Chinese Journal of Catalysis 37 (2016) 1396–1402

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

Article

Characterization of a novel marine microbial esterase and its use to make D-methyl lactate

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ARTICLE INFO

Article history:
Received 25 May 2016
Accepted 22 June 2016
Published 5 August 2016

Keywords:
Esterase
Marine microorganism
Biocatalysis
Kinetic resolution
D-methyl lactate
Hydrolysis

ABSTRACT

A novel marine microbial esterase PHE14 was cloned from the genome of *Pseudomonas oryzihabitans* HUP022 isolated from the deep sea of the western Pacific Ocean. Esterase PHE14 exhibited very good tolerance to most organic solvents, surfactants and metal ions tested, thus making it a good esterase candidate for organic synthesis that requires an organic solvent, surfactants or metal ions. Esterase PHE14 was utilized as a biocatalyst in the asymmetric synthesis of D-methyl lactate by enzymatic kinetic resolution. D-methyl lactate is a key chiral chemical. Contrary to some previous reports, the addition of an organic solvent and surfactants in the enzymatic reaction did not have a beneficial effect on the kinetic resolution catalyzed by esterase PHE14. Our study is the first report on the preparation of the enantiomerically enriched product D-methyl lactate by enzymatic kinetic resolution. The desired enantiomerically enriched product D-methyl lactate was obtained with a high enantiomeric excess of 99% and yield of 88.7% after process optimization. The deep sea microbial esterase PHE14 is a green biocatalyst with very good potential in asymmetric synthesis in industry and can replace the traditional organic synthesis that causes pollution to the environment.

1. Introduction

The enantiomers of drugs have dramatically different biological activities and toxicities. Chiral drugs are more clinically acceptable as they reduce the potential toxicity of racemic drugs. Chiral intermediates are crucial building blocks for the synthesis of valuable chiral drugs and the synthesis of the chiral intermediates of drugs is important in the pharmaceutical industry [1–3].

Chiral α-hydroxy acids, e.g. chiral lactic acid, are important building blocks in the synthesis of many drugs, pesticides and polymers [4,5]. The preparation of chiral lactic acid is of great importance in both the pharmaceutical and material industries. The synthesis of chiral chemicals like chiral lactic acid is not easily achieved by traditional organic synthesis due to the low enantio-selectivity of traditional metallic organic synthesis [5].

The synthesis of enantiomerically enriched chemicals can also be achieved by biocatalytic methods using enzymes, which

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This work was supported by the Strategic Priority Research Program of the Chinese Academy of Sciences (XDA11030404), the National Natural Science Foundation of China (21302199), Guangzhou Science and Technology Plan Projects (201510010012) and Key Project from the Chinese Academy of Sciences (KGZD-EW-606).
DOI: 10.1016/S1872-2067(16)62495-6 | http://www.sciencedirect.com/science/journal/18722067 | Chin. J. Catal., Vol. 37, No. 8, August 2016

avoid the harsh working conditions and pollution by metallic organic synthesis [6,7]. Due to the existence of one hydroxy group and one acid group in lactic acid, the synthesis of enantiomerically enriched lactic acid can be accomplished by the asymmetric reduction of ketone precursors using dehydrogenases. However, the biocatalytic method using dehydrogenases require expensive co-factors such as NADH and NADPH [8]. Another biocatalytic method is by asymmetric hydrolysis of racemic esters using esterases or lipases. L-lactic acid is not expensive because currently L-lactic acid can be directly obtained using large scale fermentation instead of an organic or enzymatic method [9,10]. D-lactic acid, which is a chiral chemical of great demand in industry, cannot be directly obtained using the inexpensive fermentation method and is thus more expensive [4]. Biocatalytic methods are the major methods for the preparation of D-lactic acid and its ester derivatives with high enantiomeric excess.

Here, we identified a novel marine microbial esterase, PHE14, from the deep seas of the western Pacific Ocean and characterized its functionalities. We also utilized esterase PHE14 as a biocatalyst for the enzymatic synthesis of D-methyl lactate. The desired chiral product D-methyl lactate was obtained with high enantiomeric excess and yield after process optimization.

2. Experimental

2.1. Microorganisms and reagents

E.coli DH5α and *E.coli* BL21 (DE3) were used as the host strains for gene cloning and protein expression, respectively. Plasmid pET-28a (+) (Novagen, USA) was used as the expression vector of the enzymes. TransStart® FastPfu DNA PolyMerase, ligases and restriction enzymes were all purchased from TransGen Biotech (Beijing, China). *p*-nitrophenyl (*p*-NP) esters were obtained from Sigma (St. Louis, MO, USA). Racemic methyl lactate and the corresponding chiral enantiomers were all purchased from Aladdin Industrial Corporation (Shanghai, China). Other chemicals were analytical grade.

2.2. Gene cloning and expression vector construction

The strain *Pseudomonas oryzihabitans* HUP022 was isolated from the sediments obtained from the deep sea of the western Pacific Ocean. The sequencing of the whole genome of *Pseudomonas oryzihabitans* HUP022 was completed by Genewiz Inc. The coding DNA sequence of an esterase (named PHE14) was amplified from the genomic DNA of *Pseudomonas oryzihabitans* HUP022 with a pair of primers (forward: 5′-CACGAATTCGTGCTGGAATCGCCTAGC-3′, reverse: 5′-CCGCTCGAGTTATTTTTTGCCGAGACGTGCC-3′). The primer sequences contained the *EcoR* I and *Xho* I (underlined) restriction sites. The PCR products were cloned into the *EcoR* I and *Xho* I sites of the expression vector pET-28a (+). Recombinant plasmids were confirmed by sequencing from Majorbio Ltd. (Shanghai, China) and then transformed into *E. coli* BL21 (DE3) according to standard protocols.

2.3. Expression and purification of PHE14

The *E. coli* and recombinant *E. coli* strains were incubated at 37 °C and 200 r/min in Luria-Bertani medium (recombinant strains with 50 μg/mL kanamycin). As the cell density reached a turbidity of 0.6 at 600 nm, 0.3 mmol/L isopropyl-beta-D-thiogalactopyranoside (IPTG) was added to the culture for protein induction. After 18 h induction at 20 °C, the cells were harvested by centrifugation at 7500 r/min for 10 min, washed with phosphate buffer (50 mmol/L, pH 7.5) two times and then re-suspended in the same buffer. The cells were disrupted by sonication on ice for 10 min and centrifuged (11500 r/min, 20 min, 4 °C). The recombinant protein PHE14 was purified and desalted using nickel-nitrilotriacetic acid agarose resin (GE Healthcare Life Science, China) and PD-10 desalting columns (GE Healthcare Life Sciences, China) according to the manufacturer's instructions. The protein concentration was measured by the method of Bradford, with bovine serum albumin as the standard. Purified PHE14 was examined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) under denaturing conditions.

2.4. Enzyme assay

The hydrolytic activity of PHE14 was measured at 405 nm using the assay procedure described by Deng et al [11]. A standard reaction system containing 10-μL substrate (10 mmol/L, dissolved in acetonitrile), 10-μL (0.8 μg/μL) enzyme, 940-μL phosphate buffer (50 mmol/L, pH 7.5), and 40-μL ethanol was incubated at 35 °C for 5 min. The esterase activity was analyzed by detecting the absorbance of *p*-NP at 405 nm. One unit of enzyme activity was defined as the amount of esterase that liberated 1 μmol of *p*-NP per minute.

2.5. Biochemical characterization of purified PHE14

The substrate specificity was determined using different substrates (*p*-NP C2-C10) under standard reaction conditions. The optimum pH for PHE14 was determined using *p*-PN acetate (C2) as the substrate in different pH ranges: 50 mmol/L NaAc/HAc (pH 5.0–6.0), 50 mmol/L Na_2HPO_4/NaH_2PO_4 (pH 6.5–8.0), 50 mmol/L Tris/HCl (pH 8.5–9.0) and 50 mmol/L Glycine/NaOH (pH 9.5–10.0). The pH stability was characterized by incubating purified PHE14 at 4 °C for 12 h in the buffer with different pH values. The optimum temperature was measured for the temperature range of 20 to 70 °C under the optimal pH value. The thermo-stability was measured by incubating PHE14 at different temperatures (from 20 to 60 °C) for different times (0–60 min) using *p*-PN acetate (C2) as the substrate under optimal pH value. The effect of metal ions (Li^+, Na^+, K^+, Mg^{2+}, Ca^{2+}, Fe^{2+}, Ni^{2+}, Mn^{2+}) on the activity of PHE14 was determined by incubating PHE14 in the presence of the metal ions (1 mmol/L) for 12 h at 4 °C. The effects of different organic solvents and surfactants were investigated in the presence of thirteen different organic solvents (10%, *v*/*v*)and four different surfactants (1%) by incubating PHE14 for 12 h at 4 °C. The effect of salt concentration on PHE14 activity was studied by