered to be the sweetest mogroside. Additionally, the taste of siamenoside I was reported to be preferred over mogrosides IV and V (Matsumoto et al., 1990; Zhou et al., 2014); however, the amount of siamenoside I in natural mogroside extracts is limited (Makapugay, Nanayakkara, Soejarto, & Kinghorn, 1985; Matsumoto et al., 1990). Thus, isolation, purification or enrichment methods that can yield high concentrations of natural siamenoside I would be beneficial. Many attempts, including chemical hydrolysis, enzyme treatment and microbial fermentation, have been made to convert mogroside V to siamenoside I. Unfortunately, the complexity of glucose branching side-chains in mogrosides makes the production or isolation of pure siamenoside I extremely challenging.

Exg1p in *Saccharomyces cerevisiae* is a glycoside hydrolase family 5 (GH5) enzyme, which is known to hydrolyze O-β-D-glycosidic linkages at the nonreducing end of polymer chains, resulting in the release of

Abbreviations: LHK, Lo Han Kuo; r*Db*Exg1, recombinant *Dekkera bruxellensis* Exg1; *Sc*Exg1, *Saccharomyces cerevisiae* Exg1; *p*NPG, *p*-nitrophenyl *β*-glucoside; *o*NPG, *o*-nitrophenyl *β*-glucoside

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glucose (Chiu, Wang, Lee, Lo, & Lu, 2013; Dealdana et al., 1991; Wang et al., 2015). Schmidt et al. revealed that substrates for Exg1p include flavonoid glucosides, such as naringenin 7-O-β-glucopyranoside and luteolin 7-O-glucoside (Schmidt, Rainieri, Witte, Matern, & Martens, 2011). We then further identified Exg1p as the major exo-(1, 3)-β-glucanase involved in the initiation of mogroside bioconversion (Chiu et al., 2013; Wang et al., 2015). During fermentation, Exg1p hydrolyzes mogroside V to create a mixture of siamenoside I and mogroside IV intermediates, with a higher preference for mogroside IV. In wild-type *S. cerevisiae*, mogroside III E, is eventually generated as an end product (Chiu et al., 2013; Wang et al., 2015). Zhou et al. extensively searched for commercial enzymes that could bioconvert mogroside V into siamenoside I (Zhou et al., 2014). A total of 62 different enzymes were collected and screened for mogroside bioconversion, and out of those 62 enzymes, only *β*-galactosidase from *Aspergillus oryzae* (Sigma G5160) and dextranase from *Penicillium* sp. (Worthington DEXC) could enrich siamenoside I in mogroside extracts. The enrichment was reported to be up to 44% and 31% of all mogrol glucosides when the extract was treated with the specific microbial enzymes at 37 °C, pH 5 for 7 h or at 37 °C pH 6 for 24 h, respectively (Zhou et al., 2014). However, it is likely that these enzymes eventually convert mogroside V into MG III E or other mogrosides as end products. Another study showed that acid or alkali treatment of mogroside V produces nonspecific stereoisomeric mixtures of mogrosides and other byproducts, which are not desirable and exhibit low yields (Chen et al., 2011).

Since the family of GH5 enzymes is widely distributed across bacteria and fungi, with broad sequence diversity and protein specificity (Aspeborg, Coutinho, Wang, Brumer, & Henrissat, 2012), we aimed to identify an Exg1-like enzyme from bacteria or yeast that may specifically produce siamenoside I from LHK mogroside extracts.

2. Materials and methods

2.1. Screening for mogroside conversion

Eighteen yeast strains and 13 lactic acid bacteria (LABs) (Table S1) were obtained from the Bioresource Collection and Research Center at the Food Industry Research and Development Institute (Hsinchu, Taiwan) for mogroside conversion screening. Mogroside extracts, containing 25.9% mixed mogrosides, were purchased from Changsha Huir Biological-tech Co., Ltd. (Hunan, China). Yeast or LABs were activated in yeast extract-peptone-dextrose (YPD, Difco, Sparks, MD, USA) or Man–Rogosa–Sharpe (MRS, Difco, Sparks, MD, USA) media, respectively. Media containing mogrosides were prepared by dissolving 1% (w/v) mogroside extracts in YPD or MRS broth. The yeast were aerobically cultured at 25 °C in an orbital shaker at 200 rpm, while LABs were anaerobically incubated at 37 °C. The mogroside conversion was stopped by adding one volume of pure methanol to the culture media, and the mogroside content was analyzed by HPLC-MS/MS.

2.2. Mogroside analysis by HPLC-MS/MS

Aliquots of fermentation media were first purified using solid phase extraction cartridges (C-18, 500 mg/3 ml, Chrome expert, Sacramento, CA, USA). The unbound impurities were washed with 45% methanol, and the mogrosides were eluted with pure methanol. Fractions of the eluted mogrosides were collected for HPLC-ESI-MS/MS (YMC Hydrosphere C18 analytical column, YMC, Kyoto, Japan; Thermo Finnigan model LXQ linear ion trap mass spectrometer, San Jose, CA, USA) analysis was as described by Chiu et al. (2013), with minor modifications. Ten microliters of the prepared samples were injected for mogrosides analysis. The following electrospray ionization (ESI) parameters were used: −4.8 kV spray voltage, 400 °C capillary temperature, 25 arb of sheath gas, 8 arb of auxiliary gas, 20% collision energy, and 2.0 Da isolation width. The mass scan range was 50–2000 *m/z*. Xcalibur 2.0.7 software was used for data analysis (San Jose, CA, USA). The dominant molecular ions in the ESI/MS^n spectra for mogrosides are $[M+Na]^+$; sequential glucose loss ($-162\,m/z$) was observed in the fragmentation pattern in the collision-induced dissociation mode. Mogroside V, siamenoside I, mogroside IV, and mogroside III E were identified based on the presence of ions with *m/z* values of 1309, 1147, 1147, and 985 for $[M+Na]^+$, respectively (Chiu et al., 2013).

2.3. Molecular cloning, expression, and purification of recombinant protein

Exg1-like *β-glucosidase* from *Dekkera bruxellensis* (*DbExg1*) was cloned with the Gateway cloning system. Briefly, the coding region of *DbExg1* was first amplified by PCR. The forward primer was 5′-GGGGACAAGTTTGTACAAAAAAGCAGGCTTCACCATGAAGTTTATTTTATTGTC-3′ (underline indicates gene coding region). The reverse primer was 5′-GGGGACCACTTTGTACAAGAAAGCTGGGTCGAAGCTACACTGGTTAGG-3′ (underline indicates gene coding region). Genomic DNA extracted from *D. bruxellensis* was used as the template. Yeast expression vectors (pYES-DEST52) were further introduced to generate galactose-inducible plasmids carrying the correct *DbExg1* sequence. The yeast vectors were then amplified and transformed into *Exg1*-deleted *S. cerevisiae* (*exg1Δ* mutants) for the galactose-induced DbExg1 protein expression assay. The *DbExg1* gene was also integrated into (*exg1Δ* mutants) with a *GPD* promoter. For protein purification, the culture media was concentrated with a 30 kDa MWCO (molecular weight cutoff) ultrafiltration membrane (Vivaspin 20, GE Co., Taipei, Taiwan). The extracellular protein concentrates were purified by Ni^{2+}-affinity chromatography. Protein homogeneity in each fraction was assessed by SDS-PAGE and silver staining.

2.4. β-Glucosidase activity and mogroside V conversion by recombinant DbExg1

The optimal conditions for β-glucosidase activity were determined. The optimum pH was obtained by monitoring enzyme activity at a range of pH levels (from pH 3 to pH 9) in the reaction buffer. Incubation was carried out at 60 °C for 30 min. To analyze enzyme stability, the same buffer system and pH range were used, however, the reactions were performed at 4 °C for 1 h. The optimum temperature was determined in 20 mM acetate buffer at pH 5 in a range of temperatures from 20 °C to 90 °C. For temperature stability analysis, β-glucosidase was extracted and activity was monitored following 1 h incubation in 20 mM acetate buffer, pH 5. Freshly purified r*Db*Exg1 was added to 10 mM of *p*-nitrophenyl β-D-glucoside (*p*NPG), *o*-nitrophenol β-D-glucoside (*o*NPG) or authentic mogroside V (final concentration of 2 mg/ml) in acetate buffer solution. The enzyme and substrate mixture were incubated under optimum enzymatic reactions conditions of 60 °C, pH 5. The β-glucosidase activity was determined by measuring the levels of *p*-nitrophenol (*p*NP) or *o*-nitrophenol (*o*NP) released from *p*NPG or *o*NPG after the glucose was hydrolyzed by glucosidase. The released *p*NP or *o*NP was detected at a wavelength of 405 nm and calibrated with standard curves (Kuo et al., 2018). Mogroside conversion was stopped by the addition of one volume of pure methanol at specific time points. The converted products were then purified for HPLC-MS/MS analysis.

3. Results

3.1. Screening for mogroside-converting strains

Several microorganism strains (18 yeast and 13 LABs) were selected for mogroside conversion testing, according to previous reports of glucosidase activity in the biotransformation of isoflavone (Chien, Huang, & Chou, 2006). Mogroside extracts that mainly contained mogroside V were used as the bioconversion substrate (Fig. 1A). Surprisingly, none of the selected LABs exhibited mogroside bioconversion activity. In contrast, most of the yeast strains, which potentially play roles in bioflavoring (Holt, Mukherjee, Lievens, Verstrepen, &