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Enzymatic hydrogenation of diverse activated alkenes. Identification of two *Bacillus* old yellow enzymes with broad substrate profiles

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ABSTRACT

By whole cell transformation, 32 out of 71 strains showed OYEs activity toward maleimide in the first round screening. Among them, a *Bacillus* strain was identified to be active toward a selection of substrates with different electron-withdrawing groups. Two OYE homologous genes, *bac-oye*1 and *bac-oye*2 were cloned from this strain and overexpressed in *Escherichia coli* BL21(DE3). The recombinant enzyme Bac-OYE2 showed a broader pH range (6.0–10.5), while Bac-OYE1 was so sensitive to pH that it lost most of the enzyme activity below pH 6.0 or above pH 9.0. The reaction temperature exerted similar effects on the activities of both enzymes, but the stability of Bac-OYE2 was more sensitive to the temperature than Bac-OYE1. In addition to α,β-unsaturated aldehydes, ketones, nitroalkenes, and the double activated carboxylic acids, esters, nitriles and cyclic imides, Bac-OYE1 and Bac-OYE2 also exhibited activities toward the "borderline" substrates such as unsaturated lactones, mono carboxylic esters, showing their broader substrate scopes. These enzymes also had excellent enantioselectivity as evidenced by the reductions of several α,β-unsaturated cyclic ketones, α-substituted α,β-unsaturated carboxylic esters and 2-methyl maleimide. For example, methyl 2-acetamidoacrylate was reduced by Bac-OYE1 with >99% conversion and >99% ee.

1. Introduction

In recent years, enone/enoate-reductases (also called "Old Yellow Enzyme, OYE) have been attracting the interest of chemists for its high potential in creating two stereogenic centers in asymmetric reduction of C=C bonds. The OYE-catalyzed asymmetric reduction proceeds via hydride transfer from the N_5 of reduced FMN onto C_β of the substrate with a solvent-derived proton being transferred to C_α, and finally results in a *trans*-fashion addition of the [^{2}H] [1,2], which is contrary to the *cis*-hydrogenaration by transition-metal-based homogeneous catalysts. The *trans*-hydrogenation function makes these enzymes important complementary tools for existing chemical catalysts.

It has been found that OYE-like enzymes could catalyze the reduction of a variety of activated alkenes, including α,β-unsaturated aldehydes, ketones, nitroalkenes, carboxylic acids and derivatives (esters, lactones, cyclic imides, cyclic acid anhydrides) [3–6] and are widely distributed in biological sources, including plants [7,8], bacteria [9], fungi [10,11], and protozoa [12]. However, there was no single conserved physiological role has been attributed until now. It was suggested that a general role of OYEs was the detoxification of a broad spectrum of electrophilic compounds [13]. Studies suggested that the physiological roles of OYE-containing organisms may resulted from their specific metabolic requirements and environmental pressures [14]. For example, OYE from *Arabidopsis* leaves metabolized α,β-unsaturated carbonyl compounds produced during insect attract and bacterial pathogenesis [15].

Because of the exquisite stereoselectivity and broad acceptance of various activating groups of OYE-like enzymes, some novel OYEs have been discovered to expand the tool-box [16–19]. However, many of the reported enzymes have either low activity or low stereoselectivity toward some specific substrates [20–22]. Therefore, it is necessary to seek new OYEs with broad substrate profile. As

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such, the available strains stored in our laboratory were screened in two rounds with maleimide and a set of α,β-unsaturated compounds with diverse structural features as substrate, respectively, and a *Bacillus* strain was identified. Two OYE genes from this strain were cloned and overexpressed. The recombinant enzymes showed activity toward a diversity of substrates, especially for the less activated alkenes (or called "borderline substrates") such as methyl acrylate.

2. Materials and methods

2.1. Chemicals

All of the chemicals were commercially available, except for racemic (5*S*)-2-methyl-5-(prop-1-en-2-yl) cyclohexanone (P3), dimethyl 2-methylsuccinate (P15), methyl 2-acetamidopropanoate (P16) and 3-methyl-2,5-Pyrrolidinedione (P30), which were obtained by Pd/C-catalyzed hydrogenation of (*S*)-carvone (S3), dimethyl itaconate (S15), methyl 2-acetamidoacrylate (S16) and 3-methyl maleimide (S30), respectively [23].

*Nde*I, *Xho*I and *Nco*I were purchased from Fermentas (Germany). Solution I ligase was purchased from Takara (Japan). Taq DNA polymerase was purchased from TIANGEN (Beijing, China). KOD DNA polymerase was purchased from TOYOBO (Japan). InsTAclone PCR cloning kit was from Thermo scientific (USA). Expression vectors, pET32a(+) and pET28a(+), were purchased from Novagen (Merck, Germany). Host cells, *Escherichia coli* Top10 and *E. coli* BL21(DE3) were stored in our laboratory.

2.2. Screening of microorganisms for ene-reductase

Seventy-one strains, which were isolated from contaminated soils, were stored in 15% glycerin in our laboratory. For cultivation, the complete medium (pH 7.0) containing 1.5% glucose·H_2O (*w*/*v*), 0.5% peptone (*w*/*v*), 0.5% yeast extract (*w*/*v*), 0.1% NaCl (*w*/*v*), 0.1% KH_2PO_4 (*w*/*v*), 0.1% $K_2HPO_4{\cdot}3H_2O$ (*w*/*v*) and 0.05% $MgSO_4{\cdot}7H_2O$ (*w*/*v*) was used and 0.5% of the strains were inoculated into 50 mL complete medium. All cultures were shaken at 30 °C for 24 to 48 h at 200 rpm and the cells were harvested by centrifugation at 12,000 × *g* for 10 min.

Maleimide was used in the first round screening, and 2-cyclohexen-1-one, *trans*-2-Hexen-1-al, crotonic acid, *trans*-β-nitrostyrene, methyl 2-acetamidoacrylate, dimethyl itaconate and benzylidenemalononitrile were used in the second round screening. For the first round screening, the resting cells (200 mg mL^{-1} wet cell) and substrate maleimide (1 g L^{-1}, final concentration) were mixed in 1 mL of phosphate buffer (50 mM, pH 7.0) containing 0.5% (*w*/*v*) glucose [18,24]. The resulting mixtures were incubated at 30 °C for 96 h at 200 rpm, 0.9 mL of ethyl acetate was added to extract the products and unconverted substrates. Organic extracts were dried with anhydrous Na_2SO_4, and analyzed by thin layer chromatography (TLC) with ethyl acetate and petroleum ether (1/2, *v*/*v*) as mobile phase and chiral GC analysis, which was performed on an Agilent 7890 gas chromatography equipped with FID detector, CP ChiraSil DEX Column (25 m × 0.25 mm × 0.25 μm, Varian, USA) and He as carrier gas (2 mL min^{-1}), and using the following column temperature program: 10 °C min^{-1} from 100 to 180 °C, 180 °C for 5 min. Standard product succinimide was used as reference.

2.3. 16S rDNA sequencing determination

Genomic DNA of functional strain WX16 was extracted according to the protocol of TIANamp Bacteria DNA Kit (Tiangen Biotech, China). The 16*S* rDNA was amplified by PCR with Taq polymerase using universal primers 27F (5′-AGAGTTTGATCCTGGCTCA-3′) and 1492R (5′-GGTTACCTTGTTACGACTT-3′) and then sequenced at BGI (China). The PCR was performed with the following conditions: 95 °C for 3 min, one cycle; 94 °C for 30 s/55 °C for 30 s/72 °C for 90 s, 30 cycles; 72 °C for 10 min, one cycle. The 16*S* rDNA sequence was submitted to Genbank of NCBI for BLAST search. A phylogenetic tree was constructed with the top 10 strains of the blast result by using ClustalX (Version 1.83) and MEGA (Version 4.0) based on the homologous 16*S* rDNA sequences.

2.4. Cloning of ene-reductase genes

The *Bacillus* ene-reductase genes were amplified by two rounds of PCRs without whole genomic sequence. Round 1: The nucleotide sequence of the NADH: flavin oxidoreductase from *Bacillus vallismortis* DV1-F-3 (NCBI reference sequence: NZ_JH600221.1) was chosen as template for primers design, which were Bac-F: 5′-ATGGCCAGAACATTATTCACAC-3′ and Bac-R: 5′-TTACCAGCCTCTCTCATATTG-3′. With Genomic DNA of strain WX16 as template, PCR was performed with KOD Plus Polymerase under the following conditions: 94 °C for 2 min, one cycle; 94 °C for 20 s/50 °C for 30 s/68 °C for 70 s, 30 cycles; 68 °C for 5 min, one cycle. The products were performed a A-addition reaction before ligating onto pTZ57R/T vector by using Ex Taq Polymerase at 72 °C for 30 min. The constructed vector were transformed into *E. coli* Top10 for TA cloning and then sequenced.

Round 2: Nucleotide sequences of NADPH dehydrogenase NamA (NCBI reference sequence: AGF27164) and putative NADH-dependent flavin oxidoreductase (NCBI reference sequence: AGF27122.1) from *Bacillus amyloliquefaciens* IT-45 were used as template to redesign primers for the amplifications of *bac-oye*1 and *bac-oye*2, respectively. The primers were as follows: for *bac-oye*1, F: 5′-ATGGCGAGAAAATTATTCACACCATGGAC-3′ and R: 5′-TCACCATGCTCTGTCGTATTGGACCG-3′; for *bac-oye*2 F: 5′-ATGAAGCAAACTTATAAACCGCTGTTTGAAC-3′ and R: 5′-CTACTTTTCAAACGGCACCCAGCCG-3′. PCRs were performed by following the protocol described above except that the annealing temperatures were 52 °C for both *bac-oye*1 and *bac-oye*2. The two nucleotide sequences of *bac-oye*1 and *bac-oye*2 were deposited in the GenBank database with accession no. KJ577134 and KJ577135, respectively.

For the sake of heterologous expression, the encoding genes of Bac-OYE1 and Bac-OYE2 were re-amplified from the recombined pTZ57R/T vectors with the following primers: for Bac-OYE1 gene, the primers were *bac-oye*1-F, 5′-CATG*CCATGG*GG**CATCATCATCATCATCAC**GCGAGAAAATTATTCACACC***G***TGGAC-3′ and *bac-oye*1-R: 5′-CCG*CTCGAG*TCACCATGCTCTGTCGTATTGGACCG-3′ with *Nco*I and *Xho*I as restriction sites (italic) and N-terminal His-tag (bold); for Bac-OYE2 gene, primers were *bac-oye*2-F: 5′-CGC*CATATG***CACCATCATCATCATCAT**AAGCAAACTTATAAACCGCTGTTTGAAC-3′ and *bac-oye*2-R: 5′-CCG*CTCGAG*CTACTTTTCAAACGGCACCCAGCCG-3′ with *Nde*I and *Xho*I as restriction sites (italic) and N-terminal His-tag (bold) [9]. The 25th nucleotide A of *bac-oye*1 was changed into G (bold and italic) without amino acid replacement in order to produce the recognition sequence of *Nco*I. The open reading frames of *bac-oye*1 and *bac-oye*2 were ligated into pET28a(+) and pET32a(+), respectively.

2.5. Expression and purification of recombinant enzymes

The recombinant plasmids were transformed into *E. coli* BL21(DE3) cells. Expressions of the genes were induced using 0.1 mM isopropyl-β-D-1-thiogalactopyranoside (IPTG) at 37 °C when the optical density at 600 nm (OD_{600}) was 0.6–0.8. Cells harvested by centrifugation were suspended in 20 mM Tris–HCl (pH 7.4, containing 250 mM NaCl) and then broken with high pressure