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Immobilization–stabilization of the lipase from *Thermomyces lanuginosus*: Critical role of chemical amination

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ABSTRACT

This paper describes the immobilization and stabilization of the lipase from *Thermomyces lanuginosus* (TLL) on glyoxyl agarose. Enzymes attach to this support only by the reaction between several aldehyde groups of the support and several Lys residues on the external surface of the enzyme molecules at pH 10. However, this standard immobilization procedure is unsuitable for TLL lipase due to the low stability of TLL at pH 10 and its low content on Lys groups that makes that the immobilization process was quite slow. The chemical amination of TLL, after reversible immobilization on hydrophobic supports, has been shown to be a simple and efficient way to improve the multipoint covalent attachment of this enzyme. The modification enriches the enzyme surface in primary amino groups with low p*K*b, thus allowing the immobilization of the enzyme at lower pH values. The aminated enzyme was rapidly immobilized at pH 9 and 10, with activities recovery of approximately 70%. The immobilization of the chemically modified enzyme improved its stability by 5-fold when compared to the non-modified enzyme during thermal inactivation and by hundreds of times when the enzyme was inactivated in the presence of organic solvents, being both glyoxyl preparations more stable than the enzyme immobilized on bromocyanogen.

1. Introduction

The use of enzymes is essential in many industrial areas by different reasons, among other things because they catalyze the most complex reactions under the mildest experimental conditions [1]. However, enzymes, to be used as industrial biocatalysts, in many instances need to be improved. For example, enzymes usually require to be immobilized and stabilized in order to facilitate the design of the reactor and to allow for enzyme recovery and reuse [1–4].

The combination of immobilization and stabilization would be extremely interesting. In this context, the enzyme immobilization via multipoint covalent attachment is one of the most powerful tools for this purpose [5,6]. However, really many covalent attachments are not easily accomplished, requiring the selection of a suitable immobilization support and proper immobilization conditions [7]. Glyoxyl agarose beads have been shown to produce very high stabilization factors (from 2 to 5 magnitude orders) when used to immobilize several different enzymes [8]. The enzymes only become immobilized on the support when at last two simultaneous enzyme-support attachments are produced. Moreover, enzymes become immobilized on these supports only at pH values over 10, when the ε-amino of Lys residues present in their external surfaces are reactive enough [9]. Moreover, the degree of the enzyme-support multipoint covalent reaction is strongly dependent on the amount of reactive groups in both the support (aldehyde) and the enzyme (amines) [10].

The enrichment of enzyme surfaces with amino groups through different techniques has been shown to successfully improve the process of multipoint covalent immobilization [10,11]. For example, the chemical amination of the protein surface via reaction of the carboxylic groups, such as the side group of Asp and Glu, with ethylenediamine after activation with carbodiimide, is a well described and easy-to-control reaction [12–16]. More recently, the chemical amination of lipases reversibly immobilized on hydrophobic supports via interfacial activation has been reported to be a way of simplifying this chemical amination previous to its covalent attachment [17].

One further advantage of this chemical amination is the reactivity of the new amino group. The new amino group has a p*K* of 9.2 [18], much lower than the p*K* of the ε-amino of Lys (around 10.7). This allows to immobilize an aminated protein on glyoxyl supports at pH below 10 (e.g., pH 9). The possibility of

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immobilizing a protein at lower pH may be critical when the enzyme intended for immobilization presents a very low stability at pH 10.

In this research, the strategy of chemical amination was used to achieve a stabilized–immobilized preparation of the lipase from *Thermomyces lanuginosus* (previously *Humicola lanuginosa*) (TLL) on glyoxyl agarose beads. 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 [19–22]. Its structure has been solved at 3.25 Å [23] and more recently for another crystal form at 1.8 Å [24]. Lipases, and TLL among them, share a common fold of the alpha/beta-hydrolase type, and the structure usually contains a small alpha-helix or loop, referred to as lid, or flap which covers the active site pocket. This conformation is termed the closed conformation. When the lipase is adsorbed to an interface, the lid is displaced so that the active site becomes accessible to substrate. This conformation is termed the open conformation [25–27]. It maintains activity reasonably well at 55–60 °C [28]. This lipase tends to form bimolecular aggregates, being necessary to use diluted solutions of the enzyme in the presence of detergents to have the monomeric form of it [29].

2. Materials and methods

2.1. Materials

Lipase from *Thermomyces lanuginosus* (TLL) was obtained from Novozymes (Denmark). Ethanolamine hydrochloride, 1-ethyl-3-(dimethylaminopropyl) carbodiimide (EDC), hexadecyltrimethylammonium bromide (CTAB), and *p*-nitrophenyl butyrate (p-NPB) were from Sigma. 1,4-Dioxane and 1,2-ethylenediamine (EDA) were from Fluka. Octyl-sepharose CL-4B and cyanogen bromide activated Sepharose 4B (CNBr) were purchased from GE Healthcare (Uppsala, Sweden). Cross-linked agarose (10 BCL) was kindly donated by Hispanagar S.A. (Burgos, Spain) and its modification to glyoxyl agarose (activated with 200 μmol/g of support) was performed as described elsewhere [9]. 2,4,6-Trinitrobenzensulfonic acid (TNBS) was from Fluka. Other reagents and solvents were of analytical or HPLC grade.

2.2. Methods

2.2.1. Lipase 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 spectrum with continuous magnetic stirring and a thermostatized cell. To initiate 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 (U) under the conditions described above. Supernatant of suspensions containing supports were obtained using a pipette-tip-filter, suspensions were assayed using cut-pipette-tips.

2.2.2. Purification of TLL

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 [30]. At different times, the activities of suspensions and supernatants were measured by using the pNPB assay. After enzyme adsorption, the lipase preparation was vacuum filtered using a sintered glass funnel and washed with an excess of distilled water. TLL was desorbed from octyl-sepharose by suspending the immobilized enzyme in a ratio of 1/10 (w/v) in 25 mM sodium phosphate at pH 7.0 containing 0.6% (v/v) of CTAB for 1 h at room temperature.

2.2.3. Chemical amination of immobilized TLL

In order to fully modify all exposed carboxylic groups of the protein, the following procedures were used [12–16]. A total of 1 g of immobilized lipase either covalently bound on CNBr agarose beads as described below, or through adsorption on octyl-sepharose beads was added to 10 mL of 1 M EDA at pH 4.75 under continuous stirring. Solid EDC was added to the suspension to a final concentration of 10 mM. After 90 min of gentle stirring at 25 °C, the immobilized-modified preparations were vacuum filtered using a sintered glass funnel and incubated for 4 h in 0.1 M hydroxylamine at pH 7 and 4 °C to recover the EDC-modified tyrosines [31]. The enzyme preparations were filtered and washed with 25 mM sodium phosphate at pH 7.5 and with an excess of distilled water. The aminated TLL (TLL-A) preparations, immobilized on CNBr or octyl-sepharose (used to obtain the soluble aminated enzyme) were stored at 4 °C.

2.2.4. Immobilization of TLL on CNBr-activated support

TLL was desorbed from octyl-sepharose as described in Section 2.2.2. The immobilization of TLL on CNBr-activated support (10 mg of the purified enzyme per g of support) was performed for 15 min at 4 °C and pH 7 in order to reduce the possibility of multipoint covalent attachments between enzyme and support [9]. During the immobilization and further blocking of the support, the suspension was submitted to continuous gentle stirring. The enzyme-support reaction was ended by incubating the support with 1 M ethanolamine at pH 8 for 2 h. Finally, the immobilized TLL preparation was vacuum filtered using a sintered glass funnel and washed with abundant distilled water in order to remove any traces of detergent. This immobilized enzyme system was named CNBr-TLL. These preparations, with just some few enzyme-support bonds [7,9] use to have a stability very similar to the free enzyme, therefore it is a good reference of the behavior of the soluble enzyme, but avoiding the problems of aggregations or other intermolecular problems.

2.2.5. Immobilization of TLL on glyoxyl-agarose beads

Both TLL and TLL-A were desorbed from octyl-sepharose as previously described and the pH was adjusted to 9 or 10 with 1 M sodium bicarbonate to a final concentration of 100 mM of this buffer. The immobilized enzyme derivatives were prepared using 1 g of glyoxyl-support and 10 mL of purified TLL or TLL-A. The biocatalysts were prepared to obtain 10 mg of protein per g of support. The mixture was maintained at the desired temperatures during the desired times. Samples of supernatant and suspension were withdrawn and their activities and/or protein concentration determined using the Bradford's method [32]. Reduced glyoxyl-agarose was used as a control in order to discard unspecific adsorptions. As reaction end-point, to transform the aldehyde in inert hydroxyl groups and the imine bonds in very stable secondary amino bonds, solid sodium borohydride was added to a concentration of 1 mg/mL to the immobilization suspension [8] and the mixture was maintained at 25 °C under gentle stirring. After 30 min, the immobilized and reduced derivatives were washed thoroughly with distilled water.

The glyoxyl biocatalysts prepared were the following: Gx-TTL was prepared by immobilization of TLL during 15 h at 4 °C and pH 10; Gx(9/10)-TLL-A was prepared by immobilization of TLL-A on glyoxyl-agarose at 25 °C and pH 9 (8 h) and further incubated at pH 10 and 25 °C overnight; Gx(10)-TLL-A was prepared by immobilization of TLL-A during 20 h at 25 °C and pH 10.

2.2.6. Thermal inactivation of different TLL immobilized preparations

The different TLL preparations were incubated in 25 mM sodium phosphate at pH 7.0 and 70 °C. Samples were withdrawn at different times using a pipette with a cut-tip and under vigorous stirring to have a homogeneous biocatalyst suspension. The activity was measured by the pNPB assay, above described. Activity was measured immediately after withdrawn the sample to determinate in the possible the state of the enzyme induce by the temperature and not the likely reactivation cause by re-incubation at low temperature. The experiments were carried out in triplicates and the standard error was under 5%.

2.2.7. Inactivation of different TLL immobilized preparations in the presence of organic cosolvent

Enzyme derivatives were washed with 60% dioxane/50 mM sodium acetate aqueous solution at pH 5 and 4 °C. Subsequently, the enzyme derivatives were resuspended in the same solution and incubated at 4 °C. Samples were withdrawn at different times, and the activity was checked. Experiments were carried out in triplicates, and standard error was under 5%.

2.2.8. Titration of the amino groups using picrylsulfonic acid

Primary amines residues were titrated using the picrylsulfonic acid methodology [33,34]. To perform these tests it was used the immobilized preparations of TLL and TLL-A adsorbed on octyl-sepharose. One hundred milligrams of each derivative was suspended in 0.4 mL of 100 mM sodium bicarbonate at pH 9. The suspension was incubated at 25 °C, and 0.1 mL of picrylsulfonic acid (5% (w/w) solution) was added; after 60 min, the colored derivatives were filtered and washed with a saturated NaCl solution, distilled water, and with 100 mM sodium bicarbonate at pH 9. A total of 50 mg of the colored preparations were then resuspended in 2 mL of 100 mM sodium bicarbonate, pH 9, and their spectra were determined. As control, the enzyme-free support was treated in a similar way.

3. Results and discussion

3.1. Immobilization of TLL on glyoxyl agarose

TLL is rapidly inactivated under the standard conditions of immobilization on glyoxyl agarose (pH 10 and 25 °C) [35], suggesting a very low stability of the enzyme under these conditions (Fig. 1a). Moreover, it can be seen that the enzyme is slowly immobilized on the support, very likely as result of the low content of Lys residues of this protein (only 7) [24], and its