

## Glutaraldehyde Cross-Linking of Lipases Adsorbed on Aminated Supports in the Presence of Detergents Leads to Improved Performance

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Lipases from *Candida rugosa* (CRL) and lipase isoforms A and B from *Candida antarctica* (CAL-A and CAL-B) were adsorbed on aminated supports in the presence of detergents to have individual lipase molecules. Then, one fraction was washed to eliminate the detergent, and both preparations were treated with glutaraldehyde. The presence of detergent during the cross-linking of the lipases to the support permitted an increase in the recovered activity (in some instances, even by a 10-fold factor). This activity was higher even than that exhibited by the just adsorbed lipases, suggesting that it was not a result of some protective effect of the detergent in the enzyme activity during glutaraldehyde chemical modification. Moreover, the enantioselectivity of the different enzyme preparations was very different if the glutaraldehyde was offered in the presence or in the absence of detergent, in some cases increasing the *E* value (even by a 7-fold factor in the case of CAL-A in the hydrolysis of (±)-2-hydroxy-4-phenylbutyric acid ethyl ester), in other cases even inverting the enantio preference (e.g., in the case of CRL). The irreversible chemical inhibition of the enzyme that was immobilized and cross-linked with glutaraldehyde in the presence of detergents was more rapid than that in the other preparations (by more than a 10-fold factor). This experiment reveals an exposition degree of the active serine in the preparation cross-linked with the support in the presence of detergent that is higher than that in the other preparations. The results suggested that different enzyme structures were “stabilized” by the glutaraldehyde treatment if performed in the presence or in the absence of detergent, and that, in the presence of detergent, a form of the lipase with the serine residue more exposed to the medium and much more active could be obtained. This strategy seems to be of general use to improve the lipase activity to be used in macroaqueous media.

### Introduction

Lipases are very likely the most used enzymes in organic chemistry, based on their broad specificity coupled with a high enantio and regio selectivity.<sup>1–7</sup> These enzymes present a complex catalytic mechanism, called interfacial activation. Thus, in homogeneous systems, the enzyme exists in equilibrium between two forms: the closed conformation, where the active center is secluded from the medium being inactive, and the open form, where the active center is exposed to the reaction medium.<sup>8–11</sup> In the presence of hydrophobic surfaces (e.g., drops of nonmiscible substrates, hydrophobic surfaces), the open form becomes adsorbed to it via the hydrophobic areas surrounding the active center, shifting the equilibrium toward the open form and promoting the “interfacial activation of the enzyme”.<sup>12–16</sup>

Lipases immobilized on porous supports and used in aqueous media may not suffer this interfacial activation, exhibiting activity that corresponds to the open–close equilibrium. One possible solution would be to increase this activity by adsorbing the lipases on hydrophobic supports at low ionic strength. This has permitted researchers to greatly increase the lipase activity against medium-small hydrophobic substrates,<sup>17–23</sup> but the activity is not very high using hydrophilic and/or very large substrates because of the proximity of the very highly hydro-

phobic surface that may reduce the accessibility for large and hydrophilic compounds.<sup>24</sup>

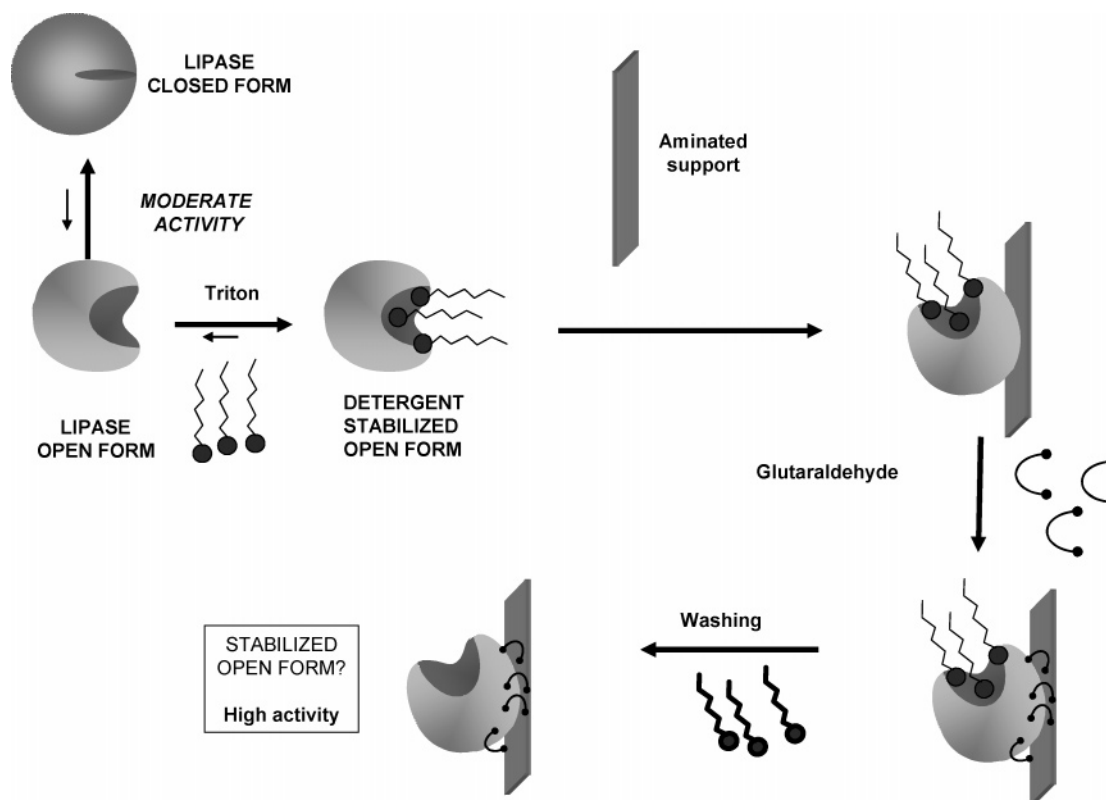
Thus, the design of protocols that could permit the immobilization of lipases presenting the open conformation is an exciting goal.

Detergents have been proposed as being activators of lipases,<sup>25–28</sup> suggesting that they would be able to shift the lipase conformational equilibrium toward the open form. Using as a hypothesis that detergents may really be able to stabilize the open forms of lipases,<sup>29</sup> we intend to develop strategies to stabilize this open form induced by the action of detergents. If this is possible, lipases should keep high levels of activity, even when the detergent has been eliminated. This idea was previously used by Mingarro and co-workers,<sup>29b</sup> lyophilizing the enzymes in the presence of detergents and achieving a hyperactivated form of the lipase. Unfortunately, this methodology was only useful if the enzyme remained in organic media; if water was added, the protein recovered its flexibility, and the hyperactivation effect was lost.

Here we propose the treatment of lipases, previously adsorbed on aminated supports, with glutaraldehyde in the presence of detergents to produce immobilized lipases with a stabilized open form, permitting the recovery of a higher activity. This methodology has proved to be very effective for cross-linking the enzymes and the supports via multipoint covalent attachments, increasing the overall rigidity of the protein molecules.<sup>30</sup> That is, the supports were used as a rigid and multifunctional cross-linking reagent, able to establish many bonds with the

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**Scheme 1.** Stabilization of the Open Form of Lipases by Cross-Linking of the Enzymes with Aminated Supports in the Presence of Detergent

enzyme. In an ideal situation, if we are able to get a rigidification of the tertiary structure of the lipase, this should reduce the mobility of the lipase lid, maintaining the form that each molecule of lipase presented during the immobilization (Scheme 1).

This strategy has been utilized with different lipases: those from *Candida antarctica* (isoforms A and B) (CAL-A and CAL-B)<sup>31–34</sup> and from *Candida rugosa* (CRL).<sup>35–37</sup> CAL-A and CRL present a large lid<sup>34,38</sup> and suffer interfacial activation.<sup>39,40</sup> Both lipases are able to give lipase–lipase aggregates.<sup>41</sup> CAL-B has a small lid,<sup>34</sup> but is still able to become adsorbed on hydrophobic supports at low ionic strengths.<sup>20</sup>

### Experimental Part

**Materials.** Lipases from CAL-B (Novozym 525L) and from CAL-A (Novozym 868) were generously donated by Novo Nordisk (Denmark). CRL, Glutaraldehyde, Triton X-100, methyl butyrate (**1**), (±)-mandelic acid methyl ester (**2**), *p*-nitrophenyl propionate (pNPP) and diethyl-*p*-nitrophenyl phosphate (D-pNP) were obtained from Sigma. The enzymes were purified using octyl-agarose adsorption at low ionic strength, yielding just one protein band.<sup>20</sup> (±)-2-Hydroxy-4-phenylbutyric acid ethyl ester (**3**) was generously donated by VITA INVEST SA (Barcelona, Spain). (±)-Glycidyl butyrate (**4**) was kindly donated by Dr. Terreni from the University of Pavia (Milan, Italy). Other reagents and solvents used were of analytical or high-performance liquid chromatography (HPLC) grade.

**Methods.** All experiments were performed at least in triplicate, and the experimental error was never over 10%.

**Immobilization of Lipases on MANAE–Agarose Support.** Ten grams of monoaminoethyl-*N*-aminoethyl (MANAE)–agarose beads<sup>42</sup> was added to 100 mL of 5 mM sodium phosphate buffer lipase solution at pH 7, containing 0.5% Triton X-100. The mixture was then shaken at 4 °C and 250 rpm for 4 h. After that, the supernatant was removed by filtration, and the supported lipase was washed several times with

distilled water to eliminate the detergent. In some cases, detergent was added to the preparation after immobilization.

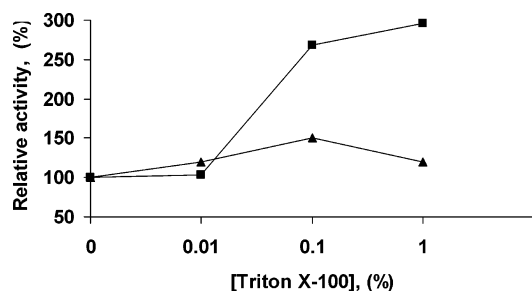
**Cross-linking of Immobilized Lipases by Glutaraldehyde.** One gram of the MANAE–lipase immobilized preparation was added to a 10 mL sodium phosphate buffer 5 mM solution of 0.5% (v/v) glutaraldehyde at pH 7.<sup>30</sup> The mixture was shaken at 25 °C and 250 rpm for 90 min, washed with distilled water, and then stored at 4 °C.

**Spectrophotometric Activity Assay.** This assay was performed by measuring the increase in absorbance at 348 nm produced by the release of *p*-nitrophenol in the hydrolysis of 0.4 mM pNPP in 25 mM sodium phosphate at pH 7 and 25 °C. To initialize the reaction, 0.1 mL of lipase solution or suspension was added to 2.5 mL of substrate solution. An international unit of pNPP activity was defined as the amount of enzyme necessary to hydrolyze 1 μmol of pNPP/min under the conditions described above.

**Enzymatic Hydrolysis of Esters.** The activities of different lipase-immobilized preparations on the hydrolysis reaction of different esters were performed by adding 0.1 g of lipase preparation to 150 mL of 10 mM of substrate **1**, 0.2 g of lipase preparation to 10 mL of 10 mM of substrate **2**, 0.5 g of lipase preparation to 6 mL of 2 mM substrate **3**, and 0.5 g of lipase preparation to 10 mL of 10 mM substrate **4** with 5% CH<sub>3</sub>CN (v/v) under mechanic stirring. All experiments were performed in 25 mM sodium phosphate at pH 7 and 25 °C.

It was determined that, in all cases, the concentrations of substrates were in the first order region of the different enzymes and enzyme preparations; therefore, the differences in activities directly reflected differences in  $V_{\max}/K_m$ .

During the reaction, the pH value was kept constant by automatic titration, and the enzymatic activity (a micromoles of substrate hydrolyzed per minute per milligram of immobilized protein) was evaluated from NaOH consumption using a pH-stat instrument. The degree of hydrolysis was confirmed via reverse-phase HPLC (Spectra Physic SP 100 coupled with a UV detector Spectra Physic SP 8450) on a Kromasil C18 (25 × 0.4 cm) supplied by Analisis Vinicos (Spain). The elution was isocratic with a mobile phase of acetonitrile (30%) and 10 mM ammonium phosphate buffer (70%) at pH 2.95 for substrates **2** and **3**,



**Figure 1.** Effect of Triton X-100 on the activity of different preparations of CAL-A. The substrate was pNPP. Experiments were carried out as described in the Experimental Section. Squares: soluble enzyme, Triangles: agarose–MANAE-adsorbed CAL-A.

and a mobile phase of acetonitrile (35%) and 10 mM ammonium phosphate buffer (65%) at pH 2.95 for substrates **1** and **4** at a flow rate of 1.5 mL/min. The elution was monitored by recording the absorbance at 225 nm (substrates **1** and **4**) or 254 nm (substrates **2** and **3**).

**Determination of Enantiomeric Excess.** At different conversion degrees, the enantiomeric excess (ee) of the release acid was analyzed by chiral reverse-phase HPLC. The column was a Chiracel OD-R, and the mobile phase was an isocratic mixture of 5% acetonitrile and 95% NaClO<sub>4</sub>/HClO<sub>4</sub> 0.5 M at pH 2.1 for substrate **2** and an isocratic mixture of 20% acetonitrile and 80% NaClO<sub>4</sub>/HClO<sub>4</sub> 0.5 M at pH 2.1 for substrate **3**. The analyses were performed at a flow rate of 0.5 mL/min by recording the absorbance at 225 nm.

The ee of the remaining ester **4** was determined by chiral phase HPLC analysis. The column was a Chiracel OD, the mobile phase was an isocratic mixture of 2-propanol and hexane (2:98 v/v) at a flow rate of 0.4 mL/min, and UV detection was performed at 225 nm.

**Calculation of *E* Values.** *E* values were calculated as the ratio between the percentage of hydrolyzed R and S isomers (from racemic mixture) at hydrolysis degrees around 15%.

**Irreversible Inactivation of Immobilized Lipases in the Presence of D-pNP.** Different lipase-immobilized preparations (0.4 g) were suspended in 5 mL of 25 mM sodium phosphate buffer solution at pH 7 and 25 °C. Then D-pNP was added up to a concentration of 1.45 mM. Samples of this suspension were withdrawn periodically, and their activities were checked using the p-NPP assay.

## Results

**Lipase from CAL-A.** Purified CAL-A was adsorbed on MANAE–agarose, and the enzyme kept its activity against pNPP (activity recovery was over 90%) almost unaffected.

Figure 1 shows that the activity of both free CAL-A and the lipase adsorbed on MANAE–agarose became strongly increased by the addition of detergent during activity determination. The increase in the enzyme activity was more significant in the case of the free enzyme, very likely because here there are two effects working in a simultaneous way: the breaking of the lipase–lipase dimers<sup>41a–d</sup> and the direct interactions between the detergent and the individual enzyme molecules. Using lipases immobilized under dissociation conditions, only direct effects of the detergents on individual lipase molecules may occur.

Using our hypothesis that the positive effect of detergents may be derived from a shifting of the equilibrium toward the open form, we compared the properties of three different immobilized preparations:

1. the enzyme is adsorbed on MANAE–agarose in the presence of detergent to ensure the adsorption of monomers of the enzyme, then washed to eliminate the detergent;
2. preparation **1** is treated with glutaraldehyde to have a multipoint covalent attachment between the enzyme and the support, in the absence of detergent; and

**Table 1.** Enzymatic Activity of Different CAL-A Preparations<sup>a</sup>

CAL-A preparations	substrates			
	1	2	3	4
MANAE–CAL-A GLUTA	60 ± 5	140 ± 10	180 ± 15	80 ± 10
MANAE CAL-A	125 ± 10	160 ± 20	1100 ± 30	120 ± 5
TRITON–GLUTA				

<sup>a</sup> Experiments were performed as described in the Methods section. Activity is expressed in relative values, giving a value of 100 to the activity observed using the just adsorbed enzyme.

**Table 2.** Enantioselectivity of Different CAL-A Preparations<sup>a</sup>

CAL-A preparations	substrates		
	2	3	4
MANAE–CAL-A	1.2 (S)	2.5 (S)	2.7 (S)
MANAE–CAL-A GLUTA	3.2 (S)	2.5 (S)	2.2 (S)
MANAE CAL-A TRITON–GLUTA	3.2 (S)	15.0 (S)	1.4 (S)

<sup>a</sup> Experiments were performed as described in the Methods section.

3. preparation **1** is cross-linked with glutaraldehyde in the presence of 1% Triton X-100 (v/v).

Preparations **2** and **3** cannot be desorbed from the support by incubating in 500 mM sodium phosphate, while preparation **1** was almost fully desorbed under these conditions. Thus, the establishment of enzyme-support bonds may be probed.

Table 1 shows that the treatment with glutaraldehyde of the adsorbed enzyme in the absence of detergent presented a different effect on the enzymatic activity for these enzyme preparations, depending on the substrate. The activity slightly decreased with substrates **1** and **4**, while an opposite behavior was observed with substrates **2** and **3**. That is, the treatment with glutaraldehyde did not promote a significant effect on the enzyme activity with the different substrates assayed.

The treatment with glutaraldehyde of the adsorbed enzyme in the presence of detergent permitted a significant increase in the enzyme activity, mainly with substrate **3** (by a 6-fold factor) and more moderately with all other substrates (by a factor of around 1.5), when compared with the lipase adsorbed and cross-linked with glutaraldehyde under standard conditions. In fact, these new preparations presented higher activity than the enzyme just ionically adsorbed on the support in all cases (even by an 11-fold factor in the case where substrate **3** is used).

Table 2 shows the enantioselectivity of the enzyme in the resolution of a racemic mixture of substrates **2**, **3**, and **4**. In all cases, the S isomer was preferred. Adsorbed CAL-A presented a low enantioselectivity value with all the assayed substrates (no higher than 3), and the treatment with glutaraldehyde did not promote a great effect in the *E* values, neither in the presence or in the absence of detergent (a slight increment for substrate **2** and a slight worsening for substrate **4**). An exception was the case of substrate **3**, where the *E* value of the enzyme cross-linked in the presence of detergent increased up to a value of 15.

To ensure that there were no effects of detergent molecules retained in the immobilized preparations, preparation **2** was, in some instances, incubated with 1% Triton X-100 and further washed with distilled water to eliminate the detergent, similar to what was performed when producing preparation **3**. The results were identical to those observed with unstarted preparation **2**. That is, if the detergent was offered after the glutaraldehyde cross-linking, no significant “permanent” effect was found, and the washing of the detergent permitted the recovery of a similar preparation.

Moreover, preparation **3** was used in 10 cycles without detecting any alteration on the enzyme properties. Incubation

**Table 3.** Activity of Different CAL-B Preparations<sup>a</sup>

CAL-B preparations	substrates			
	1	2	3	4
MANAE–CAL-B GLUTA	67 ± 5	110 ± 10	25 ± 5	60 ± 5
MANAE CAL-B	185 ± 10	700 ± 10	150 ± 10	180 ± 5
TRITON–GLUTA				

<sup>a</sup> Experiments were performed as described in the Methods section. Activity is expressed in relative values, giving a value of 100 to the activity observed using the just adsorbed enzyme.

**Table 4.** Enantioselectivity of Different CAL-B Preparations

CAL-B preparations	substrates		
	2	3	4
MANAE–CAL-B	9.0 (R)	3.4 (R)	2.5 (R)
MANAE–CAL-B GLUTA	9.5 (R)	2.9 (R)	2.9 (R)
MANAE CAL-B TRITON–GLUTA	9.0 (R)	3.1 (R)	4.5 (R)

<sup>a</sup> Experiments were performed as described in the Methods section.

**Table 5.** Activity of Different CRL Preparations<sup>a</sup>

CRL preparations	substrates		
	1	2	3
MANAE–CRL GLUTA	40 ± 2	220 ± 10	15 ± 3
MANAE CRL TRITON–GLUTA	50 ± 3	500 ± 10	25 ± 3

<sup>a</sup> Experiments were performed as described in the Methods section. Activity is expressed in relative values, giving a value of 100 to the activity observed using the just adsorbed enzyme.

of this preparation in the absence of detergent by 1 week at 25 °C did not affect the enzyme performance.

**Lipase from CAL-B.** The same immobilized preparations described above were prepared using CAL-B. Again, covalent immobilization in preparations 2 and 3 was probed by incubation at high buffer concentrations. In this case, the treatment with glutaraldehyde in the absence of detergent produced a significant decrease in the enzyme activity against all substrates except for substrate 2 (Table 3).

However, if the treatment with glutaraldehyde was performed in the presence of Triton X-100, in all cases, more activity was recovered, even more than when using the just adsorbed enzymes. For substrate 2, this increment was 7-fold, whereas, for the other substrates, this increase was less than 2-fold.

Regarding the *E* value (Table 4), all preparations presented similar values, preferring the R isomer, with an *E* value of around 9 for substrate 2 and 3 for substrate 3. In the case of substrate 4, a slight increment in the *E* value from 2.5 to 4.5 could be found if the glutaraldehyde treatment was performed in the presence of detergent.

Again preparation 2 was incubated with detergent and then washed. We did not find any effect of the detergent in the enzyme properties.

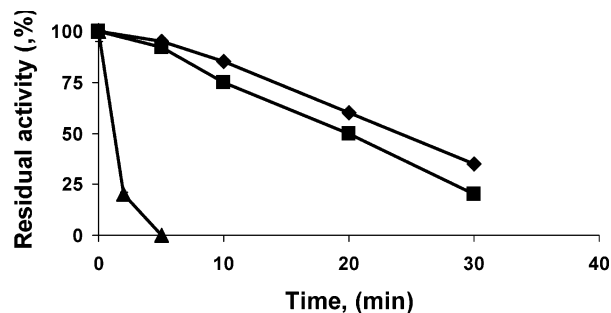
Moreover, preparation 3 was used in 15 cycles without detecting any alteration on the enzyme properties. Incubation of this preparation in the absence of detergent for 20 days at 4 °C did not affect the enzyme performance.

**Lipase from CRL.** We prepared the same three different immobilized preparations. Covalent immobilization in preparations 2 and 3 was checked by incubation of the immobilized preparations at high buffer concentrations. The treatment with glutaraldehyde in the absence of detergents presented a more negative effect when compared to the other lipases studied, reducing the activity up to 40% for substrate 1 and up to 15% for substrate 3 (Table 5). However, the glutaraldehyde treatment,

**Table 6.** Enantioselectivity of Different CRL Preparations<sup>a</sup>

CRL preparations	substrates	
	2	3
MANAE–CRL-	15.0 (S)	7.0 (S)
MANAE–CRL GLUTA	1.0	23.0 (S)
MANAE CRL TRITON–GLUTA	4.0 (R)	10.0 (R)

<sup>a</sup> Experiments were performed as described in the Methods section.



**Figure 2.** Inactivation of different CAL-A preparations by D-pNP at pH 7 and 25 °C. The experiments were carried out as described in the Experimental Section. MANAE–CAL-A cross-linked in the presence of Triton (triangles), MANAE–CAL-A cross-linked in the absence of detergent (squares), and MANAE–CAL-A (diamond).

even in the absence of detergent, increases the enzyme activity against substrate 2, increasing the activity by a 2-fold factor.

The presence of detergent during the glutaraldehyde treatment of the adsorbed enzymes reduced the negative effects in substrates 1 and 3 and increased the activity by a 5-fold factor in the case of substrate 2 (Table 5).

The studies on the *E* values offered surprising results (Table 6). Adsorbed CRL preferred the S isomer of substrates 2 and 3, with *E* values of 15 and 7, respectively. The treatment with glutaraldehyde in the absence of detergent annulled the enantioselectivity of the enzyme toward substrate 2, whereas it significantly increased the *E* value using substrate 3 (reaching a value higher than 20). The presence of Triton during the glutaraldehyde cross-linking produced the inversion of the preferred isomer, giving an *E* value of 4 for substrate 2 and 10 for substrate 3, but toward the R isomer.

Again, preparation 2 was incubated in the presence of Triton and washed with water. This treatment did not have any effect on the enzyme properties.

Moreover, preparation 3 was used in eight cycles without detecting any alteration of the enzyme properties. Incubation of this preparation in the absence of detergent for 1 week at 4 °C did not affect the enzyme performance.

**Irreversible Inhibition of Different Preparations of CAL-A by D-pNP.** Irreversible serine–hydrolase inhibitors inactivate the enzyme by direct reaction with the reactive serine; that way, the inhibition rate of lipases by this kind of compound is a direct consequence of the exposition of the serine. If the lipase is in an open form, the inhibition should proceed in a more rapid fashion. The three different preparations of the three different enzymes were incubated in the presence of an excess of D-pNP. In all cases, inhibition of the lipase cross-linked in the presence of detergent was more rapid than the inhibition of the other two immobilized preparations.

Figure 2 shows the case of the CAL-A: the enzyme treated with glutaraldehyde in the presence of detergent was fully inactivated in less than 5 min, while the other two preparations maintained around 20–30% of the activity after 30 min. That is, lipases adsorbed on aminated supports and treated with



glutaraldehyde in the presence of detergents presented a much higher exposition of the reactive serine residue to the medium.

### Discussion

Detergents are able to increase the activity of free and immobilized lipases.<sup>25–28</sup> Detergents are suggested to be able to shift the open/close equilibrium of lipases toward the open conformation, by coating the hydrophobic areas of the lipases that surround the active center of the enzyme. This coating would reduce the negative interactions between this large and highly hydrophobic pocket and the environment.

It has been found that the glutaraldehyde cross-linking of the lipases with aminated supports in the presence of detergents permits a significant increase in the activity of the lipases (even by more than a 10-fold factor) in homogeneous aqueous medium and also alters or even reverses its enantioselectivity, compared with both adsorbed or adsorbed and cross-linked in the absence of detergent immobilized preparations. This effect is only significant if the detergent is presented in the moment of the cross-linking: the adsorption on aminated supports is performed in the presence of detergent to avoid lipase dimers.<sup>41a–d</sup> Moreover, incubation in the presence of detergent of the adsorbed enzymes previously treated with glutaraldehyde did not have a significant effect.

The fact that irreversible inhibition with D-pNP was much more rapid on the enzymes treated with glutaraldehyde in the presence of detergents suggested that the reactive serine residue was more exposed to the medium in this case than it was in the other two immobilized preparations.

Thus, our results suggest that the glutaraldehyde enzyme-support cross-link, reported to be able to produce an intense enzyme-support reaction that leads to a great increase in the enzyme stability,<sup>30</sup> may be able to keep the conformational changes induced by the detergent and permit the production of enzyme molecules with higher activity and, in some cases, very different enantioselectivity. The changes induced by the presence of the detergent seem to present a slightly different shape of the active center of the lipases, yielding an enzyme form with an increased activity and altered selectivity (in a similar way to the altered specificity obtained by immobilizing lipases using different strategies).<sup>43</sup>

Mingarro et al. found an interfacial activation-based molecular bioimprinting of lipase while lyophilizing lipases in the presence of amphiphiles.<sup>29b</sup> The lack of a covalent stabilization of these “open” structures made certain that the hyperactivated forms achieved only remained in anhydrous media, where enzyme flexibility was low. Incubation in an aqueous medium promotes the loss of this “hyperactivated” form. Our cross-linking strategy permits one to keep these hyperactivated forms of lipases, even in aqueous media, for long time periods. On the other hand, the alteration of the lipase properties by the presence of detergents (altering both activity and enantioselectivity) has been recently published.<sup>29,44</sup>

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