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Biochemical properties and potential applications of an organic solvent-tolerant lipase isolated from *Serratia marcescens* ECU1010

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Abstract

An extracellular lipase was purified to homogeneity with a purification factor of 5.5-fold from a bacterial strain *Serratia marcescens* ECU1010. The purified lipase is a dimer with two homologous subunits, of which the molecular mass is 65 kDa, and the p*I* is 4.2. The pH and temperature optima were shown to be pH 8.0 and 45 °C, respectively. Among *p*-nitrophenyl esters of fatty acids with varied chain length, the lipase showed the maximum activity on *p*-nitrophenyl myristate (C_{14}). The lipase was activated by some surfactants such as Gum Arabic, polyvinyl alcohol (PVA) and Pg350me, but not by Ca^{2+}. The enzyme displayed pretty high stability in many water miscible and immiscible solvents. This is a unique property of the enzyme which makes it extremely suitable for chemo-enzymatic applications in non-aqueous phase organic synthesis including enantiomeric resolution. Several typical chiral compounds were tested for kinetic resolution with this lipase, consequently giving excellent enantioselectivities ($E = 83 \sim >100$) for glycidyl butyrate (GB), 4-hydroxy-3-methyl-2-(2-propenyl)-2-cyclopenten-1-one acetate (HMPCA), naproxen methyl ester (NME) and *trans*-3-(4′-methoxyphenyl) glycidic acid methyl ester (MPGM).

Keywords: Lipase; *Serratia marcescens*; Organic solvent tolerance; Enantiomeric resolution; Chiral compounds; Biochemical properties

1. Introduction

Lipases or triacylglycerol hydrolases (E.C. 3.1.1.3) are enzymes that hydrolyze ester bonds of triglycerides at oil–water interface. In recent years, lipases have emerged as key enzymes in swiftly growing biotechnology, owing to their multifarious properties [1–3]. The main application fields of lipase include detergents, dairy, diagnostics, oil and lipid processing, and biotransformation. Recently, special emphasis is lying on the production of chiral chemicals which serve as basic building blocks in the production of pharmaceuticals and agrochemicals [4].

Substrates of lipase are often insoluble or partially soluble in water and thus the use of organic solvents or organic–aqueous solutions is in favor of some reactions. Use of organic solvents also provides many advantages [5], including: (1) relatively high solubility of substrates; (2) relative ease of products recovery in organic phase; (3) possibility of reducing the degree of undesirable substrate and/or product inhibition in organic solvent-water two-phase systems; and (4) ability to shift the reaction equilibrium toward synthetic direction by continuously removing products with organic solvents in biphasic systems.

Unfortunately, despite the advantages that biocatalysis in organic solvent-based systems can bring, the catalytic activities of enzymes in these systems are typically much lower than those in aqueous solutions [6]. Furthermore, in an apparent paradox, protein stability is lower in water-miscible solvents than in hydrophobic solvents. The poor stability in hydrophilic solvents represents a problem for the use of lipases in reactions involving the hydrolysis of esters because of the production of alcohol or acid. Therefore, it is anticipated that the solvent-tolerant enzyme will be applicable and beneficial as catalysts for reactions in the presence of organic solvents.

In our laboratory, an extracellular lipase produced by *Serratia marcescens* ECU1010 was shown to be a potentially useful biocatalyst for the kinetic resolution of *trans*-3-(4′-methoxyphenyl)glycidic acid methyl ester [(±)-MPGM], as reported previously [7].

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To date, although there were some reports about organic solvent-tolerant lipases, such as lipase from *Bacillus* species 205y [5] and lipase from *Bacillus megaterium* CCOC-P2637 [8], no report is available on the organic solvent-tolerant lipase by *Serratia* strains. In this paper, we report the results on the lipase purification and its properties, especially the enzyme stability against various organic solvents. In addition, the lipase was supposed to be useful for biocatalytic resolution of some chiral compounds based on our previous results on enatioselective hydrolysis of (±)-MPGM [7] and ketoprofen [9]. Therefore, further utilization of this lipase for kinetic resolution of more chiral esters was also attempted, affording similar or better enantioselectivities than those reported with other lipases.

2. Materials and methods

2.1. Materials

All kinds of *p*-nitrophenyl esters [10] were prepared from *p*-nitrophenol and various acids. Naproxen methyl ester [11], *trans*-3-(4′-methoxyphenyl)glycidic acid methyl ester [(±)-MPGM] [12], and glycidyl butyrate [13] were also synthesized in our laboratory as described previously.

All other chemicals were purchased from various commercial sources and with the highest purity available. DEAE-Toyopearl 650M and Phenyl-Toyopearl 650 M were from Tosoh (Tokyo, Japan). Sephadex G150 was from Pharmacia Fine Chemicals Co. Ultrafiltration membrane system was from Millipore Co.

The strain of *S. marcescens* ECU1010 used in this work was a Gram-negative bacterium stored in our laboratory [7] and currently also deposited at China General Microbiology Collection Center, with an accession number of CGMCC No. 1219. The culture medium was designed according to a reference medium [7]. The culture broth was centrifuged to obtain a clear supernatant, which was used subsequently for enzyme purification.

2.2. Preparation of purified lipase

The culture supernatant was concentrated from 1.5 l to 300 ml using an ultrafiltration membrane system (membrane pore size: 30 kDa) after centrifugation of the culture broth at 12,000 rpm for 10 min. Solid powder of ammonium sulfate was slowly added to the concentrated culture supernatant to 35% saturation and then the solution was gently stirred for 1 h. After standing overnight, the resultant precipitate was collected through a micro-filtration membrane (Ø 50 μm). Then the precipitate was dissolved in Buffer A (Tris–HCl, 10 mM, pH 7.5) and applied to a preequilibrated DEAE-Toyopearl 650 M column (Ø 2.5 cm × 30 cm), eluted with NaCl gradient from 0.1 to 0.5 M. The fractions with lipase activity were collected, combined, and concentrated to about 1.0 ml by ultrafiltration with Amicon® Ultra-15 Centrifugal Filter Devices (Millipore). The concentrated solution was added to a Sephadex G150 column (Ø 1.6 cm × 100 cm) which has been preequilibrated with the Buffer A. After elution, the fraction possessing the highest activity was pooled, mixed with $(NH_4)_2SO_4$ up to 2% saturation, and put onto a Phenyl-Toyopearl 650 M column (Ø 0.8 cm × 10 cm), already preequilibrated with Buffer A containing $(NH_4)_2SO_4$ at 2% saturation. The bound protein was eluted from the column with Buffer A, then mixed with lactose, and lyophilized to form a powder. The powder was used for all the following experiments.

2.3. Assay of lipase activity

Lipase assay method: lipase was assayed using *p*-nitrophenyl butyrate (*p*NPB) as the substrate. Lipase or blank solution (100 μl) was added to 2.870 ml of 100 mM potassium phosphate buffer (KPB, pH 7.0). After preincubation at 30 °C for 3 min, the reaction was initiated by a quick mixing of the reaction mixture with 30 μl of 100 mM *p*NPB solution in dimethyl sulfoxide (DMSO) and the variation in absorbance at 405 nm was recorded. One unit of lipase activity was defined as the amount of enzyme releasing 1.0 μmol of *p*-nitrophenol per minute under such conditions.

Hydrolytic activities on various *p*-nitrophenyl esters were measured by a modified lipase assay method. The *p*-nitrophenyl butyrate was replaced with various *p*-nitrophenyl esters, respectively. Particularly, for *p*-nitrophenyl laurate, myristate and palmitate, lipase or blank solution (100 μl) was added to 2.570 ml of 100 mM potassium phosphate buffer (KPB, pH 7.0). After preincubation at 30 °C for 3 min, the reaction was initiated by a quick mixing of the reaction mixture with 300 μl of 10 mM *p*-nitrophenyl laurate, myristate or palmitate solution in DMSO and the variation in absorbance at 405 nm was recorded.

2.4. Protein concentration and molecular mass determination

Protein concentration was determined according to Bradford method [14] using bovine serum albumin as a standard.

The molecular mass of the lipase subunit was measured using the method of Laemmli [15] in a 10% (w/v) polyacrylamide slab gel. The molecular mass of the native lipase was measured by gel filtration with TSK-GEL G3000SWXL (30 cm × 7.8 mm I.D., 5 μm particles) using FPLC as recorded previously [16]. The reference proteins were: rabbit actin 43 kDa, bovine serum albumin 67 kDa, rabbit phosphorylase b 97 kDa, calmodulin-binding protein 130 kDa, and myosin 200 kDa.

2.5. Activity staining

The activity staining of lipase was carried out according to the reference [17]. After electrophoresis, the gel was soaked in 100 mM Tris–HCl buffer (pH 7.5) containing 0.5% Triton X-100 for 1 h in order to exchange the SDS with Triton X-100. By incubating the gel for 30 min in a 1:1 mixture of Solution A (where 8 mg of 1-naphthyl acetate was dissolved in 2 ml acetone and then mixed with 18 ml of 100 mM Tris–HCl buffer, pH 7.5) and Solution B (20 mg of Fast red TR salt suspended in 20 ml of 100 mM Tris–HCl buffer, pH7.5), the red bands corresponding to the lipase appeared against a transparent background.

2.6. Two-dimensional electrophoresis (2-DE)

Two-dimensional electrophoresis was performed as described previously [16].

2.7. Effect of temperature and pH on the lipase activity and stability

The powder of the purified lipase was dissolved with Tris–HCl buffer (100 mM, pH 7.5), and 100 μl aliquots (8.8 U/ml, on *p*NPB) were withdrawn and tested for activity at desired temperatures or pH as described for the lipase assay.

For stability tests, 0.5 ml aliquots (25 U/ml, on *p*NPB) of the above lipase was incubated at the desired temperatures or pH for 1 h. After that the residual activity was tested with the lipase assay method.

2.8. Effect of metal ions and surfactants on the lipase stability

After incubating a lipase solution (25 U/ml on *p*NPB) with various 1 or 10 mM metal ions at 30 °C for 1 h, the lipase activity was measured as the lipase assay, using the Tris–HCl buffer (100 mM, pH 7.5) instead of potassium phosphate as reaction buffer.

Incubating a lipase solution (25 U/ml on *p*NPB) with various surfactants at 30 °C for 1 h, then the lipase activity was measured as the lipase assay.

2.9. Effect of various organic solvents on the lipase stability

Effect of various organic solvents at concentrations of 10% (v/v) (0.1 ml of organic solvent plus 0.9 ml of the purified enzyme solution with 30 U on *p*NPB) and 50% (v/v) (1 ml of organic solvent plus 1 ml of the enzyme solution with