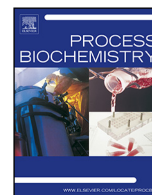
Process Biochemistry xxx (2015) xxx–xxx

Contents lists available at ScienceDirect

Process Biochemistry

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



Evaluation of divinylsulfone activated agarose to immobilize lipases and to tune their catalytic properties

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A R T I C L E I N F O

Article history:
Received 6 February 2015
Received in revised form 9 March 2015
Accepted 23 March 2015
Available online xxx

Keywords:
Lipase immobilization
Divinylsulfone
Enzyme stabilization
Lipase modulation
Multipoint covalent attachment
Lipase from *Thermomyces lanuginosus*

A B S T R A C T

Divinylsulfone (DVS) activated agarose beads have been used to immobilize several lipases, those from *Pseudomonas flourescens*, *Rhizomucor miehei*, and *Thermomyces lanuginosus* (TLL), as well as the artificial chimeric phospholipase Lecitase Ultra. The best results in terms of activity recovery and immobilization yield were achieved using TLL. This enzyme could be immobilized at pH from 5 (with poor yield) to pH 10 (with 100% yield). The incubation of the immobilized enzymes for 72 h at pH 10 before the blocking step (using ethylenediamine) improved the enzyme stability whatever the immobilization pH value, but the stabilization achieved in each case depended on the immobilization pH value, and also on the inactivation conditions.

The enzyme activities versus different substrates were very dependent on the immobilization protocol and the conditions of activity determination. That way, TLL immobilized at pH 5 on DVS activated support was the most active versus methyl mandelate, even more active than TLL immobilized on octyl-agarose (between 3 and 7 fold factors depending on the pH of measure). Using ethyl hexanoate the most active preparation was the octyl-TLL preparation. The most active among the enzymes immobilized in DVS-activated supports was that just immobilized at pH 5 if the activity was determined at pH 7 or 8.5, while at pH 5 the most active was that enzyme but after incubation at pH 10.

The results show that this support may be very useful for tuning lipase properties just by altering the first immobilization pH value, and that the further incubation at pH 10 improved enzyme stability, and in some cases, even increased activity.

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1. Introduction

Enzyme immobilization is a requirement in many industrial applications for these interesting biocatalysts [1–4]. This tool solves the problem of enzyme solubility and permits enzyme recovery and simplifies reactor design [5–10]. Moreover, immobilization, if properly designed, may be utilized to avoid other enzyme limitations or to improve some enzyme features, such as their stability [11,12] activity, selectivity or specificity (e.g., versus non-physiological substrates) [13,14]. Multipoint covalent attachment is one of the possibilities that the researcher may achieve if a proper immobilization system is utilized [10,13–18]. This permits the rigidification of the enzyme structure, as all enzyme groups involved in the immobilization cannot alter their relative positions (if the support is rigid and the spacer arm is short) [13]. If we can alter the orientation of the enzyme on the support by changing the experimental conditions, this may permit the tuning of the enzyme catalytic properties by rigidification of specific areas of the enzyme structure [14,17,19]. Epoxy [20,21], glyoxyl [22,23] or glutaraldehyde [24] groups have been proposed as support moieties with good prospects to reach this goal, but they have their own limitations. Thus, the search of new reactive groups able to give multipoint covalent attachments during immobilization remains a challenge for enzyme technology in the XXI century. In this context, divinylsulfone (DVS) activated supports have been used in

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http://dx.doi.org/10.1016/j.procbio.2015.03.018
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Please cite this article in press as: dos Santos JCS, et al. Evaluation of divinylsulfone activated agarose to immobilize lipases and to tune their catalytic properties. Process Biochem (2015), http://dx.doi.org/10.1016/j.procbio.2015.03.018

the immobilization of some enzymes [25–33], mainly in biosensors field. These supports have been recently identified as suitable ones to stabilize enzymes via multipoint covalent attachment, using chymotrypsin as a model enzyme [34]. The vinyl sulfone group is very stable in a broad range of pH values (from 4 to 10) [34], and it is able to react with primary and secondary amine, hydroxyl, phenyl, thiol or imidazol groups in different pH conditions [27,33,35]. The further long time incubation at alkaline pH value of the immobilized enzyme permitted to increase the number of enzyme-support linkages, increasing the enzyme rigidity and avoiding changes on enzyme structure induced by distorting agents [34]. The reactivity of the protein groups with the support depends on the pH, and that way the orientation of the enzyme molecules on the support may be altered by changing the immobilization pH [34]. The final blocking of the support with nucleophiles (e.g., ethylenediamine) permitted to eliminate the chemical reactivity of the support avoiding undesired enzyme-support covalent bonds and being an useful reaction end point [34].

On the other hand, lipases are the most utilized enzymes in biocatalysis due to their wide substrate specificity, high stability under a wide range of conditions and reaction media (aqueous, organic solvent, neoteric solvents) and broad range of reactions that they are able to catalyze (hydrolysis, esterifications, aminations, acydolysis, transesterifications, and also other promiscuous reactions such as perhydrolysis or C–C bond synthesis) [36–44].

Lipases properties, including selectivity, specificity and activity are very easily modulated by almost any change in the enzyme structure (including genetic manipulation, medium engineering or chemo-physic modifications (including immobilization)) or by small changes in the reaction media. [14,17,19,45–51]. Even promiscuous activities have been altered by immobilization [52]. This is due to the flexibility of their active center and the conformational changes that the lipases suffer during catalysis, involving the movement of an oligopeptide chain (lid or flat) that usually isolates the active center of lipases from the medium [53,54]. The open form of the lipases becomes strongly adsorbed on their natural substrates (drops of oils) and any other hydrophobic surface [39,55,56].

In this new research, we will explore the prospects of using activated divinyl sulfone agarose beads as a matrix to immobilize different lipases and tuning their catalytic properties during this immobilization. We have immobilized on DVS activated agarose the lipases from *Thermomyces lanuginosus* (TLL) [57], from *Rhizomucor miehei* (RML) [58] and from *Pseudomonas fluorescens* (*PFL*) [59,60]. They have a proper lid and are very likely some of the most utilized lipases after CALB [61,62]. We have also included Lecitase Ultra in these studies, a commercial chimeric phospholipase built from the gen of the lipase from *Thermomyces lanuginosus* (to obtain good stability) and that of the phospholipase from *Fusarium oxysporum* (to get the phospholipase activity) [63]. As reference, we have immobilized the lipases on octyl agarose [64], this immobilization strategy permit to keep the open structure of the lipases in absence of any other external hydrophobic interface.

2. Materials and methods

2.1. Materials

Lipase from *Thermomyces lanuginosus* (TLL) (36 mg/mL), lipase from *Rhizomucor miehei* (RML) (13.7 mg/mL), and phospholipase A1 (Lecitase Ultra) (16 mg/mL) were obtained from Novozymes (Spain). Lipase from *Pseudomonas fluorescens* (PFL) (in powder) was from Amano. Divinyl sulfone, *p*-nitrophenyl butyrate (*p*-NPB), triton X-100, ethylenediamine (EDA), cetyltrimethylammonium bromide (CTAB), sodium dodecyl sulfate (SDS), methyl mandelate, methyl phenylacetate and ethyl hexanoate were from Sigma Chemical Co. (St. Louis, MO, USA). Crosslinked agarose beads 4BCL and octyl agarose beads 4BCL were from GE Healthcare. All reagents and solvents were of analytical grade.

All experiments were performed by triplicate and the results are reported as the mean of this value and the standard deviation (usually under 10%).

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 [65] 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 active after incubation for the assay time at any of these pH values.

2.3. Immobilization of TLL on octyl-agarose beads

Lipase was immobilized on octyl-agarose beads at low ionic strength [64]. Immobilization was performed using 1 mg of protein per g of octyl-agarose beads. A corresponding volume of commercial TLL was diluted in 100 mL of 10 mM sodium phosphate at pH 7 and 25 °C. Then, the support was added. The activity of both supernatant and suspension was followed using as substrate *p*-NPB (see Section 2.2). After 3 h at 25 °C under gentle stirring, the immobilization suspension was filtered and the supported lipase was washed several times with distilled water.

2.4. Immobilization on DVS-agarose beads

2.4.1. Preparation of DVS-agarose beads

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 left under gentle agitation for 35 min. After, the support was washed with an excess of distilled water and stored at 4 °C.

2.4.2. Immobilization of lipases on DVS-agarose

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 of sodium acetate at pH 5, sodium phosphate at pH 7 or sodium carbonate at pH 10 at 25 °C. In some instances, 0.01% triton X-100 (for RML or PFL), 0.01% of SDS (for Lecitase) or 0.01% of CTAB (for TLL) were added.

In some instances, 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 EDA at pH 10 and 25 °C for 24 h to block the remaining reactive groups (this was the optimal blocking reagent using chymotrypsin and this support) [34]. Finally, the immobilized preparation was washed with an excess of distilled water and stored at 4 °C.

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