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Purification and functional characterization of the first stilbene glucoside-specific β-glucosidase isolated from *Lactobacillus kimchi*

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ABSTRACT

This study aimed to develop viable enzymes for bioconversion of resveratrol-glucoside into resveratrol. Out of 13 bacterial strains tested, *Lactobacillus kimchi* JB301 could completely convert polydatin into resveratrol. The purified enzyme had an optimum temperature of 30–40 °C and optimum pH of pH 5.0 against polydatin. This enzyme showed high substrate specificities towards different substrates in the following order: isorhaponticin » polydatin » mulberroside A > oxyresveratrol-3-*O*-glucoside. Additionally, it rarely hydrolyzed astringin and desoxyrhaponticin. Based on these catalytic specificities, we suggest this enzyme be named stilbene glucoside-specific β-glucosidase. Furthermore, polydatin extracts from *Polygonum cuspidatum* were successfully converted to resveratrol with a high yield (of over 99%). Stilbene glucoside-specific β-glucosidase is the first enzyme isolated from lactic acid bacteria capable of bio-converting various stilbene glucosides into stilbene.

1. Introduction

Resveratrol (3,4′,5-trihydroxystilbene) is a natural polyphenolic compound found in grapes, peanuts, a variety of mulberries, and other plants, especially in the medicinal plants *Polygonum cuspidatum* (Polygonaceae) and *Rheum undulatum* (Rheum). *P. cuspidatum* has the highest content of resveratrol and polydatin (resveratrol-3-*O*-β-glucoside), whereas *R. undulatum* has the highest amount of methoxy resveratrol derivatives [1]. Resveratrol is known to possess many pharmacological properties including anti-inflammatory, cardio protective, antioxidant, and anticancer properties [2,3]. Recently, it is gaining scientific attention as a longevity promoter. These valuable properties make resveratrol useful for the production of pharmaceuticals, cosmetics, and nutraceuticals as a potential health-functional compound. Therefore, there is increasing need to develop better and more effective production methods.

Until now, many methods have been designed to chemically synthesize resveratrol, but the production costs are high, and the processes are environmentally unfriendly [4]. In order to produce resveratrol at reasonable prices, the bioconversion of polydatin to resveratrol has been found to be excellent method. Previously, various methods to convert polydatin to resveratrol have been investigated, including acid hydrolysis, heating and enzymatic transformation techniques [5–7]. However, all these methods are time-consuming and uneconomical. Alternative approaches like biotechnological methods can be used to produce resveratrol from plants and yeast cells. Although resveratrol has been produced successfully from metabolically engineered *Saccharomyces cerevisiae* using tyrosine [8] and *p*-coumaric acid [9], production at a commercial scale (gram level per liter) was not achieved [8,9]. For these reasons, presently, resveratrol is mainly obtained by extraction from plant tissues. In *P. cuspidatum* grown in Hanzhong, China, the content of polydatin is usually six-fold higher than that of

Abbreviations: HPLC, high-performance liquid chromatography; HPLC-MS, high-performance liquid chromatography-mass spectrometry; NAB, sodium acetate buffer; *p*NPG-β, *para*-nitrophenyl β-glucoside; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; TLC, thin-layer chromatography.

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resveratrol [10] but the resveratrol was higher than its glucosides on the aspect of the industrial demand [11]. If polydatin was converted into resveratrol, the yield of resveratrol would increase dramatically. Therefore, bioconversion of polydatin from *P. cuspidatum* was carried out by enzymatic catalysis or microbial fermentation. For example, β-glucosidases from *Aspergillus oryzae* [1,12], *Aspergillus niger*, *yeast* [13], and snailase [14] were used to transform polydatin in the *P. cuspidatum* extract to resveratrol. Enzymes were also added into the powdered raw material to improve the yield of resveratrol. Although these methods reportedly increase the yield of resveratrol, the high cost of the enzymes and production of toxic compounds during spontaneous fermentation limit their industrial applications. To help the industrial production of resveratrol, it needs to be enzyme acting towards stilbene glucoside or methoxy glucoside derivatives found in *P. cuspidatum* and *R. undulatum*. Due to these reasons, we tried to screen for only those polydatin-bioconversion bacterial strains that have food safety clearance.

In this study, a novel polydatin-converting lactic acid bacterium was selected to produce resveratrol from polydatin and extracts of the plant *P. cuspidatum*. Considering the food safety aspect of the microorganism, screening of enzymes from 13 lactic acid bacteria strains was performed, and the ability of these enzymes to convert polydatin to resveratrol was also evaluated. Further, purification, substrate specificities, and kinetic parameters of the enzymes were investigated in detail. The bioconversion process would provide a feasible strategy for the production of resveratrol from plant like *P. cuspidatum*, which has high polydatin content, or *R. undulatum*, which has high methoxy resveratrol content.

2. Materials and methods

2.1. Chemicals and materials

Polydatin and resveratrol were purchased from Sigma-Aldrich (St Louis, MO, USA). The rhizomes of *P. cuspidatum* were purchased at an herbal market in Daejeon, Korea and its dried rhizomes (0.24 kg) were extracted with methanol (1 L) for 7 d at room temperature. The methanol extract was evaporated *in vacuo*, yielding a residue (45 g), which was re-suspended in distilled water and used for further studies. All other chemicals were of reagent grade.

2.2. Isolation of strains and culture conditions

Thirteen kimchi, Korean fermented food, samples were collected from traditional markets in Jeonju. They were thoroughly resuspended in a 50 mM phosphate buffer (pH 7.0) and spread on modified MRS agar plates (modified MRS agar contained 18 g of nutrient broth, 5 g of sodium acetate trihydrate, 4 g of yeast extract, 2 g of di-potassium phosphate, 1 g of sodium citrate dehydrate, 0.2 g of magnesium sulfate, 0.05 g of manganese (II) sulfate, and 15 g of agar powder per liter). The plates were aerobically incubated at 37 °C for 2 weeks. Single colonies were first transferred onto new modified MRS agar plates. To screen for strains capable of converting polydatin into resveratrol, isolated colonies were tested for their ability to hydrolyze polydatin. These bacterial strains were incubated in MRS broth containing 1 mg/mL of polydatin without glucose at 37 °C for 6 days. At 24 h intervals, 50 mL aliquots were collected and analyzed by thin-layer chromatography (TLC). Strain JB301 was positive for the desired polydatin converting β-glucosidase activity, and hence selected for further study.

2.3. 16S rRNA gene sequencing and phylogenetic analysis

Extraction of genomic DNA from the 13 strains, including *L. kimchi* JB301, was carried out using a commercial genomic DNA extraction kit (Core Biosystem, Daejeon, Korea). The 16S rRNA gene was PCR amplified from the chromosomal DNA using the universal bacterial primer pair 9F and 1512R, and the purified PCR products were sequenced by GenoTech (Daejeon, Korea). The 16S rRNA gene sequence of the type strain was obtained from EzTaxon server (http://www.eztaxon.org) [15]. Multialignment of related strains was done using clustal W [16]. 5′ and 3′-gaps were edited via BioEdit [17]. Neighbor-joining [18] and maximum parsimony methods [19] from the PHYLIP suit program (http://evolution.genetics.washington.edu/phylip.html) were used for construction of the phylogenetic tree. Bootstrap values were calculated with data restricted close to 1000 times and marked into branching point. Evolutionary distance matrix was estimated according to Kimura two-parameter model [20].

2.4. Enzyme purification

For the purification of enzyme, the bacterial strain was incubated in MRS broth containing 1 mg/mL of polydatin without glucose at 37 °C for 6 d. Purification of β-glucosidase was carried out using a TOYOPEARL DEAE-650 M (Tosho, Tokyo, Japan) chromatography column according to the manufacturer's protocol. The enzyme solution (3.5 mg/mL) was loaded onto the TOYOPEARL DEAE-650 M (3.2 × 20 cm, 160 mL) column equilibrated using 20 mM sodium acetate buffer solution (NAB, pH 6.0). The absorbed proteins were eluted with a 0–1 M sodium chloride linear gradient at a flow rate of 1 mL/min. Finally, the active fractions were dialyzed against 20 mM NAB (pH 6.0) and the final purified protein was concentrated using an Amicon Ultra 10,000 MWCO centrifugal filter (Millipore, Billerica, MA, USA). The enzyme purity and molecular weight were estimated by 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) using Novex Sharp standards proteins (Life Technologies, Carlsbad, CA, USA).

2.5. Enzyme assay

The enzyme activity was determined by measuring the release of glucose from *para*-nitrophenyl β-glucoside (*p*NPG-β) (concentration, 0.2–5 mM), which was used as the substrate. The reaction was stopped by addition of sodium carbonate solution (final concentration, 0.67 M) to the enzyme reaction mixture. The amount of 4-nitrophenol liberated from *p*NPG-β was determined by measuring absorption at 400 nm in a cuvette (length, 1 cm) and using a molar extinction coefficient of 55,560 M^{-1} cm^{-1}. The enzyme activity was determined by the release of resveratrol or stilbene from polydatin or other stilbene glucosides by high-performance liquid chromatography (HPLC) analysis. The activity of enzyme was determined by the release of glucose from β-glucobioses. One unit of enzyme activity was defined as the amount of enzyme capable of hydrolyzing 1 μmol of glucose from stilbene glucosides per minute. Protein concentration was measured by the Bradford method using bovine serum albumin as the standard [21].

2.6. Effects of pH and temperature on enzyme activity

The enzyme (3.28 μg) was incubated at 37 °C in a 32 mM Britton–Robinson buffer solution (pH 2–11) with 10 mM polydatin to determine the optimum pH. In addition, the enzyme was also incubated at 4 °C for 8 h in a 32 mM Britton–Robinson buffer solution (pH 2–11), and the residual enzyme activity was examined to determine the pH stability. The optimal temperature was determined by incubating the enzyme at 20–70 °C for 5 h in 20 mM NAB (pH 6.0). The thermal stability was ascertained by incubating the enzyme at 20–70 °C for 10 h in 20 mM NAB (pH 6.0), and the residual enzyme activity was examined using 10 mM polydatin at 35 °C.

2.7. Analytical methods of hydrolysis products

The products of polydatin hydrolysis from a variety of bacterial strains were analyzed by TLC and HPLC. The 13 isolated strains from kimchi were incubated with 10 mM polydatin in the culture broth at 37 °C for 24 h. At the pre-determined time intervals, the reaction mixture was placed in a water bath for 5 min to halt enzyme activity. Aliquots (10 μL) were removed, the reaction products were analyzed by TLC using pre-coated silica gel 60 F_{254} plates (Merck, Darmstadt, Germany) and developed in a solvent system consisting of acetonitrile and water [85:15 (v/v)], with resveratrol and polydatin as the standard compounds. The plates were dipped in 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 [22], and then visualized.

Chromatographic separation for quantitative analysis was achieved using a 1200 series quaternary HPLC system (Agilent Technologies, Palo Alto, CA, USA) consisting of a G1311A quaternary pump with a G1322A vacuum degasser, a G1329A thermostatted autosampler, a G1316A column oven set at 30 °C, a ZORBAX SB-Aq (5 μm, 4.6 × 150 mm), and a G1314B DA detector set at 320 nm. After desalting with Amberlite MB-3 (Organo, Tokyo, Japan), the resultant digests were separated on a C_{18} column with a stepwise methanol gradient (0–100%). The fractions containing the reaction products were collected and desalted again with Amberlite MB-3, followed by lyophilization. High performance liquid chromatography-mass spectrometry (HPLC-MS)-based analysis for the isolated compounds was performed using an HCT ultra PTM Discovery Ion Trap system (Bruker Daltonik, GmbH, Bremen, Germany) in the negative electrospray ionization mode.

3. Results

3.1. Screening for polydatin-converting strain from fermented food sources

We screened 13 bacterial strains from a Korean fermented food, kimchi, to identify an enzyme capable of converting polydatin into resveratrol (Table 1). Each culture broth was incubated with 10 mM polydatin for 24 h, and the hydrolysis product was analyzed by TLC