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Modulation of the activity and selectivity of the immobilized lipases by surfactants and solvents

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ABSTRACT

Most of lipases are in equilibrium between a majority inactive closed form and a minority active open form in aqueous media. Perhaps, a certain stabilization of these open forms of lipases could be achieved in the presence of cosolvents or surfactants in the reaction medium. Three commercial lipases were studied (from *Thermomyces lanuginosa* (TLL), *Candida Antarctica* fraction B (CALB) and Lecitase (LEC)). Different derivatives were tested: TLL and LEC were adsorbed on an anionic exchanger and their activity strongly depends on the equilibrium between their open and closed form and CALB was adsorbed on a hydrophobic support when the open form was already stabilized by the support. Derivatives ionically adsorbed were hyperactivated by surfactans as well as by cosolvents: the activity of LEC increased 12 times in the presence of 15–20% of ethanol. CALB adsorbed on hydrophobic supports was hardly hyperactivated and even it was inhibited. The modification of the rate of covalent modification of the catalytic Ser seems to confirm that the observed hyperactivations were due to a stabilization of the open form of the adsorbed lipases (TLL and LEC). The hydrolysis of sardine oil was also studied in the presence or absence of surfactants and cosolvents. An interesting improvement in the ability of derivatives to discriminate the release of eicosipentaenic acid (EPA) and docosahexaenicacid (DHA) was found.

1. Introduction

Enzymes are biological catalysts that act on the chemical transformations of biomolecules. They are able to catalyze many processes in the biologic cells and are essential to life. Industrial reactions catalyzed by enzymes occur under mild conditions with high degree of specificity, reducing the production of byproducts, which are undesirables many times. This makes them an interesting and important tool of biotechnological concernment [1]. Studies about the enzymatic technology emerged in the 1960s, with the immobilization of enzymes for chemical processes [2]. The advent of enzymology represents an important step for biotechnological industry. The enzymes market is dominated mainly by hydrolytic enzymes, such as proteases, amylases, amidases, esterases and lipases [3].

Lipases (triacylglycerol acylhydrolases, E.C. 3.1.1.3) are enzymes that catalyze the hydrolysis and synthesis of ester bonds [4,5]. These enzymes are attractive in organic chemistry due to their enantioselectivity, regioselectivity and stereoselectivity. These characteristics are interesting industrially, with many applications in the synthesis of organic compounds [6]. The lipases show an interfacial activation mechanism due to a hydrophobic amino acids chain that covers their active site, called lid, which confers the open–closed form of enzyme. In presence of interface polar–nonpolar, such as bubble of gas and oil drops, important conformational changes occur; the lid is dislocated and the enzyme shows its opened and active form [7–11].

An important application of lipases on the organic compounds synthesis is the production of long-chain polyunsaturated fatty acids (PUFAs), especially docosahexaenoic acid (DHA, C22:6$n-3$) and eicosapentaenoic acid (EPA, C20:5$n-3$), that exert

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important beneficial effects on the human health, mainly on fatal cardiovascular disease, and reduce the symptoms of rheumatoid arthritis. The oil fish is the main source of PUFAs compared to other oils commercials, with high degree of unsaturation, high content of long-chain omega-3 type PUFAs and a variety of fatty acids presents in the tryacilglycerol molecules [12,13].

The enzymes, in general, are advertised at a high cost, and the possibility of reuse becomes very interesting to cheapen the process. When the enzyme is immobilized on an inert support, it can be separated and reused many times, since that its enzymatic activity is monitored [14]. Furthermore, immobilization procedure may avoid some factors that decrease the activity of soluble enzyme, as aggregation of enzymes structure, inhibition by external factors, low stability, effect of medium partition, diffusional limitations and assists the selectivity [15]. However, the immobilization of enzymes is not always the better tool to increase the enzymatic activity, and other alternatives are required, such as compounds able to promote the enzymatic hyperactivation.

Due to the big non-polar surface of the lipases, they are easily immobilized on hydrophobic supports by selective adsorption. Hydrophobic supports are specifics to enzymes with a big chain of non-polar aminoacids, and they are applied in the immobilization, stabilization, hyperactivation and purification of lipases. There are many reports in literature that show the simplicity and effectiveness of the immobilization and purification process of these enzymes [16–20]. A particular behavior and a disadvantage of the lipases is their tendency to aggregation, and the formation of these dimmers affects negatively the enzymatic activity. This may be settled and the enzymatic activity can be increased by actuation of agents able to promote the dissociation of these molecules, for example, detergents [15,21].

In this paper, we have chosen three known lipases (from *Candida antarctica* fraction B (CaL-B), *Thermomyces lanuginose* (TLL) and Lecitase (LL)) and we have immobilize them by absorption on inert supports. The CaLB was immobilized by hydrophobic adsorption and Lec and TLL were immobilized on ionic supports. Besides the hyperactivation caused by immobilization process, we evaluated the effect of solvents and surfactants on the selectivity and activity of these immobilized lipases in the hydrolysis of a synthetic and natural substrates, increasing the hyperactivation of these immobilized enzymes.

2. Materials and methods

2.1. Materials

The lipases from *C. antarctica* fraction B (CaLB), *T. lanuginose* (TLL) and Lecitase (Lec), all the resins used as carriers, the substrate *p*-nitrophenyl butirate (*p*-NPB) and the inhibitor diethyl-*p*-nitrophenyl phosphate (D-*p*NP) were purchased from Sigma-Aldrich. Coomassie protein assay reagent was acquired from Thermo Scientific (USA). The fish oil was acquired in a local market and the other reagents used were of analytical grade.

2.2. Activity assay

Lipase activity was determined by measuring the increase in absorbance at 348 nm produced by the release of *p*-nitrophenol (*p*NP) from hydrolysis of 0.4 mM *p*NPB in 25 mM sodium phosphate buffer pH 7 at 25 °C, as described in the literature [9]. One international unit was defined as the amount of enzyme necessary to hydrolyze 1 μmol of *p*NPP per minute (IU) under the assay conditions described above.

2.3. Total protein determination

Protein concentration was determined by a modified Bradford's Method (1976). A standard curve was taken by adding a different concentration of BSA (bovine serum albumin) on Coomassie protein reagent, with an end volume at 1.0 mL. After 10 min of reaction between BSA and reagent, absorbance was measured at 595 nm.

2.4. Reversible immobilization

This method includes the hydrophobic adsorption in octyl-sepharose for CaL-B and ionic immobilization in PEI-sepharose for TLL and LL, with a load of 20 mg per gram of support and incubation at 10 mM sodium phosphate buffer pH 7 at 25 °C. Yield immobilization was determined by checking the lipase activity on the supernatant after 60 min of incubation. The amount of immobilized proteins was calculated after measuring protein concentration. Derivatives were washed with water and filtered under vacuum.

2.5. Solvent and surfactants effects on activity enzymatic

Activity of the derivatives was evaluated in the presence of organic solvents and surfactants, in order to know those effects on enzyme activity. Thus, 0.1 g of derivative was added to 1 mL of 10 mM sodium phosphate buffer pH 7 containing 0.05, 0.1 or 0.2% of surfactants (CTAB—cetyl trimethylammonium bromide, SDS—sodium dodecyl sulfate, Triton X-100) or 5, 10 or 15% of organic solvents (ethanol, dioxano, DMS—dimethyl sulfoxide). After 5 min of incubation, the enzymatic activity was determined. It was considered the activity of enzyme at absence of these agents as 100%.

2.6. Covalent inhibition of immobilized lipase

Covalent inhibition was performed by using more active derivatives. The derivatives were incubated for 30 min in presence of 1 μM of covalent inhibitor D-*p*NP, followed by the measurement of the enzymatic activity at 25 °C and 25 mM sodium phosphate buffer pH 7 using *p*-NPB as substrate.

2.7. Thermal inactivation of immobilized lipases

Thermal inhibition was performed with the incubation of immobilized enzymes for 6 h in 10 mM sodium phosphate buffer pH 7 at 55 °C and the activity was measured by hydrolysis of *p*-NPB.

2.8. Fish oil hydrolysis

The derivatives were used to hydrolysis reaction of sardine oil. The reaction was performed in an organic and aqueous two-phase system, with 4.5 mL of cyclohexane, 5.0 mL of 0.01 M sodium phosphate buffer at pH 7 and 0.5 mL of sardine oil. This system was incubated at 25 °C with 0.3 g of derivative (20 mg/g), and the reaction suspension was stirred at 150 rpm. Concentration of free fatty acids was determined at various times by HPLC (C8 column) using the mobile phase acetonitrile 80%, water 20% and 0.1% acetic acid with a flow rate of 1.0 mL/min. The elution was monitored by recording the absorbance at 215 nm and calculated rates of hydrolysis (EPA + DHA) and the EPA/DHA ratios.

3. Results and discussion

3.1. Effect of the solvents on immobilized lipases activity

Table 1 shows the derivatives used and the results of immobilization obtained after 60 min of incubation between the enzymes