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Stabilizing hyperactivated lecitase structures through physical treatment with ionic polymers

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ABSTRACT

Lecitase Ultra has been covalently immobilized on cyanogen bromide cross-linked 4% agarose (CNBr) beads, maintaining 70% of the initial activity. The activity of the immobilized enzyme was improved in the presence of Triton X-100, sodium dodecyl sulfate (SDS), and cetyltrimethyl ammonium bromide (CTAB) (e.g., up to 800% when using CTAB). However, CTAB and Triton X-100 presented a negative effect on enzyme stability even at low concentrations, and SDS cannot be used for a long time at 1% concentration. To maintain the hyperactivated conformation of the enzyme in the absence of detergent, ionic polymers were added during incubation of the immobilized enzyme in the presence of detergents. Coating the immobilized enzyme with polyethylenimine in aqueous buffer (PEI) produced a 3-fold increase in enzyme activity. However, in the presence of 0.1% SDS (v/v), this coating produced a 50-fold increase in enzyme activity. Using PEI and 0.01% (v/v) CTAB, the Lecitase activity decreased to 10%. Using irreversible inhibitors, it could be shown that the PEI/SDS-CNBr-Lecitase preparation allowed its catalytic Ser to be more accessible to the reaction medium than the unmodified CNBr-Lecitase.

1. Introduction

The improvement of enzyme activity is one of the most interesting targets of biocatalyst design. This increase in activity has been achieved by modifying the enzyme features using several tools, such as site-directed mutagenesis [1], directed evolution [2,3] and chemical modification [4,5], among others.

However, it must be considered that, in most cases, enzymes are used at the industrial level in an immobilized form to permit their reuse (if the immobilized enzyme is stable enough) [6,7]. Thus, it may be interesting to develop strategies to improve enzyme activity during the preparation of the biocatalyst, which will be compatible with the strategy of directly improving enzyme performance via genetic tools [5,8]. Immobilization is, in many cases, associated with a decrease in enzyme activity for various reasons (e.g., enzyme distortion, diffusion problems, steric problems). However, a proper immobilization protocol can maintain, and even increase, the activity of the enzyme [9]. The improvement in activity may be found in some cases by preventing some of the reasons for a reduction in enzyme activity, such as inhibition or distortion, while in other cases by producing a more active enzyme conformation [10].

Lipases have a catalytic mechanism called interfacial activation [11–13]. The active center is isolated from the reaction medium by an oligopeptide chain called "flap" or "lid" [14,15]. The internal face of the lid is hydrophobic and interacts with the hydrophobic areas surrounding the active center. This lid can be moved out exposing this very hydrophobic pocket to the medium. This conformation is unstable in an aqueous medium, causing the enzyme molecules to be in the "closed" conformation (i.e., without activity) most of the time. In the presence of its natural substrate (a drop of oil), the hydrophobic pocket permits the adsorption of the enzyme on its surface, stabilizing the open and active conformation of the lipase, and enabling the lipase to act in the interface of the oil drop and the aqueous system.

Lipases have also been hyperactivated during biocatalyst preparation by using conditions in which the enzyme structure is open and able to be further stabilized in this conformation. In

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these cases, the open conformation has typically been produced by using detergents; these amphipathic molecules stabilize the open conformation of the lipases [16,17] but may also act as inhibitors [18], leading to its inactivation [19,20]. Thus, lipases have been lyophilized [21], precipitated and cross-linked [22], ionically adsorbed [23], or cross-linked with glutaraldehyde [4] in the presence of detergents to permit the freezing of the open form, so that the active and open conformation will persist after the detergent is eliminated.

Additionally, the solid-phase physical-coating of enzymes with ionic polymers is reportedly a quite simple and rapid method for the modification of enzyme activity [5]. This coating with ionic polymers has been proposed as a way to improve enzyme stability under certain conditions [24] (e.g., presence of organic solvents or oxygen by secluding these hydrophobic reagents from the enzyme environment [25,26], preventing enzyme dissociation [27]). In some cases, the polymer coating of lipases has been reported to enhance the catalytic behavior of the enzyme (e.g., specificity) [28–30].

Lecitase Ultra is a commercial phospholipase A_1 developed mainly for degumming processes [31], although phospholipases A_1 may have different uses [32–36]. This enzyme has been obtained from the fusion of the genes of the lipase from *Thermomyces lanuginosus* (to achieve good stability) and the phospholipase from *Fusarium oxysporum* (to obtain the phospholipase activity) [31]. This enzyme has been found to behave as a standard lipase, with the capacity to be adsorbed on hydrophobic surfaces (e.g., hydrophobic supports) [37] and to present broad specificity [38–46].

Thus, in this paper we have tried to obtain the stabilized open conformation of Lecitase Ultra, immobilized on cyanogen bromide agarose by covalent attachment, through physical modification with ionic polymers, "via ionic crosslinking" after activation by detergents.

2. Materials and methods

2.1. Materials

Lecitase was a kind gift from Novozymes (Spain) (16 mg protein/mL having a pNPB activity of 5.6 U/mg protein). Cyanogen bromide cross-linked 4% agarose (CNBr) beads were from GE Healthcare (Pollards Wood, UK). Polyethyleneimine (branched PEI, Mn 10,000, Mw 25,000 Da), dextran sulfate (DS, average Mw, 9000–20000 Da) *p*-nitrophenyl butyrate (p-NPB), Triton X-100, sodium dodecylsulphate (SDS), cetyltrimethylammonium bromide (CTAB) and diethyl p-nitrophenylphosphate (D-pNPP) were from Sigma Chemical Co. (St. Louis, MO, USA).

2.2. 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 the lipase solution or suspension was added to 2.5 mL of the 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. In some instances, the studied detergents (to a maximum concentration of 0.1% v/v) were added before proceeding with the activity determination. Protein concentration was determined using Bradford' method [47] and bovine serum albumin was used as the reference.

2.3. 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% (v/v) SDS at pH 7 and 4 °C. Then, 15 g of wet CNBr-support was added. Activities of supernatant and suspension were 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. The immobilization yield was over 90% and the expressed activity was 70% [48].

2.4. Coating of immobilized Lecitase by ionic polymers

The previously optimized protocol was been followed for coating of immobilized Lecitase with ionic polymers [49]. 10 g of wet, immobilized Lecitase were added to 100 mL of PEI at pH 7 or DS at pH 5 (1 mg/mL). In some instances, the detergent at the desired concentration was added 2 min before adding the polymer, keeping this mixture under gentle stirring for a maximum of 24 h. Activity was followed during the incubation by the p-NPB activity protocol as described above.

2.5. Analysis of the effect of the detergents on the stability of different CNBr-Lecitase

To check the stability of the enzyme derivatives in the presence of detergents, 1 g of immobilized enzyme was suspended in 5 mL of 10 mM sodium phosphate at pH 7 at 25 °C. Periodically, samples were withdrawn, and the activity was measured using pNPB. A suspension of CNBr-Lecitase in the absence of detergent was used as a reference. The activity value obtained using the reference suspension and adding to the reaction mixture the equivalent amount of detergent in each sample (to discriminate inhibitory/activating effects of the detergent present in the problem suspensions) was taken as 100% activity.

2.6. Irreversible inactivation of immobilized Lecitase in the presence of D-pNPP

Different lipase-immobilized preparations (0.8 g) were suspended in 5 mL of 100 mM sodium phosphate buffer solution at pH 7 and 25 °C. Then, D-pNPP was added up to a concentration of 1 mM. Samples of this suspension were withdrawn periodically, and their activities were checked using the p-NPP assay.

3. Results

3.1. Effect of the detergent in enzyme properties

Three detergents with different characteristics were tested: CTAB, a cationic surfactant; SDS, an anionic surfactant; and Triton X-100, a nonionic surfactant. Fig. 1 shows the effects on enzyme activity of each detergent. In the range of concentrations studied, CNBr-Lecitase was hyperactivated in most cases. The highest hyperactivation was detected using CTAB (almost 8-fold), and the effect increased with the increase in detergent concentration. SDS had a positive effect at low concentrations (increasing the activity by twofold at 0.01%). However, at 0.1% SDS, the activity was lower than in the absence of SDS. Triton X-100 showed a low effect on enzyme activity at low detergent concentrations but at 0.1%, the activity was almost 3-fold higher than the control.

Furthermore, the immobilized preparation was incubated in the presence of these detergents to determine their effect on the stability of the enzyme (Table 1). Using 0.1% SDS, the observed activity after 24 h was almost 100%, while with 0.1% Triton X-100,