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Vinyl sulfone-amino-alkyl supports: heterofunctional matrixes to prevent enzyme release and stabilize lipases via covalent immobilization

Pedro Abellanas-Perez [a,1], Diandra de Andrades [a,b,1], Andres R. Alcantara [c], Javier Rocha-Martin [d], Maria de Lourdes Teixeira de Moraes Polizeli [b], Roberto Fernandez-Lafuente [a,*]

[a] Departamento de Biocatálisis, ICP-CSIC, C/Marie Curie 2, Campus UAM-CSIC Cantoblanco, 28049 Madrid, Spain
[b] Department of Biology, Faculty of Philosophy, Sciences and Letters of Ribeirão Preto, University of São Paulo, Ribeirão Preto 14040-901, SP, Brazil
[c] Departamento de Química en Ciencias Farmacéuticas, Facultad de Farmacia, Universidad Complutense de Madrid, Plaza de Ramón y Cajal, s/n, Madrid 28040, Spain
[d] Department of Biochemistry and Molecular Biology, Faculty of Biology, Complutense University of Madrid, José Antonio Novais 12, Madrid 28040, Spain

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ABSTRACT

New trifunctional supports were prepared (amino-octyl-vinyl sulfone (VS)- and amino-hexyl-VS-agarose) and compared to octyl-VS-agarose. They were utilized to immobilize the lipases A and B from *Candida antarctica* (CALA and CALB). After incubation to generate some enzyme-support bonds and blocking with different nucleophiles, SDS-PAGE analyses showed that all enzyme molecules become covalently immobilized on the support. In all VS biocatalysts, the blocking reagent presented a great effect in the properties of enzymes. The best blocking agents promoted a significant enzyme stabilization compared to the enzyme stability using the amino-alkyl-agarose supports, higher than that using octyl-VS-agarose supports, although these remained the most stable ones in most cases, as the octyl-biocatalysts were significantly more stable than the enzyme immobilized on amino-alkyl-support. Enzyme activities and specificities could be also greatly tuned by the immobilization in the new trifunctional supports, with enzyme activities in many instances enhancing that of the best non-covalently immobilized enzyme. That way, the results on this paper show that the properties of the enzymes when immobilized on these new trifunctional supports may be significantly tuned by the nature of the acyl chain in the support and the nature of the reagent used to block the reactivity of the remaining VS groups.

1. Introduction

Enzyme, and specifically lipase immobilization, may have many advantages as, if properly designed, it can improve enzyme stability, activity, selectivity, specificity, or even couple immobilization to enzyme purification [1–8]. Although conventional supports may be useful in many instances, the use of magnetic supports in the enzyme immobilization may be advantageous in certain circumstances (e.g., when the substrate is a suspension and the filtration to recover the biocatalyst is not possible) [9]. Many different magnetic supports have been used to immobilize lipases [10–15]. Final immobilized enzymes, if properly designed, in many instances present better features than the free enzymes [16–21].

Lipases are enzymes exhibiting a peculiar feature; they are interfacial enzymes [22,23], able to perform their catalytic function at the interface of their natural substrates (oil drops) [24–26]. This specific catalytic behavior of lipases is due to their specific structure: lipases have two different conformational states [27–30]. In the closed form, the active center is isolated from the medium (in most cases) by a polypeptide chain called lid or flat, giving a fully inactive enzyme structure (in most cases). This structure is in equilibrium with the lipase open form, where the lipase lid moves and exposes a large hydrophobic pocket to the medium, this is the active form of the lipases [27–30]. These large hydrophobic pockets promote the adsorption of the lipase on the hydrophobic drop of oils, their natural substrates, in a specific way [31–34]. The lid may be small and may not fully isolate the lipase active center to the medium in some instances (as it is the case for lipase B from *Candida antarctica*, (CALB)) [35] or may be a very complex structure (as the double lid exhibited by the lipase from *Bacillus thermocatenolatus*) [36].

This mechanism of interfacial activation is a specific feature of

* Corresponding author at: Departamento de Biocatálisis, ICP-CSIC, C/Marie Curie 2, Campus UAM-CSIC, Cantoblanco, 28049 Madrid, Spain.
E-mail address: rfl@icp.csic.es (R. Fernandez-Lafuente).
[1] Both authors have worked evenly in this paper

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lipases, and it has been used to immobilize lipases via a very simple protocol, as lipases can become adsorbed via a similar interfacial activation mechanism on any hydrophobic surface, including hydrophobic supports [16]. Lipase immobilization on these supports at low ionic strength enables the one-step immobilization/purification of the enzyme [37]. Moreover, as the lipase open form adsorbed on hydrophobic surfaces is more stable than the lipase in conformational equilibrium, this immobilization causes a high enzyme stabilization, even improving the values obtained using multipoint covalent immobilization [38–40].

However, lipase immobilization following this protocol is just via (multiple) hydrophobic interactions, thus being a reversible protocol. This may be an advantage, as it enables the release of the enzyme after its inactivation during operation and the reuse of the support to immobilize the fresh enzyme [41–44]. However, this also permits enzyme release during operation under certain conditions, such as high temperatures, presence of high concentrations of organic cosolvents, or components of the reaction media with detergent properties [45,46]. These enzyme release risks limit the use of lipases immobilized following this protocol [47,48].

The possibilities of undesired enzyme release during operation may be reduced via lipase intermolecular crosslinking, which may be covalent (using bifunctional reagents such as glutaraldehyde or chemically reactive polymers such as aldehyde dextran) [49–52] or physical (using ionic polymers) [53,54]. Another alternative is the use of supports exhibiting not only hydrophobic features, but also other groups able to establish different interactions with the enzyme. If this is via additional ionic interactions, the immobilization may maintain the reversibility even though enzyme release after enzyme inactivation may become harder [55,56]. This way, the support may be reused after enzyme inactivation and release, but still some risk of enzyme desorption during operation exists under certain conditions. If chemical reactive groups, such as glutaraldehyde [57], glyoxyl [45,58–62] or vinyl sulfone (VS) [63–66] groups are used to activate the hydrophobic support and at least a covalent enzyme-support (in monomeric enzymes) bond is established, enzyme release will no longer be possible. This kind of heterofunctional supports enables the use of immobilized lipases under conditions where the enzyme would be desorbed from standard hydrophobic supports [47,48].

Among them, acyl-VS supports have proved to be very effective in producing covalent bonds after lipase immobilization via interfacial activation in supports bearing an acyl layer able to promote the lipase interfacial activation [63–66]. There are other heterofunctional supports that may also provide some advantages, but may have some drawbacks compared to these new ones. For example, the use of supports bearing immobilized chelates and chemically reactive groups, permit the enzyme orientation and stabilization of lipases [67,68]. However, the metal should be removed after enzyme immobilization to prevent undesired release during operation or prevent undesired catalytic properties of them. Epoxy-hydrophobic supports may be similar, even enabling the change of the enzyme environment as they can be also be blocked [69], but the lower reactivity of the epoxy groups make harder to get the covalent bonds.

In this new paper, we have used a recently reported new hydrophobic heterofunctional support, prepared by the modification of glyoxyl supports by amino-acyl chains [70]. This support is a bifunctional one (bearing anion exchange and hydrophobic moieties) and after its further modification with divinyl sulfone, it becomes a trifunctional support (Fig. 1). The presence of a secondary amino group near the support surface permits that the DVS activation may involve this group, perhaps this may facilitate the promotion of some enzyme-support covalent bonds. This new amino-acyl support was found to exert positive effects on enzyme activity, but to be negative on enzyme stability when compared with standard octyl agarose supports. It may be expected that the modification of this secondary amino group with divinyl sulfone can hinder any enzyme-amino group interaction and that way, this can reduce the negative effects of the presence of this cationic group on the enzyme stability. The vinyl sulfone groups in the support can react with the adsorbed enzymes, involving Tyr, Lys, His, Cys or the terminal amino group during immobilization [71–78]. It has been showed by fluorescence how the blocking agent can alter the final enzyme conformation [63,65].

As model enzymes, we have selected the lipases A and B from *Candida antarctica* (CALA and CALB). CALB is among the most utilized enzymes [79–88]. The enzyme has a small lid [89,90] but is still able to become interfacially activated versus hydrophobic surfaces [35,91,92]. CALA is an enzyme with a larger lid and a more standard behavior [92,93], and that also has multiple applications [94–96].

2. Material and methods

2.1. Materials

Novozymes (Madrid, Spain) kindly provided CALA and CALB in liquid formulations. GE Healthcare supplied Sepharose and octyl-Sepharose® 4BCL beads (agarose beads). Tokyo Chemical Industry Europe (Zwijndrecht, Belgium) purposed divinyl sulfone (DVS). Sigma-Aldrich (Madrid, Spain) supplied triacetin, 1-hexylamine, 1-octylamine, sodium borohydride, sodium periodate, glycidol, ethylenediamine (EDA), ethylamine, aspartic acid (Asp), cysteine (Cys), glycine (Gly) and

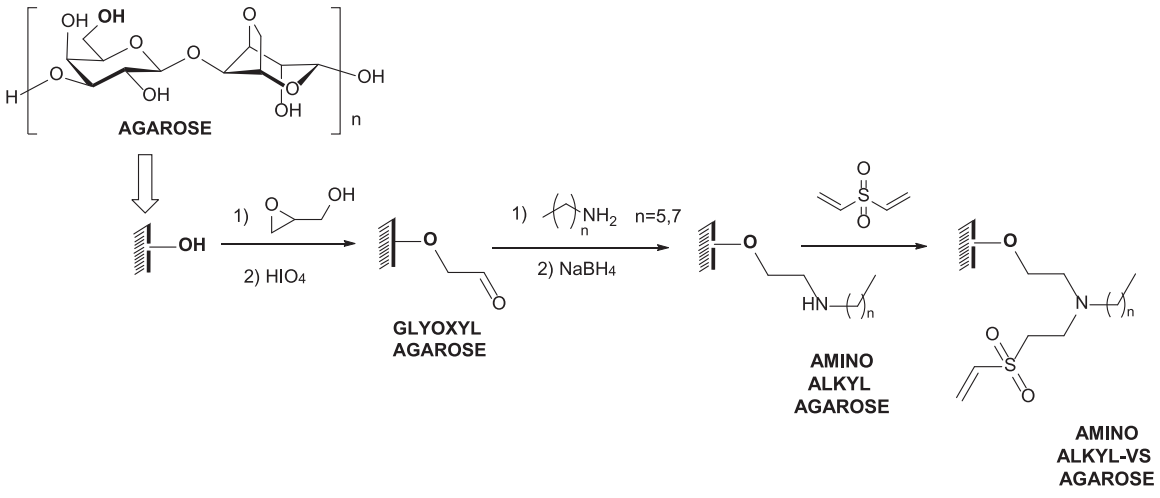

Fig. 1. Graphical representation of the modification with divinyl sulfone of the amino-acyl supports.

ρ-nitrophenyl-butyrate (ρNPB). (*R*)-and (*S*)- methyl-mandelate were acquired from Thermo-Fisher (Alcobendas, Spain). Protein concentration was determined using the method described by Bradford [97]. Glyoxyl support was prepared as previously described [98,99]. Elemental analyses studies were performed by CAI de Microanálisis Elemental, Universidad Complutense, using a Leco 932 CHNS combustion microanalyzer.

2.2. Methods

The results are presented as the mean values of triplicate experiments with their corresponding standard errors.

2.2.1. Preparation of the supports

2.2.1.1. Amino-octyl-agarose beads. 30 g of glyoxyl-agarose (washed in water) were added to 120 mL of 4 M 1-octylamine dissolved in 1,4-dioxane solution. This suspension was gently agitated for 24 h. Next, solid sodium borohydride was added to get a concentration of 10 mg/mL, and after 2 h, the support was vacuum filtered, and washed with 50 % (v/v) dioxane/water solution, 100 mM sodium carbonate at pH 9.0, 100 mM sodium acetate at pH 4, and with an excess of distilled water using a sintered glass filter.

2.2.1.2. Amino-hexyl-agarose beads. 30 g of glyoxyl-agarose was added to 120 mL of 4 M 1-hexylamine in water (the pH value of this solution was previously adjusted to 10.05), and the suspension was gently agitated. After 24 h, solid sodium borohydride was added to reach a concentration of 10 mg/mL. The reduction was left to proceed for 2 h, then the support was vacuum filtered using a sintered filter, washed with 100 mM sodium carbonate at pH 9.0, 100 mM sodium acetate at pH 4, and with an excess of distilled water.

2.2.1.3. Activation of the supports with DVS. 7.5 mL of DVS was added to 200 mL of 333 mM sodium carbonate buffer at pH 11.5, and this was stirred until a homogeneous solution was obtained [100,101]. A mass of 10 g of octyl-agarose, amino-hexyl-agarose or amino-octyl-agarose was added to this solution, and the suspensions were gently stirred for 2 h. Finally, the activated VS supports (octyl-VS-, amino-hexyl-VS- or amino-octyl-VS-agarose) was vacuum filtered, washed extensively with distilled water, and stored at 4 °C before their use.

2.2.2. CALA and CALB immobilization on different supports

CALA and CALB solutions were immobilized at a protein loading of 1 mg/g of support. The enzymes samples were diluted in 5 mM sodium acetate at pH 5.0 and 25 °C (0.1 mg/mL), and subsequently, the supports were added. The immobilization course was followed by measuring the lipase activity in the suspension, supernatant, and a reference suspension (where inert agarose was added), at different time intervals, using ρNPB as substrate [102]. After 1 h, the biocatalysts were filtered, washed with abundant distilled water, and resuspended in 50 mM sodium bicarbonate at pH 8.0 and 25 °C to permit the establishment of some covalent enzyme-support bonds [63–66]. After 3 h of reaction, the biocatalysts were washed with distilled water, and resuspended in 2 M of different blocking agents (Asp, Cys, Gly, EDA, imidazole, or ethylamine) for 24 h at pH 8, while using Asp and ethylamine the blocking step was performed at pH 9 and at 25 °C. Finally, the immobilized enzymes were vacuum filtered, washed extensively with distilled water, and stored at 4 °C.

2.2.3. Lipase activity assays

One unit of activity (U) refers to the amount of enzyme that hydrolyzes one micromole of substrate per minute under the specified conditions.

2.2.3.1. Hydrolysis of ρNPB. A sample of 50 μL of soluble enzyme solution or immobilized enzyme suspension was added to the reaction mixture. This was composed of 50 μL of 10 mM ρNPB dissolved in acetonitrile and 2.5 mL of 25 mM sodium phosphate at pH 7.0, recording the increase of absorbance at 348 nm (isobestic point of ρ-nitrophenol, ε is 5150 $M^{-1} cm^{-1}$ under these conditions) [103]. The reaction was performed at 25 °C for 90 s at using a Jasco V-730 spectrophotometer (Jasco, Madrid, Spain) supplemented with magnetic stirring. Activity is given as micromoles of ρNP released to the medium per minute calculated from the increment in absorbance per minute.

2.2.3.2. Hydrolysis of triacetin. A mass between 100 and 250 mg of immobilized enzyme was added to 3–10 mL of 50 mM triacetin dissolved in 50 mM sodium acetate at pH 5.0. The reaction was conducted under gentle stirring at 25 °C. The enzyme activity was assessed by measuring the percentage of hydrolysis at various time intervals. The formed 1,2-diacetin can suffer acyl migration, creating a mixture that contains 1,3-diacetin but this migration is nullified at pH 5 [104]. The concentrations of the reagents were analyzed using HPLC with a Kromasil C18 column (15 cm × 0.46 cm) connected to a UV detector set at 230 nm. The mobile phase consisted of 15 % (v/v) acetonitrile and 85 % (v/v) Milli-Q water, with a flow rate of 1 mL min^{-1} [105]. Activity is given as micromoles of formed diacetin per minute, calculated from the increase of the diacetin peak and the decrease of the triacetin peak.

2.2.3.3. Hydrolysis of (D) or (L)-methyl mandelate. The reaction suspension consisted of 0.02 to 0.5 g of biocatalysts and 3 to 5 mL of 50 mM (*D*) or (*L*)-methyl mandelate dissolved in 50 mM sodium acetate at pH 5.0. This mixture was submitted to gentle stirring at 25 °C. The concentrations of the reaction products were analyzed using HPLC (Shimadzu) coupled to a UV/VIS detector settled at 230 nm, employing a Kromasil C18 column (15 cm × 0.46 cm). The mobile phase consisted of 35 % acetonitrile and 65 % 10 mM ammonium acetate (v/v) at pH 2.8 [105]. Activity is given as micromoles of formed mandelic acid per minute, calculated from the increment of the mandelic acid peak and the decrease of the ester peak.

To evaluate the operational stability of the biocatalysts, some selected ones were reused in the hydrolysis of (*D*)-methyl mandelate, using 0.5 g of biocatalyst and 10 mL of substrate solution, prepared as described above in a syringe with a filter in the way out. The reaction was left to proceed performed for 1 h, and then the biocatalysts were recovered by filtration, washed 3 times with10 volumes of 50 mM sodium acetate at pH 5.0 and reused in a new reaction cycle. After 10 reaction cycles, the initial activities of the biocatalysts were calculated as defined above.

2.2.4. Thermal inactivation

The different lipase biocatalysts were incubated in 100 mM Tris-HCl, 100 mM of sodium phosphate, or 100 mM of Tris-HCl/1 M or 3 M of NaCl solutions at pH 7.0 in a water bath at different temperatures to have a reasonable inactivation rate of all the preparations in the same experiment. Samples were periodically collected, and its residual activities were measured using the previously described ρNPB assay. The initial activity of each preparation was defined as 100 %, with the activities of the other samples expressed as a percentage of this reference value.

2.2.5. SDS-PAGE analysis

SDS-PAGE was performed using a BioRad Mini-PROTEAN® system and a 12 % polyacrylamide gel as described by Laemmli [106] with some modifications. The protein samples were diluted in 4 % SDS (*w/v*) and 10 % mercaptoethanol (v/v) (the rupture buffer), and boiled for 8 min. Next, the suspension was centrifuged at 10,000 rpm for 2 min to discard the support and 15 μL aliquots of the supernatants of each sample were applied to the gel. Aliquots of 5 μL molecular standard

(LMW-SDS Marker 14.4–97 kDa) was also applied, and was run at 100 V. Finally, gels were stained using Coomassie brilliant blue stain.

3. Results and discussion

3.1. Activation of the supports with DVS

The confirmation of the activation of the different supports with DVS was performed by elemental analysis (Table 1). The presence of S on the support can be attributed to the modification of the enzyme with this reagent (Table 1). In fact, octyl-agarose beads present very low amounts of N and S, while after modification with DVS, the presence of the VS group increase the amount of S to almost 1 milimol per g of dried agarose. Using the amino-alkyl-agarose beads, as expected, the amount of N is very significant in the initial support, consequence of the amino group, and very similar for both supports. After the modification with DVS, the amount of S in the support greatly increases, surpassing the values using octyl-agarose by almost 50 %. This could be related to the higher reactivity of the secondary amino group when compared to that of the hydroxyl groups that are the ones involved in the activation of octyl-agarose. It is remarkable that the amount of S surpasses the amount of N, suggesting some polymerization of the DVS on the support or the implication of some other group of the support in the reaction. In any case, the results confirm that the supports have been modified with DVS and we have generated the desired supports: octyl-VS-agarose and amino-acyl-VS-agarose beads. These supports have been used in further experiments.

3.2. Immobilization of CALA and CALB on heterofunctional hydrophobic vinyl sulfone supports

Fig. 2 shows the immobilization course of CALB on the different supports prepared in the previous section. Immobilization was very rapid in all cases, and promoted an increase of the *p*NPB activity of all biocatalysts, lower using octyl-agarose (around 15 %) and maximum using amino-hexyl-agarose (around 60 %).

Fig. 3 shows a similar experiment using CALA. Although immobilization courses are similarly fast, the increase in enzyme activity is only detectable using octyl-VS-agarose, the other biocatalyst just maintaining the initial activity.

These results suggest that the VS groups can initially react with the nucleophilic groups of some of the immobilized enzyme molecules even at acid pH value, and this can explain why the expected CALA hyperactivation is not detected in this instance. Next, the immobilized enzymes were incubated at pH 8 for 3 h to reach some enzyme-support covalent immobilization and later the remaining VS groups were blocked with different nucleophiles as described in methods. Fig. 4 shows the SDS-PAGE studies performed with the biocatalysts of CALA and CALB produced using the initial supports and after their activation with DVS. It can be clearly visualized how while all immobilized enzyme molecules may be released from both octyl- and amino-acyl-agarose supports, no enzyme molecules could be released from the supports activated with DVS even after boiling it in SDS, with the 3 supports and both enzymes. That way, the existence of at least one enzyme-support covalent bond may be ensured.

Fig. 1S shows the structures of CALA and CALB, showing the amino acids able to react with VS supports that are placed in the face of the active center. In the case of CALA, 5 Tyr are susceptible of reacting with the support (positions 17, 22, 105, 281 and 317) and also there are 7 Lys (positions 146, 259, 265, 306, 347, 352 and 427). Among these, Lys 146, 259 and 265 are located near the active center and fully exposed, being good candidates to participate in the covalent immobilization of CALA to the support.

In the case of CALB, there are 2 no very exposed Cys (positions 22 and 293), that way, even being very reactive the thiol groups with VS, its surface position and exposition do not make then a first option to participate in the enzyme immobilization. Thera are also 3 Lys in this face, and Lys 290 seem to be a very good candidate to participate in the covalent binding of the enzyme. Tyr 82 is poorly exposed and far from the active center, and also Tyr reactivity is lower than that of Cys or Lys, but its participation in the covalent immobilization cannot be fully discarded. Whatever the groups participating in the immobilization, it is clear that there are multiple options, very likely the covalent binding can involve several groups in each enzyme molecule.

The loading capacity of the new supports coincide with that of the octyl agarose bead, as it is conditioned by the specific area of the supports and that is not modified by the activation. That way, maximum loading capacity of all the supports used in this paper was 11.5–12.5 mg/g using both enzymes.

Table 1
Elemental analysis of the different supports.

	μmol N/g	μmol S/g
Octyl-VS	57.1 ± 3.8	956.25 ± 9.84
Amino-hexyl-VS	1178.57 ± 13.9	1403.13 ± 15.8
Amino-octyl-VS	1007.14 ± 11.0	1393.75 ± 16.2

3.3. Optimization of the blocking step

The enzyme blocking can exert drastic influence on the final properties of the biocatalyst, as it may determine the enzyme-support interactions. In a first approach, octyl-VS- and amino-hexyl-agarose CALA and CALB have been blocked with 7 different reagents and their stabilities have been evaluated.

Fig. 5 shows the results using CALB. The inactivation courses show that the immobilization on octyl-VS-agarose and their further blocking permits to improve enzyme stability in a significant way compared with octyl-VS-agarose, with one exception, the blockage of octyl-VS-agarose-CALB with EDA. That gave a biocatalyst with a lower stability than the octyl-agarose-CALB biocatalyst as a result. This suggests that this enzyme immobilized via interfacial activation decreased its stability if the support can permit some interactions between the ionic groups in the enzyme and the cationic groups in the support, and can be related to the bad results on stability of the enzyme immobilized on amino-acyl-agarose supports. This negative effect of the use of EDA as blocking agent was not so clearly visualized using amino-alkyl-VS supports, although the biocatalyst blocked with EDA was among the least stable ones. This biocatalyst was clearly more stable than the amino-hexyl-agarose-CALB biocatalysts.

The other blocking reagents produced stabilities with small differences. The highest stabilities were observed using octyl-VS-agarose-CALB when employing Gly, Cys or imidazole, while using amino-hexyl-agarose-VS the most stable ones were those blocked with Asp, Gly or ethyl amine. We can assume that the good results using amino-hexyl-agarose-VS and Asp can come from the reduction of undesired ionic interactions of the enzyme with the amino groups in the support chain.

Using CALA (Fig. 6), results were not very different, except that now even the blocking with EDA of the VS supports gave more stable biocatalysts than octyl-agarose-CALA, although the octyl-VS-EDA-agarose-CALA was the least stable biocatalysts prepared on VS support. The blocking agents that gave the highest stabilization were Cys and Asp using octyl-VS-agarose. Using amino-hexyl-VS-agarose, the least stable biocatalyst was the one blocked with EDA, while the highest stabilities were observed when blocking with imidazole, Asp and Gly. Asp and Gly were also among the optimal blocking agents for the CALB biocatalyst. Figs. 2S and 3S compare the results obtained using amino-hexyl-VS and amino-octyl-VS agarose supports, results were fairly similar.

Considering that the interaction with cationic groups seemed to be deleterious for enzyme stability in both enzymes, we assayed the stability at high ionic strength (1 and 3 M of NaCl), to reinforce the

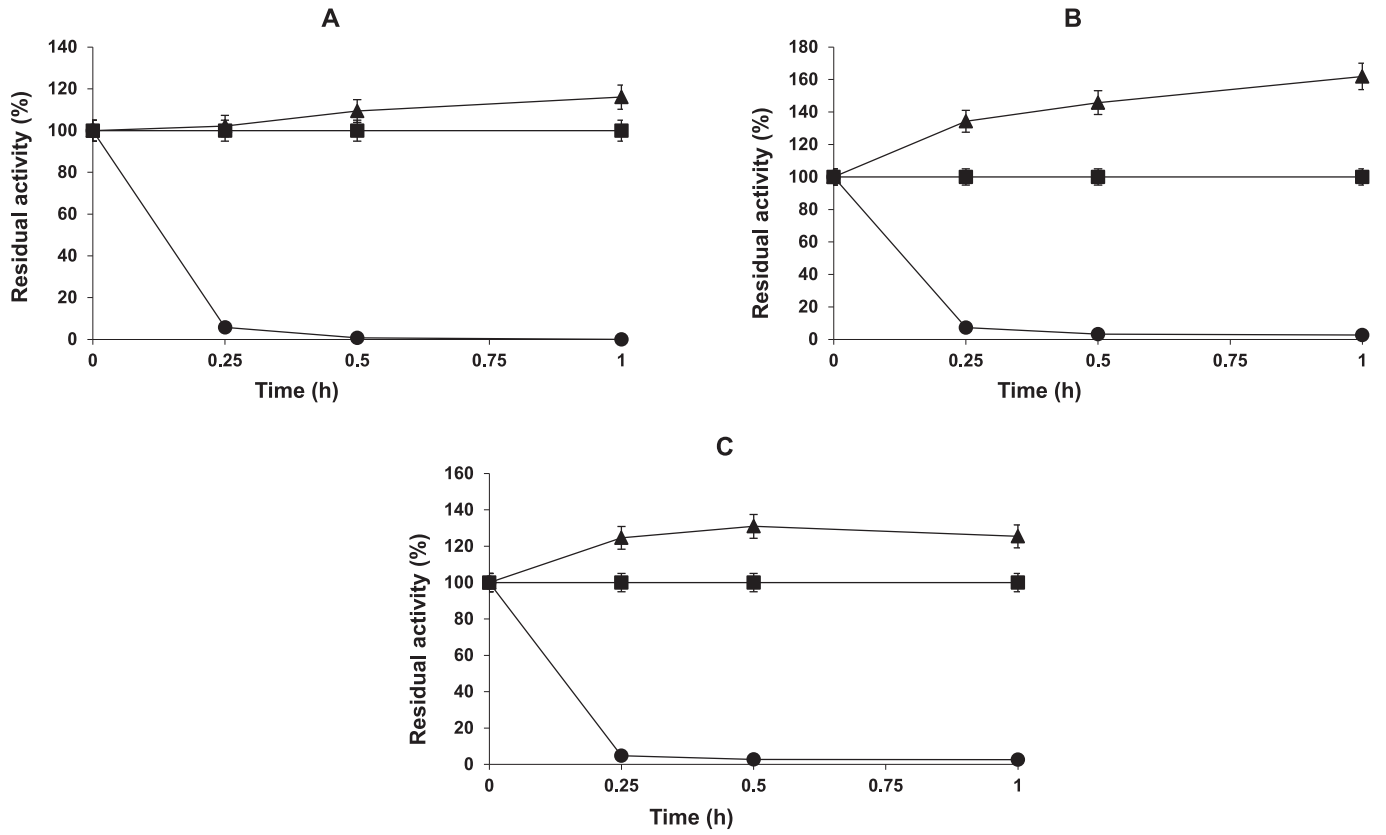

Fig. 2. Immobilization course of CALB on different supports (A): octyl-VS (B): amino-hexyl-VS, and (C): amino-octyl-VS agarose. The experiments were performed using 5 mM sodium phosphate at pH 7.0 and 25 °C. Other specifications may be found in the Methods sections. Solid triangles: suspension; solid squares: reference; and solid circles: supernatant.

hydrophobic interactions and decrease the ionic ones. In this study we have selected the biocatalysts with the highest stabilities (that blocked with Gly) and with the lowest stabilities (that blocked with EDA), comparing the octyl- and amino-hexyl-agarose biocatalyst. In the case of CALB, Fig. 7A evidences that amino-hexyl-agarose-CALB is far less stable than octyl-agarose-CALB, and that octyl-VS-Gly-agarose-CALB is slightly more stable than the unmodified biocatalyst. The second most stable biocatalyst is amino-hexyl-VS-Gly-agarose-CALB, that becomes quite similar to octyl-agarose and exemplified the huge stabilization achieved by the use of amino-hexyl-VS support. Both biocatalysts blocked with EDA presented almost with identical stabilities, clearly under that of the octyl-agarose-CALB.

The effect of the NaCl concentration is very interesting. Using octyl biocatalyst, stabilities increase using 1 M NaCl (Fig. 7B), but more for octyl-VS-Gly-agarose-CALB than became clearly more stable than octyl-CALB, and octyl-VS-EDA-agarose-CALB approached the stability of the latter. Using 3 M NaCl, the stability of octyl-VS-EDA-agarose-CALB surpassed that of the octyl-agarose-CALB (Fig. 7C), becoming its stability similar to that of the octyl-VS-Gly-agarose-CALB. That is, the cationic nature of this reagent could explain the negative effects on the enzyme stability, as when the ionic interactions between the enzyme and the support are reduced, enzyme stability is significantly increased.

Using the amino-hexyl-agarose biocatalysts, the situation is quite different. The increase on the ionic strength seemed to have a marginal positive effect for the biocatalyst prepared on amino-hexyl-agarose, but the effect is negative for the VS biocatalysts; in fact there are no differences on the Gly and EDA blocked biocatalyst in the presence of 1 or 3 M NaCl. This suggested that the effect of the amino group in the chain is not directly related to the ionic enzyme-support interactions, but to a change in the adsorption of the enzyme in this less hydrophobic chain, in a certain sense similar to a solid cationic detergent.

Fig. 8 shows similar experiments with the CALA biocatalysts, in this instance using imidazole and Asp as blocking reagents. Similarly, at low ionic strength, octyl-agarose-CALA biocatalyst were more stable than amino-hexyl-agarose CALA, although the biocatalysts performed just by interfacial activation presented not so different stabilities as in the case of CALB.

The use of VS support approached the stabilities of the CALA biocatalyst prepared in octyl and amino-hexyl supports, in fact the inactivation courses of the amino-hexyl-VS-agarose biocatalysts became next to those of the octyl-agarose-CALA. The incubation in 1 M NaCl decreased the stability of the octyl-agarose-CALA, and made the stability of the octyl-VS-Asp-agarose-CALA clearly higher than that of the other biocatalysts. Curiously, this is just the opposite of the results obtained using amino-hexyl-VS-agarose biocatalyst, although both decreased their stability, the stability of the biocatalyst blocked with imidazole became more stable than that of the one blocked with Asp.

Using 3 M NaCl, the situation changed again. Octyl-agarose-CALA improved its stability while the VS counterpart decreased it; thus, the stability became close to that of the octyl-VS-Asp-agarose-CALA. Using amino-hexyl-agarose supports, while the unmodified support provided biocatalysts with similar stability in 1 M NaCl, in the VS activated support a decrease in enzyme stability is observed, the stabilities of both biocatalysts became almost identical. This shows the complexity of the effect of the ionic strength on the stabilities of immobilized enzymes, many factors may be acting simultaneously to produce the final observed stability, making it impossible to give simple answers to the causes of the observed effects. In any case, the difference between octyl and amino-hexyl biocatalysts did not decrease in the presence of high ionic strengths, that way, the amino groups in the chain effect on the stability of the enzyme could be unrelated to the existence of enzyme-support ionic interactions. Results using amino-octyl supports were

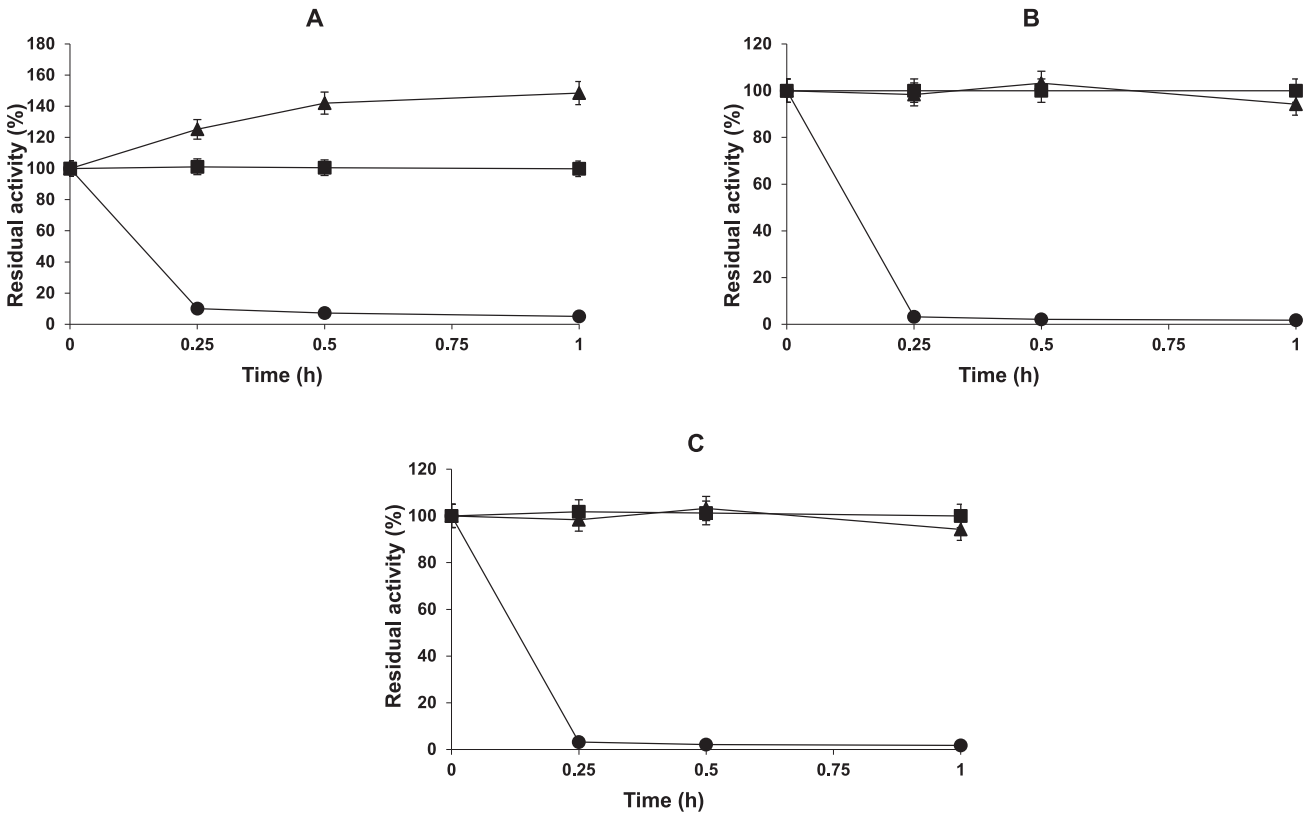

Fig. 3. Immobilization course of CALA on different supports (A): octyl-VS, (B): amino-hexyl-VS, and (C): amino-octyl-VS agarose. The experiments were performed using 5 mM phosphate buffer at pH 7.0 and 25 °C. Other specifications may be found in the Methods sections. Solid triangles: suspension; solid squares: reference; and solid circles: supernatant.

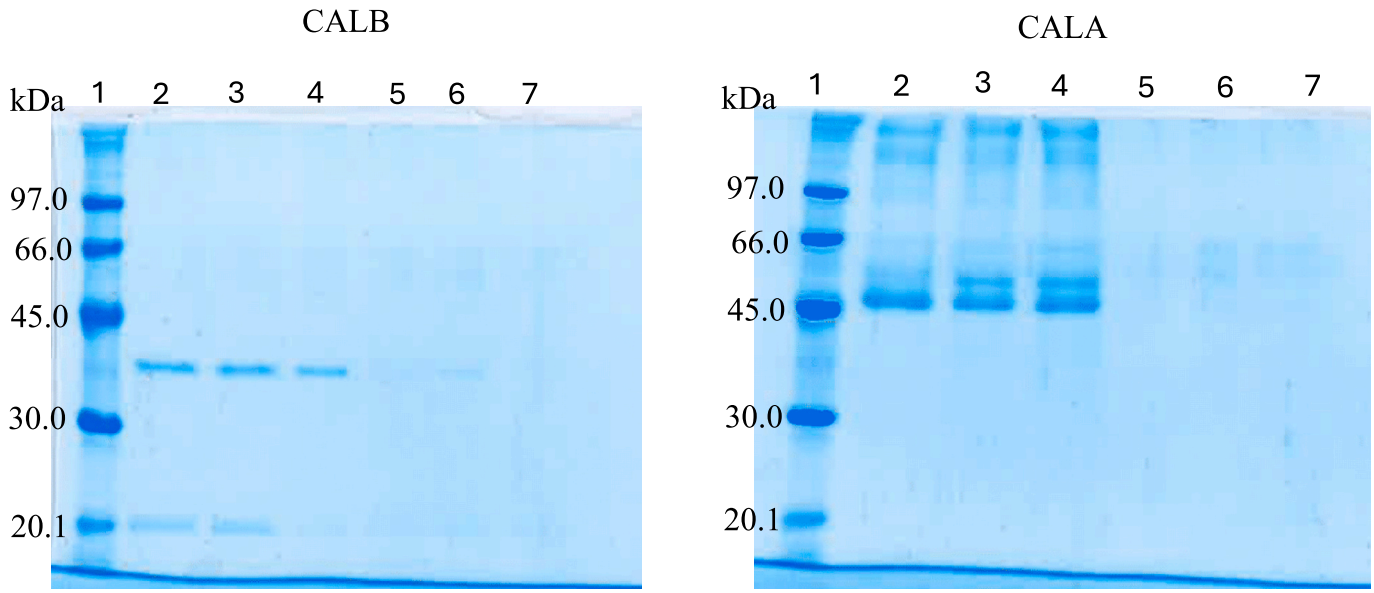

Fig. 4. SDS-PAGE analysis of CALB and CALA immobilized on different supports. Lane 1: Low molecular weight standard. Lane 2: Enzyme immobilized on octyl agarose. Lane 3: Enzyme immobilized on amino-octyl agarose. Lane 4: Enzyme immobilized on amino-hexyl agarose. Lane 5: Enzyme immobilized on octyl-VS agarose. Lane 6: Enzyme immobilized on amino-octyl-VS agarose. Lane 7: Enzyme immobilized on amino-hexyl-VS agarose. Other specifications may be found in the Methods section.

similar to the ones presented using amino-hexyl supports (Figs. 4S–7S) at both ionic strengths.

Finally, we analyzed the effect of the phosphate anions in the inactivation buffer for the different biocatalysts. This has been reported to be very negative for the stabilities of the lipases immobilized via interfacial activation [107–109]. Fig. 9 shows that the stability of the octyl-agarose-CALB decreased in the presence of this salt, and this permits the octyl-VS-EDA-agarose-CALB to reach very similar stability to octyl-agarose-CALB. In fact, both amino-hexyl–VS-agarose biocatalysts became more stable (being more stable the one blocked using Gly) than

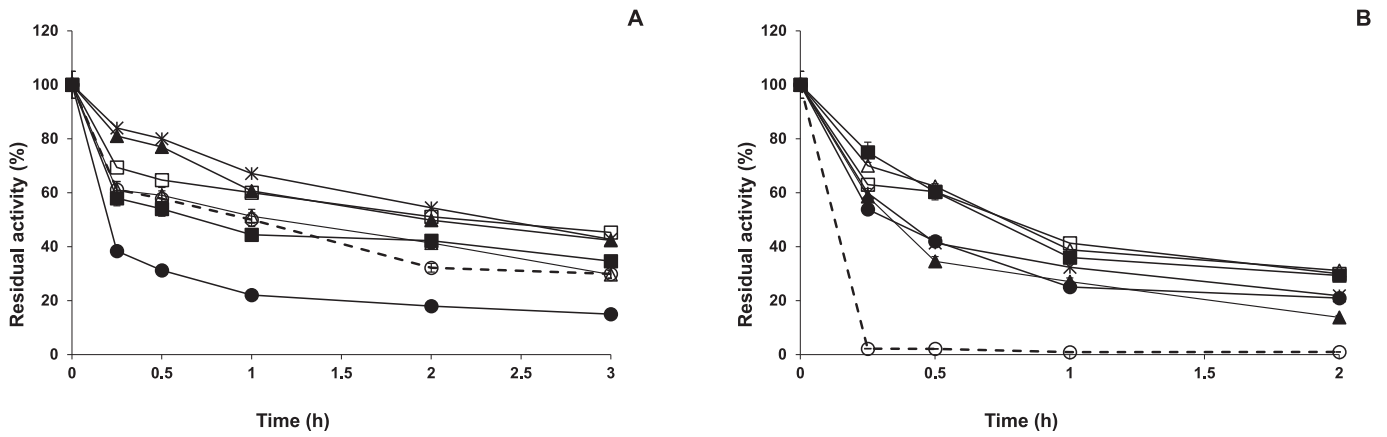

Fig. 5. Thermal inactivation courses of CALB immobilized on octyl-VS (A), and amino-hexyl-VS agarose(B) at 76 °C using 25 mM Tris at pH 7.0. Symbols: Empty circle: CALB immobilized on octyl (A) or amino-hexyl agarose (B). Full circles: blocked with EDA. Empty triangle: blocked with ethylamine. Full triangle: blocked with Cys. Empty squares: blocked with Gly. Full squares: blocked with Asp. Asterisk: blocked with imidazole. Other specifications are described in the Methods section.

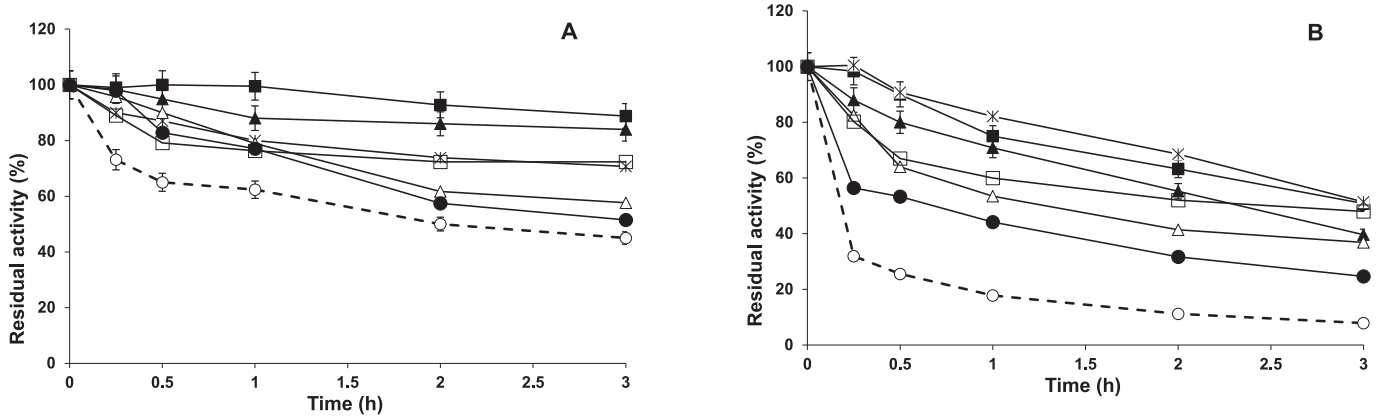

Fig. 6. Thermal inactivation courses of CALA immobilized on octyl-VS (A) and amino-hexyl-VS (B) at 76 °C using 25 mM Tris at pH 7.0. Symbols: Empty circles: CALA immobilized on octyl (A), or amino-hexyl agarose (B). Full circles: blocked with EDA. Empty triangle: blocked with ethylamine. Full triangle: blocked with Cys. Empty squares: blocked with Gly. Full squares: blocked with Asp. Asterisk: blocked with imidazole. Other specifications are described in the Methods section.

octyl-agarose-CALB, only octyl-VS-Gly-agarose remained more stable. Fig. 10 shows the results using CALA, in this instance all biocatalysts based on octyl-agarose remained more stable than the ones prepared using amino-hexyl-agarose, and the biocatalysts blocked with imidazole are more stable than the ones blocked with Asp. Again, similar results were observed using amino-octyl based supports (Figs. 8S and 9S).

3.4. Activity of the different biocatalysts with different substrates

3.4.1. Activity of CALB biocatalysts

Table 2 shows the activities of CALB biocatalysts with *p*NPB, triacetin and both isomers of methyl mandelate. Starting with the activity versus *p*NPB, and the octyl biocatalysts, the covalent immobilization seemed to have scarce effect on the activity of the enzyme. The situation is different using triacetin to determine the enzyme activity, while octyl-VS-Gly-agarose-CALB shows very similar activity than octyl-CALB, octyl-VS-EDA-agarose-CALB almost duplicate the activity. The highest activity of all CALB biocatalysts was found using (*D*)-methyl mandelate as substrate. The blocking agent once again influenced the effect of the use of octyl-VS supports, while octyl-VS-EDA-agarose-CALB was almost 40 % more active than octyl-agarose-CALB, octyl-VS-Gly-agarose-CALB almost maintained the same activity (95 %). The activity of the 3 biocatalysts decreased using the (*L*)-isomer as substrate, but in this instance both octyl-VS biocatalysts were slightly more active than octyl-agarose-CALB (by <20 %).

Using amino-hexyl-VS-agarose-CALB (Table 2), both biocatalysts presented similar activity using *p*NPB and were more active (by >15 %) than amino-hexyl-VS-agarose-CALB-EDA, which was also more active than octyl-agarose- CALB (by 45 %). This increase on enzyme activity using *p*NPB was higher using amino-octyl-VS-agarose-CALB (with both biocatalysts presented very similar activity) when compared to amino-octyl-agarose-CALB (almost by 40 %). However, amino-octyl-agarose-CALB presented only 55 % of the activity of amino-hexyl-agarose-CALB. That way, both amino-octyl-VS-agarose-CALB biocatalysts ere more active than all octyl-agarose biocatalysts, but less active than all amino-hexyl-agarose-CALB.

Using triacetin as substrate (Table 2), the situation was quite different. In this instance, amino-octyl-agarose-CALB was 1.8 fold more active than octyl-agarose-CALB, while amino-hexyl-agarose-CALB showed <90 % of the activity of octyl-CALB with this substrate. Amino-hexyl-VS-Gly-agarose-CALB was 2.3 fold more active than amino-hexyl-agarose-CALB while amino-hexyl-VS-EDA-agarose-CALB was 2.8 more active, becoming the most active among the studied CALB biocatalyst with this substrate because both amino-octyl-VS-agarose-CALB biocatalysts reduced its activity when compared to amino-octyl-agarose-CALB (by <10 %).

Moving to the results obtained using (*D*)-methyl mandelate, the initial activities of both amino-alkyl-biocatalysts greatly increased the

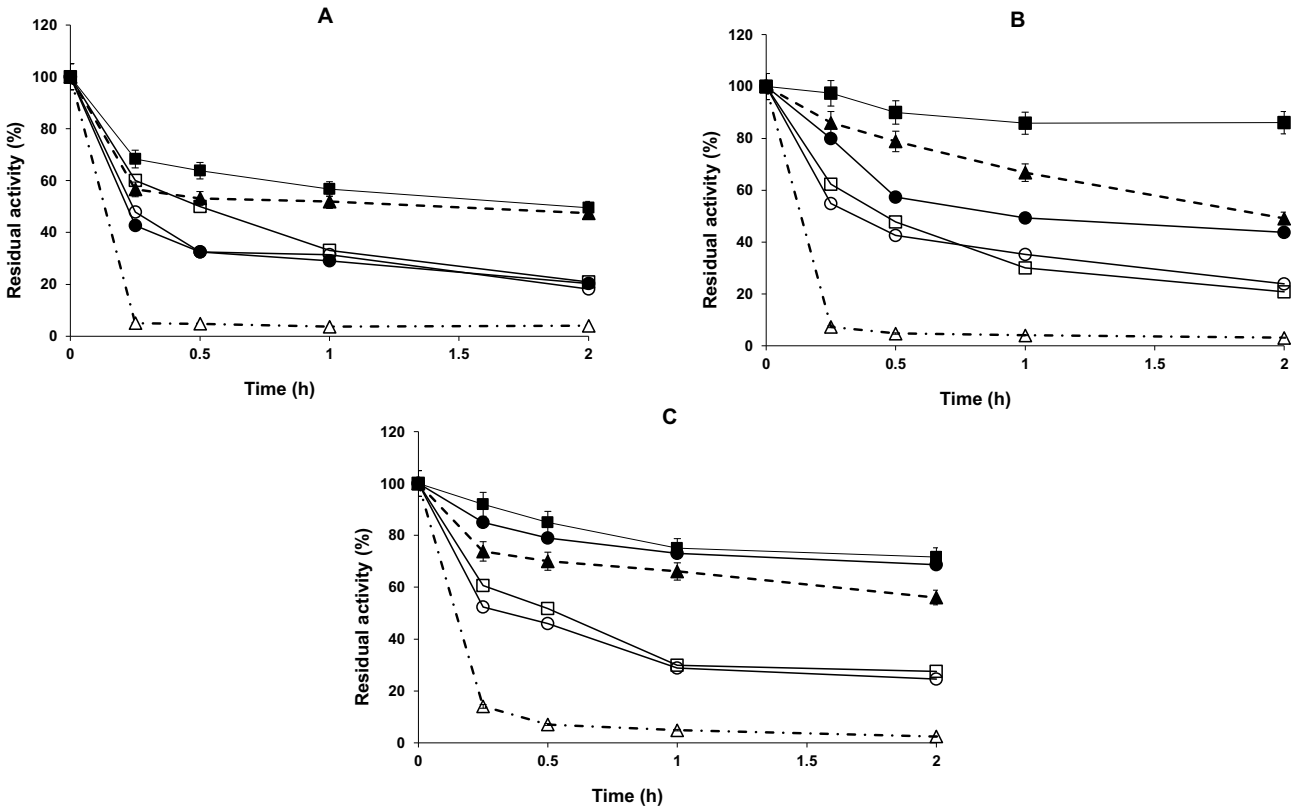

Fig. 7. Thermal inactivation courses of CALB immobilized on amino-hexyl agarose and amino-hexyl-VS agarose (empty symbols), and octyl and octyl-VS agarose (solid symbols) at pH 7.0 and 76 °C. (A): Using Tris 25 mM. (B): Using Tris 25 mM and 1 M NaCl. (C): 25 mM Tris and 3 M NaCl. Symbols: Full triangle: CALB immobilized on octyl. Empty triangle: CALB immobilized on 1-helylamine. Full circles: octyl-VS-EDA. Empty circles: amino-hexyl-VS-EDA. Full squares: octyl-VS-Gly. Empty squares: amino-hexyl-VS-Gly. Other specifications are described in the Methods section.

activity (>5 fold using amino-hexyl and almost 4 using amino-octyl) when compared to the octyl biocatalyst. The use of amino-hexyl-VS-agarose supports had a small effect on the activity versus this substrate (with both blocking agents giving similar activities). Results were very different using amino-octyl-VS-agarose supports, both gave similar activities but decreasing them to just over 35 % of amino-hexyl- agarose, although they were still more active than the all octyl biocatalysts. Using the (*L*)-isomer as substrate, both amino-alkyl biocatalysts were slightly more active than octyl-agarose-CALB (almost by 30 % for amino-hexyl-agarose-CALB and 10 % for amino-octyl-agarose-CALB). The use of VS supports increased the activity in all cases, the highest activity is obtained (almost 2.6 fold more than amino-hexyl-agarose-CALB) using amino-hexyl-VS-Gly-agarose-CALB, while amino-hexyl-VS-EDA-agarose-CALB gave 2.1 fold more activity. Amino-octyl-VS-agarose CALB also increased the activity, but to a lower extent (by 26 % when blocked with Gly and by <5 % using EDA).

This means that the effects on enzyme activity of the different immobilization protocols fully depends on the used substrate, the "best" biocatalyst is only the best for some substrates. This suggested that the enzyme could present different conformations depending on the support, and these "new" enzymes could have very different performance, as previously reported [63,65,70].

3.4.2. Activities of CALA biocatalysts

The results are shown in Table 3. Using this enzyme, the highest activity was obtained using *p*NPB in all cases. Using this substrate, the use of amino-alkyl-supports to immobilize CALA slightly improved the activity obtained using CALA (reaching a 17 % increase using amino-octyl-CALA, the most active of the just absorbed biocatalysts). Octyl-VS-Gly-CALA decrease around 1/3 the activity, while the blocking with EDA presented an activity very similar to octyl-CALA. Using amino-alkyl-VS-CALA, the activity decreased compared to the corresponding only physically adsorbed biocatalyst, more significantly using Gly (with both biocatalysts showing very similar activities) than using EDA (amino-octyl-VS-EDA being more active than amino-hexyl-VS-EDA).

Using triacetin as substrate, the use of amino-alkyl supports gave CALA biocatalysts with lower activity than octyl-supports, more significantly using amino-hexyl-CALA that gave only 10 % of the activity of octyl-CALB. The use of octyl-VS supports gave CALA biocatalysts with a significant decrease in activity, deeper using octyl-VS-Gly (<15 %). Using both amino-alkyl-VS, the immobilization produced a decrease of activity compared to the respective amino-alkyl supports, giving less activity using Gly as blocking agent (around 10 fold).

Using (*D*)-methyl mandelate, again octyl-CALA was more active than both amino-alkyl-CALA biocatalysts, although in this instance the decrease in activity is not as profound as in the case of triacetin (the lest active amino-hexyl-CALA biocatalyst showing >50 % of the activity of the octyl-CALA). The use of octyl-VS also produced a decrease in activity with this substrate, just under 50 % for the least active one, octyl-VS-Gly-CALA. The biocatalyst amino-hexyl-VS-Gly-CALA presented a further reduction of the activity compared to the amino-hexyl-Gly-CALA, however, amino-hexyl-VS-EDA-CALA increased the activity, even becoming more active than octyl-CALA (by 30 %). Using amino-octyl-VS supports, the effects were similar, but with a higher intensity in the decrease of activity using Gly as blocking agent and with a lower intensity in the increase of activity using EDA, that remains slightly under the activity observed for the octyl-CALA.

Again, the situation was different using the (*L*) isomer. In this instance, using the supports without VS groups, the least activity was octyl-CALA while the most active was amino-hexyl-CALA (by almost 60 %). The use of octyl-VS support depended on the blocking agent, compared to octyl-CALA, Gly slightly increased the activity, while EDA

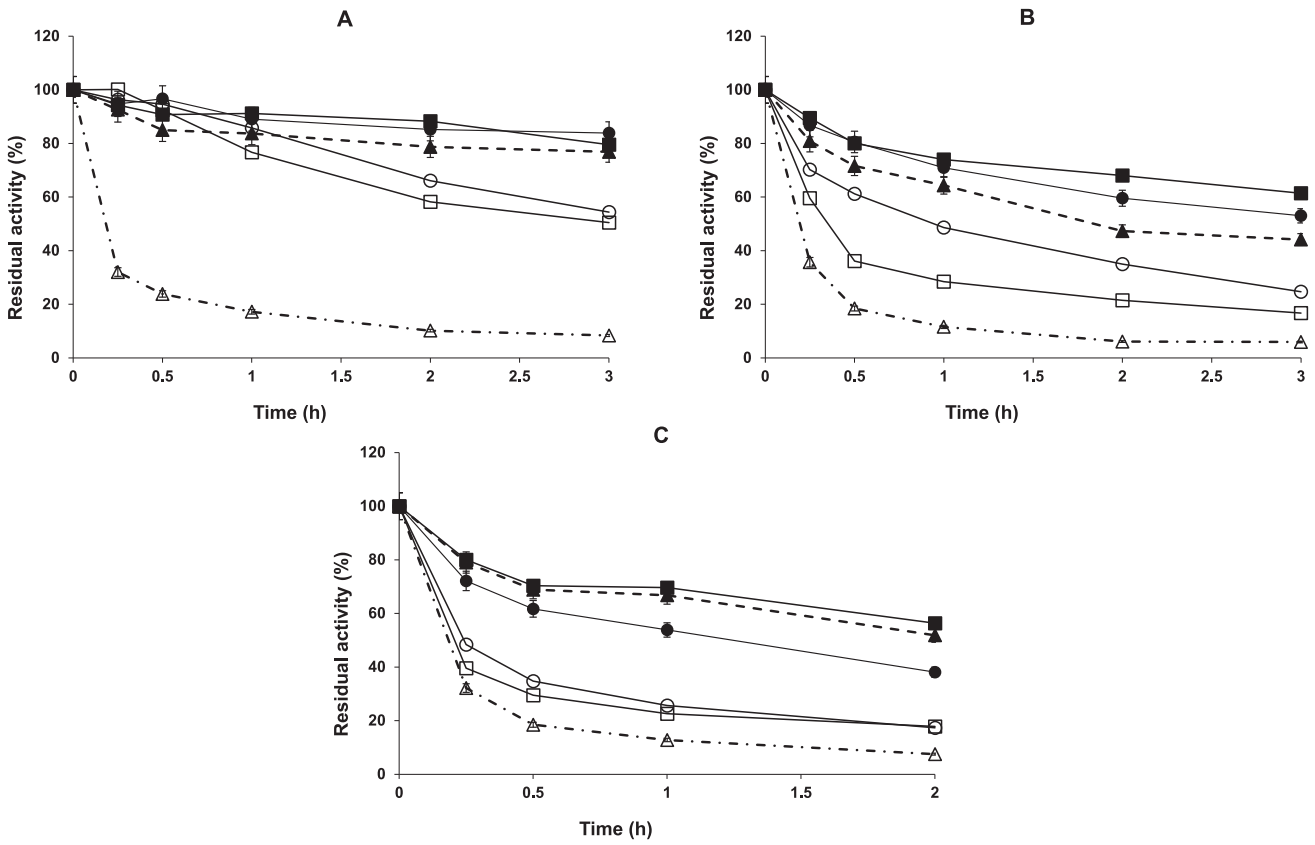

Fig. 8. Thermal inactivation courses of CALA immobilized on amino-hexyl agarose and amino-hexyl-VS agarose (empty symbols), and octyl and octyl-VS agarose (solid symbols) at pH 7.0. (A): Using 25 mM Tris at 78 °C. (B): Using 25 mM Tris and 1 M NaCl at 80 °C. (C): Using 25 mM Tris and 3 M NaCl at 80 °C. Symbols: Full triangle: CALA immobilized on octyl. Empty triangle: CALA immobilized on hexylamine. Full circles: octyl-VS-imidazole. Empty circles: amino-hexyl-VS-imidazole. Full squares: octyl-VS-Asp. Empty squares: amino-hexyl-VS-Asp. Other specifications are described in the Methods section.

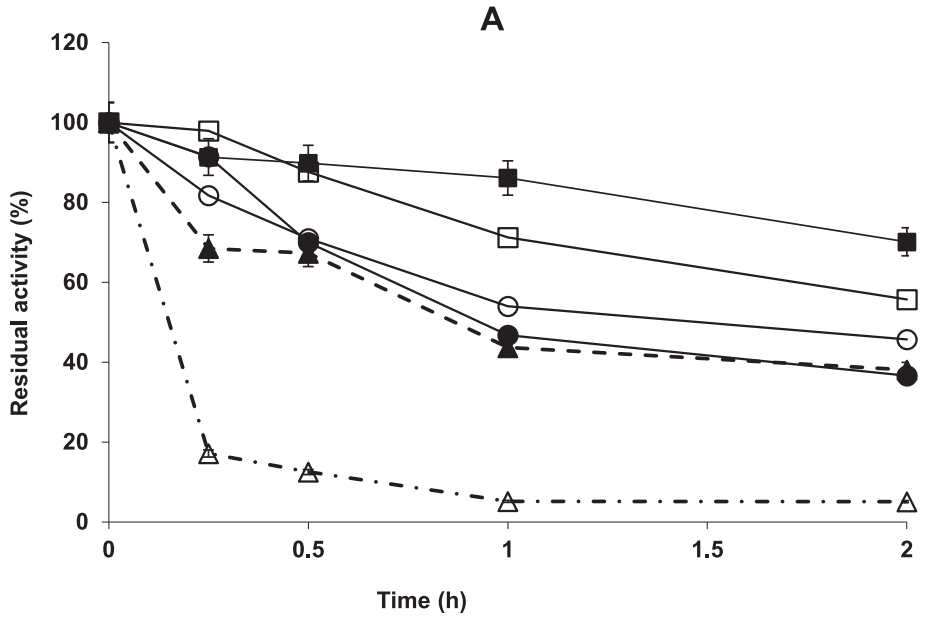

Fig. 9. The thermal inactivation courses of CALB immobilized on different supports at 70 °C using 100 mM sodium phosphate at pH 7.0. Amino-hexyl and amino-hexyl-VS agarose (empty symbols), and octyl and octyl-VS agarose (solid symbols). Symbols: Full triangle: CALB immobilized on octyl. Empty triangle: CALB immobilized on amino-hexyl agarose. Full circles: octyl-VS-EDA. Empty circles: amino-hexyl-VS-EDA. Full squares: octyl-VS-Gly. Empty squares: amino-hexyl-VS-Gly. Other specifications are described in the Methods section.

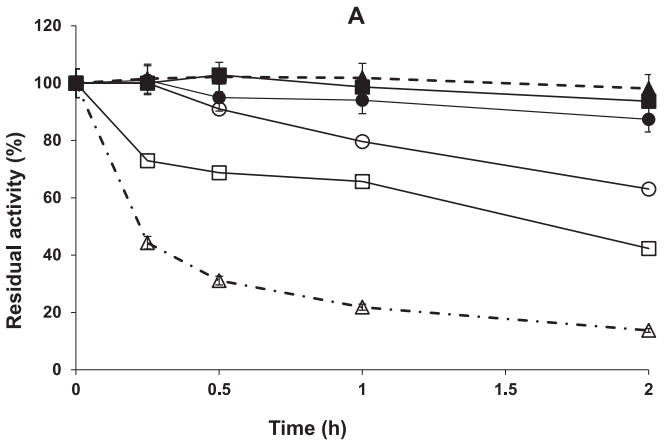

Fig. 10. The thermal inactivation courses of CALA immobilized on different supports at 70 °C using 100 mM sodium phosphate at pH 7.0. Amino-hexyl and amino-hexyl-VS agarose (empty symbols), and octyl and octyl-VS agarose (solid symbols). Symbols: Full triangle: CALA immobilized on octyl. Empty triangle: CALA immobilized on amino-hexyl agarose. Full circles: octyl-VS-Imidazole. Empty circles: amino-hexyl-VS-Imidazole. Full squares: octyl-VS-ASP. Empty squares: amino-hexyl-VS-ASP. Other specifications are described in the Methods section.

Table 2
Activity of the CALB immobilized on octyl-VS, amino-hexyl-VS and amino-octyl-VS with different substrates at 25 °C. ρNPB activity at pH 7.0 and triacetin and methyl mandelate at pH 5.0. The experiments were conducted as described in Methods section. Activity is given per gram of biocatalyst.

Support	Activity (U g^{-1}) with 0.4 mmol L^{-1} ρNPB	Activity (U g^{-1}) with 50 mmol L^{-1} triacetin	Activity (U g^{-1}) with 50 mmol L^{-1} (*D*)-methyl mandelate	Activity (U g^{-1}) with 50 mmol L^{-1} (*L*)-methyl mandelate
Octyl	6.61 ± 0.33	1.70 ± 0.085	17.15 ± 0.87	1.90 ± 0.17
Amino-hexyl	9.62 ± 0.48	1.49 ± 0.074	74.84 ± 2.75	2.45 ± 0.19
Amino-octyl	5.35 ± 0.27	3.07 ± 0.15	67.55 ± 2.67	2.1 ± 0.25
Octyl-VS-Gly	6.23 ± 0.31	1.62 ± 0.081	16.35 ± 0.8	2.25 ± 0.115
Octyl-VS-EDA	6.89 ± 0.35	3.06 ± 0.15	23.75 ± 1.2	2.22 ± 0.11
Amino-hexyl-VS-Gly	11.21 ± 0.56	3.46 ± 0.17	70.15 ± 3.5	6.24 ± 0.31
Amino-hexyl-VS-EDA	11.40 ± 0.57	4.25 ± 0.21	79.25 ± 3.95	5.05 ± 0.25
Amino-octyl-VS-Gly	7.44 ± 0.37	2.82 ± 0.14	25.05 ± 1.2	2.61 ± 0.13
Amino-octyl-VS-EDA	7.43 ± 0.37	2.88 ± 0.14	24.60 ± 1.25	2.11 ± 0.105

more than duplicated the activity. Using amino-alkyl-VS supports, the blocking with Gly produced a slight decrease in enzyme activity (while remaining more active than octyl-CALA), while the EDA blocking produced a significant increase in enzyme activity. Amino-hexyl-VS-EDA-CALA is the most active with this substrate, more than triplicating the activity of octyl-CALA and more than doubling the activity of amino-hexyl-CALA. This increase in activity is also relevant using amino-octyl-VS-EDA-CALA (130 % compared to amino-octyl-CALA), becoming the second more active with this substrate.

Table 3
Activity of the CALA immobilized on octyl-VS, amino-hexyl-VS and amino-octyl -VS with different substrates at 25 °C. ρNPB activity at pH 7.0 and triacetin and methyl mandelate at pH 5.0. The experiments were conducted as described in Methods section. Activity is given per gram of biocatalyst.

Support	Activity (U g-1) with 0.4 mmol L − 1 ρNPB	Activity (U g-1) with 50 mmol L − 1 triacetin	Activity (U g-1) with 50 mmol L − 1 (*D*)-methyl mandelate	Activity (U g-1) with 50 mmol L − 1 (*L*)-methyl mandelate
Octyl	61.96 ± 3.10	8.59 ± 0.43	0.215 ± 0.016	0.291 ± 0.029
Amino-hexyl	64.42 ± 3.22	0.82 ± 0.041	0.116 ± 0.012	0.458 ± 0.046
Amino-octyl	72.61 ± 3.63	4.12 ± 0.21	0.150 ± 0.015	0.355 ± 0.036
Octyl-VS-Gly	41.34 ± 2.07	1.20 ± 0.060	0.105 ± 0.005	0.345 ± 0.015
Octyl-VS-EDA	62.69 ± 3.13	5.65 ± 0.28	0.205 ± 0.011	0.630 ± 0.031
Amino-hexyl-VS-Gly	10.97 ± 0.55	0.12 ± 0.01	0.085 ± 0.004	0.399 ± 0.004
Amino-hexyl-VS-EDA	38.91 ± 1.95	1.19 ± 0.06	0.28 ± 0.015	1.012 ± 0.050
Amino-octyl-VS-Gly	10.17 ± 0.51	0.27 ± 0.014	0.09 ± 0.0045	0.332 ± 0.016
Amino-octyl-VS-EDA	44.10 ± 2.21	2.57 ± 0.13	0.19 ± 0.01	0.542 ± 0.054

Amino-hexyl-VS-Gly-CALB and octyl-VS-Gly-CALA were utilized in 10 cycles of 1 h in the reaction hydrolysis of (*D*)-methyl mandelate. Both biocatalysts showed intact activities after these 10 cycles (99.5 % ± 2.1 % for CALB biocatalyst and 101.3 % ± 2.5 for CALA biocatalyst).

4. Conclusion

This paper shows that the use of alkyl heterofunctional supports bearing VS is very efficient to prevent enzyme release from the support after lipase immobilization by interfacial activation, increasing that way the possibilities of use of the biocatalysts. Moreover, the blocking step enables a last opportunity to tailor the enzyme features. Using amino-acyl supports, the stabilization promoted by the covalent immobilization is higher than the one obtained using octyl-VS-supports, but still the last ones are more stable in most situations due to the negative effect that the amino group has when it is presented in the acyl chain. The use of cationic groups in the blocking step is also negative for the enzyme stability, but this may be reverted using high ionic strength, while the amino-acyl chain effect is just partially reduced. This suggests that is the amphipathic nature of the acyl chain the responsible of the negative effects on the enzyme stability more that the cationic interactions. However, the effects on enzyme activity and stability are multiple, one modification in the support may be positive for one substrate and negative for other, making it impossible to give a simple mechanistic explanation for the observed results with the current knowledge on the effects of the interactions between the enzyme and support surface interaction on the enzyme properties.

CRediT authorship contribution statement

Pedro Abellanas-Perez: Writing – review & editing, Writing –

original draft, Investigation, Formal analysis, Data curation. **Diandra de Andrades:** Writing – review & editing, Investigation, Formal analysis, Data curation. **Andres R. Alcantara:** Writing – review & editing, Formal analysis, Data curation. **Javier Rocha-Martin:** Writing – original draft, Visualization, Conceptualization. **Maria de Lourdes Teixeira de Moraes Polizeli:** Writing – review & editing, Funding acquisition, Formal analysis. **Roberto Fernandez-Lafuente:** Writing – review & editing, Writing – original draft, Visualization, Supervision, Formal analysis, Data curation, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ijbiomac.2025.143305.

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