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Biochemical properties of free and immobilized *Candida viswanathii* lipase on octyl-agarose support: Hydrolysis of triacylglycerol and soy lecithin

Alex Fernando de Almeida[a], César Rafael Fanchini Terrasan[b], Cárol Cabral Terrone[b], Sâmia Maria Tauk-Tornisielo[c], Eleonora Cano Carmona[b,*]

[a] Bioprocess Engineering and Biotechnology, Federal University of Tocantins – UFT, Rua Badejós, Chácaras 69/72, Zona Rural, Gurupi, Tocantins, 77402-970, Brazil
[b] Biochemistry and Microbiology Department, Bioscience Institute, São Paulo State University, IB/UNESP, Av. 24-A, 1515, Bela Vista, Rio Claro, São Paulo, 13506-900, Brazil
[c] Environmental Studies Center, São Paulo State University, CEA/UNESP, Av. 24-A, 1515, Bela Vista, Rio Claro, São Paulo, 13506-900, Brazil

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ABSTRACT

Microbial lipases are important enzymes in food and pharmaceutical industries. In this work, a *Candida viswanathii* lipase was purified by hydrophobic interaction chromatography on octyl Sepharose. The purification presented 78.4% yield and the enzyme was 8.7-fold purified, with specific activity 700.4 U/mg protein and 69 kDa molecular weight. Immobilization of the enzyme on the same support presented 72.5% yield and a derivative with 101% expressed activity (109.2 U/g support), indicating hyperactivation of the enzyme. Optimal activity for both free and immobilized lipase was observed at pH 4.0 and 45 °C. The free and immobilized lipase showed broad-range stability from acid to neutral pH, and apparent activation and stability on organic solvents. The derivative was 60-fold thermostabilized in relation to the free enzyme and fully retained its activity after four cycles of *p*-nitrophenyl palmitate hydrolysis. Slight activation was observed with dithiothreitol and *β*-mercaptoethanol. The free and immobilized lipase efficiently hydrolyzed monoesters, simple and mixed long chain triacylglycerols, as well as soy lecithin. The activity and stability in acid pH, the organic solvent tolerance and the lecithin hydrolysis indicate high potential application of the enzyme and its derivative in textile, food and pharma industries and for chemical synthesis.

1. Introduction

Microbial lipases are excellent biocatalysts with important properties such as high specificity and selectivity, promoting reactions under mild conditions [1]. These enzymes are used in numerous industrial applications, including detergent formulation for the degradation of oils and fats, synthesis of pharmaceuticals and esters responsible for flavor and taste, as well as in cosmetics [2]. Lipases used in processes such as biodiesel production, oil and fats hydrolysis, effluent treatment and detergent production may be used as heterogeneous or partially purified mixtures, while food, pharmaceutical and cosmetic industries require purified preparations [3].

Lipases (triacylglycerol acyl hydrolases, E.C. 3.1.1.3) naturally catalyze the hydrolysis of ester bonds from long-chain triacylglycerols in organic-aqueous interfaces by a peculiar mechanism, the so-called interfacial activation. In homogenous media, most lipases have their active center covered by a polypeptide chain called lid, which isolate the molecule from the reaction medium (closed form); in the presence of hydrophobic surfaces, however, the enzyme becomes adsorbed on it, changing the structure to the open form in which the active center become fully exposed, allowing the lipases to hydrolyze drops of oils [4]. Their physiological role is to catalyze hydrolysis and sequential synthesis of triacylglycerols, providing diacylglycerol, monoacylglycerol, free fatty acids and glycerol [5]. The equilibrium displacement towards hydrolysis or synthesis is controlled by the amount of water in the reaction medium, *i.e.*, in water presence hydrolysis is the main reaction, while esterification occurs in the presence of organic solvents [6].

Apart from the inherent solubility of the proteins, lipases usually present low stability even at mild conditions, and the high cost for a single use limits therefore its industrial application. Enzyme immobilization may provide an effective method to circumvent these issues not only by improving catalytic properties and operational stability of enzymes, but also by facilitating multiple reuse and ease separation of the biocatalyst, enabling continuous industrial operation [7]. The choice of an immobilization technique for successful lipase

* Corresponding author.
E-mail address: ecarmona@rc.unesp.br (E.C. Carmona).
URL: http://mailto:ecarmona@rc.unesp.br (E.C. Carmona).

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immobilization should consider several concerns: the enzyme should be stable during the immobilization process; the procedure should be robust, reproducible, cost-effective and scalable; the materials and equipment should apply to food grade regulations if the immobilized lipase is intended to be used in food applications; the immobilization should preferably stabilize the enzyme in terms of temperature, pH, organic solvents and operational reusability; and the biocatalyst should be physically robust and rather applicable in both batch and fixed bed processes [8].

Immobilization of lipases using interfacial adsorption on hydrophobic supports has been proposed as a simple method to prepare robust derivatives [9,10]. This method take into account a simple adaptation of the Michaelis-Menten-Henri kinetic model to the interfacial hydrolysis of oil drops, in which the first step correspond to the fixation of a water-soluble enzyme to the lipid-water interface via a reversible adsorption-desorption mechanism. The penetration/adsorption step lead to a more favorable energy state of the enzyme present at the interface, which binds to substrate molecule, resulting in the formation and subsequent dissociation of the enzyme-substrate complex [11,12]. By using hydrophobic supports that resembles the surface of natural substrates and very low ionic strength, lipases can be selectively immobilized on the support. This mechanism proved to be advantageous allowing immobilization of lipases *via* an "affinity-like" strategy that can also significantly enhances enzyme activity [10]. Manoel et al. [4] immobilized lipases from *Thermomyces lanuginosus* and *Pseudomonas cepacea* on octyl and cyanogen bromide agarose, confirming that the octyl agarose derivatives present their open form stabilized while the covalent preparation maintains the closing/opening equilibrium.

Lipases from the *Candida* genus, including those from *Candida rugosa*, *Candida antarctica*, *Candida cylindracea*, present molecular weight ranging from 33 to 64 kDa and multiple isoforms [13–16]. These lipases normally show similar biochemical properties such as optimal activity in the pH range 7.0–8.0 and at 30–60 °C [13–16]. Lipases from *C. antarctica* (CALB), *C. rugosa* and *Mucor miehei* were immobilized by adsorption on hydrophobic supports as a quick and inexpensive alternative to obtain purified samples, rendering high yield and, in some cases, activation of the enzyme [17]. Adsorption technique allows the regeneration of the support, easing enzyme immobilization, and reducing costs and industrial wastes [18].

A *Candida viswanathii* strain grows and efficiently produces lipase when cultivated in liquid medium containing natural triacylglycerols, under agitation and temperature control [19,20]. Partial characterization showed that the crude enzyme is an acid and organic solvent tolerant lipase that could potentially be applied in bioprocesses. The aims of this study were to purify and immobilize this lipase on hydrophobic support. The purified free-enzyme and the derivative were biochemically characterized and its potential for hydrolyzing triacylglycerol and soy lecithin were also evaluated.

2. Materials and methods

2.1. Strain and maintenance

C. viswanathii strain is available in the Culture Collection of the Environmental Studies Center − CEA/UNESP, Brazil. *C. viswanathii* was routinely cultivated on malt extract agar (MEA) for 3 days at 28 °C and then stored at 4 °C. Cultures were also performed in MEA slants at the same conditions for inoculum preparation.

2.2. Liquid culture and protein extract preparation

Modified Vogel liquid medium [21] was prepared using 1.5% (w/v) olive oil and 0.2% (w/v) yeast extract as carbon and nitrogen sources, respectively. Erlenmeyer flasks (125 mL) containing 25 mL of the medium were inoculated with 1.0 mL of cells suspension (1×10^7 cells/mL). Cultivation was carried out at 27.5 °C, 210 rpm for 72 h [20].

Biomass was removed by filtration using 0.45 μm cut-off cellulose acetate membrane followed by centrifugation (8500 *g*, 20 min, 4 °C). The supernatant was used as source of lipase for purification and immobilization experiments.

2.3. Lipase activity

Lipase activity was assayed with *p*-nitrophenyl palmitate (p-NPP) (Sigma-Aldrich) as substrate [20]. *p*-NPP was initially dissolved in 0.5 mL of dimethyl sulfoxide (DMSO), then diluted to 0.5 mM with McIlvaine buffer pH 3.5 containing 0.5% (w/v) Triton X-100. The hydrolysis of *p*-NPP was determined discontinuously by measuring the released *p*-nitrophenolate (*p*-NP) at 40 °C. After 5 min pre-incubation of 0.9 mL of the substrate solution in a water bath, the reaction was started by adding 0.1 mL of appropriately diluted enzyme solution. The reaction was stopped after 1 and 2 min by heat shock (1 min, 90 °C), followed by the addition of 1 mL of saturated sodium tetraborate solution. The absorbance was read at 405 nm and the activity was determined using a *p*-NP standard curve ($\varepsilon = 1.8 \times 10^4\ M^{-1}\ cm^{-1}$). Controls were prepared without enzyme. One enzyme unit (U) was defined as the amount of enzyme that releases 1 μmol of product per min.

2.4. Protein

Protein was determined with bicinchoninic acid (BCA) [22], using bovine serum albumin as standard. Protein was followed by reading absorbance at 280 nm during purification chromatography.

2.5. Purification

The crude extract was previously dialyzed against 0.05 M ammonium acetate buffer pH 6.9 (8 h, 3 changes, 4 °C). The dialyzed extract was applied to a hydrophobic octyl column (Hiprep™ 16/10 Octyl Sepharose FF fast flow, GE Healthcare) previously equilibrated in the same buffer; at 2 mL/min flow rate. The column was washed with 50 mL of the same buffer and 3.0 mL fractions were collected. Elution of bounds proteins was performed with 100 mL of a 0.0–1.0% (w/v) Triton X-100 linear gradient prepared in the same buffer. Fractions with lipase activity were pooled and sample purity was evaluated by SDS-PAGE. All purification procedures were carried out at 4 °C.

2.6. Immobilization

The crude extract was previously dialyzed against 0.05 M ammonium acetate buffer pH 6.9 (8 h, 3 changes, 4 °C). The dialyzed extract was applied to 10 g of octyl Sepharose Fast Flow (GE Healthcare) previously packed in a column (1.5 × 12.0 cm) and equilibrated in the same buffer; at 2 mL/min flow rate. The column was washed with 50 mL of this buffer and the resin containing the immobilized lipase (derivative) was manually collected and stored at 10 °C.

Immobilization efficiency was presented by the efficiency factor (η):

$$\eta = \frac{V_{immobilized}}{V_{free}}$$

where, $V_{immobilezed}$ is maximum reaction velocity of the derivative and V_{free} is maximum reaction velocity of the free enzyme.

2.7. Derivative reuse

Successive cycles of 0.5 mM *p*-NPP hydrolysis were carried out in batch mode using McIlvaine buffer pH 3.5 at 40 °C. After each 1 min-cycle, the immobilized lipase was recovered from the reaction medium by centrifugation (8000 *g*, 10 min, 4 °C) and abundantly washed with 0.05 M ammonium acetate buffer pH 6.9. Then, fresh reaction medium