Enzyme and Microbial Technology 77 (2015) 1–7

Contents lists available at ScienceDirect

Enzyme and Microbial Technology

journal homepage: www.elsevier.com/locate/emt

Tuning the catalytic properties of lipases immobilized on divinylsulfone activated agarose by altering its nanoenvironment

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ARTICLE INFO

Article history:
Received 19 March 2015
Received in revised form 29 April 2015
Accepted 6 May 2015
Available online 12 May 2015

Keywords:
Divinylsulfone support
Tuning lipase properties
Lipase activity
Enzyme stability
Lipase
Enzyme nano-environment

ABSTRACT

Lipase from *Thermomyces lanuginosus* (TLL) and lipase B from *Candida antarctica* (CALB) have been immobilized on divinylsulfone (DVS) activated agarose beads at pH 10 for 72 h. Then, as a reaction end point, very different nucleophiles have been used to block the support and the effect of the nature of the blocking reagent has been analyzed on the features of the immobilized preparations. The blocking has generally positive effects on enzyme stability in both thermal and organic solvent inactivations. For example, CALB improved 7.5-fold the thermal stability after blocking with imidazole. The effect on enzyme activity was more variable, strongly depending on the substrate and the experimental conditions. Referring to CALB; using p-nitrophenyl butyrate (p-NPB) and methyl phenylacetate, activity always improved by the blocking step, whatever the blocking reagent, while with methyl mandelate or ethyl hexanoate not always the blocking presented a positive effect. Other example is TLL-DVS biocatalyst blocked with Cys. This was more than 8 times more active than the non-blocked preparation and become the most active versus p-NPB at pH 7, the least active versus methyl phenylacetate at pH 5 but the third one most active at pH 9, versus methyl mandelate presented lower activity than the unblocked preparation at pH 5 and versus ethyl hexanoate was the most active at all pH values. That way, enzyme specificity could be strongly altered by this blocking step.

1. Introduction

Lipases are among the most used enzymes in biocatalysis [1–6]. They are used in a great diversity of reaction medium (aqueous medium [7], organic solvents [8,9], neoteric solvents [10–12]), exhibiting a high activity and stability in many cases. Lipase specificity is very wide, accepting many different substrates, some very far apart from the natural ones (oils and fats). Lipases may be used in many different reactions, like modification of oils [13,14], resolution of racemic mixtures (via different strategies) [15], esterifications [16], transesterifications [17,18], etc. In fact, lipases are among the enzymes where more promiscuous activities have been described [19]. Lipases have a mechanism of catalysis that differentiates them from standard esterases and permits that they act in interfaces of insoluble drops of substrates: the interfacial activation [20,21]. The active center may be close by a polypeptide chain, which can move giving two different forms of lipases: the open and active form, or the closed and many times inactive one [22–24]. The open form of lipases tends to become adsorbed on any hydrophobic surface via the huge hydrophobic pocket formed by the active center and the internal face of the lid [20,21].

Even with these very good possibilities, lipase properties, like those of most enzymes, need to be improved in many instances before its industrial implementation [25]. For example, in most cases it is convenient to have the enzyme in a heterogeneous form, as a simple way to separate the enzyme from the reaction media and to reuse it [26,27]. The use of immobilized enzymes also permits to enlarge the range of reactors to be utilized [28–30].

That way, considering that in many instances the use of immobilized enzymes is a requirement to use them as industrial biocatalysts, a great effort has been performed to couple immobilization to the improvement of other enzyme features [31–36].

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http://dx.doi.org/10.1016/j.enzmictec.2015.05.001
0141-0229/

Stabilization of enzymes after immobilization is a usually pursued goal (via multipoint attachment [37–39], multisubunit immobilization [40] or generation of favorable environments [41,42]), but immobilization may improve many other features if properly performed, like activity, selectivity, specificity, resistance to inhibitors [34,43].

Lipases, due to the flexibility of their active center may be very easily tuned via genetic modification [44–47], physical or chemical modification [48–51] or immobilization [34,43]. Immobilization has revealed itself as a potent tool to tuning lipase properties, even although the results are difficult to predict and are based on the building of a large library of biocatalysts immobilized following different protocols (trial and error) [34,43]. Any alteration on the movements of the lid may produce changes in the enzyme properties, e.g., by distorting different areas of the protein, or increasing the rigidity that may avoid some conformational changes. This has been usually performed using different immobilization protocols that alter the enzyme orientation or the intensity of the enzyme support attachment [34].

In this research, we have utilized agarose beads activated with divinylsulfone (DVS) to immobilize several lipases under identical conditions. This support can react with different moieties of the proteins, and produce some rigidification, and as a final enzyme–support reaction end point, the blocking of the support may be performed with different amino or thiol compounds [52]. Thus, we should have lipase molecules with identical orientation and degree of enzyme support multipoint covalent attachment. In a previous paper, the properties of the enzyme penicillin G acylase immobilized on epoxy activated supports were tuned in a kinetically controlled synthesis of antibiotics by changing the blocking reagent [53]. Results were explained mainly as partition of the substrates and products, the enzyme immobilization involved the areas near to the active center [54].

An immobilized enzyme will interact with the support surface except if it is fully chemical inert, and that is very difficult, e.g., DVS groups are already moderately hydrophobic moieties. The hypothesis on this paper is that if the physical properties of the support change, it is possible to alter the interactions between the support and the lipase molecule and that way to produce conformational changes on the enzyme molecule that can produce changes in the final functional properties. Using DVS activated supports, this may be achieved using different blocking reagents. This way, even although the orientation of the enzyme and the number of enzyme–support attachments are identical (because the multipoint covalent immobilization has been performed before the blocking step, that is the only difference), the functional properties of the enzyme may be greatly altered. Considering that the exact orientation of the enzyme molecules on the support is unknown, this is an empiric approach, but the preparations and screening of the biocatalyst with better properties is rapid. This way, the size of the library of lipase biocatalysts may increase and the possibilities of finding a lipase biocatalyst useful for a particular process will also enhance [43].

As lipases, we have utilized two of the most utilized in academic and industrial studies that can be immobilized on these DVS agarose supports with good yields and activity recovery [55,56], that from *Themomyces lanuginosus* [57], and the form B from *Candida antarctica* [58,59].

2. Materials and methods

2.1. Materials

Lipase B from *C. antarctica* (CALB) (6.9 mg of protein/mL) and lipase from *Thermomyces lanuginosus* (TLL) (36 mg of protein/mL) were a kind gift from Novozymes (Spain). Methyl mandelate, ethyl hexanoate, methyl phenylacetate, *p*-nitrophenylbutyrate (*p*NPB) and diethyl p-nitrophenylphosphate (D-*p*NPP) were from Sigma Chemical Co. (St. Louis, MO, USA). All other 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 (isobestic point) produced by the released *p*-nitrophenol in the hydrolysis of 0.4 mM *p*-NPB in 100 mM sodium phosphate at pH 7.0 and 25 °C ($\varepsilon = 5150\ M^{-1}\ cm^{-1}$ under these conditions). To start the reaction, 50–100 μL of lipase solution or suspension was added to 2.5 mL of substrate solution. One 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's method [60] and bovine serum albumin was used as the reference.

2.3. Immobilization on DVS-agarose beads

2.3.1. Preparation of DVS-agarose beads

DVS-agarose beads support was prepared as previously described [52]. A volume of 1.5 mL DVS was stirred in 40 mL of 333 mM sodium carbonate at pH 12.5, giving a concentration of 0.35 M DVS, until the medium becomes homogeneous, then 2 g of agarose beads was added and leave under gentle agitation for 35 minutes. After, the support was washed with an excess of distilled water and stored at 4 °C.

2.3.2. Immobilization of lipases on DVS-agarose

The immobilization was performed as previously described [55,56]. A 10 g portion of support (DVS-agarose) was suspended in 100 mL of solutions of proteins (maximum protein concentration was 1 mg/mL) the immobilization was performed in 100 mL of 10 mM sodium carbonate at pH 10 at 25 °C in the presence of 0.01% triton X100 (CALB), or 0.01% of CTAB (TLL). The detergents were used to avoid lipase adsorption on the support or to break enzyme dimers [61–64]. The immobilized enzymes were incubated in 100 mL of 100 mM bicarbonate at pH 10.0 and 25 °C for 72 h (washing the preparations to eliminate the detergent). As a reaction end point, all the immobilized biocatalysts were incubated in 1 M of different nucleophiles (ethylenediamine (EDA), mercaptoethanol, imidazole, hexylamine, aminoacids, etc.) at pH 10 and 25 °C for 24 h to block the remaining reactive groups. Finally, the immobilized preparation was washed with an excess of distilled water and stored at 4 °C.

2.4. Thermal inactivation of the different enzyme immobilized preparations

To check the stability of enzyme derivatives, 1 g of immobilized enzyme was suspended in 5 mL of 10 mM of sodium phosphate at pH 7 at different temperatures. Due to the very different stability of the different enzymes, we showed the half-lives at Temperatures that gave a reasonable and reliable value. Periodically, samples were withdrawn and the activity was measured using *p*NPB. Half-lives were calculated from the observed inactivation courses. The values given are those where the inactivation rate is adequate to perform a proper comparison.

2.5. Stability assays in the presence of dimethylformamide of the different enzyme immobilized preparations

Enzyme preparations were incubated in mixtures of 90% dimethylformamide/10% 100 mM Tris at pH 7 and at different