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Reversible immobilization of lipases on octyl-glutamic agarose beads: A mixed adsorption that reinforces enzyme immobilization

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ABSTRACT

A new octyl-glutamic(OCGLU) heterofunctional agarose bead has been prepared. It has been compared to octyl-agarose (OC) in their performance to immobilize 5 different lipases, those from *Candida antarctica* (A (CALA) and B (CALB)), from *Thermomyces lanuginosus* (TLL), from *Rhizomucor miehei* (RML) and from *Candida rugosa* (CRL) and a phospholipase (Lecitase ultra, LU). The immobilization rate was very similar using both supports, and the increase of activity versus *p*-nitrophenyl butyrate were also very similar. The effects on enzyme stability of the immobilization on OCGLU compared to the conventional OC was quite diverse, in some cases reducing the enzyme stability while in other examples the enzyme stability improved more than hundredfold. Curiously, the highest stabilizations were found under pH conditions where the free enzyme could not be adsorbed on a support just bearing glutamic groups on its surface, suggesting that the mechanism of stabilization may be a quite complex one that should consider the hydrophilization of the support surface, the cationic and anionic groups of glutamic, the likely partition of organic solvents from the support surface, positive and negative enzyme-support interactions, etc. Even though the lipases adsorption was very strong, the support could be regenerated and reused by incubation in ionic detergents.

1. Introduction

Enzymes are very promising biocatalysts, and lipases may be considered outstanding among them due to their broad specificity coupled to a high enantio or regio selectivity [1–8].

However, for many industrial applications of the enzyme, they need to be purified and immobilized before use [8]. Immobilization may not only solve the problem of enzyme recovery, but may also improve many other enzyme features if properly designed (stability, activity, selectivity, etc.) [9–18].

The immobilization of lipases via interfacial activation on hydrophobic supports is a very useful method to immobilize and purify lipases in one step [19]. Moreover, the use of hydrophobic supports to immobilize lipases allows stabilizing the open form of the lipase [20], in many cases producing an increased enzyme activity and a stabilization of the enzyme [21–23]. This interesting immobilization method has as main problem that the lipase molecules may be released to the medium under drastic conditions (high T, presence of organic solvents), reducing the range of conditions where the enzyme may be applied [24,25].

Recently, heterofunctional glyoxyl-octyl agarose has been proposed as an alternative immobilization method that may permit to fully prevent enzyme desorption after immobilization on octyl

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agarose under any experimental condition, as the open form of the lipase was covalently attached to the support [26,27]. After immobilization via interfacial activation versus the support surface at neutral pH values, the conditions of the suspension immobilization were moved at alkaline pH values to permit the reaction of the enzyme primary amino groups with the glyoxyl groups of the support [28].

The strategy worked in some instances, producing some further lipase-octyl stabilization at a small to moderate activity cost, but it transformed the reversible immobilization in an irreversible one [26]. Some problems (not covalent attachment of all lipase molecules, enzyme inactivation by incubation at alkaline pH) were solved by chemical amination of the enzymes [29], but still the immobilization protocol becomes irreversible. That means that, after enzyme inactivation, both support and enzyme need to be discarded if lipase reactivation is not possible [27,30].

In this new paper, we present a new heterofunctional support [31] to increase the range of conditions where the lipase molecules immobilized on octyl supports may be used, but without renouncing to the reversibility of the process. It consists of the introduction of cationic/anionic groups (using glutamic acid) on octyl-agarose (OCGLU), to permit the establishment of ionic bridges between the face of the enzyme involved in the interfacial activation of the enzyme versus the support and the glutamic groups in the support. The net charge at neutral pH per Glu molecule is −1, leaving mainly a cationic exchange character to the support surface. That way, if the hydrophobic adsorption is suppressed by the presence of non-ionic detergents or organic solvents, the enzyme should remain adsorbed on the support via ionic exchange, and very likely the lipase open form should be maintained by steric reasons. However, the support may be re-used after enzyme inactivation by washing the biocatalyst with ionic detergents or guanidine, keeping the advantage of a reversible immobilization.

Most enzymes have acidic isoelectric points, that means that their tendency to become adsorbed on cation exchangers will be limited to the acid region (pH 5 or below) [8,32–43]. In our study, this facilitates to get a first immobilization of the lipase on octyl-glutamic acid agarose via interfacial activation (the key point of the advantages of the lipase immobilization on octyl-agarose) [20] leaving the establishment of enzyme-support ionic bonds for a second step. However, this hinders the promotion of a cationic exchange between the immobilized enzyme and the support. The immobilization of enzymes on a support via ionic exchange requires the establishment of several enzyme-support ionic exchanges [44–47]. In this case, after adsorption of the open form of the lipases on OCGLU (via the octyl groups layer), the only protein area of interest to establish the ionic exchange with the support is the lipase surface surrounding the active center, the balance between Glu and Asp groups of the enzyme with that of Arg and Lys (among the most relevant groups) will be very important, because this will give the balance between repulsive and attractive forces that may permit to improve or to worsen the strength of the immobilization of the enzyme on the OCGLU matrix compared to OC support.

In this paper, we have assayed the immobilization on OCGLU of some of the most employed lipases. Lipases A (CALA) and B (CALB) [48–51] from *Candida antarctica*, lipases from *Thermomyces lanuginosus* (TLL) [52], from *Rhizomucor miehei* (RML) [53,54] and from *Candida rugosa* (CRL) [23,55] have been utilized. The phospholipase Lecitase Ultra (LU) has been also included in these studies, it is a commercial chimeric enzyme constructed from the gen of the lipase from *T. lanuginosus* and that of the phospholipase from *Fusarium oxysporum* [56] than also may be immobilized on octyl-agarose [57,58].

2. Materials and methods

2.1. Materials

Solutions of lipase A from *C. antarctica* (CALA) (19.9 mg of protein per mL), lipase B from *C. antarctica* (CALB) (10.5 mg of protein per mL), lipase from *T. lanuginosus* (TLL) (36 mg of protein per mL), lipase from *R. miehei* (RML) (13.7 mg of protein per mL) and the phospholipase Lecitase Ultra (LU) (16 mg of protein per mL) were a kind gift from Novozymes (Spain). Octyl-agarose beads support was from GE Healthcare. *p*-Nitrophenyl butyrate (*p*-NPB), L-Glutamic acid, triacetin and lipase from *C. rugosa* (CRL) (in powder form, 40% of protein content) were from Sigma Chemical Co. (St. Louis, MO, USA). All reagents and solvents were of analytical grade.

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 25 mM sodium phosphate at pH 7.0 and 25 °C (ϵ under these conditions is 5150 M^{-1} cm^{-1}). To initialize the reaction, a volume of 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 hydrolyzes 1 μmol of *p*-NPB per minute under the conditions described previously. Protein concentration was determined using Bradford's method [59] and bovine serum albumin was used as the reference.

2.3. Preparation of glutamic supports

OCGLX and GLX agarose supports were prepared following the standard protocol described in Ref. [26]. 20 g of each GLX support were resuspended in 100 mL of 3 M glutamic acid solution at pH 10.5. The suspensions containing the supports and the glutamic acid were gently stirred for 4 h at 25 °C. To end the aminoacid-support covalent reaction, solid sodium borohydride was added to a solution with a concentration of 2 mg/mL to the OCGLU and GLU suspensions (at pH 10.5) and 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 groups [60]. Finally the reduced supports were filtered, washed with abundant distilled water and stored at 4 °C. A wet support is defined as the agarose beads with the pores full of aqueous medium, but without interparticle water (dried using vacuum filter).

2.4. Immobilization of enzymes

2.4.1. Immobilization of enzymes on octyl (OC) and octyl-glutamic (OCGLU) supports

The immobilization was performed using 1 (for activity and stability studies) or 10 mg (for SDS-PAGE analysis) of protein per g of wet support. A maximum of 20 pNPB units of enzyme was sued to prevent diffusional problems. The commercial samples of the enzymes were diluted in the corresponding volume of 50 mM sodium phosphate at pH 7. Then 20 g of support were added to 200 mL of enzyme solution at 25 °C under gentle stirring. The activities of both supernatant and suspension were followed using *p*-NPB. After immobilization the suspension was filtered and washed several times with distilled water and stored at 4 °C.

In the case of OCGLU, the immobilized enzyme was filtered, washed and resuspended in 5 mM sodium acetate buffer at pH 4 and 25 °C for a minimum of 12 h, to permit the enzyme-support ionic interaction.

2.4.2. Immobilization of enzymes on GLU support

The immobilization was performed using 1 or 10 mg of protein per g of wet support (see above). The commercial samples of the enzymes were diluted in the corresponding volume of 5 mM sodium acetate at pH 4 or pH 5 or sodium phosphate at pH 7. Then, 20 g of support were added to 200 mL of enzyme solution at 25 °C under gentle stirring. The activities of both supernatant and suspension were followed using *p*-NPB. After immobilization the suspension was filtered and washed several times with the same buffer of immobilization and stored at 4 °C.

2.5. Thermal inactivation of different enzymatic preparations

The stability of the enzyme preparations was determined by suspending 1 g of immobilized enzyme in 5 mL of 5 mM sodium acetate at pHs 4 and 5 or Tris HCl at pH 7 at different temperatures. Periodically, samples of the inactivation suspension were withdrawn and the activity was measured using *p*-NPB. Half-lives were calculated from the observed inactivation courses.

2.6. Inactivation of different enzymatic preparations by incubation in organic co-solvents

A solvent and concentration where the inactivation rate of the biocatalyst permitted to have accurate measurements was chosen for each enzyme. Enzyme preparations were incubated in mixtures of dimethyl sulfoxide (DMSO), acetonitrile (ACN), or 1,4-dioxane/5 mM sodium acetate pH 4 or Tris–HCl pH 7 at 30 °C. Periodically, samples of the suspension were withdrawn and the activity was measured using *p*-NPB 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 on enzyme activity (results not shown).

2.7. Desorption of the enzyme from the supports using Triton X-100

OC and OCGLU preparations of the different enzymes were incubated at pH 4 or 5 in Triton X-100 (1% (v/v) for CALA and CALB, 0.3% (v/v) for RML and 0.5% (v/v) for CRL). This treatment releases the enzyme molecules adsorbed by interfacial activation [26,27]. The activity of the enzyme in the supernatant was determined and compared to the activity of the enzyme submitted to the same treatment.

2.8. SDS-PAGE analysis

SDS-polyacrylamide gel electrophoresis (SDS-PAGE) was performed according to Ref. [61] 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 were resuspended in 1 mL of rupture buffer (2% SDS and 10% mercaptoethanol), boiled for 10 min and a 20 μL aliquot of the supernatant was used in the experiments. Gels were stained with Coomassie brilliant blue. Low molecular weight markers from Fermentas were used (10–200 kDa).

2.9. Hydrolysis of triacetin

Solutions of 5 mM triacetin in 20 mM sodium acetate/acetonitrile (20% v/v) were prepared, and their pH values were adjusted at 5.0 using NaOH. Samples of 0.25 g of wet OCGLU CALB and RML were added to 40 mL of the triacetin solutions and the reaction suspensions were gently stirred in a shaker at 250 rpm and 22 °C. Periodically, samples were withdrawn, the biocatalyst was discarded by centrifugation and the concentration of reaction products was analyzed by HPLC. Diacetin and triacetin were analyzed using 10% acetonitrile/90% water (v/v) as mobile phase at a flow rate of 1 mL/min and a 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, retention volumes were 32.0 mL for triacetin, 5.8 mL for 1,2 diacetin, 4.8 mL for 1,3 diacetin. The pH value decreased during the reaction due to the production of acetic acid, but the pH was not controlled to avoid risks of acyl migration, the final reaction pH was 4.4.

3. Results

3.1. Immobilization of enzymes on GLU supports

First, it was necessary to find conditions where the enzymes do not become immobilized on GLU supports to ensure that all enzyme molecules are first immobilized via interfacial activation when using OCGLU supports and not via ion exchange. As expected by the isoelectric point of the enzymes, none of them became adsorbed on the GLU support at pH 7 (not shown results). In fact, at pH 5 the immobilization of all the studied enzymes on the GLU support still was negligible (not shown results). This enabled to have a wide range of pH where immobilizing these lipases on OCGLU without considering the risks of an undesired initial ion exchange.

On the other hand, an enzyme just ionically exchanged on the support may be interesting as reference in our study, and also it was convenient to find conditions where the ionic exchange may occur, as our objective was to finally have a mixed adsorption on the OCGLU supports. Fig. 1 shows the immobilization of the different enzymes on GLU supports at pH 4. At this pH value, some of the enzymes were at least partially immobilized. Using CALA and CALB around 80% or 55% of the offered protein was immobilized without a decrease in enzyme activity. CRL and TLL immobilization were far lower, around 15% and 25% respectively, and the suspension reduced the activity while the free enzyme remained fully active, suggesting that the immobilization was responsible for a reduction in the enzyme activity. LU was partially immobilized on GLU supports, but the immobilized enzyme became inactivated (while the free enzyme remained active under these conditions), after washing the preparation activity was negligible. RML became inactivated at pH 4, moreover it did not become significantly immobilized on GLU support. Thus, only CALA, CALB, CRL and TLL were immobilized on GLU support, at least for comparison purposes.

It should be considered that is not the same situation to adsorb the enzyme from the medium using a GLU support, where a direct multipoint ionic exchange must be produced and may affect any region of the enzyme that to achieve an ionic exchange of the enzyme adsorbed on OCGLU, where the enzyme is very near to the support surface, but only the groups on the face of the active center may interact with the support.

3.2. Immobilization of the enzymes on OCGLU and OC supports

The six enzymes were immobilized on OC and OCGLU (Fig. 2). We could not detect significant differences in the immobilization speed on both supports. It has been described that the immobilization of lipases on hydrophobic supports at low ionic strength produces the involvement of the open form of the lipase, producing a hyperactivation of the enzyme upon immobilization on octyl supports [20]. The hyperactivations observed using OC or OCGLU were fairly similar, and fitted with previous reports [62]. CALB did not show a significant hyperactivation (this enzyme has a very small lid that does not seclude the active center from the medium) [51], RML and CRL multiplied their activity by 5. Thus, the presence of the Glu groups on the support surface did not significantly alter the

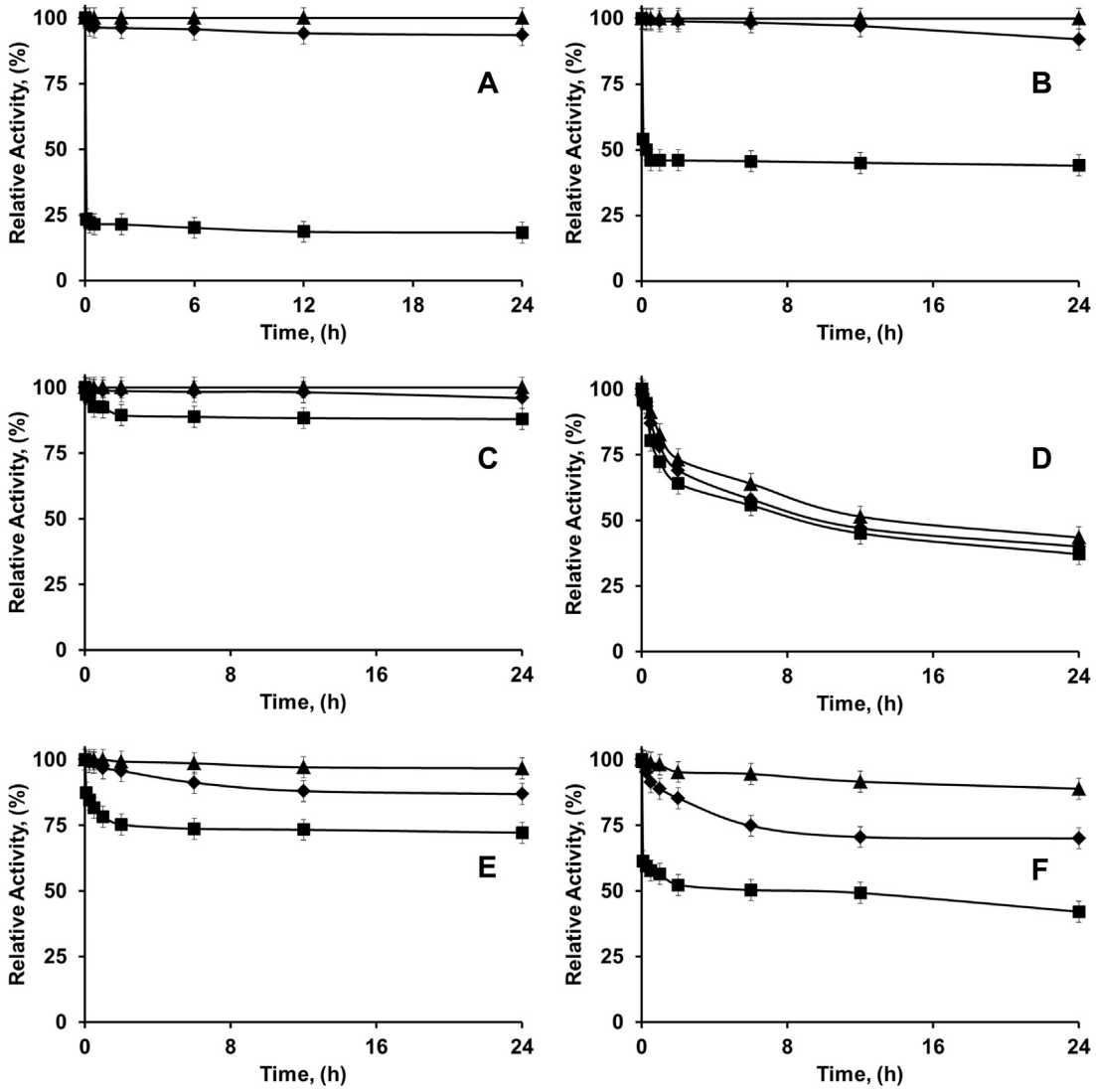

Fig. 1. Immobilization courses of the different enzymes on GLU support in 5 mM sodium acetate at pH 4 and 25 °C. Panel A: CALA, Panel B: CALB, Panel C: CRL, Panel D: RML, Panel E: TLL and Panel F: LU. Rhombus (suspension), Squares (Supernatant) and Triangles (Soluble enzyme). Other specifications are described in Section 2.

lipase immobilization on OC supports at pH 7. Incubation at pH 4 neither altered the enzyme activity.

3.3. Desorption of OC and OCGLU supports by incubation in Triton X-100

Detergents have been described to be able to release the enzymes from hydrophobic supports, as these amphipathic molecules may coat the hydrophobic pocket of lipases [20,21] The incubation of the immobilized enzymes in Triton X-100 produced the full desorption of CALB, CALA, CRL and RML adsorbed on OC. TLL was fully inactivated by this detergent and LU remained fully adsorbed on the OC support.

Using OCGLU support, less than 5% of the *p*-NPB activity of CALB, CALA, CRL and RML could be detected in the supernatant. This improved the results obtained glyoxyl agarose, where usually at least 10% of the enzyme activity was desorbed [26,27], now almost 100% of the enzyme activity remained attached to the support in the presence of non-ionic detergents. That suggested that at least in these enzymes, adsorption was now not only a hydrophobic one, and that when the hydrophobic adsorption was broken by the detergent, the ionic exchange was enough to keep the enzyme molecules adsorbed on the support. To check this point, 200 mM of NaCL was added to the suspensions in the presence of 1% of detergent, and most of 90% of the enzyme activity could be detected in the supernatant in all cases. This salt concentration (just the salt) also desorbed the enzyme adsorbed in GLU supports.

Thus, the main goal of our strategy seemed to be accomplished.

3.4. Thermal stability of the different enzyme preparations

The fact that a mixed adsorption has been established may have relevance to avoid enzyme desorption, but that is not guarantee that the enzyme stability increased, as now we had a support with more capacity to promote interactions with the immobilized enzyme and with the distorted forms of the enzyme, and this may be negative for enzyme stability [63]. Thus, the thermal stabilities of the different enzyme preparations (OC, OCGLU and GLU) were studied at pH 4, conditions where the free enzymes were able to become immobilized on the GLU support, with the exception of RML, and at pH 7, conditions where none of the used enzymes were adsorbed on the GLU support (Fig. 1). Table 1 resumes the main results.

Starting with CALA, OCGLU-CALA was the most stable preparation at both pH 4 (doubling the stability of O C—CAL) and pH

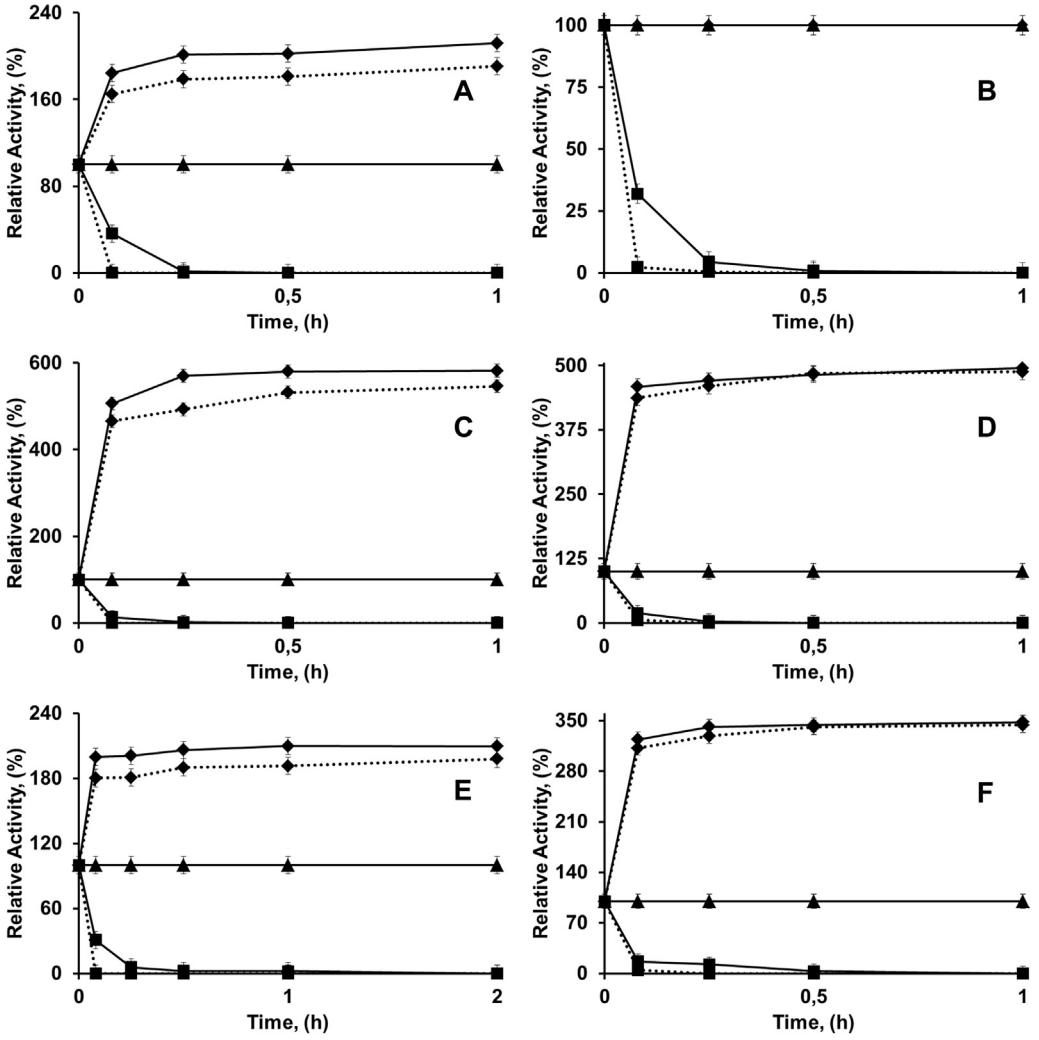

Fig. 2. Immobilization courses of the different enzymes on OC (Dotted Line) and OCGLU (Solid Black Line) supports in 50 mM sodium phosphate at pH 7 and 25 °C. Panel A: CALA, Panel B: CALB, Panel C: CRL, Panel D: RML, Panel E: TLL and Panel F: LU. Rhombus (suspension), Squares (Supernatant) and Triangles (Soluble enzyme). Other specifications are described in Section 2.

Table 1
Half-lives (minutes) of different preparation of enzymes under different conditions. Inactivation conditions are specified in the Table. Values are the mean of 3 independent experiments and are given as mean with standard error.

	pH 4, (T)	pH 7 in 5 mM buffer, (T)	pH 7 in 100 mM buffer, (T)	Organic cosolvent, pH 4 (T, concentration, solvent)	Organic cosolvent, pH 7 (T, concentration, solvent)
O C—CALA	45 ± 5 (85 °C)	240 ± 15 (80 °C)	30 ± 5 (80 °C)	2≤ (30 °C, 80%, DMSO)	3000 ± 35 (25 °C, 80% DMSO)
OCGLU-CALA	90 ± 9 (85 °C)	≥3000 (80 °C)	45 ± 8 (80 °C)	24 ± 4 (30 °C, 80%, DMSO)	3000 ± 40 (25 °C, 80% DMSO)
GLU-CALA	30± 5 (85 °C)	65 ± 5 (80 °C)	6 ± 2 (80 °C)	150 ± 20 (30 °C, 80%, DMSO)	240 ± 20 (25 °C, 80% DMSO)
O C—CALB	90 ± 9 (80 °C)	55 ± 4 (73 °C)	55 ± 4 (73 °C)	30 ± 5 (25 °C, 75%, DMSO)	110 ± 8 (25 °C, 80%, DMSO)
OCGLU-CALB	50 ± 6 (80 °C)	270 ± 15 (73 °C)	270 ± 15 (73 °C)	400 ± 35 (25 °C, 75%, DMSO)	1500 ± 80 (25 °C, 80%, DMSO)
GLU-CALB	20 ± 4 (80 °C)	2≤ (73 °C)	2≤ (73 °C)	35 ± 5 (25 °C, 75%, DMSO)	120 ± 14 (25 °C, 80%, DMSO)
O C—CRL	50 ± 9 (60 °C)	12 ± 2 (60 °C)	12 ± 2 (60 °C)	360 ± 30 (25 °C, 40%/ACN)	12 ± 3 (25 °C, 40%/ACN)
OCGLU-CRL	80 ± 10 (60 °C)	60 ± 10 (60 °C)	60 ± 10 (60 °C)	650 ± 30 (25 °C, 40%/ACN)	3000 ± 90 (25 °C, 40%/ACN)
GLU-CRL	120 ± 12 (60 °C)	100 ± 12 (60 °C)	100 ± 12 (60 °C)	120 ± 10 (25 °C, 40%/ACN)	140 ± 20 (25 °C, 40%/ACN)
OC-RML	50 ± 7 (50 °C)	240 ± 30 (45 °C)	240 ± 30 (45 °C)	8 ± 2 (30 °C, 25%, ACN)	100 ± 12 (25 °C, 25%, ACN)
OCGLU-RML	25 ± 3 (50 °C)	180 ± 12 (45 °C)	180 ± 12 (45 °C)	5 ± 2 (30 °C, 25%, ACN)	350 ± 16 (25 °C, 25%, ACN)
OC-TLL	28 (65 °C)	30 ± 3 (73 °C)	30 ± 3 (73 °C)	30 ± 3 (25 °C, 50%, Dioxane)	35 ± 3 (25 °C, 60%, Dioxane)
OCGLU-TLL	15 (65 °C)	32 ± 4 (73 °C)	32 ± 4 (73 °C)	28 ± 3 (25 °C, 50%, Dioxane)	25 ± 3 (25 °C, 60%, Dioxane)
GLU-TLL	2≤ (65 °C)	28 ± 3 (73 °C)	28 ± 3 (73 °C)	4 ± 1 (30 °C, 50%, Dioxane)	15 ± 2 (25 °C, 60%, Dioxane)
OC-LU	90 (40 °C)	140 (45 °C)	140 (45 °C)	2≤[a] (30 °C, 25%, ACN)	2≤[a] (25 °C, 25%, ACN
OCGLU-LU	180 (40 °C)	160 (45 °C)	160 (45 °C)	2≤[a] (30 °C, 25%, ACN)	250 ± 15[a] (25 °C, 25%, ACN

[a] Using 100 mM of Tris-Cl buffer.

7 (by a factor higher than 12). Glu-CALA was the least stable at both pH values (at pH 7 the enzyme was released from the support). It was surprising that the stabilization regarding the use of OC observed using OCGLU support was more significant at pH 7, conditions where the enzyme was not able to become immobilized on GLU supports. If the ionic strength was increased to 100 mM, the differences became very short (just a 50% greater half-live for the OCGLU-CALA), suggesting that some ionic interactions may be responsible of this stabilization.

OCGLU-CALB was slightly less stable than O C—CALB at pH 4 (less than 2 folds), while it was more stable at pH 7 (around 5 folds), Glu-CALB was the least stable preparation at both pH values and the enzyme was released from the support at pH 7. As in the case of CALA; the stabilization using OCGLU was higher at pH values where the free enzyme was not immobilized on the support, while under conditions where the ionic enzyme-support interaction is favored, the stability decreased (compared to the O C—CALB).

Using CRL, the most stable preparation at both pH values is Glu-CRL, while the OCGLU preparation is more stable than the OC biocatalyst (5 folds at pH 7 and less than 2 at pH 4). In this case, the enzyme release from the GLU support at pH 7 is negligible, although the enzyme cannot be immobilized on this support at this pH value.

The results were very different using RML, (it must been stressed that the enzyme did not become immobilized on GLU support even at pH 4). In this case, OC preparation was more stable than OCGLU-RML at both pH values, even though by a factor of 2 or lower. The situation was similar using TLL, OC was slightly more stable than OCGLU preparations. Curiously, Glu-TLL has a similar stability at pH 7 but it was far less stable at pH 4 than the other two preparations, when the enzyme was not able to become adsorbed on the support at pH 7 and it may be adsorbed on the support at pH 4. This suggested that a role of the amino group of the Glu group may not be discarded in the adsorption of the enzyme after the first immobilization via cation exchange. After enzyme immobilization via interfacial activation, the first additional enzyme-supports interactions seemed to be cation exchange, but the establishment of some ionic bonds between the cationic secondary amino group attached to the support and the anionic groups in the protein seemed possible at a later stage. OCGLU-LU was slightly more stable than OC-LU at pH 4, while at pH 7 stabilities became very similar. This result should be the expected results if the promotion of cation exchange was the main difference between both supports and this had a positive effect.

Thus, OCGLU permitted in certain cases a very significant stabilization compared to OC support, but this did not occur in all cases. In fact, in some instances the covalent immobilization of some lipases (e.g., TLL) on glyoxyl-octyl supports produced even a significant decrease on the enzyme stability [27]. And using this new OCGLU support the situation became even more complicated by the high number of likely effects that the 3 ionic groups may have on the enzyme stability. In fact, in some cases the lipase stabilization observed using OCGLU is higher than that observed using octyl-glyoxyl [26,27] (e.g., CALA). This occurred even though the establishment of an ionic exchange has been confirmed by the experiment of desorption with no ionic detergent for all enzymes (see above). The differences between OC and OCGLU supports may be resumed in a general lower hydrophobicity of the support surface using OCGLU, and the possibility of establishing some ionic positive or negative interactions between the Glu groups in the support (bearing two anionic groups and one cationic group) and the ionic groups of the enzyme. It cannot be discarded that although the amino group of the Glu group is not relevant in a first immobilization; the already immobilized enzyme may interact with these amino groups reinforcing the enzyme-support interactions [46,63]. Neither may it be discarded that some "repulsive" interactions may help to keep the right conformation of the enzyme while some

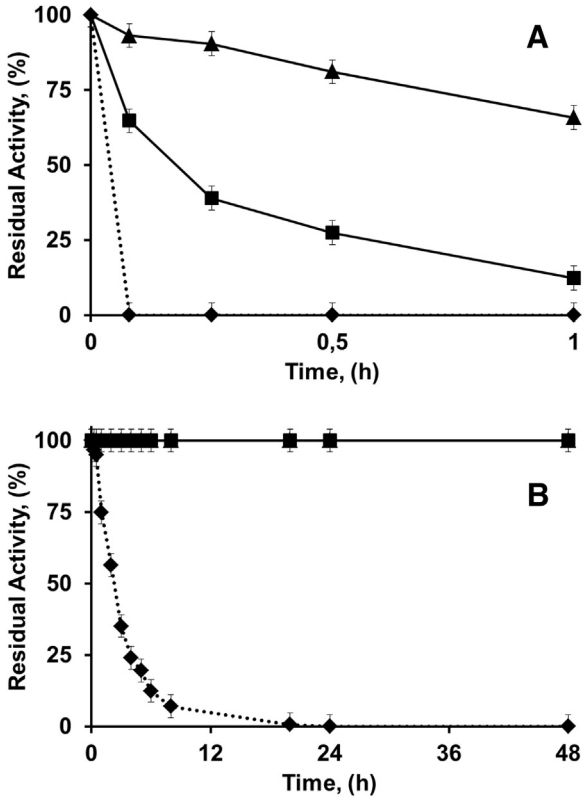

Fig. 3. Different CALA derivatives incubated in DMSO 80% (Panel A) and in DMSO 70% (Panel B) in buffer 5 mM sodium acetate at pH 4 and 30 °C. Dotted Line (Rhombus): OC, Squares: OCGLU and Triangles: GLU. Other specifications are described in Section 2.

"attractive" interactions may help the stabilization of incorrect enzyme structures. Some papers point that the presence of a surface with similar charge to that of the protein may help in the protein refolding [64–68]. Considering that the enzymes were inactivated in one drastic condition and measured under milder conditions where some reactivation is possible, the improved behavior could be derived of a more rapid reactivation. This complex situation may produce that in certain cases an enzyme may be stabilized while in other cases the enzyme become destabilized when comparing OC-lipase and OCGLU-lipase immobilized preparations.

3.5. Stability of the different preparations in organic solvents

The main problem of OC supports is that they release the lipase at high solvent concentration [24–29], and that is the problem that we intended to reduce using OCGLU supports. Thus, the enzyme preparations were evaluated at two pH values, 4 and 7, in the presence of an organic solvent at a concentration that permitted to determine the half-lives of all preparations (Table 1).

Using CALA at pH 4, the most stable preparation was the GLU one, being the OC preparation very unstable. At this pH value, the effect of the OCGLU was clear, stabilizing the enzyme by a factor higher than 12 compared to O C—CALA when using 80% DMSO. Using 70% of this solvent, the activity of OCGLU-CALA was almost fully preserved while the activity of O C—CALA was fully destroyed (Fig. 3). At pH 7, where the ionic exchange of CALA should be apparently reduced, GLU was the preparation with lower stability while OC and OCGLU-CALA stabilities became almost identical. This result suggested that at pH 7 in this concentration of solvent the ionic exchange did not play a relevant role on enzyme stability.

OCGLU-CALB was the most stable CALB preparation at both pH values, and the stabilization regarding the OC preparation was

around 13 times at both pH values. GLU and OC presented almost the same stabilities at both pH values, suggesting that the ionic interactions were maintained at pH 7.

OCGLU-CRL was slightly more stable than O C—CRL at pH 4 in acetonitrile (less than twice). Surprisingly, the OCGLU-CRL became 250 folds more stable than O C—CRL at pH 7. This agreed with the fact that Glu-CRL was more stable than O C—CRL at pH 7 and not at pH 4, but it was not the expected result considering the possibilities of ion exchange of the enzyme and the support at pH 4 and 7.

GLU-RML was the most stable RML biocatalyst at pH 4 and the less stable at pH 7 (as expected from considering just the cation exchange). OC and OCGLU-RML exhibited a very similar stability at pH 4, while at pH 7 the stability of the enzyme increased after immobilization on OCGLU (by 3.5 folds). Again, this result was difficult to explain just using the cation exchanger character of this support, but it may be related to some repulsion forces that may help to keep the right conformation of the enzyme [64–68].

TLL had similar stabilities immobilized on OC or OCGLU, while it was less stable in GLU, and this occurred at both pH values (although the difference at pH 7 was lower than at pH 4).

Both preparations of LU were very unstable at pH 4 even using moderate amounts of solvent, while at pH 7 the OCGLU-LU became several hundred folds more stable than the OC preparation (improving the results obtained using octyl-glyoxyl) [27].

Again the results were very diverse and enzyme depending and were not directly related to the capacity of the enzyme to become adsorbed on GLU supports, suggesting that several phenomena may be simultaneously playing a relevant role to produce the final stability result. In organic medium we had the same differences between OC and OCGLU supports that we commented in the thermal inactivations, and also in some cases stabilizations favorably compared to the results obtained using octyl-glyoxyl support [26,27]. The higher hydrophilicity of the support surface may produce a certain stabilization of the enzyme due to organic solvent partition away from that area of the enzyme [18,63]. Moreover, it should be considered that the presence of solvents may produce a strong increase in the pK of the carboxylic groups of the aminoacids placed both in the support and in the protein (e.g., using 80% DMSO the pK increment may be more than 3 pH units) [69]. This may completely alter the support and enzyme surface ionic character and therefore the enzyme-support interactions. At pH 4, the supports may even have a cationic character, with just one cationic group and the two carboxylic acids almost fully protonated, while at pH 7 the support regained the two anionic groups. The protein may lose many of the anionic groups at pH 4 in 80% of cosolvent. This may explain the good results at pH 7 obtained with some lipases.

3.6. Determination of enzyme release from the supports during enzyme inactivations

The idea of the OCGLU supports was defined to reduce the enzyme release of the enzymes from the OC supports without losing the reversibility of the immobilization. The very low enzyme desorption in the presence of detergents already suggested that this goal has been achieved, but we investigated if this also occurred on enzyme inactivated in the presence of organic solvents. To do this, we have boiled the immobilized preparations in breaking buffer (the anionic nature of the SDS should help to the enzyme desorption) and using the supernatant thus obtained to run SDS-PAGE experiments.

Fig. 4 shows some results obtained using OC and OCGLU-CALA after incubation in 70% DMSO at pH 4 and 30 °C for 48 h. It is clear that while the amount of protein remains identical in the OCGLU after this incubation (the enzyme remained fully active), the amount of protein in O C—CALA greatly decreased, and it is

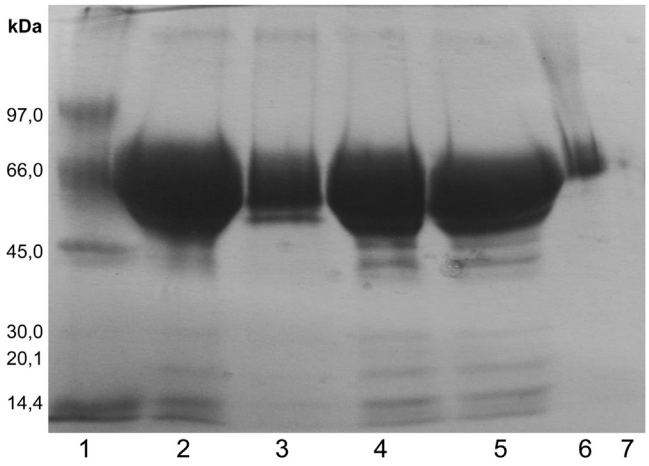

Fig. 4. SDS-PAGE of different CALA immobilized derivatives incubated in 70% DMSO/30% aqueous 5 mM sodium acetate at pH 4 and 30 °C. Lane 1: Low Molecular Weight Markers, Lane 2: O C—CALA, Lane 3: O C—CALA incubated in DMSO, Lane 4: OCGLU-CALA, Lane 5: OCGLU-CALA incubated in DMSO, Lane 6: supernatant of O C—CALA incubated in DMSO, Lane 7: supernatant of OCGLU-CALA incubated in DMSO. Other specifications are described in Section 2.

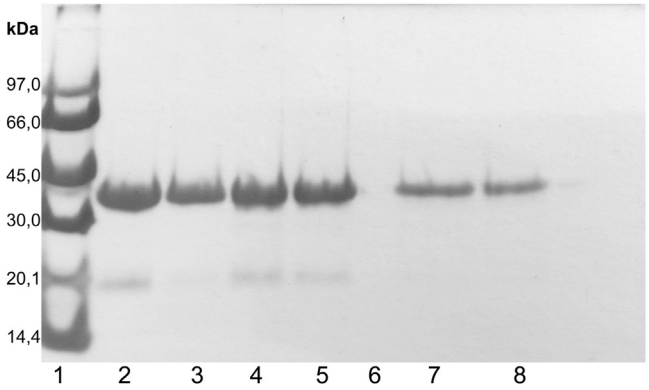

Fig. 5. SDS-PAGE of different CALB immobilized derivatives incubated in 80% DMSO/20% aqueous 5 mM TrisHCl at pH 7 and 30 °C. Lane 1: Low Molecular Weight Marker, Lane 2: O C—CALB, Lane 3: O C—CALB incubated in DMSO, Lane 4: OCGLU-CALB, Lane 5: OCGLU-CALB incubated in DMSO, Lane 7: Glu-CALB, Lane 8: Glu-CALB incubated in DMSO. Other specifications are described in Section 2.

even possible to visualize a protein band in the supernatant of the inactivation suspension.

Fig. 5 shows the results obtained after the inactivation of CALB incubated in 80% DMSO at pH 7 and 30 °C for 24 h. Again, OCGLU-CALB remained with very similar amounts of protein, while the O C—CALB band of protein severely decreased after this inactivation. This may explain the stabilization observed (Table 1). Fig. 6 offers a similar situation using OC and OCGLU-CRL incubated in acetonitrile at pH 7, conditions where a high stabilization was detected (Table 1). Thus, the results confirmed that the enzyme molecules were more strongly bound to the support using OCGLU than OC supports, and even though the enzyme may be inactivated, its desorption was greatly decreased

To visualize the usefulness of these preparations, OC-GLU CALB and RML were used in 6 consecutive reaction cycles for the production of 1,2 diacetin (a process that shows the regioselectivity and specificity of the enzyme) by hydrolysis of triacetin at pH 5 in the presence of 20% acetonitrile [70]. This chiral compound may be used as building block, and acetonitrile permitted to improve the yields, while acid pH value was a requirement to prevent acyl migration and avoided the use of any titration reagent [70]. The initial activity remained identical to that of the first cycle for both

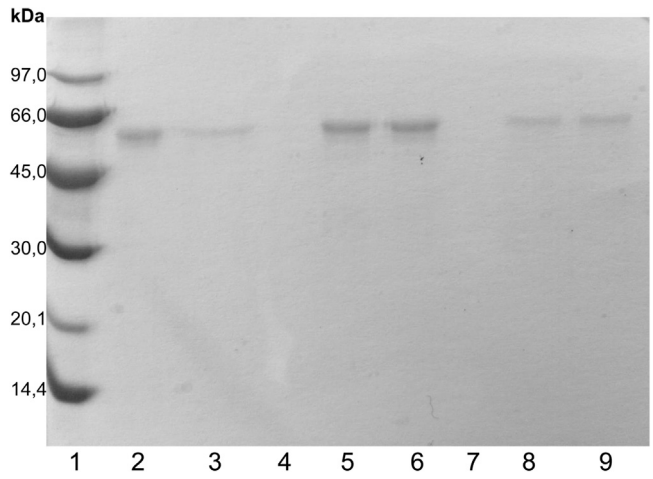

Fig. 6. SDSPAGE of different CRL immobilized derivatives incubated in 40%ACN/60% aqueous 5 mM Tris–HCl at pH 7 and 25 °C. Lane 1: Low Molecular Weight Marker, Lane 2: O C—CRL, Lane 3: O C—CRL incubated in ACN, Lane 5: OCGLU-CRL, Lane 6: OCGLU-CRL incubated in ACN, Lane 8: Glu-CRL, Lane 9: Glu-CRL incubated in ACN. Other specifications are described in Section 2.

enzymes and 93–95% 1,2 diacetin could be obtained after just one hour of reaction time using both enzyme preparations during the 6 cycles (not shown results).

3.7. *Reuse of the supports*

The reuse of this support [18,63] should be one of the advantages of this method compared to glyoxyl-octyl [26]. To this goal, all immobilized enzymes were washed 3 folds with 5 vol of 2% SDS or CTAB in 100 mM sodium phosphate at pH 7 and 37 °C and then with water. SDS-PAGE of the washed supports revealed that there is no remaining protein on the support (results not shown). These supports could be reused in the immobilization of new batches of fresh enzyme without any difference in stability or activity compared to the original OCGLU. This adsorption/desorption cycles were performed 5 times.

Thus, the immobilization on OCGLU, although stronger than using OC, permitted the reuse of the support, keeping this positive property of the immobilization on hydrophobic supports that was lost using octyl-glyoxyl supports.

4. Conclusion

OCGLU supports offer some advantages compared to the OC support, the main one that the enzymes are not desorbed to the medium during organic solvent inactivations or incubation in no cationic detergents, and also that in some instances a very significant stabilization has been achieved, even improving the results using octyl-glyoxyl supports [26,27]. All enzymes remained immobilized on the OCGLU support in the presence of detergents under conditions where all enzyme molecules were released from OC supports, while enzyme activity was not released using high ionic strength that released all enzyme from GLU supports. However, the combined use of high salt concentration and no ionic detergents permitted the release of all enzyme molecules, suggesting that all enzyme molecules are immobilized via a mixed ion exchange-hydrophobic interaction, while using octyl-glyoxyl a significant percentage of enzyme (even 30% using TLL) could be desorbed to the media by this treatment [26,27].

However, these stabilizations are not general and become difficult to predict. In general, the higher stabilizations have been found under conditions where the enzyme did not become immobilized on GLU supports, conditions where the repulsion forces should overtake to the attractive forces between the protein and the support. This result, although difficult to understand, may be very relevant, as it shows how the properties of the support surface may be a critical point in determining enzyme stability [63], but that there are not obvious rules.

This could be explained if the repulsion forces help to maintain the active enzyme structure, while the possibility of formation of new enzyme-support bonds may stabilize partially unfolded structures. The favoring of enzyme refolding caused by a similar ionic nature of enzyme and support during enzyme activity determination may be not discarded to explain these good results. The effects of the immobilization on OCGLU on enzyme stability may be decreased by increasing the ionic strength, confirming that the ionic exchange may play a relevant role on it.

OCGLU supports have a layer of hydrophilic group that should reduce the hydrophobicity of the OC support; this should produce a negative weakening of the enzyme-support interactions if an ionic interaction is not achieved. As the interaction is stronger, it is necessary to consider that the enzyme and the Glu groups on the support should be interacting. Glu has two anionic groups and one cationic group, may be not discarded that in some cases the secondary amino group (the bond that attach the Glu to the support) may establish some interactions with the anionic groups of the enzyme after adsorption, due to the proximity of both groups, interaction that is not produced on the free enzyme and by this reason cannot become immobilized.

Thus, even though it is not easy to understand the reasons for these results, OCGLU supports may be a good alternative to the octyl or even the glyoxyl-octyl lipase preparations, maintaining the reversibility of the process.

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