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Overexpression of *Serratia marcescens* lipase in *Escherichia coli* for efficient bioresolution of racemic ketoprofen

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Abstract

Lipase from *Serratia marcescens* ECU1010 was cloned and overexpressed in *E. coli*. After optimization, the maximum lipase activities reached 5000–6000 U/l and this recombinant lipase could enantioselectively hydrolyze (*S*)-ketoprofen esters into (*S*)-ketoprofen. Among six alkyl esters of racemic ketoprofen investigated, this lipase showed the best enantioselectivity for the kinetic resolution of ketoprofen ethyl ester, with an ee_p (enantiomeric excess of product) of 91.6% and *E*-value of 63 obtained at 48.2% conversion. Twelve nonionic surfactants were tested for enhancing the enantioselectivity of this lipase in the bioresolution of ketoprofen ethyl ester. A very high *E*-value of 1084 was achieved, with an optical purity of >99% ee_p and a yield of 42.6% in the presence of 3% Brij 92V. Further studies showed that the selectivity of the lipase was improved with the increase of Brij 92V concentration. The substrate (ketoprofen ethyl ester) does not inhibit the lipase activity, while the product (*S*)-ketoprofen inhibits the lipase activity to some extent. These results indicate that the *S. marcescens* lipase is very useful for biocatalytic production of chiral profens such as (*S*)-ketoprofen.

Keywords: *Serratia marcescens* lipase; Chiral resolution; Ketoprofen; Surfactant; Overexpression

1. Introduction

Lipases (EC 3.1.1.3) are ubiquitous enzymes that catalyze the hydrolysis of fats and oils, playing important roles in pharmaceutical, fine chemical industries and other industrial areas such as detergents, oil/fats, cheese making, hard-surface cleaning, leather and paper processing, etc. [1,2]. Among many successful examples of lipases application, the lipase from *Serratia marcescens* (SmL) is well-known in pharmaceutical industry for its excellent enantioselectivity in biocatalytic hydrolysis of *trans*-3-(4′-methoxyphynyl)glycidic acid methyl ester [(±)-MPGM] to produce (2*R*, 3*S*)-3-(4′-methoxyphenyl)glycidic acid methyl ester [(−)-MPGM], a key intermediate for the synthesis of diltiazem hydrochloride [3]. Recently, Bae et al. [4] have reported that a lipase from *S. marcescens* ES-2 was used for kinetic resolution of racemic flurbiprofen, giving an optically pure (*S*)-flurbiprofen (98.5% ee) with a very high enantioselectivtiy ($E = 332$). Jaeger et al. [5] tried to use the lipase from *S. marcescens* SM6 for the kinetic resolution of other racemic esters such as isopropylideneglycerol acetate in organic solvent but the reactions were failed. The lipase gene from *S. marcescens* Sr41 had been cloned by screening a DNA library encoding a protein of 613 amino acids [6]. The lipase gene from *S. marcescens* SM6 and *S. marcescens* ES-2 was also cloned and then overexpressed in *E. coli* [5,7,8].

Ketoprofen or 2-(3′-benzoylphenyl)propionic acid, belonging to the family of 2-arylpropionic acids (profens), is one kind of the nonsteroidal anti-inflammatory drugs. Its (*S*)-enantiomer has the therapeutic activity of reducing inflammation and relieving pains, while the (*R*)-enantiomer shows the gastrointestinal side effect and toxicity [9]. Various efforts have therefore been

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made to obtain optically pure (*S*)-ketoprofen. Lipases from *Candida rugosa* (CrLs) and *Acinetobacter* sp. ES-1, and many other microbial esterases such as esterases from *Trichosporon brassicae* and *Pseudomonas* sp. S34 were used for kinetic resolution of racemic ketoprofen to produce (*S*)-ketoprofen [10–18].

Recently, we have found a bacterial strain *S. marcescens* ECU1010 being able to enantioselectively hydrolyze (±)-MPGM to give a (−)-isomer with a high enantiomeric ratio ($E > 100$) [19]. In the present research, we have cloned and overexpressed the lipase gene (*lip*A) from *S. marcescens* ECU1010 in *E. coli* and firstly report the application of this lipase for the production of optically pure (*S*)-ketoprofen and the positive effect of a nonionic surfactant on the enantioselectivity of this lipase.

2. Experimental

2.1. Chemicals

Ketoprofen was provided by Xi'nan Synthetic Pharmaceutical Factory, Chongqing, China. The racemic ethyl and 2-chloroethyl esters of ketoprofen were prepared using the method described by Moreno and Sinisterra [20]. The surfactant OP-10 was bought from Tianjin Tiantai Reagent Co., China. Triton X-45, Triton X-114 and Tween-80 were from Shanghai Dazhong Pharmaceutical Factory, China. Other surfactants were from Fluka, Switzerland. All other chemicals were also obtained commercially and of analytical grade.

2.2. Bacterial strains, plasmids, and culture conditions

S. marcescens ECU1010 [19] was used as the lipase gene source for the cloning experiment. *E. coli* DH5α and BL21 (DE3) were employed as host strains in the gene manipulation and protein expression, respectively. The plasmids used for the cloning and protein expression were pMD18-T vector (Takara, Dalian, China) and pET-24a (+) (Novagen), respectively.

S. marcescens ECU1010 was grown at 30 °C in a nutrient broth (0.3% beef extract, 0.5% peptone and 0.5% NaCl). Wild-type and recombinant *E. coli* cells were cultured regularly in a Luria–Bertani (LB) medium at 37 °C, and an appropriate amount of ampicillin (25 μg/ml) or Kanamycin (25 μg/ml) was added for recombinant *E. coli* cells when needed.

Isopropyl-β-D-thiogalactopyranoside (IPTG) was added to the culture media or solid plates when needed. The cell growth was determined by measuring the optical density at 600 nm using a spectrophotometer.

2.3. DNA manipulations, sequence determination and analysis

DNA manipulations and transformations were carried out according to standard procedures [21]. The nucleotide sequence was determined by the dideoxy chain termination method [22]. Nucleotide and amino acid data were compared with annotated sequences from various genomes and protein data banks at the National Center for Biotechnological Information (NCBI).

2.4. Cloning and overexpression of lipA gene in E. coli

A 2.2-kb DNA fragment was amplified by PCR (using Pfu polymerase, Takara, Dalian, China) containing the intact *lip*A with the chromosomal DNA from *S. marcescens* ECU1010 as template. Primers were designed according to the reported *lip*A sequences from *S. marcescens* Sr41 and *S. marcescens* SM6 [6,7]: forward, 5′-CCG CATACCAATAACGTTTCATCA-3′; reverse, 5′-CAGCAGTGGTTCCGCCTTCGCAAG-3′. After adenine (A) were added to the two blunt ends, the PCR fragment was ligated to a plasmid of pMD18-T vector (Takara, Dalian, China) and then transformed into *E. coli* DH5α. Transformants were screened on tributyrin plates [23] and one lipase-producing clone, carrying a plasmid designated as pBCLipC1, which contained a 2.2-kb inserted fragment, was selected by clear-halo formation after 24 h of incubation at 37 °C. Nucleotide sequencing was performed on both strands using an automatic sequencer (ABI PRISM^{TM} 3730 × L, Perkin-Elmer). Double-stranded DNA or PCR-amplified fragments were used as the template with either universal or synthetic primers when needed.

The *lip* gene was reamplified by PCR using Pfu DNA polymerase (Takara, Dalian, China) and a combination of forward (5′-ACT*CATATG*GGCATCTTTAGCTATAAGGATC-TG-3′) and reverse (5′-TGC*AAGCTT*TTAGGCCAACACCA-CCTGATCGGT-3′) primers, where the underlines represent the NdeI and HindIII sites, respectively, and the ATG codon for the initiation of the translation and the sequence complementary to the termination codon TAA are shown in italics. The plasmid (pBCLipC1) was used as a template in this experiment. The resultant 1.8-kb DNA fragment was digested with NdeI and HindIII and ligated to the large NdeI-HindIII fragment of plasmid pET-24a (+) to create an overexpression plasmid for the *lip* gene. The resulting plasmid was designated as pBCLipE1 and an overproducing strain was constructed by transforming *E. coli* BL21 (DE3) with this recombinant plasmid.

2.5. Protein induction and expression

All liquid cultures were grown in a shaker with a shaking speed of 150 rpm. A single bacterial colony was inoculated into 4 ml of LB medium containing 25 mg/l Kanamycin at 37 °C. The overnight culture (0.6 ml) was diluted into 30 ml of a fresh LB medium containing Kanamycin in a 250-ml flask and incubated at 37 °C until turbidity of the culture reached an OD_{600} of between 0.7 and 0.8. The cell culture was induced by the addition of an inducer, either IPTG or lactose.

2.6. Enzyme assay and SDS-PAGE

The cell cultures (20 ml) were harvested by centrifugation at 4000 × *g* for 10 min, and the cell pellet was washed twice with a 0.85% NaCl solution. The cell pellet was then re-suspended in 2 ml of 50 mM Tris–HCl (pH 8.0) and was lysed by sonication. The cell lysate was centrifuged at 15,000 × *g* for 30 min. The protein expression was determined by gel electrophoresis of cell-free extract and cell debris on a SDS-PAGE as described by Laemmli [24]. Both pellet resuspension and cell-free extract