Article

JOURNAL OF THE CHINESE CHEMICAL SOCIETY

Biocatalytic Synthesis of the Anti-diabetes Agent-corosolic Acid by Whole Cells of Microorganisms

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(Received: Jan. 28, 2015; Accepted: Aug. 11, 2015; Published Online: Sept. 7, 2015; DOI: 10.1002/jccs.201500035)

Diabetes is one of the most prevalent and costly global diseases. For diabetes, frequent insulin treatment and synthetic drugs are very expensive and may cause unwanted side effects. Corosolic acid (CA), a natural product, was reported to be efficient in the treatment of diabetes, meanwhile without induction of anti-insulin antibodies and obesity. The preparation of CA attracted many researchers in the world. This study investigated the biocatalytic synthesis method of CA from ursolic acid by *Streptomyces griseus* subsp. *griseus* 4.18. LC–MS analysis demonstrated that 5 day, 125 μg/mL substrate, pH = 9 and 10% strain concentration were the appropriate conditions. It is estimated that biocatalysis will contribute to the development of green and sustainable synthetic processes with less time-consuming and more environmentally friendly.

Keywords: Biocatalysis; Synthesis; Anti-diabetes; Corosolic acid; Microbes.

INTRODUCTION

Pentacyclic triterpene acids are one group of promising natural product which distributed in many species of plants. Corosolic acid (CA), a pentacyclic triterpenoid acid, also named 2α-hydroxyursolic acid, is found in a variety of plant species. Extensive research has revealed several important pharmacological activities of CA, such as anti-cancer,[1,2] anti-inflammatory activities,[3,4] cardioprotective effect.[5] This compound, known as 'plant insulin', has attracted interests worldwide owing to its remarkable ability of anti-diabetes for application of weight-loss management and blood sugar balance on animal experiments and clinical trials.[6-8] Both the pure compound corosolic acid and the herbal product are considered as one of the good sources for a new drug or a lead to make a new drug to treat diabetes. KFDA (Korean FDA) has approved blood glucose lowering effects of banaba alcohol extract as function II grade with a restricted dose of 50-100 mg/day corosolic acid.[9] Extraction from medicinal plants is the main preparation method to obtain CA. However, CA was low in plant- based resource in nature.

Additionallly, CA and its isomers usually coexist in the same plant which also brings difficulty in separation. Besides, most of plant-originated useful materials have complicated structures having been synthesized through various stages of biosynthesis pathways. Though CA can be prepared by a chemical procedure, a green synthetic method would be desirable. The method of preparation of CA through plant cell culture techniques was developed with the low productivity and instability which prevented the method from being applied to relevant industry.[10] It is necessary to look for a new method for the preparation of CA. As more and more attentions were paid to health and environmental protection, the sustainable and green chemical synthesis is much more popular.[11,12] Biocatalysis is considered more efficient and less side-reactions. The essence of biocatalysis is the reaction of enzymes. Due to their specific three-dimensional structure, enzymes may distinguish between functional groups and other groups in different regions of the substrate molecule.[13] As a consequence, biocatalysis is of chemoselectivity, regioselectivity, enantioselectivity.

Herein, dozens of strains were screened to synthesis of CA by biotransformation and *S. griseus subsp. griseus* CGMCC 4.18 can catalyze the natural product with similar structure and low price-ursolic acid (UA) into CA. Optimization experiments were also performed.

RESULTS AND DISCUSSION

Optimization of reaction conditions

Effect of media on transformation

In two media, the PDA medium was fond to con-

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tribute to the growth of mycelia, but with no transformation product CA. Therefore, The rest experiments were performed on GYM media.

Effect of substrate concentration on transformation

Substrates (S1 to S5) of 31.25 μg/mL, 62.5 μg/mL, 125 μg/mL, 250 μg/mL, 500 μg/mL were investigated in the experiment. All the five concentrations were harvested on the 5 day. Apparently, 125 μg/mL UA was optimum for CA production; above this concentration, production of UA decreased (Fig. 1).

Time-course experiment

Every flask was harvested ever day from the second day to the eighth day with the same substrate amount. The results demonstrated that the obtained CA reached a maximum at 5 days culture time (Fig. 2). The proruction of CA reached the most yield 959.4 μg/mL.

Effect of pH on transformation

Different pH values from 6 to 9 were investigated. All the flasks were harvested on the same time. In commen, most bacteria and actinomycetes are suitable for the growth of pH in the neutral and alkaline environment. In this study, strain *S. griseus subsp. griseus* CGMCC 4.18 grew slowly and CA was difficult to be detected at pH 6. UA was converted into CA with a higher conversion rate in pH 7 and 9, while strain in the pH 8 media was polluted which was dealt with no statistically significance (Fig. 3).

Effect of strain concentration on transformation

The strain concentration also affected the catalytic activity. According to Fig. 4, when the initial concentration of was 10%, the transformation rate reached the peak. The conversion rate decreases above or below this concentration.

Separation and identification of transformation product CA

The crude extract 830 mg was subjected to column chromatography on silica gel (300–400 mesh, 20 g) with a stepwise elution with petroleum ether/acetone/acetic acid from 100:2:0.1 to acetone. The fractions were purified by

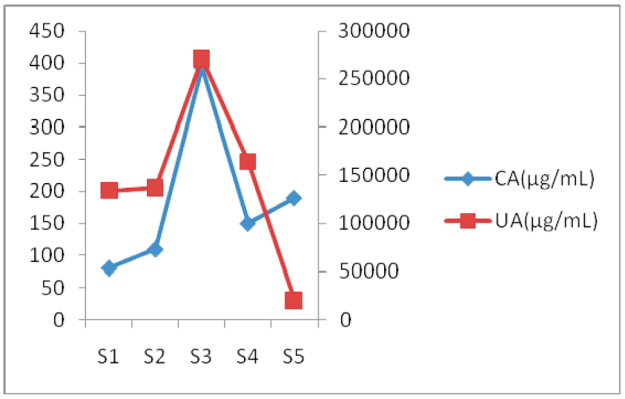

Fig. 1. Effect of substrate concentration on transformation.

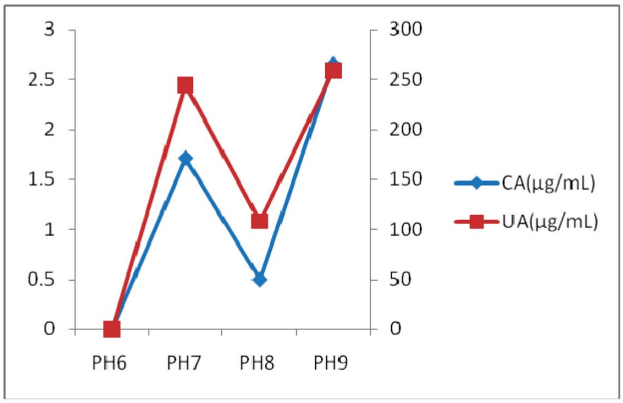

Fig. 3. Effect of pH on transformation.

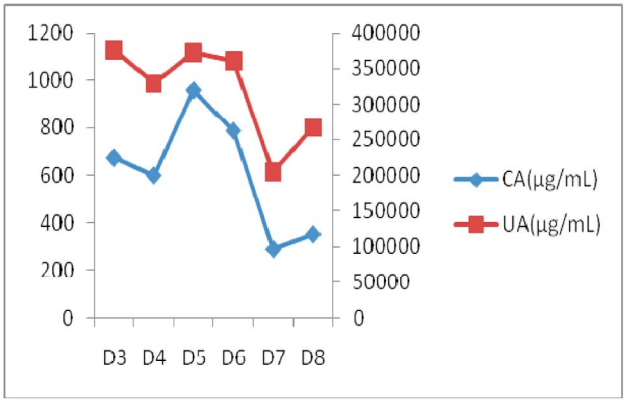

Fig. 2. Time-course experiment.

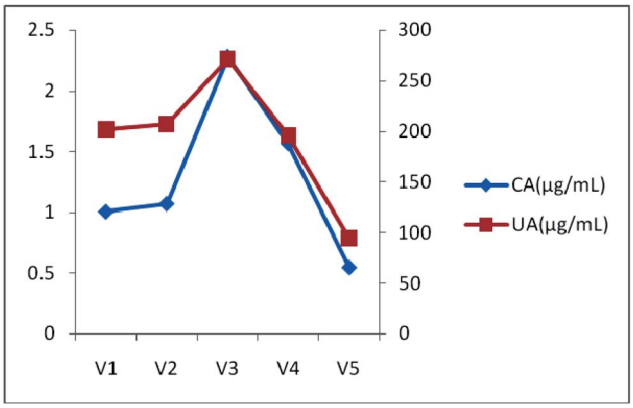

Fig. 4. Effect of strain concentration on transformation.

column chromatography on silica gel eluted with trichloromethane/THF (9:1 to 1:1) and sephadex LH-20 trichloromethane/methanol (1:1). CA (4 mg) were obtained after recrystallization. The structure of CA was confirmed by NMR (Table 1), MS (ESI-MS: *m/z*: [M-H]-: 471,) and reference.[14]

Table 1. ^{13}C NMR data and characteristic ^{1}H-NMR of CA

No	^{13}C-NMR	^{1}H-NMR
1	48.4	
2	69.0	4.11 (1H, td. *J* = 4.2, 10.2 Hz)
3	84.2	3.42 (1H, d, *J* = 9.6 Hz)
4	40.2	
5	56.3	
6	19.2	
7	33.9	
8	40.4	
9	48.4	
10	38.8	
11	24.1	
12	126.0	5.49 (t-like, 12-H)
13	139.7	
14	42.9	
15	29.0	
16	25.3	
17	48.5	
18	53.9	2.65 (1H, d, *J* = 11.4 Hz)
19	39.9	
20	39.8	
21	31.5	
22	37.8	
23	29.7	1.33 (3H, s)
24	18.1	1.23 (3H, s)
25	17.4	1.07 (3H, s)
26	17.9	1.09 (3H, s)
27	24.3	1.29 (3H, s)
28	180.2	
29	17.9	1.01 (3H, d, *J* = 6.6 Hz)
30	21.8	0.97 (3H, d, *J* = 6.6 Hz)

^{1}H-NMR (600 MHz) and ^{13}C-NMR (150 MHz) Data of CA (Pyridine-*d*5).

EXPERIMENTAL

General experimental procedure: NMR spectra were recorded on a Bruker DRX-600 spectrometer operating at 600 MHz (for $_{1}$H) and 150 MHz (for $_{13}$C) in pyridine-d5. LC-MS (AB SCIEX) was carried out on C-18 column with methanol - 0.2% FA water = 95:5 and with the condition of CUR: 40.00, CAD; Medium; IS: -4500.00; TEM: 550.00; GS1: 55.00; GS2: 55.00; DP: -140.00; EP: -10.00; CE: -40.00; CXP: -15.00. TLC experiments were performed on GF254 plates (Qingdao Oceanic Chemicals, China). Column chromatography was carried out on silica gel (300-400 mesh, Qingdao Oceanic Chemicals, China) and Sephadex LH-20. 10% H_2SO_4 in 95% ethanol spray reagent was used for TLC plated followed by heating. General solvents and reagents were purchased from Beijing Chemical Industry Company, Beijing, China. The microbe was purchased from China General Microbiological Culture Collection center (CGMCC).

Substrate and Microorganisms

The substrate ursolic acid (with purity > 98%) was purchased from Changsha Staherb Natural Ingredients Co., Ltd in China and strain *S. griseus subsp. griseus* CGMCC 4.18 was purchased from the Institute of Microbiology, Chinese Academy of Sciences (AS), Beijing, China.

Biocatalytic synthesis of CA from UA

Effect of media on transformation: The preliminary screening experiment was carried out in two media. One is PDA medium consisting of 200 g potatoes (boiling 20 min; dextrose, 20 g; peptone, 10 g; distilled H_2O, 1000 mL). The other medium is GYM medium consisting of yeast extract 4.0 g, malt extract 10.0 g, glucose 4.0 g, pH 7.3. The preliminary screening experiment of *S. griseus subsp. griseus* CGMCC 4.18 was carried out in PDA medium and GYM medium. Fermentations were carried out according to standard two-stage fermentation.[15]

Effect of substrate concentration on transformation: As previously report, the concentration of substrate played an important role in biotransformation.[16] Different concentrations of UA (from 1.25 mg to 20 mg per flask) were added in the transformation broth and the yield of CA was evaluated.

Time-course experiment: The biotransformation process was performed by a procedure similar to normal transformation examinations in 7 flasks. Reaction was terminated in only one flask every day.

Effect of pH on transformation: Different pH values from 6 to 9 were investigated. All the flasks were harvested on the same time.

Effect of strain concentration on transformation: Five strain concentrations 2.5%, 5%, 10%, 17.5%, 27.5% (1 mL, 2 mL, 4 mL, 7 mL seed broth in 40 mL media) were tested on the transformation. The incubation and other conditions were same as the pH.

Separation and identification of transformation product CA: The preparative scale biotransformation from UA to CA by *S. griseus subsp. griseus* CGMCC 4.18 was similar to the preliminary screening experiment. 600 mg substrate was added in total. The target compound was obtained after column chromatography on silica gel, sephadex LH-20, recrystallization from 830 mg crude extract. The product was separated by column chromatography and sephadex LH-20. The sructure was identified by

NMR, MS and references.

ACKNOWLEDGEMENTS

This research was financially supported by The National Natural Sciences Foundation of China (No. 21462057), Program of Ministry of Education "Chunhui Plan" (No. Z2014091), Guizhou Science and Technology Department (QKHLH-2014-7555) and Program for Guizhou Key Laboratory of Microbial Resources & Drug Development (No. GZMRD-2014-003).

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