IDENTIFICATION AND HETEROLOGOUS RECONSTITUTION OF A POLYKETIDE SYNTHASE FROM THE ENDOPHYTIC STRAIN SHIRAIA SP. LFS18

[1]LEI SUN, [2]RUOTING ZHAN, [3]QIANG LI

[1]Research Center of Chinese Herbal Resource Science and Engineering, Guangzhou University of Chinese Medicine, Guangzhou, 510006, P. R. China
[2]Key Laboratory of Chinese Medicinal Resource from Lingnan (Guangzhou University of Chinese Medicine), Ministry of Education, Guangzhou, 510006, P. R. China
[3]Joint Laboratory of National Engineering Research Center for the Pharmaceutics of Traditional Chinese Medicines, Guangzhou, 510006, P. R. China
E-mail: sunlei@gzucm.edu.cn

Abstract - A type III polyketide synthase gene (Sl-ars) was discovered from the genome of Shiraia sp. Lfs18, an endophytic fungal strain from Huperzia serrata. The intron-free gene was cloned from the cDNA and ligated respectively to two shuttle vectors pET28a and YEpADH2p-URA3 for expression in Escherichia coli BL21(DE3) and Saccharomyces cerevisiaeBJ5464-NpgA. Sl-ARS was efficiently expressed in E. coli BL21(DE3), leading to the synthesis of a series of polyketide products. Six major products were isolated and characterized as 5-alk(en)ylresorcinol with various fatty acyl side chains. Addition of IPTG at different OD600 values influenced the yields of these products. Expression of Sl-ARS in the yeast also lead to the synthesis of several 5-alk(en)ylresorcinol.

Key words - Polyketide synthase, alkylresorcinols, Escherichia coli, Saccharomyces cerevisiae

I. INTRODUCTION

Fungi are an important source of natural products and biosynthetic enzymes. Symbiotic microorganisms live closely with the hosts and are found to produce various valuable compounds. Shiraia sp. Lfs18 is an endophytic fungus which possess the ability of producing hypocrellins. As polyketide natural products,hypocrellinshave shown a good prospect of clinical application,and can be used as an effective photosensitizer for photodynamic therapy (PDT) of tumors[1-3].It is well known that polyketidesaresynthesized from acyl-coenzyme A substrates by polyketide synthases (PKSs), whichare classified into three groups based on sequences and functional mechanisms, including types I, II,and III. Type I PKS, similar to type I fatty acid synthase (FAS), is a giant assembly of multifunctional polypeptides, each consisting of a series of catalytic domains [4]. Representative bioactive compounds synthesized by type I PKSs are lovastatin (anti-cholesterol) and erythromycin (antibacterial). Type II PKSs are discrete enzymes that work collaboratively to form aromatic polyketide products, such as tetracycline (antibacterial) and doxorubicin (anticancer) [5]. Type III PKSs are self-contained enzymes that form homodimers. Type III PKSs produce a wide array of natural products such as chalcones, pyrones, acridones, phloroglucinols, stilbenes, and resorcinolic lipids[4]. All three types of PKSs are present in bacteria and type II PKSs are exclusively found in bacteria such as actinomycetes. Plants contain various type III PKSs, while fungi are known to harbor type I and type III PKSs. These PKSs are involved in the formation of various metabolites in these hosts. Compared to type I PKSs, type III PKSs are much less abundant in fungi. However, type III PKSs could have some critical roles in fungi[6]. Chalcone synthase (CHS), a type III PKSs existing exclusively in plants and bacteria, is a key enzyme in polyketide biosynthesis pathway. Here we report our research work on reconstituting the CHS gene from Shiraia sp. Lfs18 in E. coliBL21(DE3)and S. cerevisiaeBJ5464, thenanalyzing and comparing their expression.

II. METHOD

2.1 Construction of pET28a-Sl-ARS and pSUN38-Sl-ARS

Wild typeShiraia sp. Lfs18was grownin potato dextrose broth (PDB) for 3 days. The cells were harvested by filtration, from which the RNA was extracted with TRIzol Reagent (Invitrogen, USA). The extracted RNA was purified with a Direct-zol RNA MiniPrep kit (Zymo Research, CA USA). Synthesis of the cDNA from total RNA with M-MuLV Reverse Transcriptase (NEB, MA, USA). With the cDNA as a template, a 1,278-bp DNA fragment of the intron-free Sl-ars gene was amplified by PCR with a set of specific primers including JNU1-13-CHS-F-NheI (5'-aaGCTAGCatgtcccacgtggctaac-3', with a NheI site shown by underlining) and JNU1-13-CHS-R-BamHI (5'- aaGGATCCttaacctgttcgtacag-3', with anBamHIsite shown by underlining). The resulting

intron-free fragment was cloned into the pJET1.2 cloning vector, and subsequently cloned into pET28a vector between the NheIand BamHIsites, resulting in pET28a-Sl-ARS.TheSl-arsgene was amplified by PCR using another primer set including JNU1-13-CHS-F-NheI, as listed above, and JNU1-13-CHS-R-PmeI (5'- aaGTTTAAACttaacctgttcgtacagtg-3', with anPmeIsite shown by underlining). The resulting intron-free fragment was cloned into the pJET1.2 cloning vector, and subsequently cloned into pSUN38, derived from YEpADH2p-URA3 with more restriction sites added, vector between the NheI and PmeI sites, resulting in pSUN38-Sl-ARS.

2.2 Heterologous expression and Productsanalysis

E.coli BL21 Harboring pET28a-Sl-ARSwas grown in LB liquid medium supplemented with 50μg/mL kanamycin at 37°C to acertain OD600 value which was then induced by 200μM isopropyl-1-thio-β-D-galactoside(IPTG) and incubated at 28°C and 250 rpm for 12 hrs. Cell was harvest by centrifugation at 4000 rpm for 10min, then discard the supernatant. The pellet was extracted with methanol then evaporated the solvent. Dissolved the residue in methanol and analyzed using Agilent 1200 HPLC instrument (Agilent Eclipse Plus C18, 5 μm, 4.6 mm × 250 mm), eluted with a linear gradient of 75 to 90 % (v/v) acetonitrile-water (containing 0.1% trifluoroacetic acid) over 45 min at a flow rate of 1 mL min−1. The products were detected at 280 nm.

S. cerevisiaeBJ5464 harboring pSUN38-Sl-ARS was grown in SC medium (Ura-) at 28°C to an OD600 1.0,after added 1% yeast extract, 2% peptone, and grown at 28°C and 250 rpm for another 3 days. The fermentation broth was extracted with the same volume of ethyl acetate. The extracts were analyzed using the same method as described before.

2.3 Purification and structural characterization of alkylresorcinols1–6

To isolate compounds 1–6 for structural characterization, the culture was scaled up to 1 L and grown for 72 h. Briefly, 1 mL of seed culture (OD600=1.0) of E. coli BL21 /pET28a-Sl-arswas inoculated into 1 L of LB medium containing 50 μg mL−1 kanamycin, grown to OD600 of 1.0. 200 μM IPTG was then added to induce protein expression. After fermentation, the culture was extracted as described above. To isolate these compounds, a typical purification procedure was given below. The crude extract was fractionated on a silica gel-60 column, eluted with a stepwise gradient of ethyl acetate-hexane (0:100, 1:99, 2:98, 3:97, 5:95, 10:90, 15:85, 20:80 v/v, each 250 mL) to afford 8 fractions. The fractions containing the target compounds were further separated by reverse-phased HPLC (Agilent Eclipse Plus C18, 5 μm, 4.6×250 mm), eluted with acetonitrile-water at a flow rate of 1 mL min−1. Compound 1 and 2 were found to be in fraction 6 after silica gel column chromatography. This fraction was further separated on HPLC with 67% acetonitrile-water. The peak at 20.5 min and 23.7 min was collected to yield 1.7 mg of 1 and1.4 mg of 2in pure form. Compounds 3 and 4 were found to be in fraction 5 after silica gel column chromatography. This fraction was further separated on HPLC with 74% acetonitrile-water. The peaks at 32.4 min and 35.6 min were collected to yield 1.5 mg of 3and 2.1 mg of 4, respectively, in pure form. Compounds 5 and 6 were found to be in fraction 3 after silica gel column chromatography. This fraction was further separated on HPLC with 82% acetonitrile-water. The peak at 47.9 min and 50.5 min were collected to yield 0.9 mg of 5 and 1.2 mg of 6, respectively.

III. RESULTS

3.1 Characterization of the polyketide products

The E. coli BL21 was transformed with pET28a-Sl-ARS and cultured in LB broth 37°C until the OD600 reached at 1.0, then induced with 200 μMIPTG. After incubated at 28°C and 250 rpm condition overnight, we collected the cells with centrifugation at 4000rpm, and extracted the cells with sonication. With E.coli BL21 harboring empty vector pET28aas control, the results showed that Sl-ars gene from Shiraia sp. Lfs18 is responsible for the biosynthesis of a series compounds in E.coli BL21(Fig.1A). The IPTG-induced broth was extracted with an equal volume of ethyl acetate. As shown in Fig. 1A, HPLC analysis revealed that six major products (1-6, trace I) were synthesized by Sl-ARS, which were not observed in E. coli BL21-pET28a (trace II), used as negative control.

These six products were isolated from the scaled-up culture of E. coli BL21-pET28a-Sl-ARS Compounds 1-6 were obtained as yellowish oil. These compounds were identified as alkylresorcinols with either an unsaturated or saturated aliphatic side chain (Fig. 1B) based on their, UV, MS and NMR spectra and a comparison with previously published data [7]. The molecular weights of 1-6 were determined to be 264, 290, 292, 318, 320 and 346 based on their [M+H]+ ion peaks at m/z265, 291, 293, 319, 321 and 347 in the ESI-MS, respectively. Accordingly, compounds 1, 3 and 5 were deduced to be alkylresorcinols with a saturated side chain, while 2, 4 and 6 have an unsaturated side chain. The 1H and 13C NMR spectra of 2revealed two aromatic proton signals at δH 6.26 (2H, d, J = 1.8 Hz, H-4 and H-6) and δH 6.20 (1H, t, J = 1.8 Hz, H-2), suggesting that a resorcinol moiety is present in the structure. In addition, proton signals typical for a long aliphatic chain including the benzylic methylene at δH 2.51 (2H, t, J = 7.5 Hz) and terminal methyl group at δH 0.92 (3H, t, J = 6.9 Hz) were also found. Thus, 2 was identified as 1,3-dihydroxyphenyl-5-tridecane, a resorcinol derivative with a saturated C13 side chain.3 has highly similar NMR data to those of 2, whereas has an additional CH2-CH2 unit. Thus, it was characterized as 1,3-

dihydroxyphenyl-5-pentadecane that has a saturated C15 side chain. The NMR spectra of 5 also showed the aromatic proton and carbon signals which are similar to 6. However, two overlapping olefinic protons at δH 5.38-5.36 indicated that there is a double bond in the side chain. Thus, 2 was characterized as 1,3-dihydroxyphenyl-5-cis-6'-tridecene. Similarly, 4 and 6 were identified as resorcinol derivatives containing an unsaturatedC15 and C17 side chain, named 1,3-dihydroxyphenyl-5-cis-8'-pentadecene and 1,3-dihydroxyphenyl-5-cis-10'-heptadecene, respectively. These data were in good agreement with the data reported previously for 5-alk(en)ylresorcinols [7]. The thioesters of common fatty acids in E. coli[8] are proposed to be used by Sl-ARS as the starter units to synthesize 1-6. Specifically, the thioesters of unsaturated fatty acids including tetradecenoic acid (cis-7), hexadecenoic acid (cis-9), and octadecenoic acid (cis-11)are used by SlfCHSto afford 2,4 and 6, while the thioesters of myristic acid (C14:0) and palmitic acid (C16:0) were used to synthesize 1, 3 and 5, respectively.

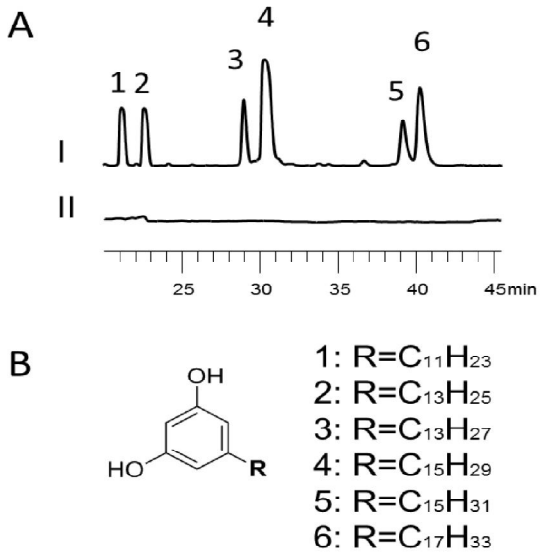


Fig.1 HPLC analysis (UV 280nm) with IPTG inducing of Ecoli.BL21 harboring pET28a-Sl-ARS (I), empty vector as control (II) (A), structures of compounds 1-6 (B)

3.2 Heterologous expression of Sl-ARS in S. cerevisiae BJ5464 and characterization of the polyketide products

The engineered strain S. cerevisiaeBJ5464 harboring pSUN38-Sl-ARS was cultured and the samples were prepared as described in the method section. The HPLC results showed that Sl-ars gene from Shiraia sp. Lfs18 is responsible for the biosynthesis of a series compoundsin S. cerevisiaeBJ5464 (Figure2). These compounds were identified as alkylresorcinols1-6 based on their, UV, MS and NMR spectra and a comparison with previousdata. The molecular weights of 1-6 were determined to be 264, 290, 292, 318, 320 and 346 based on their [M+H]+ ion peaks at m/z 265, 291, 293, 319, 321 and 347 in the ESI-MS, respectively.

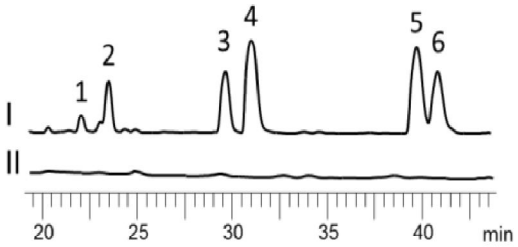


Fig. 2 HPLC analysis (UV 280nm) Sl-ARS expressed in S. cerevisiaeBJ5464 (I), empty vector as control (II).

DISCUSSION

The type III polyketide synthaseSl-ARSwas identified and heterologously reconstituted in E. coli BL21 and S. cerevisiaeBJ5464, various of alkylresorcinols were synthesized in the engineered strains. Alkylresorcinols are relatively rare in nature, with the main known sources being wheat, rye, barley, triticale (cereal grasses), the fruit of Gingko biloba Bilobol, a mollusc and some species of bacteria.The concentrations of ARs normally between 300 and 1500 μg/g in wheat and rye[9].Due to their almost exclusive presence in the outer part of wheat and rye grains among commonly consumed foods [10], ARs have been suggested and evaluated as biomarkers of whole grain wheat and rye intake in different populations [11-14].The latest research showed the dual nature of ARs,direct antioxidant properties and the ability to indirectly regulate the activity of cellular antioxidative defense mechanisms [15]. With the further research, more and moreimportant values of the ARs will be revealed. It will be getting more significantof ARs biosynthesis.

In summary, we have reconstituted Sl-arsgene in E. coli and S. cerevisiae.The type III polyketide synthase Sl-ARSis responsible for the biosynthesis of the alkylresorcinols both in E. coliand S. cerevisiae.This research is important for the research on heterologous biosynthesis of alkylresorcinols in engineered microorganisms.

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