Enzyme and Microbial Technology 73–74 (2015) 72–79

Contents lists available at ScienceDirect

Enzyme and Microbial Technology

journal homepage: www.elsevier.com/locate/emt

Studies on the function and catalytic mechanism of *O*-methyltransferases SviOMT02, SviOMT03 and SviOMT06 from *Streptomyces virginiae* IBL14

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ARTICLE INFO

Article history:
Received 20 November 2014
Received in revised form 8 March 2015
Accepted 31 March 2015
Available online 30 April 2015

Keywords:
Genome sequencing
Homology modeling
Molecular docking
O-methyltransferase
Phylogenetic analysis
Streptomyces virginiae

ABSTRACT

To identify the fuctions of the nine putative *O*-methyltransferase genes in *Streptomyces virginiae* IBL14, the evolutionary and functional relationship of these genes in its 8.0 Mb linear chromosome was set up via sequence comparison with those of other *Streptomyces* species. Further, the functions and catalytic mechanism of the three genes *svi*OMT02, *svi*OMT03 and *svi*OMT06 from this strain were studied through experimental and computational approaches. As a result, the nine putative *O*-methyltransferases belong to methyltransf_2 superfamily, amdomet-MTases superfamily, and leucine carboxyl methyltransferase superfamily, and are phylogenetically close to those of *Streptomyces sp*. C. The products of genes *svi*OMT03 and *svi*OMT06 could catalyze *O*-methylation of caffeic acid to form ferulic acid. Computational analysis indicated that the *O*-methylation mechanism of SviOMT03 and SviOMT06 proceeds from a direct transfer of the SAM-methyl group to caffeic acid with inversion of symmetry aided by a divalent metal ion in a S_N2-like mechanism. Particularly, the conservative polar amino acid residues in SviOMT03 and SviOMT06, including Lys143 that reacts with caffeic acid, Ser74, Asp140 and Tyr149 that react with S-adenosyl methionine, and His142 (SviOMT03) or His171 (SviOMT06) that transfers the 3-hydroxyl proton of substrate caffeic acid, probably be essential in their *O*-methylation.

1. Introduction

Streptomyces virginiae IBL14 is an effective degradative strain for various flavonoid compounds, including cholesterol, progesterone, isotestosterone, dihydrotestosterone, hydrocortisone and ostrone, and can methoxylate diosgenin to 6-methoxy-products, the first-ever bio-methoxylation on the B-ring of steroidal compounds, discovered in our lab [1,2]. Our previous studies demonstrated that the bio-methoxylation on the B-ring of diosgenin is probably catalyzed by a double-enzyme system (i.e., hydroxylase and *O*-methyltransferase in turn). To interpret the mechanism of 6-methoxylation on the B-ring of diosgenin by *S. virginiae* IBL14, the whole genome has been sequenced. The analyses of Basic Local Alignment Search Tool-Protein (BLASTP) based on the data of the genome sequencing demonstrated that the strain IBL14 contains 9 putative *O*-methyltransferases (OMTs, EC 2.1.1.X), 32 cytochrome P450s (CYPs), 7 ferredoxins, as well as 3 ferredoxin reductases in its 8.0 Mb linear chromosome. In the meantime, the studies on the hydroxylases and *O*-methyltransferases in *S. virginiae* IBL14 have been being carried out systematically [3].

Methylation can be divided into S-, O-, N-, and C-types and can be found in various organic compounds from small molecular phenols to macromolecular proteins and nucleic acids. OMTs, a subgroup of methyltransferases, found in diverse organisms, including plants [4], animals [5] and microbes [6,7], can transfer the methyl group via *S*-adenosyl methionine (SAM) to a hydroxyl or carbonyl group. Most of OMTs in *Streptomyces* spp. are involved in antibiotic biosynthesis [8] and those in fungi often involved in aflatoxin biosynthesis [6], which are closely related to human health. Among all the OMTs, catechol *O*-methyltransferase (COMTs) have been studied most extensively because they are involved in the inactivation of the catecholamine neurotransmitters and the reaction of catechol estrogens to form reactive estrogenic quinines (endogenous tumor initiators) [9].

Recently, much attention has been paid to caffeic acid-*O*-methyltransferase (CAOMT), one of the most important enzymes controlling lignin (a kind of biofuel) monomer production in plant

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http://dx.doi.org/10.1016/j.enzmictec.2015.03.006
0141-0229/

cell wall synthesis [10]. The phylogenetic, molecular, and biochemical characteristics of CAOMT gene family in *Brachypodium distachyon* have been reported [10]. The studies on the expression and down-regulation [11], structure-function analyses and molecular modeling of CAOMT genes [12] in plants have been conducted extensively. Further, some research work on homology modeling and/or site-directed mutagenesis of OMT have been carried out [17,18]. However, the studies on the OMTs from microorganisms, especially from *Streptomyces* spp. were limited.

In this study, we found a total of 9 putative OMTs in its 8.0 Mb linear chromosome according to the analyses of BLASTP. Meanwhile, the evolutionary relationships and the function of these OMTs were identified via sequence alignment with those from other members of *Streptomyces* species. Furthermore, the cloning, expressions of OMT genes *svi*OMT02 (Genbank Accession: KF377808), *svi*OMT03 (Genbank Accession: KF377809) and *svi*OMT06 (Genbank Accession: KF377812) from *S. virginiae* IBL14 are implemented and their functions identified experimentally. Finally, we inferred the catalytic mechanism of SviOMT03 and SviOMT06 via computational analysis and test of site-directed mutagenesis experiments.

2. Materials and methods

2.1. Strains and plasmids

S. virginiae IBL14 (CCTCCM 206045) [13] was used as a target strain for the OMT gene identification and functional analysis. *Escherichia coli* JM109, Trans1-T1 phage resistant chemically competent cell of *E. coli* (TransGen Biotech, China) and *E. coli* JM109 (DE3) were used as a host for the plasmid construction and target protein expression in the expression and functional identification of the OMT genes (*svi*OMT02, *svi*OMT03, *svi*OMT06 and *svi*OMT06S74). The vectors pET22b (for *svi*OMT02 and *svi*OMT03) and *pEASY*™-E1 (for *svi*OMT06 and *svi*OMT06S74) were used for cloning and amplifying the genes in *E. coli*. The detailed features of the bacterial strains and plasmids used in this study were listed in Table S1.

2.2. Media and chemicals

Luria-Bertani (LB) medium (10 g peptone, 5 g yeast extract and 10 g NaCl in 1 l water) was used for plasmid construction and protein expression. The medium was supplemented with ampicillin (50 μg/ml) when *E. coli* IBL16M2 [JM109 (DE3)/pET22b-*svi*OMT02], *E. coli* IBL16M3 [JM109 (DE3)/pET22b-*svi*OMT03], *E. coli* IBL16M6 [JM109 (DE3)/*pEASY*™-E1-*svi*OMT06] and *E. coli* IBL16M6S74 [JM109 (DE3)/*pEASY*™-E1-*svi*OMT06S74] were cultivated. The cultivation process of *S. virginiae* IBL14 was described previously [13]. Catechol and its derivates (Fig. S1) as substrates (over 98% purity) (Sangon Biotech Co., Ltd., Shanghai, China) were dissolved in anhydrous ethanol before being added to the medium for biotransformation. The corresponding *O*-methylated products as standards (over 98% purity) (Sangon Biotech Co., Ltd., Shanghai, China) were used for qualitative and quantitative analysis of biotransformation products.

2.3. Functional prediction and analysis of OMTs from annotated database

All the ORFs of *S. virginiae* IBL14 genome were predicted using Glimmer 3.0 and Prodigal [3]. To annotate the functional information of all possible OMT genes in *S. virginiae* IBL14, the whole genome sequence of IBL14 was compared with the SWISSPROT, TREMBL, KEGG databases by using BLASTP and with the CDD and COG databases by using Rpsblast. As a result, nine putative OMT genes were found in *S. virginiae* IBL14.

The amino acid sequences of the nine putative OMTs of *S. virginiae* IBL14 were aligned with those of OMTs from other *Streptomyces* spp., including *Streptomyces bingchenggensis* BCW-1, *Streptomyces venezuelae* ATCC 10712, *Streptomyces sp.* C, *Streptomyces auratus* AGR0001, *Streptomyces flavogriseus* ATCC 33331, *Streptomyces clavuligerus* ATCC 27064, and *Streptomyces sp.* Mg1, by using Clustal W [14]. Furthermore, the phylogenetic relationships of the OMTs among different members of *Streptomyces* spp. were constructed by neighbor-joining (NJ) methods using MEGA 5.0. Kimura-2-parameter distances were calculated from the NJ analyses and the non-parametric bootstrap analyses with 500 pseudo-replicates were performed to obtain estimates of support for each node of the NJ trees.

2.4. Cloning and construction of the expression plasmids

Three sets of specific primers (Table S2) were designed using Primer Premier 5.0 and verified by Oligo 7.0 (the same below) to amplify the genes *svi*OMT02, *svi*OMT03 and *svi*OMT06, by PCR (TransTaq™ DNA Polymerase High Fidelity, TransGen Biotech, China) using the genomic DNA of *S. virginiae* IBL14 as a template. The PCR reactions were carried out using the following conditions: 35 cycles of denaturation at 94 °C for 30 s, annealing at 66 °C (*svi*OMT02)/67 °C (*svi*OMT03)/59 °C (*svi*OMT06) for 30 s, extension at 72 °C for 90 s and a final extension at 72 °C for 10 min. The amplified products of genes *svi*OMT02 and *svi*OMT03 were digested with *Hind* III/*Xho* I, and then ligated with the pET22b vector treated previously with *Hind* III/*Xho* I. Finally, the recombinant plasmids pET22b-*svi*OMT02 and pET22b-*svi*OMT03 were transformed into host cells *E. coli* JM109 and *E. coli* JM109 (DE3), respectively. The recombinants *E. coli* IBL16M2 and IBL16M3 were obtained from the LB plate with 50 μg/ml ampicillin (In the construction of recombinant IBL16M6, the expression vector was *pEASY*™-E1 and the host was Trans1-T1 phage resistant chemically competent cell of *E. coli* JM109 (DE3)). T7 promoter and terminator primers were used to prove the validity of the mutants and further for sequence analysis. DNA sequencing in this study was carried out by Sangon Biotech Co., Ltd., Shanghai, China (the same below).

2.5. Site-specific mutagenesis

The amino acid Ser74, one of the essential amino acids for the *O*-methylation according to the analysis of homology modeling, molecular docking and protein–ligand interaction, of *svi*OMT06 was firstly selected for site-directed mutagenesis. Two oligonucleotide segments containing both mutation site of amino acid (Ala74/GCC instead of Ser74/TCC) and oppositely part sequence overlap as primer were determined as shown in Table S2. The PCR reactions for producing the recombinant plasmid *pEASY*™-E1-*svi*OMT06S74 (Easy Mutagenesis System KIT, TransGen Biotech, Shanghai, China) were performed in a total volume of 20 μl, using the recombinant plasmid *pEASY*™-E1-*svi*OMT06 as template (methylated). The conditions for PCR reactions were: pre-denaturation at 95 °C for 3 min, 30 cycles of denaturation at 95 °C for 30 s, annealing at 69 °C for 30 s, and polymerization at 72 °C for 3 min, and a final extension at 72° for 10 min. The PCR product was digested by DMT enzyme (TransGen Biotech, Shanghai, China) to remove the methylated template *pEASY*™-E1-*svi*OMT06. The recombinant plasmid *pEASY*™-E1-*svi*OMT06S74 was confirmed by gene sequencing and subsequently was transformed into *E. coli* JM109 (DE3). The recombinant *E. coli* IBL16M6S74[JM109(DE3)/*pEASY*™-E1-*svi*OMT06S74] was obtained from the LB plate with 50 μg/ml ampicillin, and further tested by PCR experiments (T7 promoter and terminator primers) and by sequence analysis, respectively. The experimental protocols were also applied to the site-directed mutagenesis experiments on the other three key amino acid residues Asp140 (Ser 140 instead of Asp 140), Lys143 (Ser 143 instead of Lys 143) and Tyr149 (Ser 149 instead of Tyr 149) of SviOMT06.

2.6. Expression of target proteins and biotransformation

Each of the 0.2 ml of the overnight cultures of *E. coli* IBL16M2, *E. coli* IBL16M3, *E. coli* IBL16M6 and *E. coli* IBL16M6S74 as seed was inoculated into an individual 30 ml LB medium (50 μg/ml ampicillin) and then cultivated at a shaking speed of 200 rpm at 37 °C. The expression of target proteins was initiated by adding isopropyl-β-D-thiogalactoside (IPTG) with a final concentration of 0.1–0.2 mM when the OD_{600} value of the culture reached 0.5–0.6. Subsequently, the culture was cultivated at 25 °C ±1 for another 24 h under the same conditions. The cells were collected by centrifugation (4000 rpm) at 4 °C for 10 min, re-suspended with 50 ml PBS (0.05 mM, pH 7.2), and lysed by sonication for 10 min in an ice-water bath. The expressed target proteins were subsequently analyzed by SDS-PAGE.

Catechol and its derivate as substrates (over 98% purity) with a final concentration of over 0.1 mM were added into each flask for biotransformation after the target protein expression of *E. coli* IBL16M2, IBL16M3, *E. coli* IBL16M6 and *E. coli* IBL16M6S74 was induced by IPTG at 25 °C ±1 for approximately 6 h. After cultivation for an additional 24 h under the same conditions, the cultures were extracted twice with half volume of 100% ethyl acetate (Sinopharm Chemical Reagent Co., Ltd.) to recover the metabolic products. The extracts were evaporated to dryness by vacuum and then re-dissolved in 1 ml anhydrous ethanol and detected by high-performance liquid chromatography (HPLC) and liquid chromatography/mass spectrometry (LC/MS). The biotransformation of each substrate by *E. coli* JM109 (DE3) and/or *E. coli* JM109 (DE3)-pET22b as control was conducted as well under the same conditions.

2.7. DNA electrophoresis and SDS-PAGE

DNA electrophoresis for the test of recombinant plasmids and products of PCR was performed in agarose gels at 110 V for 30 min [15]. SDS-PAGE with a 15% (w/v) acrylamide gel for protein identification was run at 110 V for 1.5 h and the bands of proteins were visualized by Coomassie R-250 staining [3].

2.8. HPLC and LC/MS

The operational conditions for HPLC were optimized to identify the biotransformation products. Substrates and products after biotransformation were qualitatively and quantitatively analyzed by comparing the retention time and the peak area with corresponding standard compounds under several operational conditions. In the process of HPLC, a sample of 10 μl was loaded onto a 250 mm Symmetry C18 column (4.6 mm × 250 mm, Waters Co., USA) and eluted with a mixture of the following reagents: methanol: 0.1 M NH_4COOH: anhydrous acetic