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Improving the catalytic properties of immobilized Lecitase via physical coating with ionic polymers

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ABSTRACT

Lecitase Ultra has been immobilized on cyanogen bromide agarose (via covalent attachment) and on octyl agarose (via physical adsorption on the hydrophobic support by interfacial activation). Both immobilized preparations have been incubated in dextran sulfate (DS) or polyethylenimine (PEI) solutions to coat the enzyme surface. Then, the activity versus different substrates and under different experimental conditions was evaluated. The PEI coating generally produced a significant increase in enzyme activity, in some cases even by more than a 30-fold factor (using the octyl-Lecitase at pH 5 in the hydrolysis of methyl phenyl acetate). In opposition, the DS coating usually produced some negative effects on the enzyme activity. The rate of irreversible inhibition of the covalent preparation using diethyl p-nitrophenylphosphate did not increase after PEI coating suggesting that the increase in Lecitase activity is not a consequence of the stabilization of the open form of Lecitase. Moreover, the coating greatly increased the stability of the immobilized Lecitase, for example using DS and the covalent preparation, the half-life was increased by a 30-fold factor in 30% acetonitrile. The stabilizing effect was not found in all cases, in certain cases even a certain destabilization is found (e.g., octyl-Lecitase-DS at pH 7). Thus, the effects of the ionic polymer coating strongly depend on the substrate, experimental conditions and immobilization technique employed.

1. Introduction

Lecitase Ultra is an artificial chimera phospholipase A_1 developed mainly for degumming processes [1], although phospholipases A_1 have different uses in the industry [1–5]. This enzyme has been obtained from the fusion of the genes of the lipase from *Thermomyces lanuginosus* (to obtain good stability) and the phospholipase from *Fusarium oxysporum* (to get the phospholipase activity) [6]. In some aspects, this enzyme behaves as a standard lipase, with capacity to become adsorbed on hydrophobic surfaces at low ionic strength (e.g., hydrophobic supports) [7] and presenting a broad specificity [8–16].

Lipases properties (selectivity, specificity) have been modulated via biocatalyst design [17]. For example, during immobilization their features are greatly altered. Due to the interfacial activation mechanism [18,19], their active center is very flexible and by involving specific areas of the enzyme in the immobilization, its activity, stability and even specificity and selectivity may be greatly altered [7,20,21]. This has been explained by the alteration of the closing/opening mechanism of lipases, or just by distorting the flexible active center [17]. Chemical modification is also a powerful tool to tune lipase properties, with some reports in the literature showing the potential of this strategy [22]. Chemical modification can be easily performed in previously immobilized enzyme, taking the advantage of solid phase modification and the possibility of having an enzyme with improved stability [23], being Lecitase one of the examples of catalytic properties tuning via chemical modification [24].

Another less explored possibility to alter the performance of immobilized lipases may be the coating of the enzyme surface using ionic polymers, like polyethylenimine (PEI) or dextran sulfate (DS) [25–27]. The coating via ionic exchange only requires areas of the

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protein that are rich enough on the opposite ion to permit a certain multipoint ionic exchange polymer and enzyme, in fact supports coated with both polymers are able to adsorb a very high percentage of proteins (next to 90%) even at pH 7 [28,29]. Moreover, this rapid physical coating did not involve any chemical modification of the enzyme. In biocatalyst design, together with the activation of supports, these polymers have been employed to coat free enzymes with different purposes such as stabilization of multimeric structures or preventing interactions with surfaces [30–32]. In certain cases, stabilization of enzymes is achieved just by positive effects of PEI-enzyme interactions [33,34].

The interaction of the polymer with the protein may promote different effects that can drive to a final tuning of enzyme properties. For example, it may be possible that the overall hydrophilization of the enzyme surface alters the closing/opening equilibrium. Moreover, these polymers may generate some hindrances to the movements of the lid, avoiding the full opening or closing of the lid. It is also likely that the multipoint interaction of the polymer (despite being a flexible molecule) with the enzyme surface produces some distortion of the enzyme structure. Furthermore, these polymers have been described as to be able to generate a hydrophilic micro-environment surrounding the immobilized enzyme molecules, which may also affect the enzyme performance under different circumstances (e.g., favoring partition of solvent, oxygen, etc.) [35–37]. The polymer effects can be different depending on its cationic or anionic nature, because of the area involved in the polymer/enzyme interactions. Moreover, the immobilization protocol can change the polymer effect, since different areas of the enzyme will be exposed to interaction. However, this was not hitherto studied.

Thus, in this paper we will show the changes in Lecitase Ultra activity, specificity and stability after coating with two different polymers, DS or PEI. In order to check the effect of the immobilization protocol on the modulation of enzyme features via polymer coating, we have used an immobilization strategy where the open form of the enzyme is stabilized (using reversible immobilization via interfacial activation on octyl agarose) [38,39] and another where a covalent attachment is performed (using cyanogen bromide agarose under mild conditions to reduce the number of enzyme/support linkages) [40,41].

2. Materials and methods

2.1. Materials

Lecitase was a kind gift from Novozymes (Spain). Octyl-agarose and cyanogen bromide crosslinked 4% agarose (CNBr) beads were from GE Healthcare. Polyethylenimine (Mn 10000, Mw 25000), dextran sulfate (9000, 20000 Av. wt) *p*-nitrophenyl butyrate (p-NPB), *R* and *S* methyl mandelate, ethyl hexanoate, diethyl p-nitrophenylphosphate (D-pNPP) and methyl phenylacetate were from Sigma Chemical Co. (St. Louis, MO, USA). Glutaraldehyde (25%, v/v stabilized in ethanol) was from Fluka. All reagents and solvents were of analytical grade.

2.2. Standard determination of enzyme activity

This assay was performed by measuring the increase in absorbance at 348 nm produced by the released p-nitrophenol in the hydrolysis of 0.4 mM p-nitrophenyl butyrate (p-NPB) in 100 mM sodium phosphate at pH 7.0 and 25 °C (ε under these conditions is 5150 mol^{-1} cm^{-1}). To start the reaction, 50–100 μl of lipase solution or suspension was added to 2.5 mL of substrate solution. One international unit of activity (U) was defined as the amount of enzyme that hydrolyzes 1 μmol of p-NPB per minute under the conditions described previously. Protein concentration was determined using Bradford' method [42] and bovine serum albumin was used as the reference.

In the studies of pH effects on the enzyme activity, the protocol was similar but the buffer in the measurements was changed according to the pH value: sodium acetate at pH 5, sodium phosphate at pH 6–8 and sodium borate at pH 9–10. At 25 °C, all the preparations remained fully stable after incubation for several hours at any of these pH values.

2.3. Immobilization of Lecitase on octyl-agarose beads

Lecitase was immobilized on octyl-agarose beads at low ionic strength [38,39]. 2.8 mL of commercial extract (16 mg protein/mL having a pNPB activity of 5.6 U/mg protein) was diluted in 67.5 mL of 5 mM sodium phosphate at pH 7. Then, 15 g of wet octyl-agarose beads were added. The activity of both supernatant and suspension was followed using p-NPB. After immobilization the suspension was filtered and the supported lipase was washed several times with distilled water. Immobilization yield was over 90%.

2.4. Immobilization of Lecitase on CNBr-agarose beads

A volume of 2.8 mL of commercial Lecitase was diluted in 67.5 mL of 5 mM sodium phosphate containing 0.05% (w/v) sodium dodecyl sulfate at pH 7 at 4 °C. Then, 15 g of wet CNBr- support was added. Activity of supernatant and suspension was followed using pNPB. The enzyme-support immobilization was ended by incubating the support with 1 M ethanolamine at pH 8 for 12 h. Finally, the immobilized preparation was washed with abundant distilled water. Immobilization yield was over 90%.

2.5. Coating of immobilized Lecitase by ionic polymer

A mass of 10 g of wet immobilized Lecitase was added to 100 mL of PEI at pH 7 or DS at pH 5 solutions at the desired concentration of the polymer. Activity was followed during the incubation by the p-NPB activity protocol described above.

2.6. Thermal inactivation of different Lecitase immobilized preparations

To check the stability of enzyme derivatives, 1 g of immobilized enzyme was suspended in 5 mL of 10 mM of sodium acetate at pH 5, sodium phosphate at pH 7 or sodium carbonate at pH 9 at different temperatures. Periodically, samples were withdrawn and the activity was measured using p-NPB. Half-lives were calculated from the observed inactivation courses.

2.7. Inactivation of different Lecitase preparations on presence of organic co-solvents

Enzyme preparations were incubated in mixtures of 30% acetonitrile in 100 mM Tris–HCl 7 and 25 °C to proceed to the inactivation. Periodically, samples were withdrawn and the activity was measured using p-NPB. Half-lives were calculated from the observed inactivation courses. The acetonitrile presented in the samples had not a significant effect on enzyme activity.

2.8. Hydrolysis of ethyl hexanoate

Enzyme activity was determined by using ethyl hexanoate; 200 mg of the immobilized preparations were added to 0.6 mL of 25 mM substrate in 50 mM buffer containing 50% CH_3CN. The buffer was sodium acetate at pH 5, sodium phosphate at pH 7 and sodium carbonate at pH 8.5. All experiments were carried out at 25 °C under continuous stirring. The conversion degree was analyzed by RP-HPLC (Spectra Physic SP 100 coupled with an UV detector Spectra Physic SP 8450) using a Kromasil C18 (15 cm × 0.46 cm) column. Samples (20 μL) were injected and eluted at a flow rate of 1.0 mL/min using acetonitrile/10 mM ammonium acetate aqueous