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Short communication

Development of an enzymatic synthesis approach to produce phloridzin using *Malus x domestica* glycosyltransferase in engineered *Pichia pastoris* GS115

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ABSTRACT

Phloridzin has been an attractive target for the development of preparation methodologies due to its various applications as pharmaceutical ingredient and food additive. In this study, an enzymatic synthesis approach using engineered *P. pastoris* GS115 was developed to produce phloridzin. Four factors, identifying as key factors affecting the phloridzin yield, were optimized by response surface methodology as follows: induction time 121.8 h, pH 7.0, methanol 0.75%, and a rotation speed 194 rpm. The maximal yield of phloridzin reached 41.59 mg/L (95.31 μM) and the specific bioconversion rate of substrate was up to 98.50% with 1L working volume in a 5L bioreactor under the optimal conditions. Phloridzin with purity of 93.98% and recovery of 78.14% was achieved just using affinity chromatography and preparative high performance liquid chromatography (*Pre*-HPLC). These results confirm the enzymatic synthesis developed provides an efficient alternative to traditional approach for the preparation of phloridzin *in vitro*.

1. Introduction

Phloridzin, a phloretin glycoside, is receiving a tremendous interest from the scientific and industrial community due to its impressive bioactive properties or potential pharmaceutical applications, and therefore leading to increasing demands and becoming a highly attractive target for the large-scale preparation. To date, commercial available phloridzin are mainly extracted from the plant-materials using traditional solvent-based approaches [1–5], which causes difficulty, inefficiency and high-cost in the process of isolation and purification. Besides, the traditional method also leads to high pollution owing to the use of organic solvents in large quantities [1–5].

Recently, there have been some enzymatic biocatalysts for the production of phloridzin *in vitro*, but other phloretin glycosides such as phloretin-4-*O*-glycoside, phloretin-4′-*O*-glycoside, phloretin-4′, 4-*O*-diglycoside, phloretin-6′, 4-*O*-diglycoside, or phloretin-2′, 4′, 4-*O*-triglycoside were synthesized during the reaction along with phloridzin [6–9], also interfering its subsequent isolation and purification. Therefore, a novel enzymatic synthesis approach for the large-scale preparation of phloridzin *in vitro*, preferably that cannot produce by-products during reactions, has inevitably become the main direction of industrial development with the improvement of the consciousness on the eco-friendly, commercially efficient and green technology. Purified recombinant GTs ideally fulfils the requirements, but it is not scalable as the purified enzyme is expensive and availability is limited [10–12]. As an alternative, the functional expression of glycosyltransferase (GT) in *P. pastoris*, a methylotrophic yeast generally regarded as safe (GRAS) organism and the most widely used platform microorganisms for the production of natural products [13,14], for the microbial transformation of phloretin to phloridzin is of specific interest. In this study, we developed an enzymatic synthesis approach for the production of phloridzin using an engineered *P. pastoris* GS115 strain bearing *Md*P2′GT with high region specificity regarding sugar attachment site [11] by feeding with aglycone phloretin. To enhance the bioconversion rate of substrate and the production of phloridzin, plackett-burman design (PBD) and response surface methodology (RSM), which are relatively new tools used for screening key variables and further optimizing the fermentative conditions during complex processes [15–17], were employed. In addition, effective purification and subsequent identification of phloridzin from the broth in bioreactor were

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conducted as well. To the best of our knowledge, this is the first report of that final glycosylation step being performed with a phloretin glycosyltransferase from apple in *P. pastoris*. The yield and purity of phloridzin as well as the substrate conversion are the highest reported to date.

2. Materials and methods

2.1. Materials

Golden Delicious ripe fruits were collected from the Horticultural Institute of Northwest A & F University (Yangling, Shaanxi, China). *P. pastoris* GS115 (his$^-$ mut$^+$) and pPIC9 K were purchased from Invitrogen Co. Ltd. (California, USA). PCR reagents and restriction enzymes were obtained from Takara (Dalian, China). Geneticin (G418), phloretin (purity ≥ 99%), phloridzin (purity ≥ 98%), 4-(2-Aminoethyl) benzenesulfonyl fluoride (AEBSF), and uridine diphosphate glucose (UDPG) were purchased from Sigma-Aldrich (St. Louis, MO, USA). All other chemicals and reagents were of analytical grade.

2.2. Construction and screening of engineered P. pastoris harboring MdP2′GT gene

*Md*P2′GT gene was cloned and isolated from *M. domestica Golden Delicious* peels by reverse transcription-touchdown-polymerase chain reaction (RT-TD-PCR) using the specific primers F_1: 5′-TACGTA-ATGGGAGACGTCATTGTACTGT-3′ and R_1: 5′-GCGGCCGCTTAATG-ATGATGATGA TGATGTGTTATGCTATTAACAAAGTTG-3′ that incorporated *SnaB*I/*Not*I restriction sites according to the sequence reported in our previous study [11]. The reaction mixtures were consist of 13 μl premix *Ex*Taq™ version 2.0, 1.0 μl F_1 (10 μM), 1.5 μl R_1 (10 μM), 0.5 μl cDNA (100 ng/μl) and 9 μl ddH_2O and performed on a PTC-0200 DNA Engine® peltier thermal cycler (BIO-RAD, CA, USA) with conditions of 95 °C for 1 min, 20 cycles of 94 °C for 1 min followed by annealing for 1 min (initially at 55 °C and decreasing by 0.5 °C with each cycle), with an extension step at 72 °C for 2 min; followed by another 10 cycles at 94 °C for 1 min, 46 °C for 1 min, 72 °C for 2 min; with a final extension step at 72 °C for 5 min. Purified PCR product was digested with *SnaB*I/*Not*I and ligated into the *SnaB*I/*Not*I degested pPIC9 K generating pPIC9K-*Md*P2′GT which was then sequenced by Invitrogen (California, USA) to confirm the presence and correct orientation of *Md*P2′GT gene insertion. After linearization with *Bgl*II, the correct insertion was transformed into *P. pastoris* GS115 competent cells by electroporation following the manufacturer's instructions (Invitrogen) and the multiple inserts that resist G418 at the concentration of 4.0 mg/ml were screened [17].

Each multiple insert was inoculated into 100 ml buffered minimal sorbitol medium (BMSM, 5% sorbitol replacing the glycerol in BMGY) [17–19] and cultured at 28 °C for 24 h with vigorous shaking at 180 rpm. When the optical density (OD_{600}) reached 10.0, cultures were incubated at 25 °C and methanol with final concentration of 0.25% was added every 24 h [13,14,17]. Sterile sorbitol was supplemented very 24 h to control the growth rate and maintain high cell density [18,19]. Sterile ammonia was added to maintain pH at 7.0. After 72 h induction, phloretin and UDPG (dissolved in DMSO) along with AEBSF (a protease inhibitor) were added at a final concentration of 100 μM, 100 μM and 0.5 mM, respectively. After a second 48 h induction, culture supernatants were collected to determine phloretin residues according to the established calibration curves using HPLC described in our another previous study [20]. Engineered *P. pastoris* G115 with the highest bioconversion rate toward phloretin was screened as the seed strain for the fermentative production of phloridzin, and its retentate resulting from collected supernatant with ultrafiltration tube (MWCO 3, 000, Millipore, USA) was subjected to SDS-PAGE using 8% separation gel to confirm the expression and secretion of *Md*P2′GT [21]. The engineered *P. pastoris* GS115 integrating empty pPIC9 K vector was used as a negative control for background analysis. The bioconversion rate toward phloretin of each transformant was calculated using the following formula: Bioconversion rate toward phloretin (100%) = (1-phloretin residues/100) × 100%

2.3. Extraction, purification, identification, and qualification methods for phloridzin

Culture supernatants in 2.2 were collected, lyophilized and extracted with ethanol (w/v = 1:5) using ultrasonic-assisted extraction method [2]. The extracts were concentrated to 10 ml and loaded onto a normal atmosphere column (300 × 30 mm) pre-filled with 100 ml macro-porous adsorbent resin XAD-2 (Rohm and Haas, Philadelphia, PA, USA) [22]. The column was first washed extensively with 40% ethanol and then eluted with gradient from 50%-80% ethanol for the five bed volumes. Fractions containing putative phloridzin were pooled and further purified using a prep-high performance liquid chromatography (Pre-HPLC) system with an octadecyl silica gel column (2.5 × 25 cm, Shimadzu® LC-8A, Tokyo, Japan) and a UV detector operating at 280 nm [1,4]. Fractions rich in putative phloridzin were collected, concentrated and dissolved in chromatographic grade methanol. The purified putative phloridzin was initially detected by HPLC method [20]. The HPLC peak of putative phloridzin was then subjected to Liquid chromatography-tandem mass spectrometry (LC–MS/MS) and further analyzed in a nuclear magnetic resonance (NMR) assay [11]. The content of phloridzin formed in fermentation broth was quantified using HPLC method in our previous study [20].

2.4. Optimization of conditions for the production of phloridzin in shake flask

Eight factors, including temperature (A, °C), induction time (B, h), pH (C), methanol (D, %), liquid volume in flask (E, ml/250 ml), optical density (OD_{600}, F), rotation speed (G, rpm), and AEBSF (H, mM), have potential impacts on the production of phloridzin according to our preliminary research. Each factor was first investigated by the PBD at high (+1) and low (-1) levels to screen the key factors influencing the phloridzin yield. Factors above 95% of the confidence level ($P < 0.05$) from the regression analysis of the first-order model were identified as key points. The first-order model used to fit the results of the PBD was shown as follows:

$$\beta = \lambda 0 + \sum_{i=1}^{8} \lambda i x i$$

Where β is the predicted response, λ_0 is the intercept, λ_i is the linear coefficient, and x_i is the coded independent factor.

RSM with a 4-screened factors (X_1: second induction time, X_2: pH, X_3: methanol percentage, and X_4: rotate speed)-3-level (−1, 0, 1) BBD which produced 29 experimental runs was then carried out to further optimized the conditions for the biotransformation of phloretin using the phloridzin content as a response. In order to correlate the relationship between factors and the response, a second-order polynomial equation was developed and listed as follows:

$$\theta = \theta 0 + \sum_{i=1}^{4} \theta i x i + \sum_{i=1}^{4} \theta i i x_i^2 + \sum_{i=1}^{3} \sum_{j=2}^{4} \theta i j x i x j$$

Where θ is the predicted content of phloridzin, θ_0 is the intercept, θ_i is the linear coefficient, θ_{ii} is the squared coefficient, θ_{ij} is the interaction coefficient, x_i and x_j represent independent factors in the form of coded values.

2.5. Fermentative production and purification of phloridzin

Fermentative production of phloridzin under the conditions optimized by RSM was conducted in a 5 l bioreactor (Applikon® Biotechnology, Foster City, USA) pre-equipped with a dissolved oxygen