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Easy stabilization of interfacially activated lipases using heterofunctional divinyl sulfone activated-octyl agarose beads. Modulation of the immobilized enzymes by altering their nanoenvironment

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ABSTRACT

Octyl-agarose is a support that permits the one step immobilization, stabilization and purification of lipases. However, the enzyme may be released from the support under drastic conditions. This paper describes a new heterofunctional support, octyl agarose beads activated with divinyl sulfone, that has proved to be useful to produce very stable and active biocatalysts of lipases from *Candida rugosa* (CRL), *Rhizomucor miehei* (RML) and *Thermomyces lanuginosus* (TLL), able to work under any reaction conditions without risking enzyme desorption. The three enzymes failed in immobilization on glyoxyl-octyl supports for different reasons. The immobilization at pH 5 permitted to keep the good properties of octyl agarose. Further incubation at pH 8 permitted to establish at least one covalent enzyme-support bond per enzyme molecule (preventing the risk of enzyme desorption), avoiding the inactivation produced at pH 10, and the final result is that all three new biocatalysts are more active than the octyl-glyoxyl counterparts and much more stable (e.g., 20 using CRL). The end of the enzyme-support reaction was achieved via blocking the vinylsulfone groups with different nucleophiles (cationic, anionic, hydrophobic, etc). This not only determined the final enzyme stability, but also the activity, selectivity and even specificity of the different immobilized preparations.

1. Introduction

Lipases are among the most used biocatalysts [1–5]. These very robust enzymes require their immobilization for most industrial uses to reuse this expensive catalyst or improve some enzyme properties [6–11]. Immobilization may be specially interesting if it may be coupled to purification, another requirement for some uses of lipases [12].

Recently, the immobilization of lipases on heterofunctional octyl-glyoxyl supports has been revealed as a potent tool to reach one step immobilization, purification, hyperactivation and strong stabilization of the lipases[13–18]. It permitted to specifically immobilize the open form of the lipases on the support permitting to get a significant increment in activity after immobilization [19]. Moreover, this stabilized open form of the lipase has been

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described to be much more stable than the lipase form in opening/closing equilibrium [20,21]. In fact, lipases immobilized on octyl-agarose have proved to be much more stable than enzyme immobilized via multipoint covalent attachment, overpassing the stability of enzymes immobilized on glyoxyl or vinylsulfone activated supports[22,23]. The use of octyl-glyoxyl is based on a first immobilization at neutral pH value of the lipases via interfacial activation over the octyl layer of the support [19,24] and later, the incubation at pH 10 increased the reactivity of the ε-amino group of Lys and permitted the establishment of covalent linkages between the adsorbed lipases and the glyoxyl groups on the support [18]. The immobilization is first via interfacial activation because the glyoxyl groups are unable to immobilize standard proteins at neutral pH values [25]. This strategy improved the stability of some enzymes keeping the advantages of the octyl supports, permitting even at least a partial reactivation of the inactivated enzymes via unfolding/refolding strategies [15,26]. These biocatalysts were more stable than the just octyl preparations, that was previously stated to be among the most stable preparations described in literature, even more than covalently attached lipases preparations [22,24,27]. Although this immobilization method was very useful, it presented several limitations. The enzyme needs to be stable at alkaline pH value to get the enzyme-support reaction [18], and that did not occur with all lipases (e.g., lipase from *Rhizomucor miehei* lost more than 50% of the activity during alkaline incubation) [18]. Moreover, some lipases have very few primary amino groups in the face of the active center, and in some instances a significant percentage of enzyme molecules is not covalently attached to the support (e.g., more than 30% of the lipase molecules from *Thermomyces lanuginosus* immobilized on octyl-glyoxyl released from the OCGLX support when incubated in the presence of detergents)[18] This has been solved by the chemical amination of the lipases, which has provided a simple way to increase the reactivity towards the glyoxyl groups of the support [28]. However, this strategy introduced an additional step in the biocatalyst preparation although may improve enzyme properties in certain cases[29].

In this new paper, we have prepared a new type of heterofunctional support, octyl activated with divinyl sulfone (OCDVS) to introduce vinyl sulfone groups on the support surface. The vinylsulfone group may be easily introduced by modification of the hydroxyl groups of octyl-agarose with DVS [30], and this group may react with primary and secondary amino, imidazole, thiol, phenolic groups, etc. The use of this group to immobilize proteins was first proposed by Porath [31], but this activation protocol has been scarcely utilized [32–38].

Recently, the advantages of this group in enzyme immobilization have been studied[22,30,39]. The vinylsulfone moiety is very stable (permitting long-term immobilization), it is able to react with very different moieties (primary and secondary amino groups but also hydroxyl, imidazole or thiol groups [30] These make that these supports may produce a rapid and intense multipoint covalent attachment of enzymes[30,39]. Moreover, it has been described that this support may immobilize proteins at a pH range from 5 to 10, involving different residues in the first immobilization depending on the immobilization pH value[30,39]. However, immobilization at acid pH value on this support is very slow, far slower than at neutral or alkaline pH value [30]. This is critical for our strategy, as we intended that the first immobilization of the enzyme on the support still was via interfacial activation versus the acyl-layer. Considering the rapid immobilization of lipases on octyl-agarose at any pH value [19], this new heterofunctional support may permit the first lipase immobilization via interfacial activation if the immobilization is performed at acidic pH. Later, this immobilized enzyme may get a covalent attachment with the support. To accelerate this reaction, the immobilized enzyme may be incubated at neutral or alkaline pH value to increase the enzyme-support reactivity, but it is not necessary to use pH 10 [22,30,39,27] like using octyl-glyoxyl [15,18]. The vinyl sulfone group has several advantages regarding the glyoxyl group for getting at least one enzyme-support covalent attachment: it may involve many different groups on the enzyme surface in the immobilization (Lys, but also His, Cys or Tyr), the attachment is irreversible and may be achieved under a broad range of pH values[30]. Additionally, the spacer arm is longer, reducing the steric hindrances for the covalent enzyme-support reaction caused by the layer of octyl groups when compared to the glyoxyl groups[30,39]. In fact, it has been shown that the DVS activated agarose enable a much more intense multipoint covalent attachment than the glyoxyl groups[30,39].

The possibilities using this kind of supports go further. After immobilization, and as a reaction end point, the remaining vinyl sulfone groups may be blocked using different nucleophiles[23,30]. This blocking step (using pure epoxy or DVS activated supports) has been used as an additional tool to tailor enzyme properties[23,40]. Using lipases immobilized on supports activated by DVS, it has been shown that the blocking step permits to alter the lipase properties, not only stability, but also activity, selectivity or specificity[23]. This was explained by different interactions between the enzyme and the support, which could promote some conformational lipase changes. The conformational changes of lipases make that their active center is quite flexible making relatively easy modulate the final properties (even if in an empiric way) via different tools [41–47]. Using OCDVS supports, the presence of a certain amount of blocking reagents (in a moderate spacer arm) with different physical properties may induce some additional interactions with the already immobilized lipase.

In this paper, we show the results obtained with three different lipases after the optimization of the immobilization protocol on octyl-DVS supports: the lipase from Candida rugosa (CRL)[48], *R. miehei* (RML)[49,50] and *T. lanuginosus* (TLL)[51]. These three lipases have some problems using octyl-glyoxyl (enzyme inactivation or moderate covalent immobilization yield)[15,18]. Moreover, the immobilization on agarose activated with DVS in the case of the lipase from *R. miehei* did not permitted to obtain an active enzyme preparation, while using TLL the activity recovery was satisfactory but the stability was far below that of the octyl-TLL preparations[27]. Here, we intend to evaluate if the new heterofunctional OCDVS support is able to solve these problems.

2. Materials and methods

2.1. Materials

Octyl-Sepharose beads matrix (4BCL) was from GE Healthcare. Solutions of lipase from *T. lanuginosus* (TLL) (36 mg of protein/mL) and lipase from *R. miehei* (RML) (13.7 mg of protein/mL) were kindly supplied by Novozymes (Spain). Lipase from *C. rugosa* (CRL) (in powder form, 40% protein (w/w)), divinyl sulfone (DVS), *R* and *S* methyl mandelate, methyl phenyl acetate, ethyl hexanoate and *p*-nitrophenyl butyrate (*p*-NPB) were obtained from Sigma Chemical Co. (St.Louis, MO, USA). All reagents and solvents were of analytical grade. The preparation of octyl glyoxyl-agarose (OC-GLX) was performed as previously described [18].

2.2. Standard determination of enzyme activity

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

This reaction was followed by recording the increase in absorbance at 348 nm produced by the released *p*-nitrophenol in

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