Process Biochemistry 50 (2015) 1211–1217

Contents lists available at ScienceDirect

Process Biochemistry

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

Immobilization of lipases on glyoxyl–octyl supports: Improved stability and reactivation strategies

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ARTICLE INFO

Article history:
Received 15 April 2015
Received in revised form 11 May 2015
Accepted 13 May 2015
Available online 22 May 2015

Keywords:
Immobilization of lipases via interfacial activation
Enzyme stabilization
Covalent immobilization
Enzyme reactivation
Enfolding/refolding

ABSTRACT

Lipases from *Candida rugosa* (CRL) and from *Candida antarctica* (isoform A) (CALA) have been successfully immobilized on octyl–glyoxyl agarose (OCGLX) beads and compared to the octyl–agarose (OC) or glyoxyl (GLX) beads immobilized counterparts. Immobilization on OCGLX gave similar hyperactivations than those found for the immobilization on OC supports, although the incubation at pH 10.0 for 4 h decreased the activity of both enzymes by 25%. After reduction, more than 95% of the enzyme activity was covalently attached to the support. The fraction not covalently attached was desorbed by washing with detergent. These biocatalysts were more stable than the octyl counterparts in thermal or organic solvent inactivation. More interestingly, the irreversible immobilization permitted the reactivation of CALA biocatalysts inactivated by incubation in organic solvent, after unfolding in the presence of guanidine and refolding in aqueous buffer (around 55% of the activity could be recovered during 3 successive cycles of inactivation/reactivation). GLX–CALA permitted to recover 75% of the activity, but the thermal stability and activity was much lower, and this strategy could not be applied to CRL. Neither the enzyme immobilized on cyanogen bromide nor the enzyme immobilized on OCGLX exhibited significant activity after the unfolding/refolding strategy.

1. Introduction

Enzymes are very interesting [1–7] but they also have some limitations as industrial biocatalysts [8]. This occurs for example with the moderate stability of enzymes under conditions sometimes required by industry [9,10]. The operational enzyme stability may be improved by genetic tools [11], chemical modifications [12,13], enzyme immobilization [14–18], and also by selecting adequate reaction conditions [19].

Enzyme immobilization is a requirement for many applications [20], and it is compatible with any other strategy for enzyme stabilization [13,18,21,22], and the inactivated biocatalyst may be submitted to strategies of reactivation after partial or total inactivation [23]. If the enzyme is incubated in the presence of inert organic solvents, at neutral pH value and moderate temperature, the enzyme will be inactivated mainly via the promotion of incorrect structures. If this is the case, the enzyme may be submitted to unfolding/refolding strategies trying to recover the native enzyme structure [24].

The previous immobilization of the enzymes on supports via covalent linkages may help the refolding step [25–28]. In fact, if several enzyme-support linkages are established, the refolding may be facilitated because the relative positions of these groups cannot be altered, and those may act as reference points during refolding [29]. Even heavily chemically modified enzymes could be unfolded and refolded after immobilization via multipoint covalent attachment [29,30]. The main requirements to use this strategy are that the enzyme should remain attached to the support during all the reactivation steps, and that the support is inert enough to avoid undesired enzyme-support interactions. Some examples of

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http://dx.doi.org/10.1016/j.procbio.2015.05.010
1359-5113/

reactivation of immobilized enzymes involve lipases [26,29–31]. These enzymes have a peculiar mechanism of action, the so called interfacial activation, which requires some movements of the enzyme structure between a closed and inactive structure, with a polypeptide chain (called lid) blocking the active center, and an open and active structure, with the active center exposed to the medium [32–36]. This open form has a tendency to become adsorbed on any hydrophobic surface [37]. The lipase from *Candida antarctica* (form B) (CALB) has a very small lid and does not fully isolate the active center [38], but CALB is still able to become adsorbed on hydrophobic surfaces [39] while the lipase from *Bacillus thermocatenolatus* has a double lid [40]. However, the lipases usually have a large and single lid able to fully prevent the interaction of enzyme active center and medium, as it is the case of the lipase from *Thermomyces lanuginosus* (TLL) [41].

The interfacial activation of the enzyme on hydrophobic supports, like octyl agarose [42,43] is a very useful immobilization strategy that has permitted the one step immobilization/purification/hyperactivation/stabilization of many lipases [44]. This immobilization protocol is not compatible with unfolding/refolding reactivation strategies, as this immobilization is reversible and the enzyme will be desorbed after the incubation in caotropic agents [44].

Octyl–glyoxyl agarose [45] couples the advantages of octyl agarose with those of covalent attachment, making the immobilization irreversible and producing more stable biocatalysts than the octyl-support. The irreversibility of the immobilization has the drawback that the support cannot be reused after enzyme inactivation. However, it may now be possible to submit the immobilized enzyme to unfolding/refolding reactivation strategies without risk of enzyme desorption. If this reactivation strategy is successful, it may make discarding neither enzyme nor support unnecessary [45]. A likely problem is the possibility of interaction of the hydrophobic groups of the enzyme with the octyl groups of the support during the refolding step.

In this new paper, we have extended the application of the new octyl–glyoxyl [45] to the immobilization of two new enzymes, and analyze their reactivation possibilities. The new enzymes used in this paper are the lipase A from *C. antarctica* (CALA) and the commercial lipase from *Candida rugosa* (CRL). Both enzymes have a proper lid and have been used in many different applications [46–50], After characterizing the biocatalyst, we have studied the possibilities of applying the strategy of unfolding/refolding to these biocatalysts. To our knowledge, this is the first report of reactivation of any CALA or CRL immobilized preparations. The reactivation possibilities of the new biocatalysts have been compared to that of the covalent preparations.

2. Materials and methods

2.1. Materials

Crosslinked-Sepharose beads 4BCL and Octyl-Sepharose beads 4BCL were from GE Healthcare. Lipase A from *C. antarctica* (*CALA*) (presented as a solution with 19.1 mg of protein/mL) was kindly supplied by Novozymes (Spain). *R* and *S* methyl mandelate, *p*-nitrophenyl butyrate (*p*-NPB) and lipase from *C. rugosa* (CRL) (in powder form, 4% protein (w/w)) were from Sigma Chemical Co. (St. Louis, MO, USA). All reagents and solvents were of analytical grade. The preparation of glyoxyl or glyoxyl–agarose was performed as previously described [51].

2.2. Standard determination of enzyme activity

The hydrolysis of 0.4 mM *p*-NPB was used as standard activity assay. The released *p*-nitrophenol in 50 mM sodium phosphate at pH 7.0 and 25 °C was determined at 348 nm (ε under these conditions is 5150 M^{-1} cm^{-1}). The reaction was initialized by adding 50–100 μL of lipase solution or suspension to 2.5 mL of substrate solution. One international 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. In the case of enzyme incubated in organic solvents, a similar amount of these solvents were added to the reaction mixture to determine if they have any effect on enzyme activity.

Protein concentration was determined using Bradford's method [52] using as reference bovine serum albumin.

2.3. Immobilization of enzymes

2.3.1. Immobilization of CALA and CRL on glyoxyl supports

The immobilization was carried out using 4 mg of protein per g of wet support (support where the inter-particle water had been eliminated but the internal pores are full of water by vacuum drying). The immobilization medium was 50 mM sodium bicarbonate buffer at pH 10.0. After adding the support, the suspension was submitted to gentle stirring. Periodically, samples of the supernatant and suspension were withdrawn, and the enzyme activity was measured as described above.

2.3.2. Immobilization of enzymes on octyl and octyl–glyoxyl (OCGLX) supports

10 IU of protein per g of wet support were used in the immobilization studies, using as immobilization medium 10 mM potassium phosphate at pH 7.0. After adding the support, the suspension was submitted to gentle stirring. The activities of both supernatant and suspension were followed using *p*-NPB assay. After the indicated time, the immobilized enzyme was recovered by filtered and washed several times with distilled water. When the enzyme was immobilized on octyl–glyoxyl, the washed biocatalyst was in some instances incubated at pH 10.0 for different times, to favor the enzyme-support covalent reaction [53].

2.3.3. Reduction of glyoxyl preparations with sodium borohydride

As a support-enzyme chemical reaction end point, solid sodium borohydride was added to a concentration of 1 mg/mL to the OCGLX and GLX suspensions (at pH 10.0) under gentle stirring for 30 min. This treatment reduces unreacted aldehydes groups to fully inert hydroxy groups and transforms reversible imine bonds to very stable secondary amino bonds [13,51,53,54]. The preparations were washed with Triton X-100 (1% (v/v) for CALA and 0.5% (v/v) for CRL) to release from the support the adsorbed (but non-covalently attached) enzyme molecules. Finally the biocatalysts were filtered, washed with abundant distilled water and stored at 4 °C.

2.4. Thermal inactivation of different biocatalysts

0.5 g of immobilized enzyme was suspended in 5 mL of 50 mM of potassium citrate at pH 5.0, potassium phosphate at pH 7.0 or sodium bicarbonate at pH 9.0 at different temperatures to find a temperature where the half-life were reasonable and reliably determined. Periodically, samples of the inactivation suspensions were withdrawn and the pNPB activity was determined (see above). Half-lives were calculated from the observed inactivation courses.

2.5. Inactivation of different preparations in the presence of organic co-solvents

Immobilized biocatalysts were suspended in acetonitrile (ACN) (using CRL) or dimethylsulfoxide (DMSO) (using CALA)/100 mM Tris–HCl mixtures at pH 7.0 and 30 °C. Samples were withdrawn periodically and the *p*-NPB activity was determined as described