IMMOBILIZATION OF LIPASES ON GLYOXYL-OCTYL SUPPORTS: IMPROVED STABILITY AND REACTIVATION STRATEGIES

ANGÉLICA MARÍA SUESCÚN PADILLA

UNIVERSIDAD INDUSTRIAL DE SANTANDER
FACULTAD DE CIENCIAS
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DIRECTOR
JOHN JAIRO CASTILLO LEÓN, *PhD* Química
CODIRECTOR
NAZZOLY RUEDA ARANGO, Química

UNIVERSIDAD INDUSTRIAL DE SANTANDER
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A mis padres, familia y amigos, quienes me apoyaron, me incentivaron y fueron mi ayuda incondicional en los buenos y malos momentos, para ellos este logro significa un triunfo compartido.

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CONTENTS

LIST OF FIGURES AND TABLES

RESUMEN

TITULO: IMMOBILIZATION OF LIPASES ON GLYOXYL-OCTYL SUPPORTS: IMPROVED STABILITY AND REACTIVATION STRATEGIES*

AUTOR: SUESCUN PADILLA Angélica María**

PALABRAS CLAVES: Inmovilización de lipasas mediante activación interfacial, estabilización de enzimas, inmovilización covalente, reactivación enzimática, desplegamiento/replegamiento.

DESCRIPCION:

Las lipasas candida rugosa (CRL) y candida antárctica (isoforma a) (CAL A) se han inmovilizado con éxito sobre el soporte heterofuncional octil glioxil agarosa (OCGLX) en comparación con el soporte octil agarosa (OC) y glioxil agarosa (GLX). La inmovilización sobre OCGLX permitió obtener hiperactivaciones similares a las encontradas sobre los soportes OC, aunque la incubación a pH 10.0 durante 4 h disminuyó la actividad de ambas enzimas a un 25%. Después de la reducción, más del 95% de la actividad de la enzima se unió covalentemente al soporte. La fracción no unida covalentemente se desorbió por lavado con detergente. Estos biocatalizadores fueron más estables que sus homólogos inmovilizados sobre OC en las inactivaciones térmicas y en solventes orgánicos. Aún más interesante es que la inmovilización irreversible permite la reactivación de los biocatalizadores de CAL A inactivados por incubación en solvente orgánico, después del desplegamiento de la enzima en presencia con guanidina y de replegamiento en tampón fosfato de potasio (alrededor del 55% de la actividad logró ser recuperada durante 3 ciclos sucesivos de desplegamiento/replegamiento). Por su parte GLXCAL A permitió recuperar el 75% de la actividad, pero la estabilidad térmica y la actividad fue mucho más baja, además esta estrategia no se pudo aplicar a CRL. Ni la enzima inmovilizada sobre bromuro de cianógeno ni sobre OCGLX exhibió actividad significativa después de utilizar las estrategias de despliegamiento/replegamiento.

*Trabajo de grado
**Facultad de Ciencias, Escuela de Química, Director: John Castillo, Codirector: Nazzoly Rueda.

ABSTRACT

TITLE: IMMOBILIZATION OF LIPASES ON GLYOXYL-OCTYL SUPPORTS: IMPROVED STABILITY AND REACTIVATION STRATEGIES*

AUTHOR: SUESCÚN PADILLA Angélica María**

KEYWORDS: Immobilization of lipases via interfacial activation, enzyme stabilization, covalent immobilization, enzyme reactivation, unfolding/refolding.

DESCRIPTION:

Lipases from Candida rugosa (CRL) and from C. 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.

*Bachelor Thesis

**Facultad de Ciencias, Escuela de Química, Director: John Castillo, Codirector: Nazzoly Rueda.

INTRODUCTION

Enzymes are very interesting (Ni, Holtmann, and Hollmann 2014; Tibrewal and Tang 2014; Wells and Meyer 2014; Schrittwieser and Resch 2013; Reetz 2013; Zheng and Xu 2011; Patel 2011) but they also have some limitations as industrial biocatalysts (Schoemaker, Mink, and Wubbolts 2003). This occurs for example with the moderate stability of enzymes under conditions sometimes required by industry (Iyer and Ananthanarayan 2008; Polizzi et al. 2007). The operational enzyme stability may be improved by genetic tools (Eijsink et al. 2005), chemical modifications (Wong SS and Wong LJC 1992; Rodrigues, Berenguer-Murcia and Fernández-Lafuente 2011) enzyme immobilization (Guzik, Hupert-Kocurek, and Wojcieszyńska 2014; Stepankova et al. 2013; Rodrigues et al. 2013; Hwang and Gu 2013; Fernandez-Lafuente 2009), and also by selecting adequate reaction conditions (Kumar and Venkatesu 2012).

Enzyme immobilization is a requirement for many applications (Sheldon and van Pelt 2013), and it is compatible with any other strategy for enzyme stabilization (Cowan and Fernandez-Lafuente 2011; Hernandez and Fernandez-Lafuente 2011; Fernandez-Lafuente 2009; Rodrigues, Berenguer-Murcia, and Fernandez-Lafuente 2011), and the inactivated biocatalyst may be submitted to strategies of reactivation after partial or total inactivation (Garcia-Galan et al. 2011). 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 (Mozhaev and Martinek 1982).

The previous immobilization of the enzymes on supports via covalent linkages may help the refolding step (Romero et al. 2012; Godoy et al. 2011; Bolivar et al. 2010; Guisán et al. 1993). 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 (Rodrigues, Bolivar, Palau-Ors, et al. 2009). Even heavily chemically modified enzymes could be unfolded and refolded after immobilization via multipoint covalent attachment (Rodrigues, Bolivar, Volpato, et al. 2009; Rodrigues, Bolivar, Palau-Ors, et al. 2009). 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 reactivation of immobilized enzymes involve lipases (Rodrigues, Godoy, et al. 2009; Rodrigues, Bolivar, Palau-Ors, et al. 2009; Rodrigues, Bolivar, Volpato, et al. 2009; Godoy et al. 2011). 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 (Cambillau et al. 1996; Brzozowski et al. 1991; Kim et al. 1997; Jaeger et al. 1993; Cygler and Schrag 1999). This open form has a tendency to become adsorbed on any hydrophobic surface (Verger 1997). The lipase from *Candida antarctica* (form B) (CALB) has a very small lid and does not fully isolate the active center (Uppenberg et al. 1994), but CALB is still able to become adsorbed on hydrophobic surfaces (Hernandez, Garcia-Galan, and Fernandez-Lafuente 2011) while the lipase from *Bacillus thermocatenolatus* has a double lid (Carrasco-López et al. 2009). 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) (Derewenda et al. 1994).

The interfacial activation of the enzyme on hydrophobic supports, like octyl agarose (Bastida et al. 1998; Manoel et al. 2015) is a very useful immobilization strategy that has permitted the one step immobilization/purification/hyperactivation /stabilization of many lipases (Fernandez-Lafuente et al. 1998). 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 (Fernandez-Lafuente et al. 1998).

Octyl-glyoxyl agarose (Rueda et al. 2015) 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 (Rueda et al. 2015). 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 (Rueda et al. 2015) to the immobilization of two new enzymes, and analyze their reactivation possibilities. The new enzymes used in this paper are the lipase A from *Candida antarctica* (CALA) and the commercial lipase from *Candida rugosa* (CRL). Both enzymes have a proper lid and have been used in many different applications (Benjamin and Pandey 1998; Domínguez de María et al. 2005; Domínguez De María et al. 2006; Akoh, Lee, and Shaw 2004; Dominguez de Maria et al. 2006), 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.

1. MATERIALS AND METHODS

1.1. 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 Candida 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 (J. Guisán 1988).

1.2. 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 (Bradford 1976) using as reference bovine serum albumin.

1.3. IMMOBILIZATION OF ENZYMES

1.3.1. Immobilization of CAL A 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.

1.3.2. 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 (Mateo et al. 2005).

1.3.3. 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 (Blanco, Calvete, and Guisán 1989; J. Guisán 1988; Mateo et al. 2005; Rodrigues, Berenguer-Murcia, and Fernandez-Lafuente 2011). 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.

1.4. 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.

1.5. 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 above. Half-lives were calculated from the observed inactivation courses. The organic co-solvents presented in the samples did not have a significant effect during the enzyme activity determination (results not shown).

1.6. INCUBATION IN SODIUM GUANIDINE

Immobilized CALA and CRL were incubated in 9 M guanidine at 25°C for 3 hours. Then, derivatives were filtered and washed with 100 mM phosphate buffer pH 7.0 to remove the denaturant, and resuspended in the same volume of aqueous 50 mM sodium phosphate at pH 7.0. Activity was tested until a constant value of residual activity was reached along time (a maximum of 24 h). Three consecutive cycles of unfolding/refolding of immobilized preparations were performed. The use of cross-linked agarose ensures the stability of the structure of the support when the derivatives are incubated on caotropic agents.

1.7. HYDROLYSIS OF R AND S METHYL MANDELATE

R or S methyl mandelate were also used to determine the activity of the biocatalysts. After preparing 1 mL of 50 mM substrate in 50 mM sodium phosphate at pH 7.0, a

mass of 200 mg of the lipase preparations were added and the suspension was maintained at 25 °C under continuous stirring. Organic solvents were added when indicated. The reactions were followed by RP-HPLC (Spectra Physic SP 100 coupled with an UV detector Spectra Physic SP 8450) using a Kromasil C18 (15 cm × 0.46 cm) column. Samples (20 µL) 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 retention times of the different compounds were: acid: 2.4 minutes, ester: 4.2 minutes. The enzyme activity was measured in µmol of mandelic acid per minute under the conditions described above. Activity was determined by triplicate, using a maximum conversion of 20–30%. The data are given as average values.

1.8. SDS-PAGE ANALYSIS OF CAL A AND CRL OCTYL AND OCTYL-GLYOXYL PREPARATIONS

SDS-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out according to (LAEMMLI 1970) using a Miniprotean tetra-cell (Biorad), 12% running gel in a separation zone of 9 cm × 6 cm, and a concentration zone of 5% polyacrylamide. One hundred milligrams of the immobilized enzyme samples (containing 10 mg/g for CALA or 20 mg/g for CRL) were re-suspended in 1 mL of rupture buffer (2% SDS and 10% mercaptoethanol). Then, this suspension was boiled for 5 min and a 20 µL aliquot of the supernatant was used in the SDS-PAGE analysis. The heating in the presence of SDS is enough to release all the adsorbed enzyme molecules, while it cannot break the support-enzyme secondary amino bonds (Bastida et al. 1998). Gels were stained with Coomassie brilliant blue. Low molecular weight markers from Fermentas were used (14.4–116 kDa).

2. RESULTS AND DISCUSSION

2.1. IMMOBILIZATION OF CAL A AND CRL ON DIFFERENT SUPPORT

(Figure 1) shows the immobilization of CALA on glyoxyl, OC and OCGLX supports. The immobilization of the enzyme at pH 10.0 on glyoxyl agarose is very slow. Moreover, the low stability of the enzyme at alkaline pH value makes that the activity of the immobilized enzyme suffers a significant decrease. Immobilization of the enzyme on both OC supports is very rapid, slightly more rapid in OCGLX than in OC. Moreover, both immobilized biocatalysts exhibited a slight increase in activity (40-50%), very likely due to the implication of the open form of the enzyme in the immobilization (Manoel et al. 2015). Using CRL (Figure 2), the immobilization on GLX support is not possible, the enzyme is rapidly inactivated at this pH value and the immobilization rate is too slow. However, using both OC supports the immobilization is very rapid, again more rapid using OCGLX and the observed enzyme hyperactivation is more significant than using CALA (final activity is 230-250% of that of the free enzyme). Neither CALA nor CRL were immobilized on GLX supports at pH 7.0, therefore we can ensure that the first step of the immobilization of the enzyme in the OCGLX is the interfacial activation.

Figure 1. Immobilization course of CALA on glyoxyl agarose beads (Panel A) or octyl or octyl- glyoxyl agarose beads (Panel B). Experiments have been performed as described in Section 1. Circles: suspension, Squares: Supernatant, Triangles: Soluble enzyme.

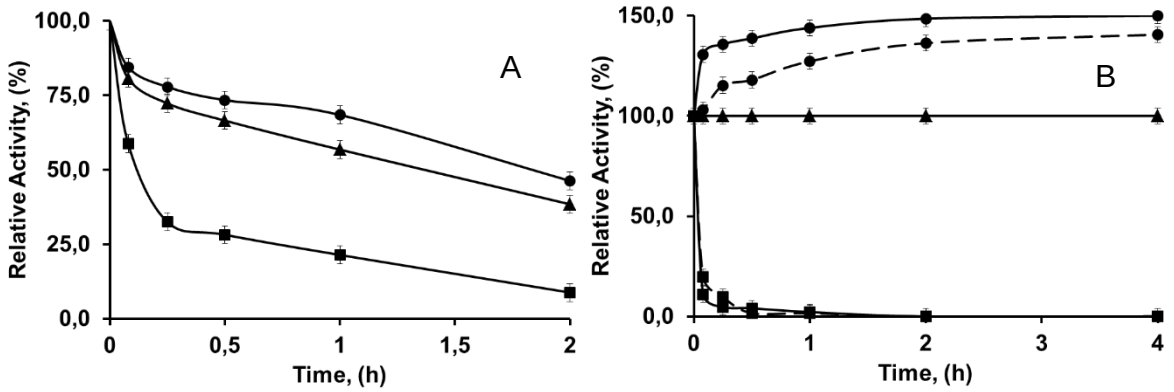
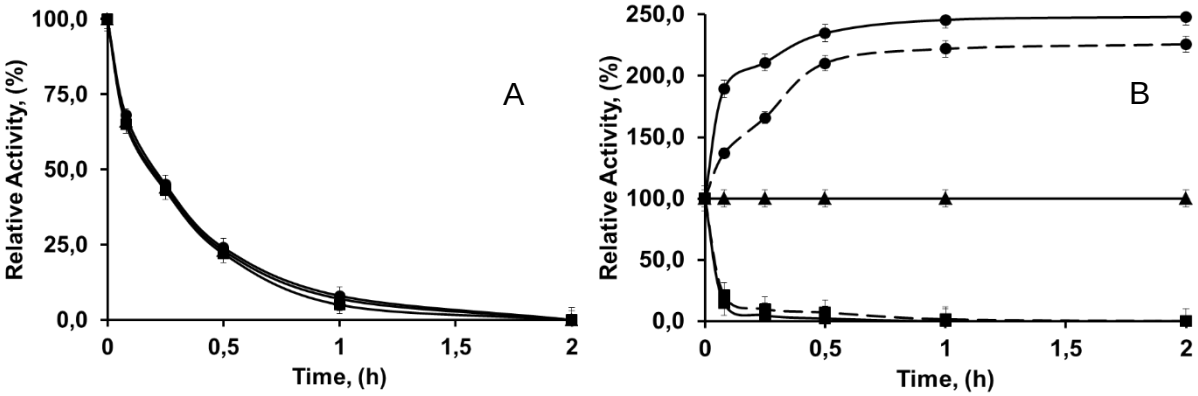
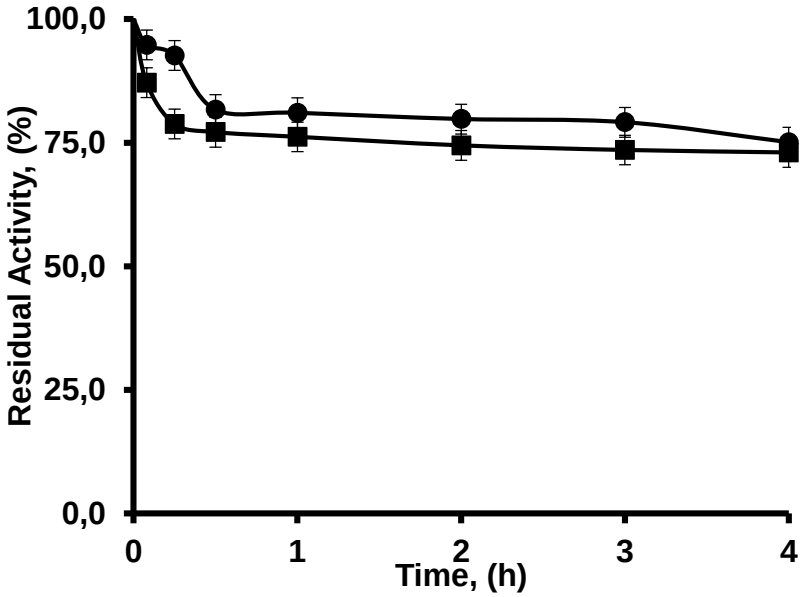


Figure 2. Immobilization course of CRL on glyoxyl (Panel A) or octyl agarose beads or octyl glyoxyl beads (Panel B). Experiments have been performed as described in Section 1. Circles: suspension, Squares: Supernatant, Triangles: Soluble enzyme.



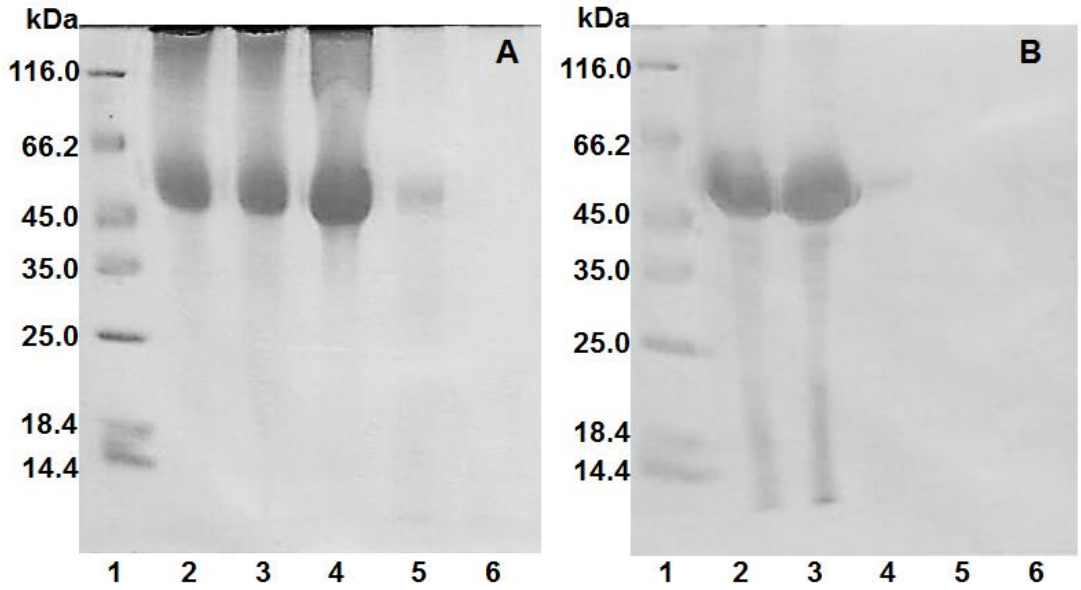
Following the results previously obtained with other enzymes, we decided to incubate the lipases immobilized on OCGLX supports at pH 10.0 for 4 h to achieve some covalent bonds (Rueda et al. 2015). This incubation produced a decrease in enzyme activity of 25% for both enzymes (Figure 3), and the final reduction did not have an appreciable effect on the enzyme activity, therefore the final preparations maintained over 100% of the activity of the free enzyme using CALA, and almost 190% using CRL. This low decrease in enzyme activity of the immobilized enzyme during incubation at pH 10.0 should be related to the high stabilization usually achieved after immobilization of lipases on hydrophobic supports that increases the retention of activity during enzyme incubation under drastic conditions (Peters et al. 1996; Liang et al. 2000; Palomo et al. 2002).

Figure 3. Effect of the incubation at pH 10.0 on the activity of enzymes immobilized on octyl-glyoxyl agarose support before reduction. Experiments have been performed as described in Section 1. Circles: OCGLXCALA, Squares: OCGLXCRL.



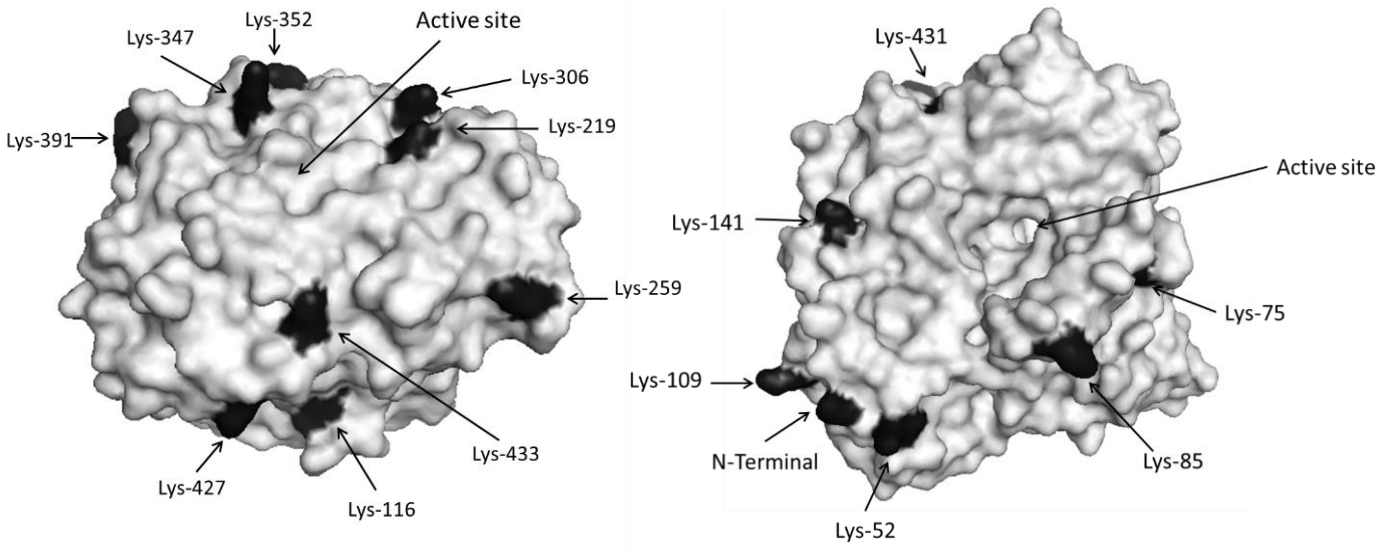
After this immobilization protocol, the OCGLX preparations were washed with Triton X-100 using concentrations that produced full enzyme desorption from octyl support (0.5% for CRL and 1% for CALA) (results on desorption from octyl support are not shown). The activity only decreased by less than a 10% after these washings, suggesting that most of the protein had been covalently attached to the support. To check this, we submitted to SDS-PAGE analysis the different preparations (Figure 4). While the non-reduced OCGLX preparations released an amount of enzyme similar to the OC preparations to the medium, the reduced samples almost did not release protein to the medium. This small fraction of enzyme that may be desorbed from the support were eliminated during the detergent washings, these preparations did not show any protein release.

Figure 4. SDS-PAGE analysis of different biocatalysts. The immobilized enzymes were submitted to the processes described in Section 1. Experiments have been carried out as described in Section 1. Panel A: CALA and Panel B: CRL. Lane 1: molecular weight marker, Lane 2: OC, Lane 3: OCGLX, Lane 4: OCGLX incubated to pH 10.0, Lane 5: OCGLX incubated to pH 10.0 and reduced with NaBH4, Lane 6: OCGLX incubated to pH 10.0, reduced with NaBH4 and washed with detergent.



(Figure 5) shows the structure of these enzymes in the area surrounding the active center, with the primary amino groups highlighted. It is evident that both enzymes have many groups on this area that may provide the enzyme covalent immobilization. These results explain the good performances obtained. However, the fact that some protein molecules may still be desorbed suggested that an intense support-enzyme reaction is quite unlikely, very likely due to the steric problems generated by the octyl groups (Garcia-Galan et al. 2011). For all further experiments, OCGLX preparations were washed to eliminate the small fraction of just adsorbed enzyme molecules that could make the understanding of the results more complex.

Figure 5. 3D surface structure model of open form of CALA (Panel A) and CRL (Panel B), PDB code 2VEO and 1CRL, respectively. The 3D surface structure was obtained using PyMol vs 0.99.



2.2. ENZYME PREPARATIONS STABILITY

Table 1 shows the half-lives of the different enzyme preparations. The most stable preparation was always the OCGLX for CALA and CRL. The stabilization achieved comparing the OC and OCGLX is much more significant using CRL than using CALA (using CALA the stabilization factor is never higher than 2, using CRL reach a 6 fold-factor at pH 5.0). In the presence of organic solvents, both enzymes are around 3 fold more stable when immobilized on OCGLX than when immobilized on OC. The GLXCALA was even less stable than the octyl preparation under termal inactivation, and still was less stable than the OCGLX in inactivations in the presence of organic solvents. CRL could not be immobilized on GLX support.

Thus, the immobilization of CRL and CALA on OCGLX not only permitted to improve the enzyme activity, but also permitted to increase their stability.

Table 1. Half-lives of the different biocatalyst under different experimental conditions (in minutes). CALA (pH 5.0 at 85°C, pH 7.0 at 80°C and pH 9.0 at 60°C), CRL (pH 5.0 at 70°C, pH 7.0 at 65°C and pH 9.0 at 45°C). All biocatalyst were incubated at 30°C in the organic solvent inactivation. Experiments have been performed as described in Section 1.

	Experimental Conditions				
Biocatalyst	**pH 5.0**	**pH 7.0**	**pH 9.0**	**ACN 45%**	**DMSO 80%**
OCCALA	90	120	80	-	60
OCGLXCALA	180	180	120	-	180
GLXCALA	-	15	-	-	120
OCCRL	10	30	30	120	-
OCGLXCRL	60	120	60	360	-

2.3. CHANGES IN ENZYME SPECIFICITY

It has been described in many instances that the immobilization via different protocols may affect the final selectivity or specificity of the lipases (Garcia-Galan et al. 2011). Thus, we have compared the activity of the different preparations of CALA and CRL (in this case, we have added the immobilized enzyme in cyanogen bromide) versus R and S methyl mandelate. Table 2 resumes the results.

Using CRL, the octyl preparation was the most active one versus the preferred isomer, S-methyl mandelate, OCGLXCRL expressed less than 9% of the activity of the OC preparation, even though the activity versus p-NPB was quite similar (just a 25% less activity the OCGLX preparation). The differences between the enzyme activities versus R and S isomer also are higher for the OC preparation (50 U versus

20 U). The results using the covalent preparation were the worst in all senses (lower activity and activity ratio).

Using CALA the picture is different. OCCALA and OCGLXCALA have very similar activities versus R-methyl mandelate. However, the activity using the S isomer is much higher using OCGLXCALA preparation. The result is that while OCCALA has only a very moderate enantiopreference for the R isomer, OCGLXCALA has a clear enantiopreference for the S isomer (by 3 fold factor). GLXCALA presented a lower decrease in activity if compared to the decrease observed using p-NPB, and presented a clear enantiopreference for the R isomer (a factor of 2).

Therefore, these are new examples on how the immobilization, even maintaining similar orientation and just causing some distortion of the enzyme structure, may exert an unexpected effect on enzyme specificity, improving activity versus some substrates and decreasing it versus other ones.

Table 2. Activity of different lipase biocatalysts versus R or S methyl mandelato. Experimental conditions were pH 7.0 and 25ºC, using 50 mM of substrate. Experiments were carried out as described in Section 1. Activity is defined as µmol of mandelic acid /min/ mg of protein.

	$V_{R\text{-methyl mandelate}}$	$V_{S\text{-methyl mandelate}}$	V_R / V_S
Biocatalyst			
OC-CALA	0.0740 ± 0.004	0.0667 ± 0.005	1.1
OCGLX-CALA	0.0696 ± 0.003	0.2083 ± 0.004	0.3
GLX-CALA	0.0536 ± 0.004	0.0268 ± 0.004	2.0
OC-CRL	0.0068 ± 0.0004	0.3750± 0.003	0.02
OCGLX-CRL	0.0017± 0.0001	0.0330± 0.0005	0.05
BrCN-CRL	0.0013± 0.0002	0.0076± 0.0003	0.18

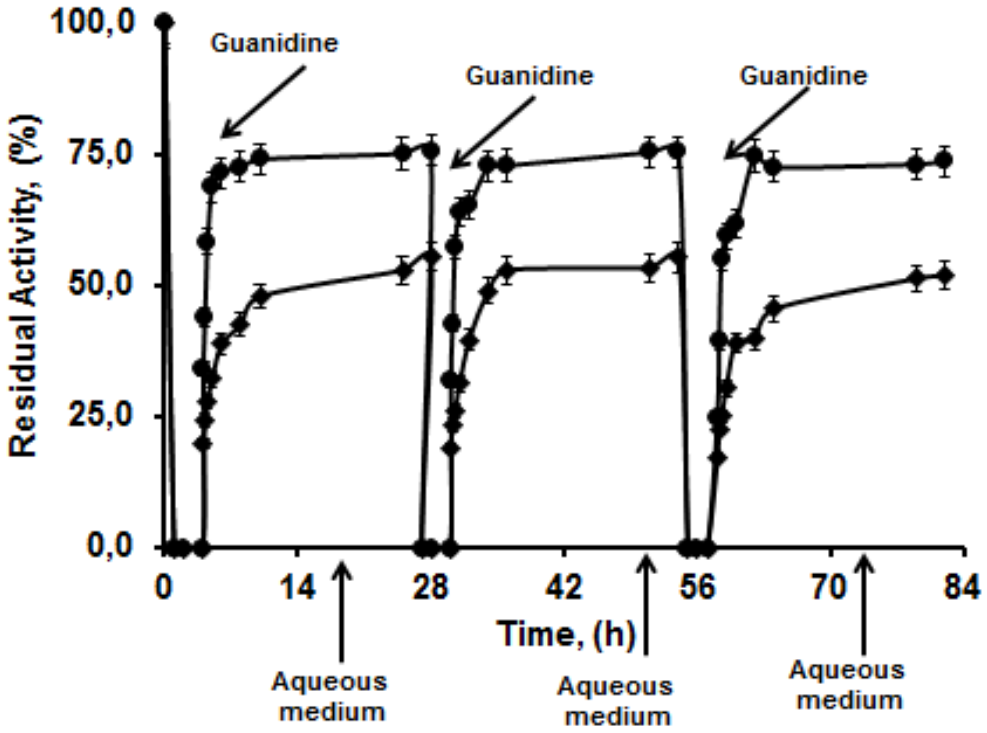
2.4. REACTIVATION OF THE IMMOBILIZED ENZYMES

The immobilization of the enzymes on OCGLX has many advantages, as the enzyme may be used under any experimental conditions without any risk of enzyme desorption, is more stable, and enables the stabilization of the open form of the lipase. However, it has a problem: the immobilization becomes irreversible; now after enzyme inactivation, both enzyme and support should be discarded, while using the OC support the support may be reused. This situation may be solved if the covalently immobilized enzyme may be at least partially reactivated.

For this purpose, OCGLX of both CALA and CRL and GLXCALA were submitted to experiments of unfolding in 9 M sodium guanidine. A covalent preparation of CRL was prepared using cyanogen bromide, as this enzyme cannot be immobilized on GLX. Both CRL preparations were fully inactivated in the presence of 9 M sodium guanidine, and their reincubation in aqueous buffer at pH 7.0 did not permit to obtain more than 6% of the initial activity. We tried to change temperature of unfolding/refolding, or perform a guanidine gradient during refolding, but we did not achieve any significant improvement on the activity recovery. Thus, immobilized CRL reactivation seems to be a quite complex problem, and that is not generated by the octyl groups in the supports as the other reference covalent preparation offered similar results. Results were very different using CALA (Figure 6). GLXCALA may be fully inactivated by incubation in guanidine solution, but after incubation in aqueous buffer 75% of the initial activity could be recovered. After 4 hours, the reactivation was almost completed. We can submit this preparation to three successive unfolding/refolding experiments with similar results.

OCGLXCALA experimented a slower reactivation, and permitted a lower reactivation (around 55% after 20 h) but a significant percentage of activity could be recovered. Again, this value was stable along successive cycles of unfolding/refolding.

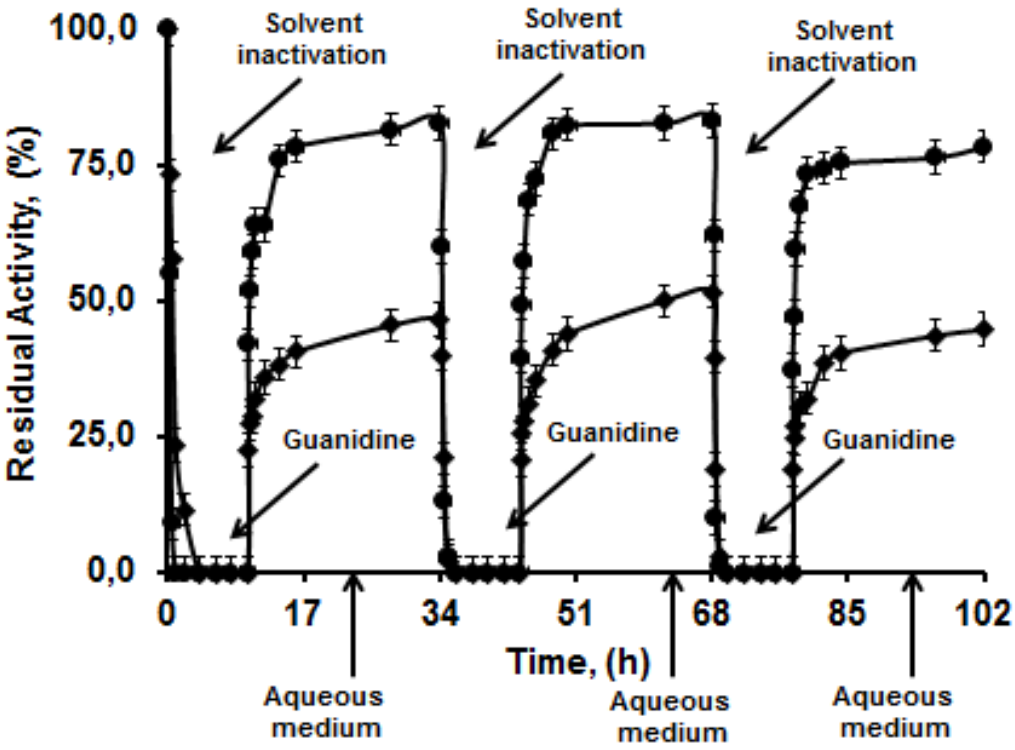
Figure 6. Cycles of unfolding-refolding of different CALA preparations. Experiments have been performed as described in Section 1. Experiments have been performed as described in Section 1. Circles: GLXCALA, Rhombus: OCGLXCALA.



To check if this strategy was really valid for enzyme reactivation, we performed a similar experiment, but before proceeding to the unfolding/refolding experiments, the enzyme preparations were fully inactivated by incubation in DMSO. We used a very high concentration (90%) of organic solvent to have a very rapid enzyme inactivation. The incubation in this solvent at neutral pH and low temperature should not affect the chemical structure of the enzyme. Thus, if the enzyme was fully unfolded by incubation in guanidine and refolded in aqueous buffer, the enzyme activity should be recovered in similar bases to the results obtained on the unfolding/refolding experiments. In fact, (Figure 7) shows that the activity of the organic solvent inactivated CALA preparations could be recovered by three successive cycles of inactivation/reactivation. Thus, OCGLXCALA preparations coupled one further

advantage to the OCCALA preparation, the possibility of enzyme reactivation after solvent inactivation.

Figure 7. Cycles of enzyme inactivation by incubation in 90% DMSO and reactivation by unfolding via incubation in 9 M guanidine and refolding by incubation in aqueous medium of different CALA immobilized preparations. . Experiments have been performed as described in Section 1. Circles: GLXCALA, Rhombus: OCGLXCALA.



3. CONCLUSIONS

This paper shows the advantages of using glyoxyl-agarose beads in the preparation of immobilized preparations of CALA and CRL. After a proper incubation at pH 10.0, the interfacially adsorbed enzymes could be covalently attached to the support. This way, we can couple the selective adsorption of lipases on hydrophobic supports via the open form of the lipases (Manoel et al. 2015) that are inherent to the octyl-agarose support, to an irreversible binding. The new biocatalysts were more stable in the presence of organic solvents and in thermal inactivations, as suggested in the previous paper (Rueda et al. 2015) very likely due to the impossibility to release the enzyme to the medium. Moreover, the enzyme preference for R or S methyl mandelate could be strongly tuned by the immobilization protocol. This irreversible immobilization permits one further advantage: using CALA the octyl-glyoxyl preparation could be submitted to 3 cycles of organic solvent inactivation/reactivation by unfolding /refolding in guanidine and recover at least 50% of the initial activity of the biocatalyst.

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