Process Biochemistry xxx (2017) xxx–xxx

Contents lists available at ScienceDirect

Process Biochemistry

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

Effect of immobilization rate and enzyme crowding on enzyme stability under different conditions. The case of lipase from *Thermomyces lanuginosus* immobilized on octyl agarose beads

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ARTICLE INFO

Article history:
Received 13 January 2017
Received in revised form 24 February 2017
Accepted 27 February 2017
Available online xxx

Keywords:
Immobilized enzyme distribution
Enzyme packing
Enzyme stability
Octyl agarose
TLL stabilizers
Glycerin

ABSTRACT

Lipase from *Thermomyces lanuginosus* (TLL) was immobilized on octyl agarose (OC). Three different TLL-OC biocatalysts were prepared: one lowly loaded using a low enzyme concentration, one fully loaded using a low enzyme concentrations, and a final one using a large excess of enzyme at a higher concentration. The activities after immobilization increased (180%), although diffusion limitations reduced the hyperactivation of the fully loaded preparations (140%). The stabilities of both preparations using low enzyme concentrations were similar under all studied conditions discounting the diffusional limitations of the biocatalyst. However, the biocatalyst prepared using a large concentration of enzyme was less stable that the other preparations at pH 7.0, more stable at pH 5.0 and with a similar stability at pH 9.0. Adding 3 M NaCl, the stability of the fully loaded preparations significantly increased; while the lowly loaded preparation slightly improved enzyme stability. This produced that the biocatalyst prepared under using high enzyme concentration become significantly more stable than the other two TLL preparations. Glycerin increased immobilized TLL stability, in this case all OC-TLL preparations became with similar stabilities. Results show that the TLL concentration during immobilization may greatly affect TLL properties, perhaps due to altering enzyme packing.

1. Introduction

Lipases are among the most used enzymes in biocatalysis due to their wide specificity, in many cases coupled to a strict regio- or enantioselectivity or specificity, robustness under many reaction media and capability of performing many different reactions, including the so-called promiscuous reactions [1–4]. As most enzymes, the use of lipases requires their previous immobilization to simplify enzyme recovery [5–8]. This immobilization step may be utilized to improve many other enzyme properties, like stability, activity, selectivity [9–13] or even enzyme purity [14].

Lipases are called interfacial enzymes [15–22] because they are able to become adsorbed on the hydrophobic surfaces of drops of oils, their natural substrates, thanks to the existence of two conformational forms. The closed form has usually the active center isolated from the medium (lipase B from *Candida antarctica* is one of the exceptions [23]) by a polypeptide chain called lid. This lid has a hydrophobic internal face that interacts with the hydrophobic surroundings of the active center [18–22]. The open form has this lid shifted and exposes a large hydrophobic pocket to the medium. This structure is unstable in aqueous homogenous media but it is strongly adsorbed on the hydrophobic surface of the drops of substrates (oils). This affinity of lipases for hydrophobic surfaces of substrates drops is extended to any other hydrophobic surface. That way, lipases may be adsorbed on other open molecule of a lipase [24,25], hydrophobic proteins [26] or hydrophobic supports [27,28].

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http://dx.doi.org/10.1016/j.procbio.2017.02.024
1359-5113/

That way, immobilization of lipases on hydrophobic supports permits the one step immobilization, purification and hyperactivation of most lipases [27], involving the open form of the lipase [29]. The method is very simple, may give enzyme stabilizations higher than multipoint covalent attachment [30,31] and has become a preferred method for lipase immobilization [32]. The main limitations of this strategy come from the possibility of lipase release under certain conditions or in the presence of some substrates/products with detergent properties [33–35]. Lipase from *Thermomyces lanuginosus* is among the most utilized lipases [36] and it has been successfully immobilized on different hydrophobic supports [36].

Immobilized lipases are considered to be dispersed on the support surface, as most enzyme molecules may become immobilized at certain distance from one another. Using bovine serum albumin (BSA), the coating of an anion exchanger with this protein permitted to develop a support able to only immobilize proteins smaller than BSA [37], confirming that BSA was dispersed on the surface of the support. However, this may be not entirely true when the immobilization rate is much higher than the diffusion rate [38–45]. In those cases, the enzyme molecules may be packed together, and some intermolecular interactions may be produced. This is the case of CALB immobilized on octyl agarose, where the immobilization is so rapid that the enzyme molecules are very near each other, enabling a small reagent such as glutaraldehyde to produce the enzyme intermolecular crosslinking [46]. This "crowding" effect has been reported to be generally stabilizing using free enzyme [47–49], but using CALB immobilized on octyl agarose it was found that when the support was fully loaded of enzyme, the stability of the enzyme decrease under certain conditions [50]. This negative effect was explained by interactions between vicinal partially unfolded enzyme molecules (perhaps exhibiting hydrophobic pockets) that prevented the recovery of the enzyme activity when measuring the enzyme activity under mild conditions during the inactivation experiments. Stabilizing effects of crowded immobilized enzyme may be derived from difficulties in the movement of the enzyme structure, if one enzyme is closely immobilized to another, when some parts of the enzyme molecule try to move, they will crash with another immobilized enzyme and thus conformational changes may be avoided.

The presence of high concentrations of salt may have several effects on the stability of OC-TLL [51–53]. From one side, the adsorption of the enzyme to the octyl support should be reinforced, if this is a key point on OC-TLL inactivation, this can increase the enzyme stability. On the other hand, the movement of hydrophobic groups from the core of the protein to the medium should be hindered, perhaps increasing enzyme stability (in this case, for any immobilized enzyme where precipitation is not possible), also high concentrations of salt may produce some order in the water molecules reducing enzyme mobility. Moreover, high concentrations of salt may reduce water activity and that may also produce some stabilization by reducing enzyme mobility. Glycerin is a polyol that in many instances has been used to stabilize enzymes with effect well described [54–59]. However, it is more hydrophobic than water, and in the case of OC-TLL, it may favor enzyme desorption from the support, making unclear the final effect of this additive on enzyme stability. However, these different physical conditions of the inactivation suspensions may drive to different inactivation ways, altering the effect of the enzyme crowding on enzyme stability [60].

We will focus on the functional properties of the enzyme (stability) to first check if it is possible to have a TLL crowding in immobilized octyl-TLL and how this really affects enzyme stability (the loading have not significant effect as described in [50]). This will have a significant impact in the development of immobilized enzyme preparations [61,62], as may be a proof that the exact immobilization conditions, even using a simple method as an adsorption of the enzyme in the support, may have finally significant effect on the enzyme performance. Moreover, these effects may be different depending on the inactivation conditions [60].

In this new paper, we have prepared fully loaded biocatalyst of TLL immobilizing the enzyme in octyl agarose to have a very rapid immobilization, and utilizing very different enzyme concentrations (this variable will determine the immobilization rate). Then, we have checked the effect on the enzyme stability of the different enzyme biocatalysts under different conditions that can alter the inactivation conformational changes (e.g., different pH values, different ionic strength or presence of glycerin). To determine any positive or negative effect of the enzyme concentration during its immobilization may have some evident impacts on the preparation of enzyme industrial biocatalysts. We do not intend to give optimal protocols for the preparation of octyl-TLL biocatalyst, but to detect likely effects of immobilized enzyme crowding on enzyme stability, and also if these effects may be different depending on the studied conditions.

Thus, the main aim of the paper is to detect if the stability of immobilized enzymes may be altered by the concentration of the enzyme utilized in the immobilization, and if these effects may depend on the inactivation conditions.

2. Materials and methods

2.1. Materials

Commercial soluble lipase from *Thermomyces lanuginosus* (16 mg of protein/mL) was kindly donated by Novozymes (Spain). Octyl-agarose CL-4B beads were from GE Healthcare (Uppsala, Sweden). *p*-Nitrophenyl butyrate (*p*-NPB) was purchased from Sigma-Aldrich (St. Louis, MO, USA). Electrophoresis purity reagents were obtained from Bio-Rad (Hercules, USA). Other reagents 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 (under 5%).

2.2. Assay of enzyme activity

This assay was performed by measuring the increase in absorbance at 348 nm (isobestic point) produced by the release of *p*-nitrophenol in the hydrolysis of 0.4 mM *p*NPB in 25 mM sodium phosphate at pH 7.0 and 25 °C (molar extinction coefficient (ε) under these conditions is 5150 mol^{-1} cm^{-1}). To start the reaction, 50–100 μl of lipase solution or suspension was added to 2.5 ml of substrate solution. One international unit of activity (U) was defined as the amount of enzyme that hydrolyses 1 μmol of *p*NPB per minute under the conditions described previously.

2.3. Immobilization of TLL on octyl agarose

The immobilization experiments were performed using 1, 6 or 30 mg of protein per gram of wet support. The commercial sample of enzyme was diluted in different volumes of 5 mM sodium phosphate at pH 7.0 (respectively, 250, 1500 or 60 ml respectively for the biocatalyst with 1, 6 or 30 mg of protein per gram of wet support). Then 10 g of the desired support were added. The activity of both supernatant and suspension was followed using *p*NPB. After immobilization, the suspension was filtered and the supported enzyme was washed 10 times with 10 vol of distilled water and stored at 4 °C until use. Washing 100 times and using 3 M NaCl or 60% glycerin (followed with 10 times washing with distilled water) did not affect the final enzyme activity and stability.

Please cite this article in press as: H. Zaak, et al., Effect of immobilization rate and enzyme crowding on enzyme stability under different conditions. The case of lipase from *Thermomyces lanuginosus* immobilized on octyl agarose beads, Process Biochem (2017), http://dx.doi.org/10.1016/j.procbio.2017.02.024