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Cloning and expression of a Baeyer–Villiger monooxygenase oxidizing linear aliphatic ketones from *Dietzia* sp. D5

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ABSTRACT

A Baeyer–Villiger monooxygenase has been identified in the genome sequence of *Dietzia* sp. D5. Sequence similarity search revealed that the enzyme belongs to a group of BVMOs that are closely related to ethionamide monooxygenase from *Mycobacterium tuberculosis* (EthA). The BVMO was expressed in *E. coli* BL21-CodonPlus(DE3)-RP and the best expression was achieved when the *E. coli* cells were cultivated in terrific broth (TB) at 15 °C and induced with 0.1 mM of IPTG. Since the purified enzyme did not show any measurable activity, the substrate scope of the BVMO has been determined using whole-cell and crude cell extract systems. The enzyme was most active towards linear aliphatic substrates. However, it has shown a moderate degree of conversion for cyclobutanone, 2-methylcyclohexanone, bicyclo[3.2.0]hept-2-en-6-one, phenylacetone and thioanisole. There was no detectable conversion of ethionamide, cyclohexanone and acetophenone.

1. Introduction

Baeyer–Villiger monooxygenases (BVMOs) catalyze the oxidation of ketones and heteroatoms (i.e. sulphur, nitrogen, phosphorous, boron and selenium) to lactones and oxides, respectively [1]. BVMOs are considered promiscuous enzymes which oxidize a wide variety of substrates. Among the ketones, small and bulky cyclic ketones, as well as ketones in multicyclic structures and linear aliphatic ketones with and without aromatic substituents can be oxidized by BVMOs. However, each BVMO presents a well defined substrate preference and can oxidize only a limited number of substrates. A systematic investigation and classification of the substrate scope of some BVMOs have been recently reported [2].

BVMOs can also be categorized on the basis of their primary sequence as Type I, Type II and Type O [3]. Among these three groups, the most studied BVMOs are Type I BVMOs which contain FAD as a prosthetic group and are NADPH dependent [4]. Several reports have suggested a further division of Type I BVMOs based on the degree of sequence similarity [5–7]. One of such subdivisions contains ethionamide monooxygenase (EthA) and other enzymes having close sequence similarity with EthA. EthA monooxygenase was isolated from *Mycobacterium tuberculosis* H37Rv and was identified to be responsible for the activation of the pro-drug ethionamide into the bio-active sulfoxide intermediate [8,9]. In addition to ethionamide, the enzyme is active towards aliphatic ketones, such as 2-octanone and 2-decanone, and phenylacetone [9]. Besides EthA monooxygenase, *M. tuberculosis* H37Rv has two other monooxygenases, MO0565c and MO3083, which are known to catalyze the sulfoxidation of thioanisole and MO3083 can also oxidize 2-octanone and bicyclohept-2-en-6-one [10]. Among the 23 BVMOs from *Rhodococcus jostii* RHA1, only two, MO13 and MO16, share significant similarity with EthA monooxygenase. MO16 oxidizes different substrates (cyclobutanone, 2-metylcyclopentanone, bicyclohept-2-en-6-one and 2-octanone) but the substrate scope of MO13 is not yet available [5,7]. A BVMO from *Pseudomonas putida* KT2440 is the other characterized enzyme that shows sequence similarity to EthA monooxygenase; the enzyme oxidizes 4-decanone with the highest efficiency [11]. A recent report shows the presence of

Abbreviations: BVMO, Baeyer–Villiger monooxygenase; BVMO3, *Dietzia* sp. D5 BVMO; EthA, ethionamide monooxygenase; MO0565c and MO3083, *Mycobacterium tuberculosis* BVMOs; MO13 and MO16, *Rhodococcus jostii* BVMOs; BVMO Pp, *Pseudomonas putida* BVMO; BVMO Ar, *Acinetobacter radioresistens* BVMO; BVMO Php, *Physcomitrella patens* BVMO; BVMO Cm, *Cyanidioschyzon merolae* BVMO; BVMO Pf, *Pseudomonas fluorescens* DSM 50106 BVMO; BVMO Pv, *Pseudomonas veronii* MEK700 BVMO; AFL 210, AFL 456 AFL 619 and AFL 838 *Aspergillus flavus* BVMOs; GDH, glucose dehydrogenase.

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another BVMO oxidizing ethionamide from *Acinetobacter radioresistens* [12].

Recently, we have sequenced the genome of a strain of *Dietzia*. Four BVMOs have been identified from the draft genome sequence and one of the BVMOs has been characterized and shown to react with sulfides and aldehydes with rare regiospecificity [13,14]. One of the *Dietzia* sp. D5 BVMOs, named BVMO3, is closely related to EthA monooxygenase and in this paper, we report the cloning, expression and substrate scope of this BVMO.

2. Experimental

2.1. Organisms, plasmids and chemicals

E. coli BL21-CodonPlus(DE3)-RP and ArcticExpress(DE3)-RP were purchased from Agilent Technologies (Santa Clara, USA). *E. coli* NovaBlue, *E. coli* BL21(DE3), *E. coli* Rosetta2(DE3) and the plasmid pET-22b(+) were purchased from Novagen (Darmstad, Germany). QIAGEN Plasmid Mini Kit and QIAEX II Gel Extraction Kit (Qiagen, Sollentuna, Sweden) were used to extract plasmids from cells and DNA from agarose gel, respectively. Genomic DNA was extracted using ZR Fungal/Bacterial DNA MiniPrep (Zymo Research, Irvine, USA) from *Dietzia* sp. D5 which was isolated in our laboratory.

All chemicals used in the study are of the highest available purity obtained from standard sources.

2.2. Sequence analysis

The multiple sequence alignment analysis of BVMO3 and the other EthA-like monooxygenases was done using the T-coffee algorithm with default parameters and the output figure was prepared using CLC Main Workbench. The multiple sequence alignment was used to derive the phylogenetic tree using Clustal W2 and visualization was obtained using FigTree.

The secondary structures of the BVMOs were predicted using the software CLC Main Workbench.

The sequence of BVMO3 has been deposited in GenBank database under the accession number AHE80562.

2.3. Cloning of BVMO3 gene

The gene encoding BVMO3 was amplified from the genomic DNA of *Dietzia* sp. D5 using a pair of primers, BVMO3-F: ATTACCATGGCTGGTAGCACCCACCTC and BVMO3-R: ATTACTCGAGTGATCGGGCCACCTCGTC, which were designed based on the BVMO3 gene sequence identified in the draft genome sequence. The forward and reverse primers had NcoI and XhoI (underlined) restriction sites, respectively. High Fidelity PCR enzyme mix (Fermentas, Gothenburg, Sweden) was used to amplify the gene following the manufacturer instructions. DMSO (5% v/v) was added to the PCR mix to improve the amplification. After purification with Qiagen PCR cleaning kit, the PCR product was digested with NcoI and XhoI and ligated to the expression vector pET-22b(+) digested with the same restriction enzymes. The ligation product was transformed into electrocompetent *E. coli* NovaBlue cells and spread on Luria Bertani (LB) agar plates containing ampicillin. Colonies were picked from the agar plates and recombinant plasmids were extracted and sequenced. The plasmid containing the correct sequence was transformed into the expression hosts *E. coli* BL21(DE3), *E. coli* BL21-CodonPlus(DE3)-RP and *E. coli* ArcticExpress(DE3)-RP.

2.4. Protein expression and purification

The recombinant *E. coli* cells were cultivated using low salt LB (tryptone 10 g, yeast extract 5 g, NaCl 5 g per litre), Terrific Broth (TB, composed of tryptone 12 g, yeast extract 24 g, glycerol 4 ml, filtered solution of 0.17 M KH_2PO_4 and 0.72 M K_2HPO_4 100 ml per litre) and M9 medium (1 M $MgSO_4$ 2 ml, 20% glucose 20 ml, 1 M $CaCl_2$ 0.1 ml, M9 salts 200 ml per litre), respectively. The M9 salts comprised $Na_2HPO_4{\cdot}7H_2O$ l64 g, KH_2PO_4 15 g, NaCl 2.5 g and NH_4Cl 5 g and water to a final volume of 1 l. The media when required contains 100 μg/ml ampicillin, 34 μg/ml chloramphenicol, and/or 20 μg/ml gentamycin.

Cultivation of *E. coli* for the recombinant protein expression was initiated by inoculating the culture media with a pre-inoculum corresponding to 5% of the final culture volume of 200 ml in 1 l flask. After 3 h of cultivation at 30 °C, the OD_{600} was about 0.6 and the protein expression was induced adding IPTG or lactose to final concentration of 0.1 mM and 10 mM respectively. During the expression phase the cells were cultivated at 15 °C for 16 h with shaking at 150 rpm. The culture was divided into five aliquots of equal volume that were harvested by centrifuging for 10 min at 4 °C and 9820 × *g* using Sorvall RC5C centrifuge. Then each aliquot was re-suspended in different buffers. The buffers used were: 50 mM sodium phosphate buffer pH 7.5, 100 mM potassium phosphate buffer pH 7.5, 100 mM potassium phosphate buffer pH 7.5 with 0.1% Triton X-100 (v/v), 100 mM potassium phosphate buffer pH 7.5 with 10% glycerol (v/v) and 100 mM potassium phosphate buffer pH 7.5 with 1 g/L Bovine Serum Albumin (BSA). The cell suspension was placed on ice and lysed in three intermittent cycles of 45 s sonication (Hierscher UP400S Ultrasonicator; amplitude 50%, cycle 0.5) with 1 min break. The sonicated cell suspension was centrifuged for 15 min at 4 °C and 15,000 × *g* to remove cell debris and the clear supernatant was used as the enzyme source.

The BVMO was purified from the clear supernatant using immobilized metal ion affinity chromatography on a Ni^{2+} bound column HisTrap™ FF (GE Healthcare, Uppsala Sweden) and eluted with an imidazole gradient from 0 to 300 mM in 100 mM potassium phosphate buffer pH 7.5 containing 200 mM NaCl and 10% glycerol. After protein elution, FAD was added to the fraction containing the purified BVMO3. Finally, desalting and concentration was performed using Vivaspin 20 MWCO 30,000 Da centrifugal concentrators (Sartorius Stedim Biotech GmbH, Goettingen, Germany).

The activity of the recombinant enzyme in the crude cell extract and in the purified form was followed by measuring the conversion of 25 mM 2-nonanone by gas chromatography as described below. One enzyme unit is expressed as the amount of enzyme that oxidizes one μmol of 2-nonanone per minute under the standard assay conditions.

2.5. Biotransformations using whole cells and crude cell extract

Whole-cell biotransformations were performed using both growing and resting cells. For growing cells, 2 ml TB medium in a 15 ml Falcon tube was inoculated with a pre-culture of *E. coli* BL21-CodonPlus(DE3)-RP cells containing the recombinant plasmid with the BVMO gene. The cells were grown for 3 h at 30 °C and with shaking at 150 rpm. Immediately after adding IPTG, the biotransformation reaction was initiated by adding the substrate to a final concentration of 5 mM. To boost the regeneration of NADPH, glucose (25 mM) was also added at the moment of induction. Biotransformations were carried out for 16 h at 15 °C and at a shake rate of 150 rpm. The level of conversion was measured by comparing *E. coli* BL21-CodonPlus(DE3)-RP expressing BVMO3 with cells from the same *E. coli* strain carrying pET-22b(+) without the BVMO3 gene, that served also as a blank for side reactions.

Biotransformation with resting cells was done using a culture grown as described above in Section 2.4. At the end of the overnight expression, the cells were harvested by centrifugation and re-suspended in an equal volume of 20 mM sodium phosphate buffer