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A novel one-step microbial transformation of betulin to betulinic acid catalysed by *Cunninghamella blakesleeana*

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ABSTRACT

Betulinic acid and its derivatives are potential bioactive compounds present in nature. This study investigated the biotransformation of betulin to betulinic acid by *Cunninghamella blakesleeana* cells. LC–MS analysis demonstrated that betulin could be transformed into at least five products from cultured *C. blakesleeana* cells, among which betulinic acid was the most important. The presented method provides an attractive alternative approach to chemical synthesis, because is less time-consuming and more environmentally friendly. *C. blakesleeana* can transform betulin into potent derivatives with high pharmacological activities.

1. Introduction

Betulinic acid, (3β)-3-hydroxy-lup-20(29)-en-28-oic acid, a triterpenoid present in many plant species, has attracted more and more attention due to its important physiological and pharmacological activities. Betulinic acid has been found in low amounts in the bark of white birch; it has been found to show remarkable effects in suppressing tumorigenesis as well as inhibiting tumours (Mullauer, Kessler, & Medema, 2010; Salvador, 2010). Betulinic acid has also been reported to have anti-HIV, antiviral, anti-leukaemia, anti-inflammatory, antimicrobial, antihelmintic and anti-feedant activities (Bastos, Pimentel, De Jesus, & De Oliveira, 2007; Mukherjee, Kumar, Srivastava, Agarwal, & Burman, 2006; Yogeeswari & Sriram, 2005). Betulinic acid shows anticancer activities mainly by means of induction of apoptosis through the activation of caspases independent of the p53 gene status and CD95 activation (Kommera, KaluperoviĆ, Kalbitz, & Paschke, 2010). Most interestingly, recent studies revealed that betulinic acid has anti-obesity potential through modulation of fat and carbohydrate metabolism, and may be a lead compound in the treatment of obesity and diabetes (Choi et al., 2009; De Melo et al., 2009). Therefore, betulinic acid is considered as a promising chemotherapeutic agent against HIV infection and other cancers cells (Cichewicz & Kouzi, 2004).

Though betulinic acid can be prepared by a chemical procedure, a green synthetic procedure would be desirable. In previous investigations, we conducted the biosynthesis of betulinic acid from betulin through cultured *Armillaria luteo-virens* Sacc cells under a normal transformation system and in an ionic liquid-containing system (Chen, Liu, Zhang, He, & Fu, 2009; Fu et al., 2011; Liu, Fu, & Chen, 2010). However, a suitable and efficient biocatalyst should be adopted to prepare betulinic acid as a therapeutic agent.

Biotransformation is defined as an enzymatic reaction catalysed by microbial cells. Biotransformation can be a viable alternative to chemical synthesis for preparation of drug metabolites (Azerad, 1999). Some reactions, which cannot be fulfilled by chemical approaches, are facile by microbial transformation (Stewart, 2000; Zhang et al., 2008). *Cunninghamella* sp. can metabolise a wide variety of xenobiotics in regio- and stereo-selective manner, which are similar to those in mammalian enzyme systems (Stewart, 2000). Nowadays, *Cunninghamella blakesleeana* is used extensively as a microbial model for biotransformation (Asha & Vidyavathi, 2009). The biotransformation of glycyrrhetinic acid was fulfilled by *C. blakesleeana* (Qin et al., 2010). A novel one-step biotransformation of cortexolone-21-acetate to hydrocortisone acetate was successfully performed using *C. blakesleeana* ATCC 8688a (Manosroi, Saowakhon, & Manosroi, 2007). Zhang, Qiu, Yao, and Qu (2007) reported that the microbial transformation of curcumol could be efficiently fulfilled by *C. blakesleeana*. The trasformation metabolism of methoxychlor was carried out with *Cunninghamella elegans,* a well-known fungal species (Keum, Lee, & Kim, 2009). *C. blakesleeana* AS 3.153 was used as a microbial model of mammalian metabolism to convert verapamil (Sun, Huang, Liu, & Zhong, 2004).

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In order to identify highly active anti-cancer substances, the microbial transformation of betulin by *C. blakesleeana* cells was undertaken in this work. The main objective of this work was to optimise the biotransformation conditions and carry out semi-preparative chromatography to separate the transformed products.

2. Materials and methods

2.1. Chemicals and apparatus

Standard betulin (99%) was obtained from Skyherb Technologies Co. Ltd., (Hangzhou, China). Betulinic acid (99%) was purchased from Tokyo Kasei Kogyo Co. Ltd., (Japan). Methanol, acetonitrile and dimethyl sulfoxide (DMSO) were HPLC grade. Other chemicals in this work were of analytical grade.

The RP-HPLC system used in this work consists of two Waters 510 pumps (Waters, Milford, MA), a sample injector (Rheodyne, Cotati, CA) with a 20 μL loop, and a Waters 996 photodiode array detector. Rotary evaporator was provided by Shanghai Yuhua Instrument Co. Ltd., (Shanghai, China). Low-speed large-capacity centrifuge was provided by Hunan XiangYi Co. Ltd., (Hunan, China). Pure water was provided by Baoer Water Treatment Co. Ltd., (Shanghai, China). The 5 L bioreactor system consists of 5 L pH-stat fermenter (B. Braun Biotech. International GmbH, Melsungen, Germany), containing 4 L fermentation medium. The identification of betulinic acid in the sample was performed using a TSQ Quantum LC/MS system (Thermo Fisher Scientific, Waltham, MA), which was equipped with an electrospray ionisation (ESI) source.

2.2. Microorganism and experimental media

C. blakesleana AS 3.910 was obtained from China General Microbiological Culture Collection centre. This fungus was maintained on potato dextrose agar (PDA) slants at 4 °C for further use. The compositions of resting-cell transformation medium consisted of 2% glucose, pH 6.0 phosphate buffer. The compositions of growing-cell transformation medium consisted of 2% glucose, 0.5% yeast extract, 0.5% peptone and 0.5% K_2HPO_4, pH 6.5. All media were sterilised at 120 °C for 15 min before use.

2.3. Biotransformation procedures of betulin

The biotransformation experiments were carried out with a shaking speed of 180 rpm at 28 °C. In all experiments, substrate controls were composed of sterile medium with betulin and incubated without fungus. Culture controls consisted of the identical medium in which fungus was grown, but without the substrate. The resting-cell transformation and growing-cell biotransformation procedures were as in our previous study (Chen et al., 2009) with minor modifications.

2.3.1. The resting-cell transformation procedure of betulin

The freshly inoculated fungus was incubated with PDA at 28 °C for 6 days, and then inoculated into the growing liquid medium (30 mL in 250 mL flask). The flasks were cultivated for 2 days at 28 °C with a shaking speed of 180 rpm, and then the culture broth was combined. The mycelia in the flasks were collected by centrifugation at 12,000*g*, and then washed three times using sterile water. The collected wet mycelia (5 g) were added into 30 mL resting-cell transformation medium in the culture flasks; 0.2 mL betulin at 7.5 mg/mL concentration (dissolved in DMSO) were then added to the culture flasks. The flasks were incubated for 3 days at 28 °C, shaken at 180 rpm.

2.3.2. The growing-cell biotransformation of betulin

The inoculated fungus was incubated on PDA at 28 °C for 6 days, and then inoculated to 30 mL growing-cell transformation medium in a 250 mL flask with 1.5×10^8 spores/mL. The flasks were cultured for 2 days at 28 °C with a shaking speed of 180 rpm. Then the pre-cultures were aseptically supplemented with 0.2 mL prepared substrate (99% betulin dissolved in DMSO, 7.5 mg/mL) and transformed for another 3 days on a rotary shaker under identical conditions. As the optimisation procedure was performed, glucose, Tween 80, betulin concentration, pH and the culture age of inoculation mycelia were varied according to the designed conditions.

2.4. Preparation and isolation of the transformed metabolites

At the end of the biotransformation procedure, the culture broth was centrifuged at 12,000*g* for 30 min, then the cultured mycelia were washed with sterilised water three times and used to measure the relevant enzyme activity. The collected supernatant, after adjusting the acidity between pH 3 and 4, was extracted three times with an equivalent volume of ethyl acetate, and all the organic layers were combined. The extracted solutions were then concentrated under vacuum at 30 °C. Finally, the collected residues were dissolved in methanol and analysed by RP–HPLC or fractionated (Chen et al., 2009; Fu et al., 2011).

2.5. Purification of transformation products by silica gel column chromatography and semi-preparative HPLC

The transformed products were extracted with ethyl acetate and the solvent was evaporated off. The residues dissolved in methanol were purified with 2× volumes of silica gel (200–300 mesh). The silica gel column chromatography was undertaken using a Biotage Isolera System. The gradient elution was detected at 210 nm, and then the effluent solution was collected and fractions containing product peaks were combined. The combined fractions were concentrated and purified through semi-preparative HPLC. HPLC chromatography conditions were as follows: mobile phase was acetonitrile–water in the ratio 91:9 (v/v) at a flow rate of 1.0 mL/min at 25 °C. The detection wavelength was 210.1 nm. ^1H and ^{13}C NMR spectra were recorded at 300 MHz on a DRX 300 Bruker instrument in deuteriochloroform ($CDCl_3$) with tetramethylsilane (TMS) as an internal standard. The physical and spectral data of transformation metabolites were consistent with reported data (Carpenter, Sotheeswaran, Sultanbawa, & Ternai, 1980).

2.6. Simultaneous determination of betulin and betulinic acid by reversed-phase HPLC (RP–HPLC)

Stock solutions of betulin and betulinic acid at 120 μg/mL and 80 μg/mL, respectively, were prepared in methanol. Standard solutions for RP–HPLC determination were prepared by a series of dilutions according to the required concentrations. All the solutions were aspirated with nitrogen and kept at −20 °C in darkness. Standard curves of betulin and betulinic acid were constructed at five different levels. The sample solution was filtered through a 0.22 μm membrane filter, and then an aliquot (10 μL) of solution was injected into the RP–HPLC system for analysis. The productivity of betulinic acid after biotransformation was calculated as the percentage of the amount of betulinic acid formed against the total amount of betulin added to the culture broth.

The RP–HPLC system used in this work consists of two Waters 510 pumps (Waters, Milford, MA), a sample injector (Rheodyne, Cotati, CA) with a 20 μL loop, and a Waters 996 photodiode array detector. The column used is a reversed-phase Symmetry C18 (250 × 4.6 mm i.d., 4 μm, Waters). Mobile phase is composed of acetonitrile–water in the ratio of 91:9 (v/v). The mobile phase is fil-