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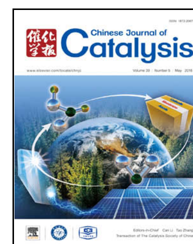

Article

Cloning, overexpression, and characterization of a novel organic solvent-tolerant lipase from *Paenibacillus pasadenensis* CS0611

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We found a novel lipase gene in the *Paenibacillus pasadenensis* CS0611 strain. The lipase gene sequence was cloned into the pET-28a expression vector to construct a recombinant lipase protein containing 6 × His tags at the C- and N-termini, respectively. High-level expression of the lipase in *E.coli* BL21 (DE3) was obtained upon induction with IPTG at 20 °C. The recombinant lipase activity was approximately 1631-fold higher than the wild type. His-tagged recombinant lipase was purified rapidly and efficiently by using Ni-charged affinity chromatography with 63.5% recovery and a purification factor of 10.78. The purified lipase was stable in a broad range of temperatures and pH values, with the optimal temperature and pH being 50 °C and 7.0, respectively. Its activity was stimulated to different degrees in the presence of metal ions such as Ca^{2+}, Mg^{2+}, and some non-ionic surfactants. In addition, the purified lipase was activated by a series of water-miscible organic solvents such as some short carbon chain alcohols and was highly tolerant to some water-immiscible organic solvents.

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1. Introduction

Lipases (E.C 3.1.1.3) catalyze the hydrolysis of medium- and long-chain triglycerides, and a major characteristic is the sharp activation at the interface generated by a water-insoluble lipid substrate in aqueous solution [1]. Lipases can also catalyze ester synthesis and transesterification reactions with high regional and stereoselectivity in nonaqueous solvent systems [2–4]. These features allow lipases to be the most widely used enzymes in synthetic detergent additives, fine chemicals, and for precursors of chiral pharmaceutical and agrochemical production [5–7].

Organic solvents are usually utilized instead of water in some lipase-catalyzed reactions. There are many advantages that anhydrous organic solvents can offer, such as shifting the equilibrium to the synthetic direction, controlling or modifying enzyme selectivity by solvent engineering, increasing the solubility of substrates and the recovery of products in the organic phase, improving the thermal stability of enzymes, and suppressing undesirable water-dependent side reactions [8,9]. However, organic solvents affect the activity and stability of enzymes to different degrees. Therefore, lipases that show high activity and stability in organic solvents are of interest [10–13].

At present, research on microbial lipases has mainly focused on strains with industrial application value such as *Rhizopus*, *Aspergillus*, *Candida*, *Pseudomonas*, and *Bacillus* [14–16]. There

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are few studies and reports on lipases from *Paenibacillus*. *P. pasadenensis* CS0611 was previously isolated from soils around chitin biological production factories in Shandong China and identified based on morphological characterization and 16s rDNA sequence analysis. The present paper describes the cloning, heterogeneous expression, and purification of a novel organic solvent-tolerant lipase from this strain. Consequently, detailed enzymatic properties of the recombinant enzyme were studied.

2. Experimental

2.1. Bacterial strains, plasmids, enzymes, and reagents

P. pasadenensis CS0611 was isolated by our group previously and stored at the China Center for Type Culture Collection (CCTCC M2014458). KOD FX (Toyobo, Japan) was used for PCR; pET-28a vector (Novagen, Germany) was used for the cloning, sequencing, and expression experiments; *E. coli* BL21 (DE3) was used as the expression host. Restriction enzymes Fast Digest Sal I and BamH I, T4 DNA ligase, and DNA and protein markers were purchased from Thermo Fisher Scientific (Wilmington, DE, USA). Synthesis of the primers and DNA sequencing were completed by Invitrogen (USA). The kits used in the construction of recombinant plasmids were purchased from Generay (Shanghai, China). Substrates (*p*-nitrophenyl fatty acid esters with varying acyl chain lengths), iso-pro-pyl-β-D-thiogalactopyranoside (IPTG), and kanamycin were purchased from Sigma-Aldrich (St. Louis, MO) or Aladdin (Shanghai, China). All other reagents and solvents were of analytical grade and used without further purification.

2.2. Cloning and sequencing of gene lp2252

The cloning and transformation of the target gene were according to the methods described by Sambrook et al [17]. *P. pasadenensis* CS0611 was grown in Luria-Bertani (LB) medium (1% tryptone, 0.05% yeast extract, 1% NaCl) at 37 °C for about 15 h, and the genomic DNA was extracted and purified using a bacterial genomic DNA Kit according to the manufacturer's instructions. Two primers F1(5'-CGCGGATCCATGCGGAAGCAAAGCGAAAAGGA-3') and R1(5'-GCGTCGACAGAGTTTGCATAAATCCACATCTTGACCG-3') were designed based on the nucleotide sequences immediately upstream and downstream the known coding sequences of lp2252 and introduced BamH I and Sal I restriction sites in the 5' and 3' ends of the complete gene, respectively (underlined sequences correspond to the restriction sites). The amplification of the lp2252 gene was carried out by KOD FX DNA Polymerase using genomic DNA as a template. The PCR amplification was performed with the following cycles: one cycle of pre-denaturation at 94 °C for 2 min; 30 cycles of 98 °C for 10 s (denaturation), 45 °C for 30 s (annealing), and 68 °C for 1.5 min (extension); followed by one cycle of final extension at 68 °C for 7 min. The purified PCR product was digested with BamH I and Sal I, and inserted into the expression vector pET-28a previously digested with the same restriction enzymes to obtain the recombinant plasmid pET-28a-lp2252. The recombinant plasmid was transformed into *E. coli* BL21 (DE3) competent cells using heat shock. The transformed cells were plated on LB agar (1% tryptone, 0.05% yeast extract, 1% NaCl, 2% agar) containing kanamycin (50 µg/mL) and incubated at 37°C overnight. The positive transformed colonies were confirmed by sequencing after the plasmid extraction (using a high purity plasmid preparation kit according to manufacturer's instructions).

2.3. Expression and purification of recombinant lp2252

The recombinant cells were cultivated in 20 mL LB medium containing 50 µg/mL of kanamycin at 37 °C, 180 r/min for 12 h. A 250-mL flask containing 100 mL LB broth medium supplemented with 50 µg/mL kanamycin was inoculated with 1% (*v/v*) of pre-cultured cells described above. Different concentrations of IPTG were added as an inducer at different OD600 values, and the cultivation continued at different temperatures to determine the optimum induction conditions. Recombinant cells were harvested by centrifugation (8000 r/min, 5 min) at 4 °C. The cell pellet was suspended in 20 mmol/L phosphate buffer, pH 7.5 (10 mL). After ultrasonic disruption and centrifugation, the supernatant was filtered through a membrane filter with a pore size of 0.45µm and applied to a Bio-Scale™ Mini Nuvia ™ IMAC Ni-charged column (5 mL, Bio-Rad, USA). The column was washed with 10 volumes of distilled water. After equilibration with 10 volumes of binding buffer (20 mmol/L phosphate buffer, 500 mmol/L NaCl, and 10 mmol/L imidazole, pH 7.5), 5 mL of the crude enzyme preparation was loaded onto the column. The column was washed with 20 volumes of washing buffer (20 mmol/L phosphate buffer, 500 mmol/L NaCl, and 20 mmol/L imidazole, pH 7.5). The target protein was eluted with a 20–500 mmol/L imidazole gradient at a flow rate of 0.3 mL/min with elution buffer (20 mmol/L phosphate buffer, 500 mmol/L NaCl, and 500 mmol/L imidazole, pH 7.5). The active fraction was collected and concentrated with an ultra-filtration membrane (10 kDa, Millipore, Billerica, MA). The purity of lp2252 was analyzed by sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE) as described by Sambrook et al. [17].

2.4. Enzyme and protein assays

Lipase activity was determined using *p*-nitrophenyl fatty acid esters as substrates [18]. The standard assay reaction mixture contained 20 mmol/L phosphate buffer (400 µL, pH 7), 10 mmol/L *p*-NPC_{16} dissolved in isopropanol (50 µL), and purified enzyme (50 µL). 500 µL of 0.5 mol/L Na_2CO_3 was added to the reaction mixture after 5 min at 50 °C to terminate the reaction. One unit (U) of enzymatic activity is defined as the amount of enzyme required to produce 1 µmol of *p*-nitrophenyl per min (ε410 nm = 0.016 µL/mol). Unless specially mentioned, all measurements of lipase activity were conducted under standard conditions. Protein concentration was determined using the Bradford method with bovine serum albumin as the standard [19]. All assays were performed in triplicate and average

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