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Production of gastrodin through biotransformation of *p*-2-hydroxybenzyl alcohol by cultured cells of *Armillaria luteo-virens* Sacc

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ABSTRACT

Gastrodin is the important ingredients of *Gastrodia elata* Blume owing to its important pharmacological properties. The aim of the current research is to introduce one-step microbial bioconversion of *p*-2-hydroxybenzyl alcohol (HBA) to gastrodin instead of chemical synthesis. The method of gastrodin measurement was firstly constructed. Structural elucidation of biotransformed compounds was based on one-dimensional and two-dimensional NMR. Nine resting-filamentous cells suspension are screened for their transforming capabilities to produce gastrodin. The microorganisms used were *Armillaria luteo-virens* Sacc (CGMCC no. 1884), *Aspergillus foetidus* ZU-G1 (CGMCC no. 1628), *Peniciuium cyclopium* AS 3.4513, *Aspergillus niger* AS 3.40, *A. niger* AS 3.429, *Trichoderma viride* AS 3.4005, *Trichoderma* sp., *Penicillium notatum*, *Mucor* sp., *Rhizopus* sp. *A. luteo-virens* Sacc U⑩ gave the highest gastrodin concentration. The optimized bioconversion conditions for *A. luteo-virens* Sacc U⑩ consisted of inoculums size 160.0 ± 10.5 g resting cells/L, amounts of precursor (HBA) 10.0 mg/ml, bioconversion system pH 6.0, 1% (v/v) Tween 80 or 0.1% oleic acid, bioconversion temperature 23 °C, shaking speed 120 rpm in darkness. Under the optimized transformation conditions, the highest gastrodin concentration was 750 ± 38 μg/100 ml at 5 days. The results of current study cannot only apply to replace the chemical synthesis process that involves the glycosylation reaction, but also apply to scale-up production.

1. Introduction

"Green chemistry" is defined as the design, development and application of chemical processes and products to reduce or eliminate the use and generation of substances hazardous to human health and environment [1–3]. Modern biocatalysis is developing new and precise tools to improve a wide range of production processes, which reduce energy and raw material consumption and generate less waste and toxic side-products [4]. There is an increasing body of information about the use of biocatalysis for selective transformations of synthetic and natural products. The principal advantages of these biocatalytic processes are their relatively mild conditions, environmental safety, and especially high selectivity. Special attention has been paid to filamentous fungi because they are able to catalyze regio- and stereo-selective hydroxylation [5–8]. Microbial transformation is defined as an enzymatic reaction catalyzed by the enzymes secreted in metabolic activities of microorganisms. It has such advantages over chemical synthesis as high stereo- and regio-selectivity, as well as mild reaction conditions, simple operation procedures, less cost and lower pollution. Some reactions that cannot be fulfilled in chemical approach are facile process by microbial transformation [8,9].

Gastrodia elata Blume belongs to the family of Orchidaceae. It can improve the circulation and is usually prescribed for treating rheumatism, paralysis, hemiplegia, lumbago, headache, vertigo, tetanus and convulsant diseases [10,11]. Gastrodin has been verified as the major active ingredient in the herb of *G. elata* Blume, and pharmacological tests show that it has sedative, anaesthesic, neuroprotective effect, memory improving, anticonvulsant effects, antioxidant and free radical scavenging activities [12,13]. However, recent studies on GAS focused mainly on the extraction, identification, quantification and its pharmaceutical activity from *G. elata* Blume [14,15]. As well known, gastrodin production from HBA was one-step by glycosylation with different glucose donor. The possible synthesis route was postulated as Fig. 1. Glycosylation is often the final step in the biosynthesis of secondary plant products resulting in the formation of an overwhelming number of natural glucosides with numerous applications. The benefits of glycosylation may be an increased solubility or a decreased volatility in comparison with the non-glycosylated molecules. Most of the research on enzymatic synthesis of glycosidic linkages has focused on the oligosaccharides biosynthesis. Glycosylation is

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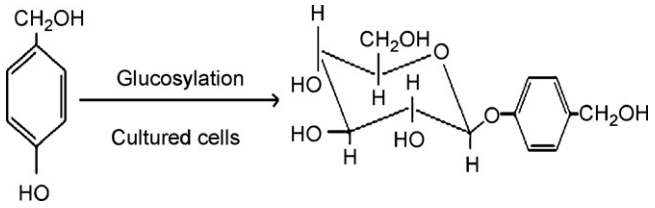

Fig. 1. Possible pathway of biotransformation from exogenous *p*-hydroxybenzyl alcohol for gastrodin production using filamentous fungi cell suspension cultures [21,22].

a characteristic biotransformation reaction in cultured plant cells because glycosyltransferases are widespread in plants [16–18]. There have many reports on the glycosylation of exogenously supplied phenolic compounds by cultured plant cells [19,20]. Glycosylation is postulated as the very important step for gastrodin biosynthesis whether in plant cells or microbial cells. To our knowledge, there have been no reports on the biotransformation of HBA to produce gastrodin by cultured microbial cells.

Less reports investigated the biotransformation of gastrodin by plant cells culture, such as *Panax ginseng* C.A. Mey [21], *Datura stram onium* [22], *Datura tatula* L. [23]. As well known, the biotransformation process by plant cells needs long-time, and the plant cells is difficult to cultivate owing to contamination. As a result, to discuss more efficient and low cost transforming ways is to our main objective in the gastrodin research field. We report in the current study the biotransformation of HBA into the corresponding monoglucosides GAS, which are more soluble and active, by cultured cells of filamentous fungi.

2. Materials and methods

2.1. Chemicals and reagents

Standard gastrodin (GAS) was purchased from Chinese Pharmaceutical Bioproduct Identification Institute (Beijing, China). High-purity HBA was purchased from Sigma–Aldrich Chemical Co. (St. Louis, MO, USA). Methanol and acetonitrile (HPLC grade) were purchased from Sanli Chemical Factory (Zhejiang, China). Water was prepared in a Milli-Q water purification system (Millipore, Bedford, MA, USA). Acetone and dimethylsulphoxide (DMSO), methanol, tetrahydrofuran and acetonitrile were HPLC grade. The other chemicals used in this study were of analytical grade.

2.2. Preparation of standard solution

Stock solutions of gastrodin and HBA in 100.0 μg/ml and 20.0 mg/ml, respectively, were prepared with a solvent of methanol. Standard solutions for HPLC measurements were prepared by serial dilutions to the required concentrations. All the solutions were filled with nitrogen and kept at −20 °C in darkness.

2.3. Preparation of calibration standards

Gastrodin calibration curves were constructed with six different gastrodin standards covering the expected concentration ranges (0.4–200.0 μg/ml for GAS). Different standard solutions were prepared with methanol solution. The mixture was deproteinized with 1 ml methanol, added with 2 ml dichloromethane, vortexes for 5 min for full deproteinization and extraction, and then centrifuged for 10 min at 4000 rpm. Calibration curves were constructed using peak-area ratios of analyte to internal standard.

2.4. Microorganisms and preparation of the cultured cells suspension

Armillaria luteo-virens Sacc QH (CGMCC no. 1884) was isolated and screened from the Qingzang Plain, China, and bred after several natural mutations. *Aspergillus foetidus* ZU-G1 (CGMCC no. 1628) was preserved at the China General Microbiological Culture Collection Center (CGMCC); *Penicinium cyclopium* AS 3.4513, *Aspergillus niger* AS 3.40, *A. niger* AS 3.429, *Trichoderma viride* AS 3.4005 were bought from CGMCC; *Trichoderma* sp., *Penicillium notatum*, *Mucor* sp. were isolated and stored in our lab. The fungus was maintained on potato dextrose agar (PDA) slants. The slant was inoculated and incubated at 23 °C for 3 days. Erlenmeyer flasks (250 ml) containing 30 ml of medium were inoculated with microorganism before 5 days of subculture. The cultures were incubated with shaking speed of 100 rpm at 23 °C for 3 days. After that, culture broth were centrifuged at 4 °C and the speed of 10,000 × *g* for 30 min, and the collected cultured cells was washed with sterilized water for three times. At last, the washed mycelia were collected and stored at 4 °C for the following experiment.

2.5. Biotransformation and structure identification

The suspension cells were cultivated in 250 ml of flask with 30 ml liquid biotransformation medium (3% glucose and sterilized water, initial pH 5–6). A 1 ml of prepared substrate solution (dissolved in absolute ethanol) was added to one flask with resting cell cultures, and one additional flask without substrate was taken as the control. After additional 5 days of incubation at 23 °C with shaking speed of 120 rpm in darkness, the cell cultures were filtered out in vacuum and washed three times with distilled water. The filtrate was collected and extracted three times by equivalent volume of EtOAc, and all the extracted solutions were concentrated in vacuum at 50 °C. Residues were dissolved in methanol and analyzed by TLC and HPLC. The mobile phase was chloroform–methanol (9:1), and detected by spraying with 10% EtOH (in H_2SO_4) followed by heating at 105 °C. The TLC chromatogram result showed that a new spot appeared in the plate compared with that of the control. For preparative biotransformation, 0.5 ml of substrate solution was added to each flask on the 3 days culture. After additional 5 days of incubation, all the media were collected, extracted and concentrated as described above. The obtained residue is separated by silica gel chromatography (200–300 mesh) eluting with chloroform–methanol (19:1) to yield product. NMR spectra (^{1}H NMR, ^{13}C NMR) were recorded in CD_3COCD_3 on INOVA-300 spectrometer and chemical shifts were recorded in ppm using TMS as internal standard.

2.6. TLC analysis

The extracts were spotted on silica gel plates which were developed by petroleum ether (60–90 °C)–ethyl acetate (1:1), and visualized by spraying with 10% H_2SO_4 solution, followed by heating at 100 °C for 5 min.

2.7. Reverse-phase HPLC (RP-HPLC) analyses

The crude samples with two-steps purification were analyzed by RP-HPLC according to the literature [24]. The RP-HPLC system used throughout the current study consisted of two Waters 510 pumps (Waters, Milford, MA, USA), a sample injector (Rheodyne, Cotati, CA, USA) with a 20 μl loop, and a Waters 996 photodiode array detector. Evaluation and quantification were made on a Millennium chromatography data system (Waters). The column used was a reversed-phase Symmetry C18 column (150 mm × 3.9 mm i.d., 5 μm; Waters).

The mobile phase was filtered, degassed by sonication and pumped through the system at a flow rate of 1.0 ml/min at 35 °C. The normal operating pressure was 90–120 MPa and the analytical time was 20 min. The mobile phase was methanol (solvent A)–0.02% phosphoric acid solution (solvent B, pH 2.6) in the aso-gradient mode as follows: 95% A and 5% B. The flow rate was 0.5 ml/min, and the effluent was monitored at 220 nm. Routine sample calculations were made by comparison of the peak area with that of the standard curve.

2.8. Residual reduced sugar and mycelia measurement

Reducing sugar was measured with the DNS method [25], pH was measured with the pH meter. Fungal mycelia was collected with centrifugation at 10,000 × *g*, and weighed at the 4 °C for further measurement.

2.9. Statistical analysis

All experiments were carried out in triplicate. Treatment effect (conditions of enzymatic hydrolysis) was analyzed using analysis of variance and Duncan multiple-range test determined the differences between treatment means of gastrodin concentration. Differences were considered to be significant at $P < 0.05$ throughout the current study.

3. Results and discussion

3.1. Quantitative analysis of GAS and HBA by RP-HPLC

Concentrated stock solutions of GAS and HBA were prepared separately at concentrations of 100.0 μg/ml and 20.0 mg/ml in methanol and was further diluted into 1.0–100.0 μg/ml and 0.5–20.0 mg/ml for the preparation of calibration standards, respectively. All solutions were stored at −20 °C in dark and sealed in glass. Pure standards prepared for the recovery calculations were diluted with methanol to appropriate concentrations. A 20 μl