Microbial synthesis of anti-tumor agent oxysophoridine through one step by filamentous fungus

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Abstract: Sophoridine and oxysophoridine are alkaloids extracted from Chinese traditional medicine with significant antitumor activities. Computer-aided design and preliminary structure-activity relationship studies showed that the biological activity of sophoridine can be improved after oxidation (oxysophoridine) while its toxicity significantly decreased. A new method to prepare oxysophoridine from natural product Sophoridine by biotransformation was established for the first time except for traditional extraction from plants and chemical synthesis. Positive biocatalytic reaction was detected by TLC. Structure of conversion product was elucidated based on NMR spectroscopy. Optimizations of reactions including substrate concentration, pH, conversion time and inoculation quantity were complemented which analyzed by LC-MS. Results showed that filamentous fungus Xylariales sp F005 (CCTCC M2014660) has the capacity of synthesizing oxysophoridine from sophoridine in the selected 37 strains. The yield of oxysophoridine reached highest at conditions pH 6.0, the third day of conversion time, 5.0 mg substrate and 5% inoculums respectively. The method for preparing oxysophoridine catalyzed by enzymes with green and sustainable synthetic processes was developed.

Keywords: biotransformation, alkaloid, fungus, natural product, condition optimization

1 Introduction

Oxysophoridine (SR), an alkaloid extracted from many traditional Chinese medicine including Sophora alopecuroides L., with high content was reported to be a variety of pharmacological activities, such as anti-tumor, anti arrhythmia,[1–4] Selectively induce apoptotic cell death in a range of human cancer cells in vitro and in vivo was also proved for SR. Results showed that SR exhibited anti-cancer activity on glioma cells by inducing cell apoptosis, inducing ROS accumulation, and activating mitochondrial signal pathways.[5] Besides, SR could down-regulate the secretion of TNF-α and IL-1β in LPS-induced RAW264.7 cells.[6] Computer-aided design and preliminary structure-activity relationship studies indicated that the biological activity of SR could be improved after oxidation (oxysophoridine, OSR) while its toxicity significantly decreased.[7] Moreover, the effect of OSR on lung cancer and intestinal cancer was found to be stronger anticancer activity than SR, while the less side effects than SR with formulations of freeze-dried powder injection, soft pill, pills and film coated controlled-release capsules and others.[8] The anti-inflammation effects on cerebral ischemia–reperfusion injury of OSR in mice revealed that OSR protected neurons from ischemia-induced injury by downregulating the proinflammatory cytokines and blocking the NF-κB pathway.[9,10] OSR could protect against focal cerebral ischemic injury by inhibiting oxidative stress and apoptosis in mice.[11] In vivo and in vitro tests improved that OSR induced the apoptotic effects on colorectal cancer cells via the Bcl2/Bax/caspase-3 signaling pathway.[12]

The traditional preparation method of OSR was extracted from plant resource in low yield. Besides, excessive excavation may lead to the decreasing of species and biodiversity, which will affect the vegetation and the environment for human survival.[13] In addition, the extraction method was difficult to achieve large-scale industrial production. While for the chemical synthesis method, m-chloroperoxybenzoic acid and dichloromethane belongs to the class limited solvent. Therefore, a new preparation method for OSR with efficient, economical and environment-friendly was desired.
Microbial transformation regarded as green, environment-friendly and sustainable has been increasing significantly in many fields. There are many advantages on biotransformation, including mild conditions, less by-products and less contamination. The biotransformation reaction was usually operated at near neutral pH, ambient temperatures and atmospheric pressures. More importantly, microbial transformation was regarded as reaction specific, enantiomer-specific and regio-specific. Therefore, biotransformation attracted more and more attentions in recent years.

Herein, dozens of strains were screened to synthesis of OSR by microorganisms and the endophytic fungus identified Xylariales sp F005 (CCTCC M2014660) can catalyze the natural product SR into OSR the first time. Optimization experiments were also investigated.

2 Materials and methods

2.1 General experimental procedure

NMR spectra were recorded on an Agilent NMR spectrometer (DDR 2400, China) in CDCl$_3$. LC-MS (AB SCIEX, USA) was carried out on C-18 column with methanol-water=85:15 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 detections were performed on silica gel GF$_2$54 plates (Qingdao Oceanic Chemicals, China). Visualization of the TLC plates was performed by Bismuth potassium iodide spray reagent. Culture with microorganisms but without substrate and culture without microorganism but with substrate were both designated as the controls and incubated in the same conditions as described above.

2.2 Substrate and Microorganisms

Substrate SR and control product OSR (with purity >98%) was purchased from Beijing Gold- moutain Chromatography Science and Technology Co. Ltd.China. Microbial strains consist of bacteria, actinomycetes and fungi. Twenty-five strains including bacteria and actinomycetes were isolated from soil in the campus of Zunyi Medical University according to dilution method and twelve fungi were endophytes isolated from plant Huperzia serrata in Guizhou province.

2.3 Screening procedures

The preliminary screening experiment by soil microbes (bacteria and actinomycetes) was carried out in LB media and screened by endophytic fungi in PDA liquid media. Fermentations were carried out according to a standard two stage protocol. The microorganisms were cultured in 100 mL Erlenmeyer flasks containing 40 mL medium that incubated at 37°C for soil microbes and 28°C for endophytes and 140 rpm on a shaker. And then 5 mg substrate dissolved in ethanol was added after 12 hours for soil microbes and the co-cultures were incubated for another 3 days. Adding 5 mg substrate in ethanol into flasks after the fungi cultured 2-3 days, and then fungi and substrate were co-cultured for another 5-7 days. Then the cultures were extracted twice with n-butyl alcohol. The extractions were evaporated under reduced pressure and were analyzed by TLC (chloroform: methanol=5:1) with improved Bismuth potassium iodide spray reagent. Culture with microorganisms but without substrate and culture without microorganism but with substrate were both designated as the controls and incubated in the same conditions as described above.

2.4 Biocatalytic synthesis of OSR from SR by strain Xylariales sp

The preparative scale biocatalysis of SR by soil microbes was carried out in 11 L medium and 1.04 g substrate in total dissolved 2.5 mL ethanol was added. After two stage fermentation, the co-cultures were extracted twice with n-butyl alcohol and were evaporated under reduced pressure at 55°C. About 9.10 g crude extracts were obtained. The crude extracts were subjected to silica gel column chromatography with pH>7 that adjusted by diethylamine and eluted with mixtures of chloroform/methanol from 40:1, 35:1, 30:1 to methanol. Fraction 1a, 1b, 1c, 1d and 1e were obtained. Fraction 1c was further purified by Sephadex LH-20 with chloroform/methanol=1:1, repeated gel column chromatography with chloroform/methanol several times and recrystallization, then the transformation product (15.8 mg) were obtained.

2.5 Optimization of conversion conditions

Effect on transformation of substrate concentration (0.3125 mg, 0.625 mg, 1.25 mg, 2.5 mg, 5.0 mg in 40 mL PDA liquid media), pH (from 6.0 to 9.0), conversion time (from the second day to eight day) and inoculums (2 mL, 4 mL, 6 mL, 8 mL, 10 mL in 40mL PDA liquid media) were investigated in our experiment. The yield of OSR was detected by LC-MS.
3 Results and discussion

3.1 Strain screening

37 Strains including bacteria and fungi were screened to synthesize OSR. Based on TLC (chloroform: methanol=5:1, colored by Dragendorff reagent), soil bacteria T009 and fungus F005 were able to transform SR into OSR (Figure 1). From Figure 1, fungus F005 showed better catalytic activity than bacteria T009. SR was almost transformed to OSR by F005 while there were surplus SR that can’t be catalyzed by T009. Thus, strain F005 was selected in the further experiment.

3.2 Preparation and isolation of conversion product OSR

Endophytic fungus F005 was applied to preparation of OSR in 11 L PDA liquid media. The crude extracts were subjected to repeated silica gel column chromatography and Sephadex LH-20. The structure of product OSR was mainly confirmed by $^{13}$C-NMR (Table 1). The chemical shift of SR and OSR was compared here (Figure 2). $^{13}$C-NMR(400 MHzCDCl$_3$) δ: 169.9 (C-15), 72.4(C-6), 70.9(C-2), 59.3(C-10), 58.1(C-11), 46.6(C-7), 34.9(C-17), 32.2(C-5), 29.9(C-14), 28.5(C-12), 27.5(C-4), 24.1(C-3), 22.1(C-8), 18.8(C-13), 18.0(C-9).

3.3 Optimization of reaction conditions

It could be found that the highest OSR production occurred when pH value was at 6 (Figure 3). Maybe it was related to the acidic environment which was suitable for fungi. However, Bacteria prefer alkaline environment and the conversion ratio was higher [16]. From Figure 4, with the increase of substrate concentration, the yield of the product OSR increased and the max substrate concentration was 5.0 mg in 40 mL media; in the time-coursing test, the product OSR was detected from the second day to eight day after co-cultured at the same time of every day. The results demonstrated that OSR reached to maximum at the third day (Figure 5). Nutrition and enzyme activity maybe was the most highest at the third day. Enzyme activity probably decreased with the reduction of nutrition. The OSR production was also influenced by strain concentration. According to Figure 6, 5% inoculums (2 mL seed solution in 40 mL media) afforded the maximum production of OSR.

Figure 2. $^{13}$C-NMR of OSR

Figure 3. Effect of pH on transformation

In the present study, we investigated the possibility of preperation for OSR except for chemical synthesis and extration from the plants. Our results demonstrated that there were enzymes that can catalyze the precursor into OSR in microorganisms. It was the first report on this method. Although the field of OSR was improved, the
Table 1. Comparison of $^{13}$C-NMR of SR and OSR

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Figure 4. Effect of substrate concentration on transformation

Figure 5. Effect of conversion time on transformation

Figure 6. Effect of inoculums on transformation

results was not satisfied. In further research, the gene-engineering technology maybe considered to increase the production by biotransformation. The similar situation was observed in our previously study. SR and OSR were both important compounds. We demonstrated the two compounds can be converted to each other by microbes.

4 Conclusions

A new method for preparing OSR from natural product by enzyme produced by fungus was developed. The precursor SR could be transformed into OSR by one-step oxidation of SR. Substrate concentration, inoculums, pH and conversion time could affect the transformation from SR to OSR. Microbial conversion can be a viable alternative to chemical synthesis for preparation of popular natural product.

5 Conflicts of interest

The authors declare there was no conflict of interest.

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