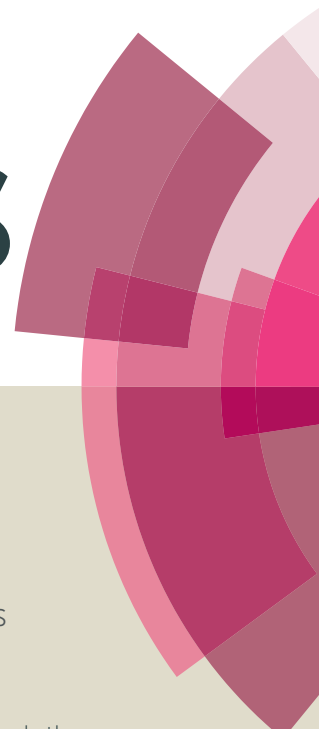


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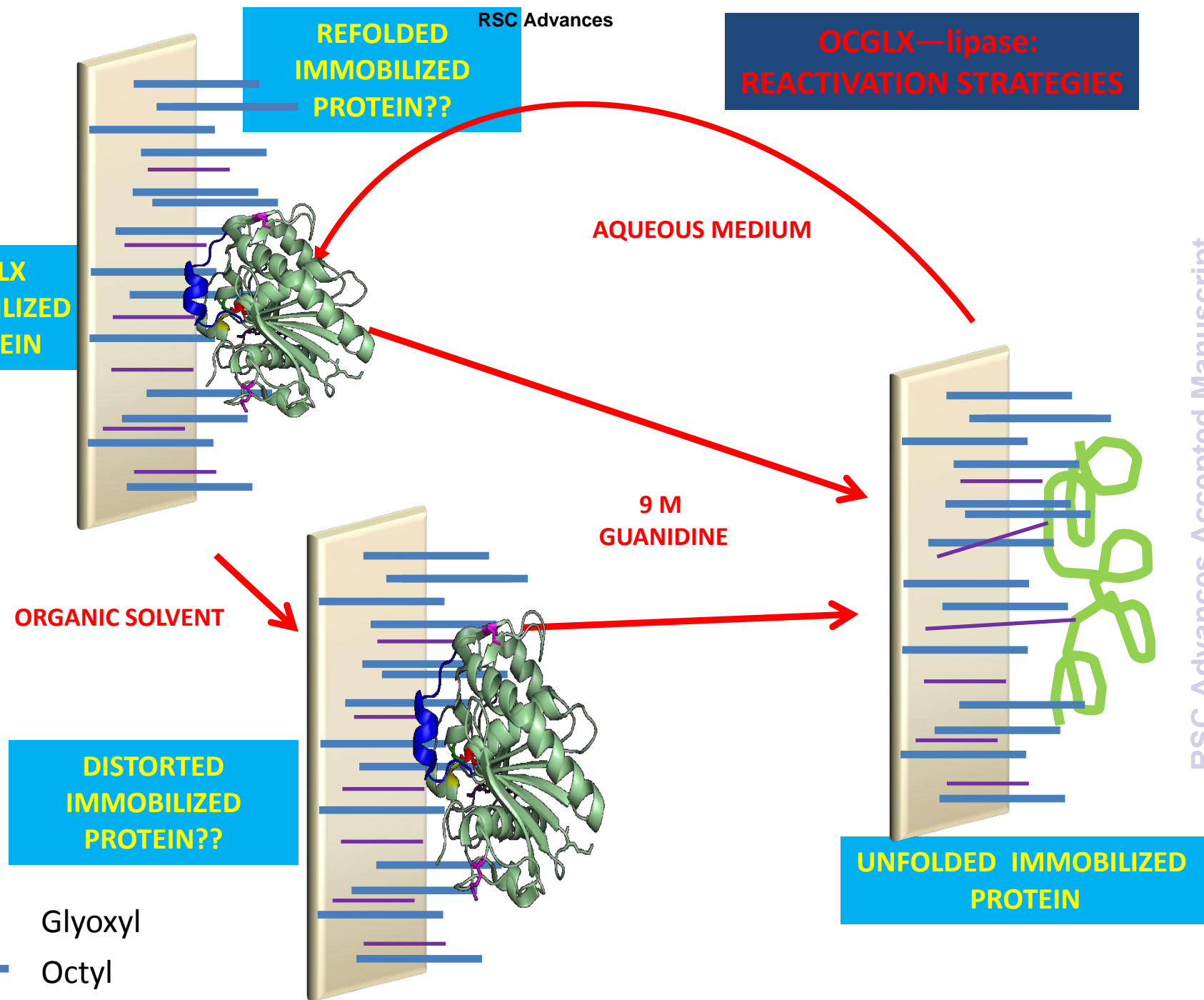


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REACTIVATION OF LIPASES VIA UNFOLDING/REFOLDING OF COVALENTLY IMMOBILIZED BIOCATALYSTS.

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Abstract

Lipases from *Candida antarctica* (isoform B) (CALB) and *Thermomyces lanuginosus* (TLL) have been immobilized covalently or via interfacial activation versus octyl support followed by covalent attachment via glyoxyl groups using octyl-glyoxyl agarose beads (OCGLX). These biocatalysts have been submitted to successive cycles of unfolding by incubation in 9 M guanidine and refolding by incubation in aqueous 100 mM phosphate buffer at pH 7, before and after total inactivation in the presence of organic solvents. The four preparations may be reactivated in some extension using this strategy, but results depended on the preparation. Glyoxyl immobilized CALB may recover 100% of activity versus the *p*-nitrophenyl butyrate, but after solvent inactivation this activity recovery was only 95%. The pure covalent TLL preparation permitted to recover around 80% of activity after or before solvent inactivation. Both enzymes offer lower activity recoveries using OCGLX, as may be expected from the hydrophobic nature of the groups in the support (60% for CALB and 45% for TLL). Moreover, using previously solvent inactivated enzymes, the results decreased in a 5-10%. These values were maintained along three successive cycles. However, using *R* and *S* methyl mandelate, it is clear that the activity recovery decreased along the reactivation cycles. Altogether, the unfolding/refolding strategy may be used to obtain part of the enzyme activity and that is relevant from an applied point of view, as this may permit to use the enzyme preparations for longer times. However, to reach the same enzyme structure in each reactivation cycle it is necessary to perform further studies that may involve from use of other supports to improve the unfolding and refolding steps of this strategy.

Key words: Enzyme reactivation, operation stability, enzyme solvent inactivation, enzyme unfolding, enzyme refolding, octyl-glyoxyl supports, heterofunctional support.

Introduction

Enzymes are very interesting biocatalysts due to their high activity in aqueous media at low temperature and pressure, and high enantio and regio selectivity and specificity¹⁻⁷.

However, enzymes have also some limitations, because the physiological optimal properties and the features required for their industrial implementation do not match in some points⁸. This occurs for example with the moderate stability of enzymes at conditions far from the physiological ones, that in many times are the ones required by industry^{9,10}. The operational enzyme stability may be improved by genetic tools¹¹, chemical modifications (e.g., crosslinking of the enzyme surface)^{12,13}, enzyme immobilization (e.g., generating adequate nano-environments or via multipoint or multisubunit immobilization)¹⁴⁻¹⁸, and also by selecting adequate reaction conditions¹⁹.

Enzyme immobilization is a requirement for many industrial applications²⁰, and it is compatible with any other strategy of enzyme stabilization^{13,18,21,22}. Moreover, the biocatalyst operational stability may be further improved if the inactivated biocatalyst may be submitted to strategies of reactivation after partial or total inactivation²³. 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. In these cases, the enzyme may be submitted to strategies of unfolding/refolding, and the native structure of the enzyme may be recovered²⁴.

Some recent papers suggest that the previous immobilization of the enzymes on supports via covalent linkages may help the refolding step²⁵⁻²⁸. Together with the simplification promoted by the use of immobilized enzymes, the immobilization prevent the enzyme aggregation during any step of the unfolding/refolding process, and if several enzyme-support linkages are established, the refolding may be facilitated because the relative positions of these groups cannot be altered, and

they may act as reference points during refolding²⁹. The main requirements to use this strategy are that the enzyme molecules remain attached to the support during the whole protocol, and that the support was inert enough to avoid undesired enzyme-support interactions. Surprisingly, even fully chemically aminated enzymes could be refolded after immobilization via multipoint covalent attachment^{29,30}.

Some of the examples of 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 structure, with a polypeptide chain (called lid) blocking the active center, and an open structure, with the active center exposed to the medium^{32–36}. This lipase open form is the only active one in many cases, and has a tendency to become adsorbed on hydrophobic surfaces³⁷. The lid may have different configurations, in the case of the popular lipase from *Candida antarctica* (form B) (CALB) the lid is very small and does not fully isolate the active center from the medium³⁸, but CALB is still able to become adsorbed on hydrophobic surfaces³⁹. In other cases, like the lipase from *Bacillus thermocatenuatus*, the enzyme has a double lid⁴⁰. However, usually the lipases have a large single lid able to fully prevent the interaction of enzyme active center and medium, like in the case of the lipase from *Thermomyces lanuginosus* (TLL)⁴¹.

On the other hand, one very useful strategy to immobilize lipases is the interfacial activation of the enzyme on octyl agarose^{42,43}. This strategy has permitted the one step immobilization / purification / hyperactivation / stabilization of many lipases⁴⁴. However, this immobilization protocol is not compatible with unfolding/refolding reactivation strategies, as this immobilization is reversible⁴⁴. In fact, the incubation of the lipase immobilized on this support in solutions having high concentration of guanidine is a strategy used to recover the support after enzyme inactivation⁴⁴. However, recently a new heterofunctional support has been proposed, octyl-glyoxyl agarose⁴⁵. This support couples the advantages of octyl agarose to those of the covalent attachment, making the immobilization irreversible. Moreover, these new biocatalysts are usually even more stable than

the standard octyl-lipase biocatalysts⁴⁵. As a drawback, it should be considered that using this support the immobilization becomes irreversible, and therefore the support cannot be reused after enzyme inactivation. However, now it may be possible to submit the immobilized enzyme to unfolding/refolding reactivation strategies without risk of enzyme desorption, making it unnecessary to discard neither enzyme nor support⁴⁵. A likely problem is the possibility of interaction of the hydrophobic groups of the enzyme with the octyl groups of the support during refolding that could avoid the full reactivation of the enzyme.

In this new paper, the reactivation possibilities of CALB and TLL covalently immobilized on standard supports (glyoxyl or cyanogen bromide) or immobilized on octyl glyoxyl supports have been studied and compared. CALB is the most utilized lipase in literature, with many applications^{46,47}, and presents a very small lid³⁸, while TLL is also very utilized in literature⁴⁸, and has a large lid⁴¹.

2. Materials and methods

2.1. Materials

Solutions of *CALB* (6.9 mg of protein /mL) and *TLL* (36 mg of protein /mL) were a kind gift from Novozymes (Spain). Cyanogen bromide crosslinked 4 % agarose (CNBr) beads and octyl-agarose beads were from GE Healthcare. R and S methyl mandelate, *p*-nitrophenyl butyrate (*p*-NPB) were from Sigma Chemical Co. (St. Louis, MO, USA). All reagents and solvents were of analytical grade. The preparation of octyl-glyoxyl and glyoxyl-agarose was performed as previously described^{45, 49}.

2.2. Standard determination of enzyme activity

This assay was performed by measuring the increase in absorbance at 348 nm 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 (ϵ under these conditions is 5150 M⁻¹ cm⁻¹). To start the reaction, 50–100 μ L of lipase solution or suspension were added to 2.5 mL of substrate solution. One international unit of activity (U) was defined as the amount of enzyme that hydrolyzes 1 μ mol of *p*-NPB per minute under the conditions described previously. Protein concentration was determined using Bradford's method²⁸ and bovine serum albumin was used as the reference.

2.3. Immobilization of enzymes

2.3.1 Immobilization of enzymes on octyl-glyoxyl (OCGLX) supports

The immobilization was performed using 1 or 5 mg of protein per g of wet support. The commercial samples of the enzymes were diluted in the corresponding volume of 5 mM sodium

phosphate at pH 7. Then, the OCGLX support⁴⁵ was 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 several times with distilled water. Then, the washed immobilized enzyme was re-suspended at pH 10 for 12 h, to favor the enzyme-support covalent reaction⁵⁰.

2.3.2 Immobilization of enzymes on glyoxyl (GLX) support

The immobilization was performed using 1 or 5 mg of protein per g of wet support. The enzymes were diluted in 50 mM sodium bicarbonate buffer at pH 10. Then, the support was suspended in the enzyme solution under gentle stirring. Periodically, samples of the supernatant and suspension were withdrawn, and the enzyme activity was measured as described above.

2.3.3 Reduction with sodium borohydride

To end the enzyme-support covalent reaction, solid sodium borohydride was added to a concentration of 1 mg/mL to the OCGLX and GLX suspensions (at pH 10) and were submitted to gentle stirring for 30 min. This treatment reduces reversible Schiff's bases to very stable secondary amino bonds and unreacted aldehydes groups to fully inert hydroxy groups⁴⁹⁻⁵¹. The preparations were washed with CTAB (TLL) or Triton (CALB) to eliminate the non-covalently attached enzyme molecules. Finally the biocatalysts were filtered, washed with abundant distilled water and stored at 4°C.

2.3.4 Immobilization of enzymes on (BrCN) support

TLL could not be immobilized on glyoxyl agarose because the enzyme was not stable enough at pH 10,^{29,48} for this reason an alternative support was utilized. A volume of 2 mL of commercial TLL was diluted in 60 mL of 5 mM sodium phosphate at pH 7. Then, 6 g of wet BrCN-

support was added. After 90 min at 4°C under gentle stirring, around 45% of lipase became immobilized on the support. The enzyme–support immobilization was ended by incubating the biocatalyst with 1 M ethanolamine at pH 8 for 2 h. Finally, the immobilized preparation was washed with abundant distilled water and stored at 4°C.

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

Enzyme preparations were incubated in mixtures of 90% (v/v) dimethylformamide (DMF) (for CALB) or 80% (v/v) 1,4-dioxane (for TLL) with 100 mM Tris–HCl pH 7 at 30°C. Periodically, samples were withdrawn and the activity was measured using *p*-NPB as described above. The organic co-solvents presented in the samples did not have a significant effect during enzyme activity determination (results not shown).

2.5. Incubation in sodium guanidine

Immobilized CALB and TLL biocatalysts were incubated in 9 M guanidine at 25°C for 12 hours. Then, the biocatalysts were filtered and washed with 100 mM sodium phosphate buffer at pH 7.0 to remove the denaturant, and resuspended in the same volume of aqueous 100 mM sodium phosphate buffer at pH 7.0. Activity was periodically determined for 24 h.

2.6. Determination of the hydrolysis of *R* and *S* methyl mandelate

Enzyme activity was also determined using *R* or *S* methyl mandelate. A mass of 200 mg of the immobilized preparations were added to 1 mL of 50 mM substrate in 50 mM sodium phosphate at pH 7 and 25 °C under continuous stirring. The conversion degree was analyzed by RP-HPLC (Spectra Physic SP 100 coupled with an UV detector Spectra Physic SP 8450) using a Kromasil

C18 (15 cm \times 0.46 cm) column. Samples (20 μ L) were injected and eluted at a flow rate of 1.0 mL/min using acetonitrile/10 mM ammonium acetate (35:65, v/v) at pH 2.8 as mobile phase and UV detection was performed at 230 nm. The acid presented a retention time of 2.4 minutes while the ester had a retention time of 4.2 minutes. One unit of enzyme activity was defined as the amount of enzyme necessary to produce 1 μ mol of mandelic acid per minute under the conditions described above. Activity was determined by triplicate with a maximum conversion of 20–30%, and data are given as average values.

3. Results

3.1. Unfolding/Refolding of immobilized TLL and CALB

Figure 1 shows that GLX-CALB may be submitted to 3 consecutive cycles of unfolding/refolding steps recovering 100% of the initial activity versus p-NPB. The reactivation was very rapid and in one hour the enzyme fully recovered the initial activity. Using OCGLX-CALB the situation is not so positive. The reactivation permitted to recover 60% of the initial activity in the first cycle, and the reactivation is slower than using GLX. In the third cycle, the reactivation gave a 55% of the initial enzyme activity.

The results point that GLX-CALB, that by the nature of immobilization on GLX supports involves a multipoint attachment⁵⁰ and provides a fully inert surface, permits a rapid and almost complete refolding of the enzyme: the multipoint covalent attachment provides some reference points to properly refold the enzyme²⁹ while the inert surface avoids any undesired enzyme-support interaction that could stabilize different enzyme structures. Using OCGLX, the reactivation is slower and did not reach the initial activity (60%). This may be mainly caused for some interactions between the unfolded enzyme structures during the enzyme refolding and the octyl groups of the support. Even if we cannot fully recover the enzyme activity, OCGLX-CALB recovered a large percentage of activity in a consistent way, and that is another advantage of this new preparation when compared to just octyl supports, where all enzyme molecules are released from the support if incubated under unfolding conditions (results not shown).

Figure 2 shows the results using TLL. This enzyme cannot be immobilized on GLX because its low stability at pH 10^{29,48}, therefore as a standard covalent reference for our studies, BrCN-agarose has been used. This preparation permitted the recovery of around 82% of the enzyme activity, and the reactivation took several hours. In the third cycle, the reactivation enables the recovering of 78% of the enzyme activity, very similar to the values of the first cycle. Using OCGLX-TLL, again a lower percentage of initial enzyme activity is recovered, the 3 cycles ranged

between 48% and 45%. Moreover, reactivation is slower than using BrCN-TLL. These results suggested that the refolding of TLL may be more complex than that of CALB, and agree with previous reports, where the addition of some detergents improved the reactivation results³¹.

3.2. Reactivation of immobilized TLL and CALB

Next, we have studied the possibilities of this strategy to get the reactivation of enzyme that have been inactivated by the action of organic solvents. If we are able to fully unfold the enzyme structure, the results should be similar to that obtained after unfolding/refolding of the unaltered immobilized enzyme.

Figure 3 shows that reactivation of GLX-CALB after full inactivation in the presence of 90% DMF (v/v) permitted to recover 95% of the enzyme activity in the first cycle and 90% in the third one. Moreover, the reactivation was slower than that shown in Figure 1. Results were similar using OCGLX-CALB; now reactivation accounted for only 50% in the first cycle and 45% in the third one, versus the 60% observed using the unaltered enzyme. In any case, the OCGLX immobilized enzyme operational life could be increased, and this may revert in part the lack of reversibility of the immobilization using this support.

Reactivation of BrCN-TLL permitted to recover similar values of enzyme activity after enzyme inactivation in the presence of 80% dioxane (v/v) (Figure 4) to those obtained when the unaltered enzyme was used (Figure 2), around 80% of enzyme activity was recovered during 3 successive inactivation/reactivation cycles. However, using OCGLX-TLL results now (Figure 4) differed from those in Figure 2: reactivation permitted to recover only around 40% of the initial activity, although this value was maintained along the 3 studied cycles.

The results were not identical comparing the unaltered immobilized enzyme and the inactivated enzymes, except for BrCN-TLL. Using GLX-CALB, one likely explanation is that we

cannot fully unfold this multipoint immobilized enzyme even in 9 M guanidine, and therefore the structures that the enzyme has when refolding starts are not identical and drive to different final structures. The presence of large octyl groups and the attachment of the enzyme via very short spacer arms may produce some additional problems to the unfolding and refolding steps of the reactivation strategy. A likely explanation for the good results using BrCN-TLL is that this preparation has very few enzyme-support attachments (very likely just one) and the unfolding and refolding is not hampered by the interactions with the support.

3.3. Activity of different treated CALB and TLL immobilized enzymes versus *R* and *S* methyl mandelate.

The proposed strategy of unfolding/refolding of enzymes covalently immobilized has proved to be quite useful, but leaves some doubts on the real form of the reactivated enzyme structure and if this form may be obtained during several reactivation cycles. *p*-NPB is a very easily hydrolyzed ester, and it has been the only substrate used in many of the published papers. Now, we have evaluated the immobilized enzymes after the different treatments in the hydrolysis of a more complex substrate, both isomers of the chiral methyl mandelate. Results are collected in Table 1. It is clear that the percentages of activity recovered using these more complex substrates are lower than those obtained using *p*-NPB, and also decreased when comparing cycles 1 and cycles 3 of the unfolding/refolding, suggesting that the structure of the enzyme that we obtain in each reactivation cycle may differ that obtained in the previous cycle. Thus, OCGLX-CALB decrease the activity versus *R*-methyl mandelate to less than 70% in the first cycle, and after 3 cycles 60% of the activity were recovered. The inactivation of the enzyme by incubation in 90% DMF did not significantly alter the results. Using GLX-CALB, in opposition with the results using *p*NPB, now a first decrease in activity to 80% is clear. After the third cycle, only 70% of the initial activity is recovered. OCGLX-TLL decrease the activity to 50% in the first cycle of unfolding/refolding experiments and

to a similar value if the enzyme was first inactivated with dioxane, after 3 cycles the recovered activity decreased slight in both cases. BrCN-TLL preparations presented a very similar behavior.

Using CALB, the decrease of enzyme activity usually is higher versus the isomer that is the worst substrate (the S isomer) and this fact produced an increase of the CALB enantiospecificity in this reaction after applying the reactivation strategy. OCGLX-CALB increased the ratios of the rate of hydrolysis of isomers R and S from 2 to a maximum of 2.7. It may be expected that the not fully recovering of the enzyme structure has a higher impact in the activity versus the less suitable substrate.

This result agrees with the discrepancy between the results obtained in the unfolding/refolding of unaltered and inactivated enzyme preparations and points that even if this simple strategy may be useful to reactivate enzymes used in simple reactions with good substrates, it may be more complex if the enzymes are used with worse substrates, as the achievement of an identically functional enzyme structure seems unfulfilled.

4.-Discussion

The strategy of unfolding/refolding may be used to reactivate inactivated enzymes by the incubation in high concentrations of organic solvents if they are covalently attached to the support. This is possible even using a support whose surface is full of large and hydrophobic octyl groups, which produces diverse problems to the unfolding/refolding of the enzyme, offering lower percentages of activity recovery. However, enzyme activity and stability in these supports is very adequate and this possibility for partial reactivation may further increase the usefulness of the immobilization method compared to the use of pure octyl supports (where reactivation is not possible) or covalent preparations (where activity and stability is lower).

However, the results point out that it is not simple to reach the initial enzyme structure, or at least an identical structure after each reactivation cycle, and that may be a problem if the enzyme is used in reactions where the enzyme determines not only the reaction rates, but also the quality of the product (e.g. in resolution of racemic mixtures). Moreover, this suggests that in studies of reactivation of enzymes, it is not enough to use just a simple and easily modifiable substrate, some substrates hardly recognized by the enzymes may be used as probes of the real reactivation of the enzyme molecules.

The lack of a full recovery of the enzyme activity or at least a similar activity during the successive cycles suggests that we are not obtaining exactly the same structure on each refolding cycle. Apparently, simpler substrates permit a higher enzyme conformation change before the activity starts to decrease. The failure in recovering similar activity in each reactivation cycle may have several explanations. First, an enzyme submitted to unfolding/refolding strategies may give a not fully renatured enzyme, even in free form, and that may produce an enzyme with different conformation and, therefore, different functional properties⁵². Moreover, Illanes's group has reported how reactivated immobilized penicillin G acylase biocatalysts really have a significant alteration of the kinetic parameters compared to the initial enzyme preparations, although they have not analyzed if this new enzyme structure was always obtained during successive reactivation cycles, as just one cycle was studied⁵³.

It has been also reported that if unfolding/refolding strategies are applied to immobilized enzymes, if the distance between enzyme molecules is not large enough to prevent interactions between unfolded enzyme structures (unfolded state has a larger volume than folded one), these interactions may prevent a correct refolding of the unfolded enzyme²⁵. This problem cannot occur in the covalent preparation, where the immobilization is very slow and the loading of the support was quite low, but immobilization of lipases on OCGLX is so rapid that can form a crown in the outer part of the support particle of enzyme molecules packed together. In fact, it has been shown

that even using very low enzyme loading, the distance between enzyme molecules immobilized on octyl supports is so short that may be crosslinked using a molecule as small as glutaraldehyde^{54,55}.

Thus, it is very likely that OCGLX preparations have diverse problems for the refolding: interactions with the octyl groups, some steric problems of the movements of the protein chain generated by the support and a close proximity of other unfolded enzyme molecules than can produce undesired interactions. However, it is clear also that the enzyme activity may be fully destroyed by enzyme distortion caused by incubation in solvents, unfolded by incubation in guanidine, and we can get a high percentage of enzyme activity after refolding in aqueous medium.

Thus, these initial promising results, showing a reactivation of lipases immobilized on OCGLX supports open new lines of research, trying to shortcut some of the problems found in this research to apply unfolding/refolding strategies as a method of reactivating lipases immobilized in this interesting support. Optimization of the reactivation medium (e.g., using detergents, thiolated compounds) may permit to further improve these promising results.

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Figure legends

Figure 1. Cycles of unfolding -refolding of different biocatalyst from CALB. Unfolding has been carried out via incubation in 9M guanidine and refolding by incubation in aqueous 100 mM sodium phosphate . Experiments have been performed as described in Section 2. Circles: OCGLX, Squares: GLX.

Figure 2. Cycles of unfolding -refolding of different biocatalyst from TLL. Unfolding has been carried out via incubation in 9M guanidine and refolding by incubation in aqueous medium. Experiments have been performed as described in Section 2. Circles: OCGLX, Squares: BrCN.

Figure 3. Cycles of enzyme inactivation by incubation in 90% DMF and reactivation by unfolding via incubation in 9 M guanidine and refolding by incubation in aqueous medium of different biocatalyst from CALB. Experiments have been performed as described in Section 2. Circles: OCGLX, Squares: GLX.

Figure 4. Cycles of enzyme inactivation by incubation in 80% dioxane and reactivation by unfolding via incubation in 9 M guanidine and refolding by incubation in aqueous medium of different biocatalyst from TLL. Experiments have been performed as described in Section 2. Circles: OCGLX, Squares: BrCN.

| BIOCATALYST | V _R -methyl mandelate | V _S -methyl mandelate | V _R /V _S |
|----------------------|----------------------------------|----------------------------------|--------------------------------|
| OCGLXCALB | 83 ± 4.2 | 42 ± 2.1 | 2.0 |
| OCGLXCALB* Cycle 1 | 57 ± 2.9 | 21.1 ± 1.1 | 2.7 |
| OCGLXCALB *Cycle 3 | 51.2 ± 2.7 | 19.7 ± 1 | 2.6 |
| OCGLXCALB ** Cycle 1 | 53 ± 2.7 | 23 ± 1.2 | 2.3 |
| OCGLXCALB ** Cycle 3 | 49.3 ± 2.5 | 20 ± 1 | 2.5 |
| GLXCALB | 23.7 ± 1.2 | 11.3 ± 0.6 | 2.1 |
| GLXCALB* Cycle 1 | 19.1 ± 1 | 7.6 ± 0.4 | 2.5 |
| GLXCALB* Cycle 3 | 17.2 ± 0.9 | 7.1 ± 0.4 | 2.4 |
| GLXCALB ** Cycle 1 | 17.1 ± 0.9 | 7.6 ± 0.4 | 2.3 |
| GLXCALB** Cycle 1 | 16.3 ± 0.7 | 7.2 ± 0.4 | 2.3 |
| OCGLXTLL | 0.034 ± 0.002 | 0.031 ± 0.002 | 1.1 |
| OCGLXTLL* Cycle 1 | 0.017 ± 0.001 | 0.011 ± 0.001 | 1.5 |
| OCGLXTLL* Cycle 3 | 0.015 ± 0.001 | 0.009 ± 0.001 | 1.7 |
| OCGLXTLL** Cycle 1 | 0.019 ± 0.002 | 0.012 ± 0.001 | 1.6 |
| OCGLXTLL** Cycle 3 | 0.018 ± 0.002 | 0.010 ± 0.001 | 1.8 |
| BrCNTLL | 0.0088 ± 0.0002 | 0.0058 ± 0.0002 | 1.5 |
| BrCNTLL* Cycle 1 | 0.0047 ± 0.0001 | 0.0031 ± 0.0001 | 1.5 |
| BrCNTLL* Cycle 3 | 0.0046 ± 0.0001 | 0.0031 ± 0.0001 | 1.5 |
| BrCNTLL ** Cycle 1 | 0.0045 ± 0.0001 | 0.0032 ± 0.0001 | 1.4 |
| BrCNTLL ** Cycle 3 | 0.0041 ± 0.0001 | 0.0028 ± 0.0001 | 1.5 |

Table 1. Activity of different biocatalysts versus R or S methyl mandelate (50 mM) at pH 7 and 25°C. Experiments were performed as described in section 2. The activity (V) is given in μ moles of substrate hydrolyzed per minute and mg of immobilized enzyme.

* The biocatalyst has been submitted to unfolding/refolding for the indicated number of cycles.

** The biocatalyst has been submitted to inactivation by incubation in solvent (90% DMF for CALB, 80% dioxane for TLL), unfolding and refolding steps during the indicated number of cycles.