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Reactivation of covalently immobilized lipase from *Thermomyces lanuginosus*

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ABSTRACT

Lipase from *Thermomyces lanuginosus* (TLL) immobilized on cyanogen bromide agarose (CNBr) may be fully inactivated when incubated in saturated solutions of guanidine. When this inactivated enzyme is re-incubated in aqueous medium, 20% of the activity may be recovered for several cycles. However, if the activity was determined in the presence of a detergent (CTAB, an activator of this enzyme), 100% of the initial activity in the presence of detergent was recovered. The enzyme was also inactivated in the presence of organic solvents and at high temperatures. Inactivations were more rapid when the activity was determined in absence of detergent. In both cases, some activity could be recovered just by incubation under mild conditions, and this increase was higher if the activity measurements were performed in the presence of CTAB. These results suggested that the opening of the lipase could be a critical step in the inactivation or reactivation of immobilized TLL. In inactivations in the presence of solvents, 100% of activity could be recovered during several cycles, while in thermal inactivations, the recovered activity decreased in each inactivation–reactivation cycle. The incubation of the enzyme inactivated by temperature in guanidine improved the results, but still 100% could not be achieved during several cycles even measured in the presence of CTAB.

Thus, the simple incubation of the partially or fully inactivated enzyme under mild conditions permitted to recover some activity (enhancing the half life of the biocatalysts), even in thermal inactivations.

1. Introduction

The use of enzymes in the presence of organic solvents [1] may have interest for different purposes: to improve the solubility of substrates or products [2,3], to shift thermodynamic equilibrium [4–6], to improve the enzyme properties [7,8] or even to prevent microbial contamination.

One problem of the use of organic solvents and cosolvents in biotransformations is the fact that enzymes may be readily inactivated in the presence of high concentrations of these compounds [9–11]. Immobilized enzymes may be very rapidly inactivated by the presence of high concentrations of inert solvents, even at neutral pH values and low temperatures. At pH 7 and low temperatures, it is unlikely that the primary structure of the enzyme can be modified. Moreover, if the enzyme is immobilized and dispersed on the support surface, aggregations will be impossible. Thus, under these chemically mild conditions, the only cause of inactivation of immobilized monomeric enzymes should be the distortion of their tridimensional structure that can drive to an incorrect and inactive conformation of the enzyme [12]. In this way, if this incorrect conformation may be reversed, the recovery of the enzyme activity could be achieved.

Lipases are one of the most interesting enzymes for use in biocatalysis [13–16]. These enzymes are monomeric proteins that exhibit a complex catalytic mechanism, called interfacial activation [17–20]. Usually, in homogenous medium, lipases mainly have their active center secluded from the reaction medium by an oligopeptide chain called flap or lid [21]. This lid has a very hydrophobic internal side that interacts with hydrophobic aminoacids near to the active center. In the presence of a hydrophobic interface (e.g., an oil drop [22,23], a hydrophobic support [24,25], a hydrophobic protein [26,27]), the lid moves exposing the active center of the lipase to the medium, and this open form becomes adsorbed to the hydrophobic surface, shifting the conformational equilibrium towards the open form.

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In this way, strategies for reactivation of immobilized lipases should regenerate not only the active center of the lipase, but also the mechanism of opening and closing of the lipases. As a result, the recovery of active forms of lipases after their inactivation may be a bit more complex than the reactivation standard proteins [28,29].

In this context, it has been described that detergents may help the lipases to give an open form [30,31]. It is suggested that these reagents may stabilize the open form of the lipase by reducing the interaction of the large hydrophobic pocket present in the open form of the lipase with the medium. In fact, the use of detergents has been proposed as a way to have hyperactivated form of lipases, just by adding the detergents to the reaction medium [32], or by preparing the immobilized lipase in the presence of detergent [33,34]. Thus, it may be interesting to analyze the recovery of the enzyme activity in the presence or absence of detergents. Moreover, there are some reports stating that some detergents could help to the correct refolding of some proteins [35,36].

Lipase from *Thermomyces lanuginosus* (TLL) is the enzyme responsible for the lipolytic activity of Lipolase®, a commercial lipase preparation supplied by Novozymes. This enzyme has been broadly used in many biotransformations [37–40]. Its structure has been solved at 1.8 Å [41], presenting a large flap that, in the closed form, isolates the active centre from the reaction medium. This lipase presents a high tendency to form bimolecular aggregates, making it necessary to use diluted solutions and the presence of detergents to have a monomeric form of the enzyme [26].

In this paper, lipase from *T. lanuginosus* immobilized on cyanogen bromide agarose (CNBr) was inactivated by heat and organic cosolvents, and later the recovery of the activity was analyzed by incubation under milder conditions. The inactivation and the activity recovery were analyzed in both, aqueous medium or aqueous-detergent medium. The effect of the previous full unfolding of the lipase by incubation in saturated guanidine hydrochloride to destroy any incorrect structure but stable lipase structure [28,29] was also studied.

2. Materials and methods

2.1. Materials

Lipase from *T. lanuginosus* was obtained from Novozymes (Denmark). Ethanolamine hydrochloride, hexadecyltrimethylammonium bromide (CTAB), and *p*-nitrophenyl butyrate (p-NPB) were from Sigma. 1,4-Dioxane, guanidine hydrochloride and urea were from Fluka. Octyl-sepharose CL-4B and cyanogen bromide activated Sepharose 4B (CNBr) were purchased from GE Healthcare (Uppsala, Sweden). Other reagents and solvents used were of analytical or HPLC grade.

2.2. Methods

The experiments were carried out at least by triplicate and the standard error was always under 5%.

2.2.1. Standard enzymatic activity assay

This assay was performed by measuring the increase in the absorbance at 348 nm produced by the released *p*-nitrophenol in the hydrolysis of 0.4 mM pNPB in 25 mM sodium phosphate at pH 7 and 25 °C, using a thermostatized spectrum with continuous magnetic stirring. To initialize the reaction, 0.05 mL of lipase solution or suspension was added to 2.5 mL of substrate solution. One international unit of pNPB activity was defined as the amount of enzyme necessary to hydrolyze 1 μmol of pNPB/min (IU) under the conditions described above.

In some instances, 0.01% of CTAB was added to the substrate solution.

2.2.2. Purification of TLL

TLL was purified prior to use. The used strategy was the interfacial adsorption on hydrophobic supports. The enzyme was adsorbed on octyl-sepharose beads under continuous stirring in 10 mM sodium phosphate at pH 7.0, following a previously described procedure [24,25]. Periodically, the activity of suspensions and supernatants was measured by using the pNPB assay. After enzyme adsorption, the lipase preparation was vacuum filtered using a sintered glass funnel and abundantly washed with distilled water. TLL was desorbed from octyl-sepharose by suspending the immobilized enzyme in a relation 1/10 (w/v) in 25 mM sodium phosphate at pH 7.0 containing 0.6% (v/v) of CTAB during 1 h at room temperature. The SDS-PAGE of this preparation revealed just one protein band.

2.2.3. Immobilization of TLL on CNBr-activated support

TLL was desorbed from octyl-sepharose as described in the purification section. The immobilization of TLL on CNBr-activated support was performed for 15 min at 4 °C and pH 7 to reduce the possibilities of getting a multipoint covalent attachment between the enzyme and the support [42]. During the immobilization and further blocking of the support, the suspension was submitted to continuous gentle stirring. The enzyme preparations were carried out by using 10 mg of the purified enzyme per g of support. The enzyme–support reaction was ended by incubating the support with 1 M ethanolamine at pH 8 for 2 h. Finally, the immobilized TLL was vacuum filtered using a sintered glass funnel and washed with abundant water, to eliminate the detergent. This immobilized enzyme was called CNBr-TLL.

2.2.4. Incubation of CNBr-TLL on caotropic agent solutions

For all inactivation/reactivation steps a relation of 1:10 (g of support/mL of solution) was used.

CNBr-TLL was incubated in 50 mM phosphate buffer containing 8 M of urea or guanidine hydrochloride at pH 7.0, 25 °C. Samples were withdrawn periodically to check the remaining activity of the immobilized enzyme, diluting 100 mL of the caotropic agent solution with 2 mL of the buffer of the substrate solution.

2.2.5. Thermal inactivation

CNBr-TLL was incubated in 50 mM sodium phosphate at pH 7.0 and 60 °C. Samples were withdrawn periodically using a pipette with a cut-tip and under vigorous stirring to have a homogeneous biocatalyst suspension. The activity was measured using the pNPB assay described above in the presence and absence of CTAB in cuvette.

2.2.6. Inactivation by organic solvent

CNBr-TLL was washed with 80% dioxane/50 mM Tris–HCl aqueous solution at pH 7 and 4 °C. Subsequently, the enzyme derivatives were resuspended in such solution and incubated at 4 °C. Samples were withdrawn periodically, and the activity was checked following the above assay, diluting 100 mL of the inactivating solution with 2 mL of the buffer of the substrate solution.

2.2.7. Reactivation experiments

Fully or partially inactivated CNBr-TLL preparations (sometimes after incubation in saturated guanidine solution) were filtered, washed with water and resuspended in aqueous buffer (50 mM sodium phosphate at pH 7) and the activity was determined along the time. When a constant value of residual activity was achieved, this was considered the maximum recovered activity. In some cases, several consecutive cycles of inactivation/reactivation of immobilized CNBr-TLL were performed.

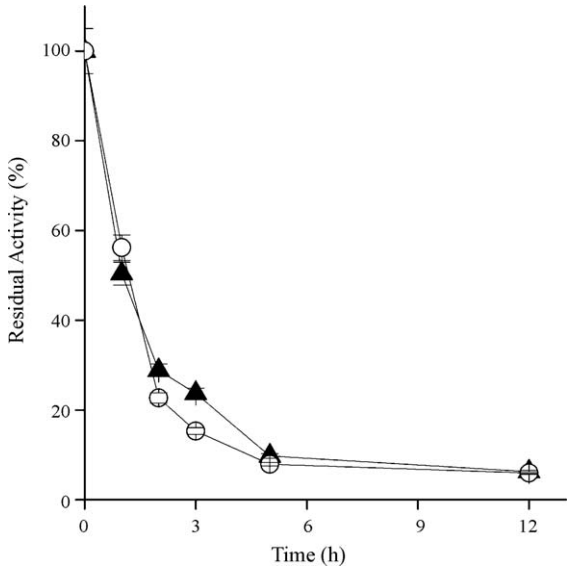

Fig. 1. Inactivation courses of soluble and immobilized TLL in 50 mM sodium phosphate at 60 °C and pH 7. (▲) Soluble-TLL; (○) CNBr-TLL.