Fusaruside Biosynthesis Using Recombinant Pichia pastoris (Postprint)

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Date: 2019-03-05T00:00:00+00:00

Abstract

Objective: To engineer a fusaruside-producing *Pichia pastoris* strain and address the supply issue of the natural small-molecule immunosuppressant fusaruside. Methods: The genes involved in fusaruside biosynthesis—Δ3(E)-SD (3-position desaturase) and Δ10(E)-SD (10-position desaturase)—were amplified from *Fusarium graminearum* PH-1; a bicistronic expression vector was constructed using the 2A peptide strategy and transformed into *Pichia pastoris* GS115 for induced co-expression of both enzymes; following methanol/dichloromethane extraction of the induced *P. pastoris* biomass, product formation was analyzed by high-performance liquid chromatography-mass spectrometry (HPLC-MS). Results: The 3-position desaturase and 10-position desaturase were successfully co-expressed in *P. pastoris*, with SDS-PAGE analysis revealing a molecular weight of approximately 48 kDa for the 3-position desaturase and approximately 65 kDa for the 10-position desaturase; HPLC-MS demonstrated that the recombinant yeast could produce fusaruside. Conclusion: Compared with the native fusaruside-producing *Fusarium* strain, this engineered yeast strain exhibited a shorter fermentation time and higher yield, thereby laying the foundation for further development and application of fusaruside.

Full Text

Preamble

China Biotechnology Journal, 2019 ChinaXiv Partner Journal
Biosynthesis of fusaruside based on recombinant *Pichia pastoris*
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Funding: This work was supported by the National Natural Science Foundation of China (21602152), Shandong Provincial Natural Science Foundation (ZR2016BB01), and the Open Project Program of Shandong Provincial Key Laboratory of Agricultural Microbiology (SDKL2017015).

Abstract

Objective: To construct a *Pichia pastoris* strain producing fusaruside and solve the source problem of this natural small-molecule immunosuppressant. **Methods:** The biosynthetic genes for fusaruside—3-position desaturase (Δ3(E)-SD) and 10-position desaturase (Δ10(E)-SD)—were amplified from *Fusarium graminearum* PH-1. A co-expression vector for both genes was constructed using a 2A peptide strategy and transformed into *P. pastoris* GS115 for dual enzyme induction expression. The induced yeast cells were treated with methanol and dichloromethane, and the products were analyzed by High Performance Liquid Chromatography-Mass Spectrometry (HPLC-MS). **Results:** The two desaturases were successfully co-expressed in *P. pastoris.* SDS-PAGE showed molecular weights of approximately 48 kDa for Δ3(E)-SD and 65 kDa for Δ10(E)-SD. HPLC-MS analysis demonstrated that the recombinant yeast could produce fusaruside. **Conclusion:** Compared with the native fusaruside-producing *Fusarium* strain, this engineered yeast has shorter fermentation time and higher yield, laying a foundation for further development and application of fusaruside.

Keywords

Pichia pastoris, desaturase, 2A peptide, co-expression, fusaruside

Introduction

Immunosuppressive agents are the primary drugs for treating transplant rejection and autoimmune diseases. However, currently used clinical immunosuppressants have various adverse effects, and long-term administration leads to severe side effects, largely due to their low selectivity for targets. Therefore, identifying new therapeutic targets for immune system diseases and developing efficient, low-toxicity, highly selective novel small-molecule immunosuppressants has become urgent [1]. Recent studies have discovered that the natural small-molecule compound fusaruside exhibits excellent selective immunosuppressive activity. It promotes phosphorylation of phosphatase SHP-2, which selectively binds to non-phosphorylated STAT1 in the cytoplasm, thereby selectively blocking the IFN- /STAT1/T-bet signaling pathway with good targeting effects [2-6], making it a highly promising clinical immunosuppressant candidate.

However, fusaruside is a cellular component of filamentous fungi *Fusarium* sp. and exists in minute quantities in microorganisms, with only 24 mg isolated from 200 g of crude extract [7]. Additionally, these compounds have complex structures and cumbersome chemical synthesis steps, and the use of toxic reagents in the synthesis process causes environmental pollution [8]. These factors severely restrict in-depth pharmacological and physiological activity studies.

Elucidating the biosynthetic pathway of natural products and, based on this, using synthetic biology techniques to reconstruct relevant biosynthetic enzymes in engineered cells enables scalable and programmable preparation of natural products [9,10]. Compared with chemical synthesis, target metabolites can be produced in large quantities through fermentation of recombinant strains, thereby reducing production costs and environmental pollution. Recent studies have found that *Pichia pastoris* contains metabolic pathways similar to those in filamentous fungi *Fusarium* sp. [11]. Compared with filamentous fungi, *P. pastoris* offers multiple advantages including rapid growth, easy genetic manipulation, and simple downstream processing. If the biosynthetic genes for fusaruside could be introduced into *P. pastoris*, the yeast might directly produce this small-molecule immunosuppressant, enabling rapid acquisition of large quantities of product.

This study employed a 2A peptide strategy to co-express fusaruside-related synthetic enzymes in *P. pastoris*, reconstructing the fusaruside biosynthetic pathway and obtaining a recombinant yeast strain capable of producing this compound, aiming to solve the source problem of this compound and lay a foundation for further development and application of fusaruside.

Materials and Methods

1.1 Materials

Pichia pastoris GS115 strain, *E. coli* DH5 strain, and pPIC3.5K plasmid were preserved in our laboratory. *Fusarium graminearum* PH-1 was purchased from the American Type Culture Collection. Plasmid Mini Kit I and Gel Extraction Kit were purchased from OMEGA Bio-Tek (USA). RNA extraction kit was purchased from Sangon Biotech (Shanghai). Restriction enzymes BamH I, EcoR I, Bgl I, and dNTP were purchased from Takara (Dalian). Glucose, peptone, yeast extract, and agar were purchased from OXOID (UK). Methanol was purchased from Sigma (USA). Other reagents were domestic analytical grade. *E. coli* culture medium LB, *P. pastoris* culture medium YPD, selection medium MD, induction expression medium BMGY and BMMY, and related culture formulations are described in the *Pichia pastoris* operation manual from Invitrogen.

1.2 Methods

1.2.1 Acquisition and Modification of Target Genes Total RNA was extracted from *F. graminearum* PH-1 and reverse-transcribed to obtain cDNA. The primers listed in Table 1 were used to amplify the 3-position desaturase (Δ3(E)-sphingolipid desaturase, Δ3(E)-SD, GenBank accession: XM_383758.1) gene using D3-F/D3-R, and the 10-position desaturase (Δ10(E)-sphingolipid desaturase, Δ10(E)-SD, GenBank accession: XP_390021.1) gene using D10-F/D10-R.

Since the selected pPIC3.5K vector has few restriction sites, and the target genes contain these sites, the genes needed to be modified. Using the primers listed in Table 1, overlap extension PCR (OL-PCR) was performed to modify the BamH I and EcoR I sites in the genes without changing the amino acid sequences, ensuring the genes no longer contained these restriction sites.

The 2A peptide used for co-expression was the most commonly used F2A peptide derived from foot-and-mouth disease virus (FMDV), obtained through chemical synthesis, with a length of 66 bp. Its sequence is shown in Table 1.

1.2.2 Construction of Co-expression Vector Using OL-PCR, the 2A peptide gene was inserted between the two desaturase genes (Δ3(E)-sd and Δ10(E)-sd) to construct the combined gene fragment Δ3(E)-sd-2a-Δ10(E)-sd. Both the combined fragment and yeast expression vector pPIC3.5K were digested with BamH I and EcoR I, then ligated and transformed into *E. coli* DH5 .

Colony PCR was performed on the resulting *E. coli* colonies, followed by extraction of plasmids from positive bacteria for verification by double digestion with BamH I and EcoR I.

1.2.3 Construction and Screening of Recombinant Strains The constructed co-expression vector pPIC3.5K-Δ3(E)-sd-2a-Δ10(E)-sd was linearized with Bgl I and dephosphorylated with alkaline phosphatase to prevent self-ligation. The processed expression vector was introduced into competent cells via electroporation as follows: the vector was gently mixed with 40 L competent cells, transferred to a pre-chilled 2 mm electroporation cuvette, placed on ice for 1 min, and then electroporated once (1.5 kV, 25 F, 200 Ω, 6 ms). Immediately after electroporation, 1 mL of ice-cold sterile 1 M sorbitol was added, and all solution was transferred to a sterile centrifuge tube in a laminar flow hood, incubated statically at 30 ℃ for 1-2 h, and 300 L was plated on YPD plates containing 1 M sorbitol and 0.25 mg/mL geneticin.

After 3-4 days of culture, several single colonies were randomly selected, cultured in YPD liquid medium with shaking for 24 h, and chromosomal DNA was extracted for PCR identification using primers D3-F/D10-R. Successfully identified strains were subjected to induction expression.

1.2.4 Induction Expression of Recombinant Strains *P. pastoris* contains the alcohol oxidase gene promoter (PAOX1). The foreign gene carried by the expression vector integrates downstream of the promoter, and PAOX1 can drive high-level expression of the downstream foreign gene under methanol induction. The selected positive strains were inoculated into test tubes containing 5 mL YPD medium and cultured at 30 ℃, 200 r/min for 1 day. The seed culture was then inoculated at 2% volume into 250 mL flasks containing 50 mL BMGY medium, cultured at 30 ℃, 200 r/min for 1 day, centrifuged to collect cells, and then transferred entirely into 250 mL flasks containing 50 mL BMMY medium for culture at 30 ℃, 200 r/min. Methanol was added every 12 h to a final concentration of 0.5%, and samples were taken every 12 h for SDS-PAGE analysis of protein expression in the cells.

1.3 Analytical Methods

1.3.1 Detection of Fusaruside The successfully induced yeast cells were dried and extracted with methanol/dichloromethane (v/v = 1:1) three times. The extract was evaporated to dryness using a rotary evaporator. A small amount of the extract was analyzed by HPLC-MS for fusaruside production, with non-recombinant *P. pastoris* as negative control and fusaruside and its precursor cerebroside B as positive controls.

The HPLC-MS instrument used was an Agilent high-resolution quadrupole time-of-flight liquid chromatography-mass spectrometer. HPLC separation conditions were: ZORBAX Eclipse Plus C18 column, 100 mm × 4.6 mm, 3.5 m; mobile phase: methanol-water (v/v = 9:1); flow rate 0.4 L/min.

1.3.2 Purification of Fusaruside After confirming by HPLC-MS that the recombinant yeast could produce fusaruside, large-scale fermentation and induction expression were performed. Yeast cells were collected by centrifugation and extracted three times with methanol/dichloromethane (v/v = 1:1). The crude extract was fractionated by reversed-phase silica gel column chromatography with elution conditions of 50%, 60%, 70%, 80%, 90%, and 100% methanol-water. Fractions containing fusaruside were further separated by gel column LH-20 with dichloromethane/methanol (1:1) as eluent, tracked by HPLC-MS.

Fractions containing fusaruside were further purified by HPLC to obtain pure fusaruside, and the chemical structure was confirmed by 1H NMR.

Results

2.1 Gene Cloning and Vector Construction

The fusaruside biosynthetic genes Δ3(E)-sd and Δ10(E)-sd were amplified using the primers listed in Table 1 . The PCR products were recovered, yielding fragments of 1,320 bp and 1,722 bp, respectively (Figure 1 [Figure 1: see original paper]).

Using OL-PCR, the combined gene fragment Δ3(E)-sd-2a-Δ10(E)-sd was constructed as shown in Figure 2 [Figure 2: see original paper], with a length of approximately 3,000 bp.

The combined fragment and yeast expression vector pPIC3.5K were digested with BamH I and EcoR I, then ligated and transformed into *E. coli.* Colony PCR verification was performed on the resulting *E. coli* colonies, and several positive strains were obtained. Plasmids extracted from positive clones were verified by restriction digestion. The schematic of the constructed co-expression vector and the digestion verification results are shown in Figure 3 [Figure 3: see original paper], indicating successful construction of the dual-enzyme co-expression vector pPIC3.5K-Δ3(E)-sd-2a-Δ10(E)-sd.

2.2 Recombinant Strain Construction and Expression

The co-expression plasmid pPIC3.5K-Δ3(E)-sd-2a-Δ10(E)-sd was introduced into *P. pastoris* GS115 via electroporation, and positive transformants were selected on medium containing geneticin. Chromosomal DNA was extracted from recombinant yeast strains, and PCR identification was performed using primers D3-F/D10-R. As shown in Figure 4 [Figure 4: see original paper], positive recombinant strains yielded a fragment of approximately 3,000 bp, consistent with the theoretical value, confirming successful integration of pPIC3.5K-Δ3(E)-sd-2a-Δ10(E)-sd into the *P. pastoris* GS115 genome.

The positive strains were inoculated into medium with glycerol as the sole carbon source, cultured at 30 ℃ for 1 day, then collected for methanol induction culture. Samples were taken every 12 h for SDS-PAGE analysis. As shown in Figure 5 [Figure 5: see original paper], after 84 h, Δ3(E)-SD (molecular weight approximately 48 kDa) and Δ10(E)-SD (molecular weight approximately 65 kDa) were successfully expressed in the recombinant yeast.

2.3 Product Analysis

2.3.1 Detection of Fusaruside The successfully induced yeast cells were dried, extracted, and concentrated. The extract was analyzed by HPLC-MS for fusaruside production. The extracted ion chromatogram is shown in Figure 6 [Figure 6: see original paper], demonstrating that the engineered strain could produce fusaruside and its precursor compound cerebroside B, while non-recombinant *P. pastoris* could not produce these compounds.

2.3.2 Purification and Structural Confirmation After confirming that the yeast could produce fusaruside, large-scale fermentation of the recombinant yeast was performed, yielding 3.6 g of crude extract for purification. Tracked by HPLC-MS, reversed-phase silica gel, gel filtration, and HPLC separation yielded 5 mg of pure fusaruside. The chemical structure was confirmed by 1H NMR. As shown in Figure 7 [Figure 7: see original paper], there were seven proton signals at 5.34-6.06, indicating four double bonds in the compound, two more than cerebroside D produced in *P. pastoris*, thus confirming the correct structure.

The fusaruside yield of this yeast strain compared with that reported for *Fusarium* sp. in the literature [7] is shown in Table 2 . The table shows that the yeast yield was 11.6 times that of the reported *Fusarium*.

Discussion

Our previous studies through gene knockout and heterologous expression experiments identified the 10-position desaturase (Δ10(E)-sphingolipid desaturase, Δ10(E)-SD) involved in fusaruside biosynthesis and elucidated the complete biosynthetic pathway of fusaruside (Figure 8 [Figure 8: see original paper]) [12]. We found that cerebroside D could be converted to cerebroside B via dehydrogenation by Δ3(E)-SD, and then to fusaruside via dehydrogenation by Δ10(E)-SD [11-13]. Since cerebroside D exists abundantly in *P. pastoris* cells, co-expression of these two enzymes in *P. pastoris* could convert cerebroside D in yeast cells to fusaruside, enabling rapid biosynthesis of fusaruside.

This study utilized the self-cleaving 2A peptide strategy for multi-gene vector construction, which avoids disadvantages such as low protein activity during multi-gene expression and offers clear advantages, representing an ideal current strategy for multi-gene expression [14]. Brazier-Hicks et al. used the 2A peptide strategy to co-express flavonoid glycoside synthase genes in *P. pastoris*, obtaining recombinant yeast capable of producing flavonoid glycosides [15]. Beekwilder et al. used this technology to reconstruct the -carotene biosynthetic pathway in *Saccharomyces cerevisiae*, obtaining -ionone [16]. Geier et al. first used the 2A peptide strategy to successfully co-express nine genes in *P. pastoris*, obtaining recombinant yeast strains capable of producing violacein and carotenoid-like compounds [17]. These successful studies demonstrate the effectiveness of this method. This study successfully constructed a co-expression vector for Δ3(E)-SD and Δ10(E)-SD using this method, obtaining for the first time a recombinant *P. pastoris* strain capable of producing fusaruside.

In this study, 5 mg of fusaruside was obtained from 3.6 g of crude extract from recombinant *P. pastoris*, representing a yield 11.6 times higher than that of the filamentous fungus *Fusarium* sp. reported in the literature [7]. Moreover, the fermentation time of recombinant *P. pastoris* was shorter (84 h) compared to 11 days for filamentous fungus *Fusarium* sp. The acquisition of this recombinant *P. pastoris* lays a foundation for further development and application of fusaruside.

Ternes et al. found that *P. pastoris* contains two metabolic pathways, where pathway A produces the fusaruside precursor cerebroside D, while pathway B generates sphingosine and inhibits pathway A, thereby suppressing cerebroside D production [11]. To relieve this inhibition and further increase fusaruside yield, the next step could involve overexpression of the glucosylceramide synthase (GS) gene in *P. pastoris*. Combined with optimization of *P. pastoris* culture and induction conditions, this could further improve fusaruside production, effectively solving the source problem for this novel immunosuppressant.

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