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Preparation and characterization of different immobilized and chemically modified preparations of lipase B from *Candida antarctica*: is it the activation energy a good indicator of the biocatalyst expressed activity?

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ABSTRACT

Lipase B from *Candida antarctica* immobilized on octyl (via interfacial activation) and octyl-vinyl sulfone (covalently attached) agarose beads via different immobilization protocols was submitted to amination and/or glutaraldehyde modifications. The catalytic performance of the resulting biocatalysts significantly varied across different substrates: using octyl-CALB with the double modification, activity increased 3.5 fold versus triacetin and decreased by 5 % using *R*-methyl mandelate, while using the covalent biocatalyst, activity increase by 2.2 or 20 %, respectively. Similarly, the stability of the biocatalysts —both in absolute and relative terms— was strongly influenced by the inactivation pH and the substrate used for residual activity determination. Under the tested conditions, activity versus substrate concentration followed first-order kinetics up to the substrate solubility limit, preventing the determination of kinetic parameters such as Kcat or Km. Activation energy (E_a) for triacetin hydrolysis was also measured for each biocatalyst under different inactivation states. Interestingly, no consistent correlation was found between E_a and enzyme activity. Generally, partial inactivation of the biocatalysts increased E_a, although some exceptions were observed. These findings suggest that E_a alone does not directly correlate with enzymatic activity, highlighting the complex interplay between structural enzyme modifications, substrate used to determine the enzyme activity, and the enzyme catalytic behavior.

1. Introduction

Enzymatic biocatalysis is becoming increasingly popular due to the extraordinary selectivity and specificity of these biological catalysts, being able to perform their function under environmentally friendly conditions (aqueous media, room temperature and atmospheric pressure) [1–4]. However, they have been designed by natural selection to fit in their physiological role, and some of their features differ from the industrial requirements, where enzymes are in many instances under unconventional media and with non-physiological substrates [5]. These discrepancies between nature and industry requirements can be solved using many different tools that are rapidly evolving, these solutions address the contradictions between industrial demands and the inherent properties of natural enzymes. Among these new solutions, we can remark metagenomics (that permits the use of any natural enzyme) [6–9], directed evolution (that permits to improve a specific enzyme feature mimicking natural evolution but in an accelerated way) [10–12], modelling, and site-directed mutagenesis (that permits to create *ex novo* active centers in inert proteins [13,14] or in enzymes, creating the so-called plurizymes [15–17]), chemical modification [18–21] or enzyme immobilization [22]. Enzyme immobilization started as a tool to permit enzyme recovery and reuse but, nowadays, it has been revealed as a powerful tool to improve many enzyme features: stability [23–29], activity [30–33], selectivity and specificity [34–37], inhibitions [38–42], etc. Enzyme immobilization can be coupled to enzyme purification if the immobilization protocol is properly designed (in some instances with the help of the genetic modification of the enzyme) [43–47]. Moreover, immobilization may be used in a coupled way with any other

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modification strategies cited above to improve the properties of an enzyme, in order to get a biocatalyst with the required properties [48–59]. One of the enzyme features that the researcher usually intends to improve via immobilization is the enzyme stability while maintaining good activity values (or even improving them), as enzyme immobilization using adequate protocols can greatly improve enzyme stability by different reasons as recently reviewed [26].

The main role of any catalyst in a reaction is to decrease the activation energy of the catalyzed reaction (Ea) [60,61]. A poorly efficient enzyme may still lower the activation energy of the reaction, but to a lesser extent compared to a highly efficient enzyme. This phenomenon would be observed in partially inactivated enzymes, which may reduce E_a less efficiently than their fully active counterparts. In addition to altering E_a, enzyme inactivation may be manifested in the modification of other kinetic parameters, such as the Michaelis constant (Km) and the catalytic constant (Kcat). These changes typically result from structural modifications. There are some literature on the comparison of the Ea in the inactivation of free and immobilized enzymes, an interesting data that may be related to the stabilization achieved by the immobilization [62–66]. There are far less papers reporting the effects of the immobilization on the Ea of the reaction catalyzed by the immobilized enzymes compared to the free enzymes. For example, purified alkaline protease from *Bacillus pumilus* Y7 exhibited an increase in the spontaneity of the biochemical reaction after immobilization [67]. However, this was no7 relate dto an increase in enzyme activity. In another paper, using as model inulinase from *Kluyveromyces marxianus* in free and immobilized forms, the analysis of the kinetic and thermodynamic parameters showed a higher optimal T for the free enzyme and the authors suggested that the operation T should be lower for booth enzyme formulations than that predicted from the optimal temperatures of a high enzyme stability was desired [68]. In another paper, the authors found that the differences on activity between free and immobilized alkaline phosphatase from the *Escherichia coli* ATCC2757 were more related from the internal pH values of the immobilized enzymes than to the temperature [68]. That way, there are very few examples focused on the changes on the Ea on the catalyzed reactions, and we have been unable to find a single paper on the effect of the enzyme thermal inactivation on the value of this parameter.

The extent of these modifications often depends on the nature of the enzyme inactivation process and the specific substrate used. Surprisingly, there are only very few papers where enzyme inactivation is followed using different substrates [70–72] and we have not been able to find any paper where relevant information such as changes in Km, Kcat and Ea are determined during the biocatalysts inactivation courses. This gap in knowledge is critical, as understanding how these parameters evolve during enzyme manipulation (e.g., immobilization, chemical modification, or inactivation) is essential for designing biocatalysts with optimized performance. The current study aims to fill this gap by exploring the impact of enzyme inactivation and modification on these key kinetic parameters. To reach this goal, we have selected one of the most used enzymes, the lipase B from *Candida antarctica* (CALB) [73–75]. This lipase has been immobilized via its interfacial activation on octyl-agarose beads [31]. Moreover, the enzyme has also been immobilized on heterofunctional octyl-vinyl sulfone (VS)-agarose beads [76]. In these supports, a first lipase immobilization is performed at low pH to reduce the enzyme-support reactivity and to prevent a direct covalent lipase immobilization, increasing the possibilities of the lipase becoming firstly immobilized via interfacial activation (as it occurs using unmodified octyl supports) [76]. After enzyme immobilization, the biocatalyst is incubated at higher pH value to facilitate the reaction of the already physically immobilized enzyme with the vinyl sulfone layer [76]. Next, the remaining VS groups in the support were blocked with ethylenediamine (EDA) to avoid any undesired enzyme-support additional and uncontrolled covalent reaction (e.g., during inactivation) and to get a support bearing primary amino groups (that can react with some bifunctional reagents, such as glutaraldehyde).

To have more biocatalyst formulations from the same enzyme to analyze, both immobilized biocatalysts were modified with EDA and/or glutaraldehyde. In the modification with glutaraldehyde, the reaction conditions were controlled to have just one glutaraldehyde molecule per primary amino group, as these amino-glutaraldehyde groups have been reported to be able to react with each other in a wide range of pH values, giving inter or intramolecular crosslinkings, or enzyme-support reactions (if the support is aminated, as it will also bear amino-glutaraldehyde groups) [77–80]. This can occur using the octyl-VS-EDA biocatalyst. However, it may be expected that, in most cases, just a one point glutaraldehyde modification of the primary amino groups is introduced, which means introducing one moderately hydrophobic moiety pending on a secondary amino group [81]. Full modification of the immobilized enzyme with EDA after carbodiimide activation of the superficial carboxylic groups is a well-established method of chemical modification of the protein surface [49,82,83], and this has been applied to both immobilized CALB biocatalysts. This should alter the ionic interactions network in the enzyme surface, changing ionic bridges by repulsion forces. Both aminated immobilized biocatalysts (those obtained from octyl and octyl-VS supports) were later further modified with glutaraldehyde under conditions that permitted to introduce just one glutaraldehyde molecule per amino group. It may be expected that now, after enrichment of the enzyme surface with amino groups, there is a higher possibility of achieving inter or intramolecular crosslinkings in the enzyme or additional enzyme-support bonds (when using the octyl-VS-support blocked with EDA) [84].

In this study, we will investigate the impact of these modifications and enzyme inactivation on the activity, kinetic parameters, and activation energy (E_a) of the biocatalysts. We will also examine how these parameters change during thermal inactivation, using different substrates of varying nature, including *p*-nitrophenyl butyrate (an aromatic compound), enantiomers of methyl mandelate (a chiral compound), and triacetin (a short-chain fatty acid triglyceride).

We will determine the activity, kinetic constants and activation energy of these very different biocatalysts and we will analyze these parameters during their thermal inactivation under different conditions (that could alter the route of the enzyme structure modifications during the biocatalyst inactivation) [70,71,85], in some instances using different substrates of very different nature: *p*-nitro phenyl butyrate, with an aromatic ring in the nucleophile part of the molecule, both enantiomers of methyl mandelate, a chiral compound with an aromatic ring in the acyl part of the molecule, and triacetin, a short fatty acid triglyceride. The main objective of this paper is to compare the activity of the different biocatalysts (that is the reason of preparing a collection of them) from the same enzyme and the activation energy on the catalyzed reactions.

2. Materials and methods

2.1. Materials

Liquid CALB (lipase B from *Candida antarctica*, 24.77 mg of protein/mL) was kindly provided by Novozymes A/S (Madrid, Spain). Octyl-Sepharose® 4 % BCL beads was from GE Healthcare (Uppsala, Sweden). Triacetin, *p*-nitrophenyl-butyrate (*p*NPB), ethylenediamine (EDA), ethylcarbodiimide hydrochloride (ECD) and glutaraldehyde (Glu) were acquired from Sigma-Aldrich (Madrid, Spain). *R*- and *S*- methyl-mandelate esters were purchased from Thermo-Fisher (Alcobendas, Spain). Divinyl sulfone (DVS) (stabilized with hydroquinone) was purchased from Tokyo Chemical Industry Europe (Zwijndrecht, Belgium). All the other reagents and solvents used were of analytical grade.

2.2. Methods

All experiments have been performed by duplicate and the results are presented as the mean value of at least 3 measurements, their standard

errors reported in this paper.

2.2.1. Preparation of octyl-vinyl sulfone agarose beads (octyl-VS)

The activation of the octyl agarose beads with DVS was performed according to the methodology optimized by Abellanas-Perez [86]. A solution of 200 mL of 0.333 M sodium carbonate at pH 11.5 was mixed with 7.5 mL of DVS under vigorous stirring until the solution became homogeneous. Subsequently, 10 g of octyl agarose beads were added, and the suspension was gently stirred for 2 h. Then, the octyl-VS support was vacuum filtered through a sintered glass funnel, washed thoroughly with distilled water, and stored at 4–6 °C.

2.2.2. Immobilization of CALB on octyl- or octyl-VS-agarose beads

CALB was immobilized using 1.0 g of support per 10.0 mL of 0.1 mg/mL enzyme solution. For the octyl-agarose support, the CALB solution was prepared in 5 mM Tris-HCl (pH 7.0), while for the octyl-VS support, the same amount of enzyme was used but in 5 mM sodium acetate at pH 5.0 [76]. The immobilization suspensions were maintained at 25 °C under gentle stirring for 30 min. Immobilization processes were monitored by measuring the activity in the suspension, supernatant, and a reference suspension (where inert agarose beads were used instead of octyl supports) using *p*NPB as a substrate. Afterwards, the biocatalysts were washed with distilled water, vacuum filtered and the octyl-CALB biocatalysts was stored at 4–6 °C. Octyl-VS-CALB was resuspended in 50 mM sodium bicarbonate at pH 8.0 for 4 h at 25 °C to facilitate the enzyme-support covalent reaction [76]. Next, the biocatalyst was recovered by vacuum filtration and resuspended in 2 M EDA at pH 8.0 and 25 °C for 24 h (using 1.0 g of biocatalyst per 10 mL of blocking solution), to block any remaining vinyl sulfone groups on the support [76,86]. Samples were periodically withdrawn to check enzyme activity during the process. Finally, the biocatalyst was washed, vacuum filtered, and stored at 4–6 °C.

2.2.3. Chemical amination of immobilized CALB biocatalysts

1.0 g of both immobilized CALB biocatalyst were suspended in 10 mL of 2 M EDA at pH 4.75 and 25 °C. Then, solid ECD was added to reach a final concentration of 10 mM, to modify 100 % of the external carboxylic acids of the enzyme [82]. The reaction suspension was magnetically stirred at room temperature for 2 h. Afterwards, the aminated immobilized enzymes were vacuum filtered, washed with distilled water, and stored at 4–6 °C until further use.

2.2.4. Modification of CALB biocatalysts with glutaraldehyde

Samples of 1 g of the biocatalysts were suspended in 10 mL of 50 mM sodium phosphate containing 1 % (*v*/v) glutaraldehyde at pH 7.0. This suspension was gently stirred for 1 h at 25 °C to ensure the complete modification of the primary amino groups of the enzyme and the support (in the case of the biocatalysts prepared in octyl-VS blocked and with EDA), involving only one molecule of glutaraldehyde per primary amino group [79]. These amino-glutaraldehyde moieties have a good reactivity with other identical groups and can lead to some covalent bonds [77]. After that, the biocatalysts were thoroughly washed with distilled water by employing a sintered filter and stored at 4–6 °C until use.

2.2.5. Determination of enzyme activity versus different substrates

2.2.5.1. Hydrolysis of pNPB. The standard enzymatic activity assay was performed using *p*NPB by measuring the increase in absorbance at 348 nm (isobestic point of *p*NP, its ε under these conditions is 5150 M^{-1} cm^{-1}) [87] over 90 s using a Jasco V-730 spectrophotometer (Jasco, Madrid, Spain). The reaction was initialized by adding 50 μL of the enzyme sample to 2.5 mL of 25 mM sodium phosphate at pH 7.0 containing 50 μL of *p*NPB solution (*p*NPB was dissolved in acetonitrile at a concentration of 20 mM) under magnetic stirring and temperature control (25 °C). The reaction was also carried out in 25 mM sodium acetate at pH 5.0 to compare the observed activity with triacetin under similar conditions (see below). It was defined that a unit of activity (U) corresponds to the conversion of 1 μmol of substrate per minute under the described conditions.

2.2.5.2. Hydrolysis of triacetin. This assay was performed by suspending 1.0 g of immobilized CALB in 10.0 mL of 50 mM triacetin dissolved in 50 mM sodium acetate at pH 5.0. The reaction suspension was kept under stirring at 25 °C using a roller mixer (Tube Roller MX-T6-S, Scilogex, USA). Samples of the reaction suspension were taken at different times, and the conversion degree was quantified by HPLC (Shimadzu, Tokyo, Japan), determining the absorbance of the different compounds at 230 nm. The mobile phase consisted of a 15 % acetonitrile-85 % Milli-Q water solution with a flow rate of 1 mL/min, and a Kromasil C18 (15 cm × 0.46 cm) (Analisis Vinicos, Tomelloso, Spain) was employed. Under these reaction conditions, the reaction product, 1,2-diacetin, does not undergo acyl migration [88]. The retention times were 4 min for 1,2 diacetin and 18 min for triacetin. Conversions between 15 % and 20 % were used to calculate the initial reaction rates. We established that a unit of activity (U) corresponds to the conversion of 1 μmol of substrate per minute under the described conditions.

2.2.5.3. Hydrolysis of R- or S-methyl mandelate. The hydrolysis of both methyl mandelate isomers was carried out by adding 1.0 g of biocatalyst to 10.0 mL of 50 mM of either *R*- or *S*-methyl mandelate in 50 mM sodium acetate at pH 5.0 and 25 °C under gentle stirring using a roller mixer (Tube Roller MX-T6-S, Scilogex, USA). The reaction products were detected by HPLC, and the compounds were quantified with a UV-VIS detector at 230 nm, by injecting 20 μL of reaction samples. The retention times were 2.4 min for mandelic acid and 4.2 min for methyl mandelate. Conversions between 15 % and 20 % were used to calculate the initial reaction rates. The column was a Kromasil C18 (15 cm × 0.46 cm) (Analisis Vinicos, Tomelloso, Spain), and a mobile phase of 35 % acetonitrile-65 % (*v*/v) 10 mM ammonium acetate in Milli-Q water at pH 2.8 was used, with a flow rate of 1 mL/min. We established that a unit of activity (U) corresponds to the conversion of 1 μmol of substrate per minute under the described conditions. Activities ratio is defined as the activity versus the *R* isomer/activity versus the *S* isomer.

2.2.6. Thermal inactivations of the different CALB preparations

The thermal inactivations of the different CALB biocatalysts were assessed at various pH values, different temperatures were employed at each pH value to determine the inactivation conditions. Due to the varying stabilities of the biocatalysts at different pH values, the temperature was selected to provide reliable inactivation courses. The biocatalysts were resuspended at pH 5.0 (10 mM sodium acetate) and 70 °C, pH 7.0 (10 mM sodium phosphate) and 62 °C, or pH 9.0 (10 mM sodium carbonate) and 55 °C. Samples were periodically taken, and their remaining activities were measured using the *p*NPB assay. Samples were withdrawn when their residual activity versus *p*NPB reached 50 % and 25 %. Afterward, the biocatalysts were vacuum filtered, washed with distilled water, and stored at 4 °C for 24 h to allow for potential enzyme reactivation before any further analysis [70,89]. The initial activity of the preparation was considered 100 %, and the activities of subsequent samples were expressed as a percentage relative to this reference value.

2.2.7. Determination of Ea and kinetic constants

To determine the activation energy (Ea) all biocatalysts were incubated in a suspension of 50 mM triacetin at pH 5 with the temperature established at 4, 25 and 45 °C and stirred at 900 rpm using a thermal mixer (Eppendorf Thermomixer C). In these experiments, samples of the reaction suspension were taken at different times, and the triacetin conversion degree was quantified by HPLC as described above.

The values of E_a, were determined from the relationship between the logarithm of the reaction rate (ln*k*) on the reciprocal of temperature (1/

T). The activation energy of the biocatalyst was calculated by taking the natural logarithm on both sides of the Arrhenius (Eq. (1))

$$\ln k = \frac{E_a}{RT} + \ln A \quad (1)$$

where E_a represents the activation energy (J.mol^{-1}), R the universal gas constant (8.3145 J.mol^{-1}.K^{-1}), T the temperature, and A the pre-exponential factor. By plotting a graph of ln *K* versus 1/T, the slope (E_a/R) and the intercept (ln *A*) could be estimated using linear regression [90].

The activities of the enzyme versus different substrate concentrations were determined in the range 0.1-solubility limit in aqueous medium (100 mM in the case of triacetin) to calculate Km and Kcat.

3. Results

3.1. Immobilization of CALB

Fig. 1 shows the immobilization courses of CALB on octyl and octyl-VS, utilizing pNPB at pH 7 to determine the activities of immobilization supernatant and suspensions, and reference suspensions. The enzyme was rapidly immobilized on both supports, with scarce effects on enzyme activity. Octyl-CALB presented 85 % of the activity versus *p*NPB of the free enzyme, while octyl-VS–EDA-CALB maintained 93 % of the initial activity (Fig. 1). The incubation of the octyl-VS-CALB biocatalyst at pH 8 to favor the formation of covalent bonds and the further blocking of EDA (octyl-VS–EDA-CALB) promoted a slight increase in enzyme activity versus *p*NPB (described for other instances using this immobilization protocol) [76,91,92], becoming this biocatalyst a 18 % more active than octyl-CALB (Table 1). SDS-PAGE study of these biocatalysts showed that all enzyme molecules were covalently immobilized on octyl-VS-EDA-CALB, as no protein bands could be visualized in the gels, whereas obviously all enzyme molecules were released from the octyl support (Fig. 2). It should be remarked that the covalent bonds between enzyme and support are secondary amino, ether or tioether, and these resisted even the hydrolysis of the proteins at high pressure, in high concentration of sulfuric acid, and this mean that the enzyme molecules cannot be released from the support without the full destruction of the enzyme and support [93,94].

3.2. Chemical modification of the immobilized enzymes

Table 1 shows the changes of the CALB activities versus the 4 assayed substrates after chemical amination of the two immobilized CALB biocatalysts. The amination increased the *p*NPB activity at pH 7 for both biocatalysts: by 1.9-fold using octyl-CALB (octyl-CALB–EDA) and by less than 1.6 times using octyl-VS-EDA-CALB-EDA. Both aminated biocatalysts presented similar activities, as octyl-VS-EDA-CALB was initially more active. The modification of octyl-CALB with glutaraldehyde (octyl-CALB-Glu) increased its activity also by 1.9 fold, while octyl-EDA-CALB-Glu left the activity almost unaltered. Now, the covalent and Glu modified preparation was less active than the just interfacially activated and modified enzyme. These differences with the effects of the glutaraldehyde modification of octyl-CALB and octyl-VS-EDA-CALB could be caused by the changes in the support surface features (increasing its hydrophobicity). Moreover, in both cases, all external primary amino groups in the biocatalysts will be modified with one molecule of Glu, and inter or intramolecular crosslinkings can be promoted by the glutaraldehyde treatment, while some enzyme-support covalent bonds may be only formed in the case of octyl-VS-EDA-CALB (that could distort the enzyme structure). This could explain the different effect of the modification with glutaraldehyde of both biocatalysts. Finally, both aminated biocatalysts were modified with glutaraldehyde. Octyl-CALB–EDA-Glu further increased its activity by almost 1.7 fold, which meant that the activity versus *p*NPB at pH 7 of octyl-CALB was enhanced by 3.2 fold after the double modification. Octyl-VS-EDA-CALB-EDA increased its activity by around 1.3 fold after Glu modification. This result is better than the one obtained when modifying with glutaraldehyde octyl-VS-EDA-CALB, although now all phenomena that we described before may be produced with more intensity. Now, the effect of the modification with glutaraldehyde of the aminated enzyme is clearly positive: the activity of octyl-VS-EDA-CALB-EDA-Glu doubles that of octyl-VS-EDA-CALB-Glu.

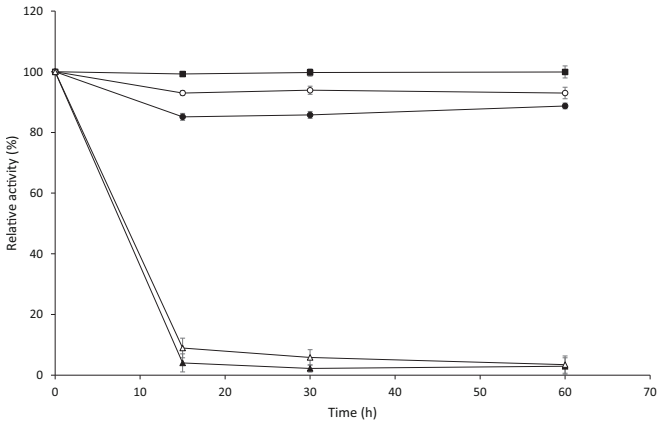

Fig. 1. Immobilization course of CALB offering 1 mg of enzyme per g of support on octyl (solid symbols) at pH 5 and octyl-VS (empty symbols) at pH 7.0 both at 25 °C. Square: reference; Circle: suspension; Triangle: supernatant.

3.3. Specificity of the different CALB formulations

To analyze in a deeper way the effects of the different treatments on enzyme activity, the biocatalyst activity has been determined using the different substrates mentioned in introduction (Table 1). As triacetin needs to be hydrolyzed at pH 5 to prevent acyl-migration [88], activity of the biocatalyst versus *p*NPB was also determined at pH 5.

Starting with the effect of the change of pH in the *p*NPB activity determination, all CALB biocatalysts were more active versus *p*NPB at pH 7 than at pH 5 (it should be remarked that we are measuring in the isobestic point of the *p*NP). However, differences were higher using octyl-CALB and their derivatives than using octyl-VS-EDA-CALB derivatives. In this last case, all 4 preparations decreased their activity by around 30 %. The *p*NPB activity of the octyl-CALB biocatalysts decreased by lowering the measure pH to 5 to 67 % for the unmodified biocatalyst. This change in the pH lowered the activity down to 48 % using octyl-CALB-EDA and octyl-CALB-Glu, being the changes in activity of the double modified biocatalyst in between (57 %). That way, the effects of the pH in activity versus *p*NPB were very dissimilar for the different CALB formulations, being this specific case apparently very relevant in the immobilization technique. While the activity at pH 7 of octyl-CALB after both single modifications greatly increased, at pH 5 the effects were much smaller (remaining similar for Glu and EDA modifications). Using octyl-VS-EDA-CALB, the modification with EDA remains very positive also measuring the activity at pH 5, and is almost neutral if using Glu. The double modification remains positive for both biocatalysts also when determining the activity versus *p*NPB at pH 5.

Analyzing the enzyme activities at pH 5 versus triacetin, the activities were lower than when using *p*NPB for all preparations, even using a much higher substrate concentration. Octyl-VS-EDA-CALB is more than 50 % more active than octyl-CALB (a higher difference than using *p*NPB as substrate). The effects of the modifications followed the same trends as using *p*NPB, but the changes presented a different magnitude. Octyl-CALB increased its activity after amination by more than 1.9 fold (similar to the changes of activity versus *p*NPB at pH 7, but much larger if comparing the activities of both biocatalysts versus *p*NPB at pH 5), however octyl-CALB-Glu only increased the activity by less than 30 %, similar to the increase in activity versus *p*NPB at pH 5. Again, the double

Table 1
Activity (U/g) of different immobilized CALB biocatalysts using different substrates under defined conditions.

Activity (U/g of Biocatalyst)					
Biocatalyst	pNPB at pH 5.0	pNPB at pH 7.0	Triacetin	*R*-mandelate	*S*-mandelate
octyl-CALB	6.08 ± 0.22	8.98 ± 0.41	2.23 ± 0.09	15.08 ± 0.57	1.36 ± 0.07
octyl-CALB-EDA	8.29 ± 0.31	17.19 ± 0.62	4.33 ± 0.25	17.70 ± 0.84	1.84 ± 0.08
octyl-CALB-EDA-Glu	16.67 ± 0.65	28.92 ± 1.43	7.88 ± 0.28	14.46 ± 0.58	1.77 ± 0.07
octyl-CALB-Glu	8.31 ± 0.26	17.14 ± 0.79	2.84 ± 0.15	16.06 ± 0.64	1.76 ± 0.04
octyl-VS-EDA-CALB	7.37 ± 0.28	10.64 ± 0.42	3.44 ± 0.19	11.65 ± 0.59	1.47 ± 0.05
octyl-VS-EDA-CALB-EDA	14.11 ± 0.55	16.80 ± 0.81	5.62 ± 0.32	11.88 ± 0.73	1.62 ± 0.05
octyl-VS-EDA-CALB-EDA-Glu	15.61 ± 0.60	22.21 ± 0.98	7.75 ± 0.28	13.94 ± 0.77	1.57 ± 0.04
octyl-VS-EDA-CALB-Glu	7.77 ± 0.24	11.20 ± 0.46	3.02 ± 0.09	15.35 ± 0.81	1.73 ± 0.06

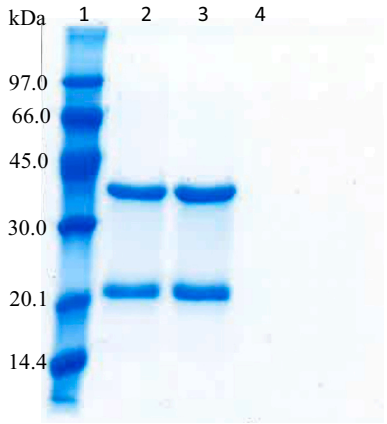

Fig. 2. SDS-PAGE analysis of free and immobilized CALB preparations. Lane 1: Low molecular weight standard, Lane 2: free CALB, Lane 3: Octyl-CALB and Lane 4: Octyl-VS-EDA-CALB. Further specifications can be found on the methodology section.

modification provided the highest activity, octyl-CALB-EDA-Glu increased the activity by more than 3.5 fold compared to octyl-CALB, more than with *p*NPB at pH 5 and even slightly more than at pH 7 with this substrate. The amination of octyl-VS-EDA-CALB produced a further increase in the enzyme activity (by 63 %), while the modification with glutaraldehyde produced a slight decrease in activity (to 88 %). These results followed the same trend than using *p*NPB at pH 5, but the values are different and now Glu modification promotes a slight decrease in enzyme activity instead of a slight increase. Octyl-VS-EDA-CALB-EDA-Glu is again the most active formulation of these biocatalysts group (increasing the activity of the aminated biocatalyst by 1.4 fold), with a final activity very similar to the double modified octyl-CALB biocatalyst. Again, glutaraldehyde modification effects on octyl-VS-EDA-CALB-EDA are quite different from the effects of this modification using octyl-VS-EDA-CALB (in the first case promoting an increase in enzyme activity while promoting a decrease in enzyme activity when using the second).

Analyzing the effects of the modifications using with both enantiomers of methyl mandate at pH 5 to determine the enzyme activity, the *R* isomer gave the highest activates in both initial biocatalyst among the studied substrates. In fact, the *R* isomer is the preferred isomer for all biocatalysts.

Going in detail, octyl-CALB hydrolyzed more than 11 times faster the *R* than the *S* isomer, while using octyl-VS-EDA-CALB this factor was 8. Using *R*-methyl mandelate, the covalently immobilized biocatalyst presented 77 % of the activity of the just adsorbed enzyme (in contraposition with the results found using the other two substrates, where the activity was higher). In fact, using the *S* isomer, the covalent biocatalyst is again more active than the just adsorbed enzyme, although by less than 10 %. The amination effects on the activity are also different than using the other substrates, octyl-CALB-EDA increased its activity versus the R isomer by less than 15 % and by 35 % using the *S* isomer. This decreased the activities ratio with both isomers to less than 10. The modification with glutaraldehyde increased the activity versus *R*-methyl mandelate of octyl-CALB by 5 % and by 30 % using the *S* isomer (this again decreased the activity rations to more than 9). The largest difference with previous results is octyl-CALB-EDA-Glu, that is less active than the aminated biocatalyst using *R*-methyl mandelate (by almost 20 %) or even than the unmodified biocatalyst (by less than 5 %) (when for the other substrates this double modification provided the highest activities). This double modification decreased the activity of the aminated enzyme by less than 5 %, but increased the activity when compared to the unmodified biocatalyst by 30 % using the *S* isomer (activities ratio decreased to around 8 after this double modification). That is, the chemical modification affected the enzyme activity versus the different substrates in a very diverse fashion, for example, using only the octyl biocatalysts, the most active biocatalyst using triacetin was octyl-CALB-EDA-Glu while using both isomers of methyl mandelate, it was octyl-CALB-EDA.

The amination of octyl-VS-EDA-CALB has a low impact in the activity of the biocatalyst using both isomers of methyl mandelate (a 2 % increase using *R*- methyl mandelate and 10 % increment using *S*-methyl mandelate) (the activities ratio decreased to 7.3), in contraposition with the higher increase in the activity using the just physically immobilized enzyme promoted by this modification. Octyl-VS-EDA-CALB-Glu was 30 % more active using the *R* isomer and only a 17 % more active using the *S* isomer (activities ratio increased to almost 9) compared to the unmodified biocatalyst. The octyl-VS-EDA-CALB-EDA modification with Glu increased the activity versus *R*-methyl mandelate (by 10 %) while it remained almost unaltered versus the S isomer (it decreased by less than 4 %) (the activities ratio increased to almost 9).

That way, the chemical modification of the immobilized CALB produced changes in the activities of the biocatalysts that differ when using different substrates, some modifications are clearly positive for the enzyme activity versus some substrates while they are almost neutral or negative when using other substrates. These changes are different depending on the initial biocatalyst (results are different using octyl or octyl-VS biocatalysts). It is like generating new lipase structures, which will have different catalytic properties that cannot be predicted if the exact new enzyme structures cannot be predicted [34].

3.4. Determination of the kinetic constants of the different biocatalyst using the different substrates

Attempts to determine the Michaelis–Menten kinetic parameters of the different biocatalysts were unsuccessful due to the observation of a strictly first-order kinetic regime across the entire range of substrate concentrations tested, within the solubility limits of the substrate. For all biocatalysts, the initial reaction rate increased linearly with substrate concentration: doubling the substrate concentration led to a proportional doubling of the observed activity. This indicates that enzyme saturation by the substrate is far from being achieved under the tested conditions and suggests that the apparent Michaelis constant (Km) is significantly higher than the maximum soluble concentration of the

substrates, likely in the molar range. Such behavior is not uncommon in immobilized lipases adsorbed via interfacial activation on hydrophobic supports, where the active site is exposed and oriented toward the support acyl layer, although it is not commonly studied [95].

In these systems, substrate partitioning between the aqueous phase and the hydrophobic acyl layer, where the active center of the enzyme is oriented, may significantly influence the effective substrate concentration at the active site. Therefore, the substrate concentration in the bulk phase does not necessarily reflect the local concentration experienced by the enzyme. Even with this problem, the enzyme activity can experience a significant increase after enzyme immobilization. Given these limitations, classical kinetic parameter estimation (Km and Vmax) was not feasible. Instead, relative activity ratios, as discussed in the previous section, provide more meaningful comparative insights. In this situation, these ratios are directly given the enzyme apparent enantiospecificity.

Furthermore, since enzyme inactivation is generally expected to result in an increase in Km due to partial active site deformation, our focus shifted to analyzing the temperature dependence of enzyme activity. In particular, we concentrated on evaluating activation energy (Ea) and residual activity with a range of structurally diverse substrates as alternative indicators of biocatalyst performance and stability.

3.5. Determination of activation energy of the different biocatalyst in the hydrolysis of triacetin

Table 2 shows the apparent activation energy Ea values calculated for the hydrolysis of triacetin catalyzed by the different biocatalysts, as determined according to the Arrhenius method described in Methods. These values were analyzed in relation to the observed catalytic activities to assess whether variations in Ea could be directly correlated to changes in enzyme performance. In some instances, a lower Ea was associated with a higher specific activity, supporting the classical interpretation that Ea reflects the energetic barrier for the rate limiting step. For example, the amination of octyl-CALB, which significantly increased enzyme activity, is correlated to a slight decrease in the Ea of the reaction. This decrease in activation energy was larger when modifying the enzyme with glutaraldehyde, although octyl-CALB-Glu presented lower activities than the aminated biocatalyst, and only slightly improved the activity compared to the unmodified biocatalyst. This result contradicts a simple inverse correlation between Ea and observed activity of the different biocatalysts: if Ea was the predominant factor of catalytic efficiency, the aminated enzyme would be expected to have a lower Ea than the glutaraldehyde-modified one, which is not the case. The double modified biocatalyst (octyl-CALB-EDA-Glu) presented an Ea similar to the biocatalyst only modified with Glu and lower than using the aminated biocatalyst. However, the activity was significantly higher using this biocatalyst than employing the one just modified with Glu, further challenging the notion that Ea alone governs observed enzyme activity. A similar lack of correlation was observed between octyl-CALB and octyl-VS-EDA-CALB: the latter displayed higher activity (~20 %) but also a higher Ea. Again, this inconsistency suggests that activation energy does not fully account for the differences in biocatalyst performance.

Table 2
Activation energy (Ea) values for different immobilized CALB biocatalysts in the hydrolysis of triacetin.

Biocatalysts	Ea (kJ/mol)
octyl-CALB	33.96
octyl-CALB-EDA	32.06
octyl-CALB-EDA-Glu	29.14
octyl-CALB-Glu	29.44
octyl-VS-EDA-CALB	41.24
octyl-VS-EDA-CALB-EDA	29.48
octyl-VS-EDA-CALB-EDA-Glu	34.55
octyl-VS-EDA-CALB-Glu	30.68

Octyl-VS-EDA-CALB-EDA was more active than the unmodified biocatalyst, and the Ea decreased in a very significant way (decreasing by 1.4 fold), in this instance Ea and activity changes seem to be correlated. Nevertheless, other examples again undermine a consistent trend: three CALB biocatalysts all exhibited Ea values around 29 kJ/mol but differed significantly in their activities (Table 2). Octyl-VS-EDA-CALB-Glu had a slightly higher Ea than the aminated version yet it was markedly less active. Octyl-VS-EDA-CALB-EDA-Glu, which displayed enhanced activity compared to the singly modified counterparts, had a higher Ea than the aminated variant.

Taken together, these observations indicate that changes in Ea cannot fully explain the variations in biocatalyst activity. This suggests that other mechanistic factors, such as the relative ease of acyl-enzyme intermediate formation, may exert a strong influence on the observed rate. Modifications that improve substrate binding or orientation—without necessarily stabilizing the transition state—could facilitate acylation steps, leading to increased activity despite a higher overall Ea. In this sense, the apparent Ea determined under first-order conditions likely reflects changes in both Kcat and the apparent Km. Since the kinetic regime was first order in all cases (see Section 3.3), direct estimation of Km was not possible; however, one can postulate that alterations in Km could mask or counterbalance changes in Kcat. Specifically, an increase in Kcat accompanied by an unfavorable shift in Km may still yield comparable or reduced activity under conditions far from substrate saturation.

Therefore, while Ea can serve as a useful descriptor of the energetic profile of the reaction, it should not be interpreted in isolation when evaluating the effect of enzyme modification on catalytic performance. The lack of direct correlation observed here undermines the complexity of enzyme–substrate interactions in immobilized systems and the importance of considering multiple kinetic and structural factors simultaneously. Indeed, apparent activation energies involved both enthalpic and entropic contributions. This has been the subject of different papers where Ea of the free and immobilized enzymes do not fit the changes in activity, giving different explanations for these results [67–69].

3.6. Enzyme inactivation of the different CALB biocatalysts at pH 5, 7 and 9 using pNPB as substrate to determine the enzyme residual activity

Figs. 3–5 shows the inactivation courses at pH 5, 7 and 9 of the

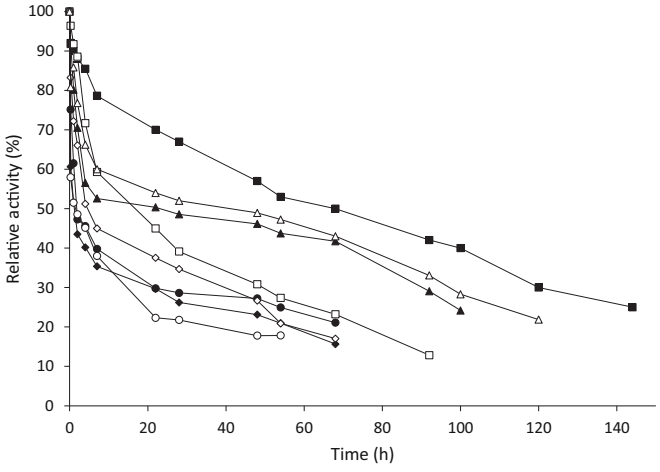

Fig. 3. Thermal inactivation courses of lowly loaded CALB immobilized on different octyl-agarose preparations, using 0.4 mM p-NPB as the substrate. The inactivation was performed using 10 mM of sodium acetate buffer at pH 5.0 at 70 °C. Symbols: Full square: octyl-CALB. Full circle: octyl-CALB-EDA. Full diamond: octyl-CALB-EDA-Glu. Full triangle: octyl-CALB-Glu. Empty square: octyl-VS-EDA-CALB. Empty circle: octyl-VS-EDA-CALB-EDA. Empty diamond: octyl-VS-EDA-CALB-EDA-Glu. Empty triangle: octyl-VS-EDA-CALB-Glu.

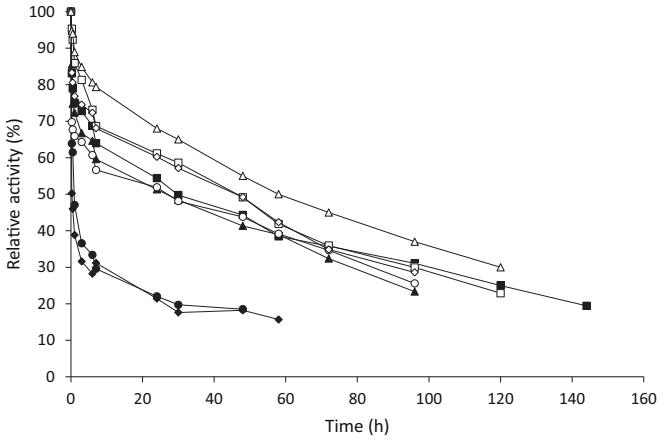

Fig. 4. Thermal inactivation courses of lowly loaded CALB immobilized on different octyl-agarose preparations, using 0.4 mM p-NPB as the substrate. The inactivation was performed using 10 mM of sodium phosphate buffer at pH 7.0 at 62 °C. Symbols: Full square: octyl-CALB. Full circle: octyl-CALB-EDA. Full diamond: octyl-CALB-EDA-Glu. Full triangle: octyl-CALB-Glu. Empty square: octyl-VS-EDA-CALB. Empty circle: octyl-VS-EDA-CALB-EDA. Empty diamond: octyl-VS-EDA-CALB-EDA-Glu. Empty triangle: octyl-VS-EDA-CALB-Glu.

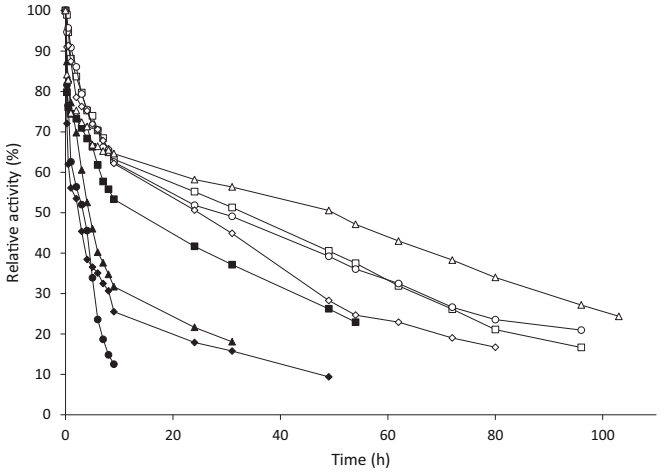

Fig. 5. Thermal inactivation courses of lowly loaded CALB immobilized on different octyl-agarose preparations, using 0.4 mM p-NPB as the substrate. The inactivation was performed using 10 mM of sodium carbonate buffer at pH 9.0 at 55 °C. Symbols: Full square: octyl-CALB. Full circle: octyl-CALB-EDA. Full diamond: octyl-CALB-EDA-Glu. Full triangle: octyl-CALB-Glu. Empty square: octyl-VS-EDA-CALB. Empty circle: octyl-VS-EDA-CALB-EDA. Empty diamond: octyl-VS-EDA-CALB-EDA-Glu. Empty triangle: octyl-VS-EDA-CALB-Glu.

different biocatalysts following the activity with *p*NPB. Not only the qualitative stability of the biocatalysts depends on the pH value, also the qualitative results are strongly dependent on the inactivation pH, changing the most and least stable biocatalysts.

Inactivating the enzyme at pH 5 (Fig. 3), the biocatalyst that lost the activity in the slowest fashion is octyl-CALB. Octyl-VS-EDA-CALB maintains around 45 % of the initial activity after 22 h, while octyl-CALB retained 70 % of the activity after that time, reaching a 45 % of residual activity after around 90 h. The effect of the chemical modification is very different for both immobilized biocatalysts. In the case of octyl-CALB, all the modifications reduced the enzyme stability, the amination presented a very negative effect on the enzyme stability, while the enzyme biocatalyst stability is almost unaffected by the further modification with Glu (this double modified biocatalyst became less stable than the octyl-VS-EDA-CALB). The modification of the adsorbed enzyme with glutaraldehyde decreases enzyme stability, but to a smaller extent than after amination. Using octyl-VS-EDA-CALB, its amination also produced a very negative effect on the enzyme stability, this is the least stable biocatalyst among all the studied ones. The modification with glutaraldehyde of this aminated biocatalyst is able to improve the enzyme stability, approaching the inactivation course to that of the unmodified biocatalyst. The most stable preparation using the covalent biocatalyst is that obtained by the modification with Glu, this became with a higher stability than octyl-CALB-Glu, but was still less stable than octyl-CALB.

As presented above, at pH 5, octyl-VS-EDA-CALB is significantly less stable than octyl-CALB. This can be related to the recently described negative effect of the presence of aminated groups in the support surface in the case of this enzyme [96,97]. A drastic enzyme destabilization is observed for both biocatalysts after amination. Using octyl-VS-EDA-CALB-EDA, amination will change possible ionic attraction forces between anionic groups in the enzyme and the cationic groups in the support by repulsion forces. At pH 5, both the EDA groups in the support and the cationic groups in the enzyme (now including the modified carboxylic groups) will be as cations, increasing the repulsion forces. Whatever the cause, CALB is clearly destabilized under these conditions. The treatment with Glu has a negative impact for octyl-CALB stability, while it is clearly positive in the case of octyl-VS-EDA-CALB. This suggested that the one-point chemical modification with Glu of the enzyme is negative (although some intra or intermolecular crosslinkings can be established, the main modification is the one point modification of the primary amino groups), but that using octyl-VS-EDA-CALB-Glu some additional covalent enzyme-support bonds are formed and these bonds will have a positive effect on the enzyme stability. Additionally, it cannot be discarded that the modification of all amino primary groups in the biocatalyst (enzyme and support) with glutaraldehyde can somehow reduce the enzyme-support ionic interactions that apparently are negative for enzyme stability. The Glu modification of the aminated preparations should facilitate the introduction of inter and more intramolecular crosslinkings [77,84]. However, it did not seem to be very effective in improving the stability of octyl-CALB-EDA, while it was very positive for the stability of octyl-VS-EDA-CALB-EDA, suggesting that the promotion of more enzyme-support bonds is more relevant than the formation of enzyme intra or inter crosslinkings.

The situation was very different if the biocatalysts are inactivated at pH 7 (Fig. 4). Now, octyl-VS-EDA-CALB is more stable than octyl-CALB, at least in the first hours of inactivation, becoming the remaining activities more similar later. The pK of the two amino groups in the immobilized EDA are around 6.8 and more than 10 [98], so at pH 7 the cationic nature of the support is reduced compared to pH 5. The amination of octyl-CALB remains a very negative effect on enzyme stability, and its modification with Glu has almost no effect (even though some inter or intra crosslinkings can be introduced in the enzyme molecules). The modification with Glu of octyl-CALB still presenting a small but negative effect on the stability of this biocatalyst. The situation is quite different studying octyl-VS-EDA-CALB. Its amination produced a slight destabilization of the enzyme, while the Glu modification improved the enzyme stability for both covalent biocatalysts, using the aminated enzyme the stability became similar to that of the unmodified biocatalyst, while using the non-aminated biocatalyst, the stability became the highest among all studied biocatalysts.

The situation is again different when inactivating the biocatalyst at pH 9 (Fig. 5). Now, all octyl-VS-EDA-CALB biocatalysts are clearly more stable than the octyl-CALB biocatalysts. Octyl-CALB-EDA was the least stable biocatalyst, in this instance the treatment with glutaraldehyde of aminated and non-aminated biocatalyst giving similar stabilities, both with lower stability than octyl-CALB. Octyl-VS-EDA-CALB-EDA was also the least stable among the covalent preparations, but under these inactivation conditions, it was more stable than octyl-CALB. The Glu treatment permitted to improve the stability of this aminated biocatalyst; the inactivation course became similar to that of the unmodified covalent biocatalyst. However, octyl-VS-EDA-CALB modification with

Glu had a very positive effect on the enzyme stability, becoming this preparation the most stable one.

That way, the effect of the chemical modification on the enzyme stability depends on the immobilization protocol and on the inactivation conditions. This, coupled to the different effect of the modifications of the enzyme activity versus different substrates, exemplifies the difficulties in selecting an optimal biocatalyst for a specific application from partial studies.

3.7. Changes on enzyme specificity during inactivation under different conditions

Next, we have evaluated the inactivation courses using all the substrates previously utilized. The activities were determined with all the substrates when the activity versus pNPB decrease to around 50 and 25 %, after leaving the samples by a minimum of 24 h at 4–6 °C to permit any enzyme refolding (references).

Regarding the recovered of activity during the storage using *p*NPB, some facts may be mentioned (Table 3). When the biocatalysts were inactivated at pH 5, octyl-CALB maintained almost the same activity that was detected during inactivation, while octyl-CALB-EDA and octyl-CALB-EDA-Glu improved their activity (by around 10 %) and octyl-CALB-Glu improved the activity from 56 % to 80 %. Using the samples with an activity around 25 %, only octyl-CALB-EDA-Glu showed a significant improvement on the enzyme activity after incubation (from 28 % to 44 %). Octyl-VS-EDA-CALB also recovered some activity during storage, octyl-VS-EDA-CALB-Glu was again the one with a higher activity recovery, although also the unmodified biocatalyst was quite reactivated. Similarly, the samples inactivated (and incubated at 4 °C) at pH 7 and 9 presented different levels of reactivation depending on the biocatalyst. This will have an effect on the "inactivation" courses presented below.

Table 3
Residual activity of different immobilized CALB biocatalysts in the inactivation courses at pH 5.0 and 70 °C, pH 7.0 and 62 °C and pH 9.0 and 55 °C, and reactivation after 24 h.

	Residual activity (%)				
		*p*NPB			
	Biocatalyst	50 % inactivation	After 24 h	25 % inactivation	After 24 h
pH 5	octyl-CALB	57.76	55.78	22.05	25.92
	octyl-CALB-EDA	47.26	56.53	27.19	26.04
	octyl-CALB-EDA-Glu	49.28	56.72	23.48	24.42
	octyl-CALB-Glu	56.53	81.47	24.18	45.62
	octyl-VS-EDA-CALB	50.65	56.86	28.49	41.92
	octyl-VS-EDA-CALB-EDA	57.96	69.99	24.32	32.95
	octyl-VS-EDA-CALB-EDA-Glu	51.43	66.22	26.97	31.29
	octyl-VS-EDA-CALB-Glu	55.52	85.71	28.28	44.47
pH 7	octyl-CALB	52.50	58.75	23.94	40.39
	octyl-CALB-EDA	58.59	65.57	27.13	21.79
	octyl-CALB-EDA-Glu	57.44	66.72	28.44	30.97
	octyl-CALB-Glu	50.61	54.64	28.57	31.86
	octyl-VS-EDA-CALB	52.57	66.08	26.35	50.37
	octyl-VS-EDA-CALB-EDA	51.79	58.67	24.86	31.29
	octyl-VS-EDA-CALB-EDA-Glu	48.74	45.08	23.35	38.19
	octyl-VS-EDA-CALB-Glu	53.11	73.53	21.50	46.63
pH 9	octyl-CALB	51.50	50.36	23.03	37.72
	octyl-CALB-EDA	49.76	56.96	24.76	29.04
	octyl-CALB-EDA-Glu	54.22	66.68	23.87	27.82
	octyl-CALB-Glu	54.98	60.66	26.54	35.00
	octyl-VS-EDA-CALB	51.87	70.75	25.17	47.93
	octyl-VS-EDA-CALB-EDA	56.75	71.00	27.49	37.07
	octyl-VS-EDA-CALB-EDA-Glu	49.57	63.28	26.90	49.47
	octyl-VS-EDA-CALB-Glu	51.12	67.95	25.22	37.64

Figs. 6–8 shows the activity recoveries of samples of the biocatalysts withdrawn when the activity versus *p*NPB at pH 7 was around 50 and 25 %, during inactivation at pH 5, 7 and 9, after 24 h of incubation at 4 °C under the same conditions using the different substrates. Figs. 1S–3S shows the comparison of the inactivation courses of each biocatalyst following the activity using the different substrates.

An obvious fact is the very different inactivation courses obtained when using different substrates to determine the residual activity, and that not all biocatalysts have the same response to the change in the substrate to follow the activity. The dissimilar change of the activity versus the different substrates indicates that the new enzyme structures generated during the inactivation (and reactivation) have different catalytic features, altering enzyme specificity. These changes depend on the enzyme formulation and inactivation conditions, suggesting that this partially inactivated enzymes may have very different conformations and that way, very different catalytic features, as it has been previously reported [70–72,85].

For example, in inactivations at pH 5, octyl-CALB presented similar activity losses using *p*NPB and triacetin, these substrates providing the lowest stability for this biocatalyst, while both isomers of methyl mandelate gave better and similar inactivation courses (Fig. 1S). Compared to the covalent preparation, there are some differences. Using this biocatalyst, its stability was not very different using the diverse substrates, although triacetin provides the lowest enzyme stability and *S*-methyl mandelate the second highest one. Using *R*-methyl mandelate, in the first inactivation time, the biocatalyst showed an increase in enzyme activity (Fig. 1S). Octyl-CALB-EDA presented similar inactivation courses with 3 of the substrates, only using triacetin the stability was lower. The modification with Glu maintained the stability with triacetin being the lowest one, using *R*-methyl mandelate in the first point a clear increase in enzyme activity was detected in the first inactivation time, being the *S*-isomer the one that provided the most similar recovered activity in the second inactivation point. Octyl-CALB-Glu gave a similar inactivation course compared to the previous one. Using the covalently immobilized enzyme, the amination promoted a higher activity recovery using the *S*-isomer compared to the initial biocatalyst, the other activity recoveries following the trend of this biocatalyst. Octyl-VS-EDA-CALB-EDA-Glu presented similar stabilities with all substrate except triacetin (that again gave the lowest stability), no hyperactivation using *R*-methyl mandelate was detected. Octyl-VS-EDA-CALB-Glu gave some hyperactivation in the first inactivation time using the R-isomer, maintaining triacetin as the substrate that produced the lowest stability (Fig. 1S). These changes produced some changes in the comparison of the different biocatalyst inactivated at pH 5 (Fig. 6). Using pNPB as substrate to determine the residual activity, the biocatalyst that lost the activity in a slower way was octyl-VS-EDA-CALB-Glu, shortly followed by octyl-CALB-Glu and octyl-CALB (this already differs from the results obtained using the direct measurement of the activity without reactivation), the least stable biocatalyst was octyl-CALB-EDA-Glu, shortly followed by octyl-VS-EDA-CALB-EDA. Using triacetin, octyl-CALB is the most stable preparation, and octyl-CALB-EDA is the preparation with the lowest stability. Using *R*-mandelate, many of the biocatalyst show an increase in the activity for the first time. Focusing on the activity retention in the second point, octyl-CALB is the most stable preparation followed by octyl-VS-EDA-CALB-Glu, while octyl-CALB-EDA is the biocatalyst with the lowest stability shortly followed by both double modified biocatalysts. Using *S*-methyl mandelate to determine the

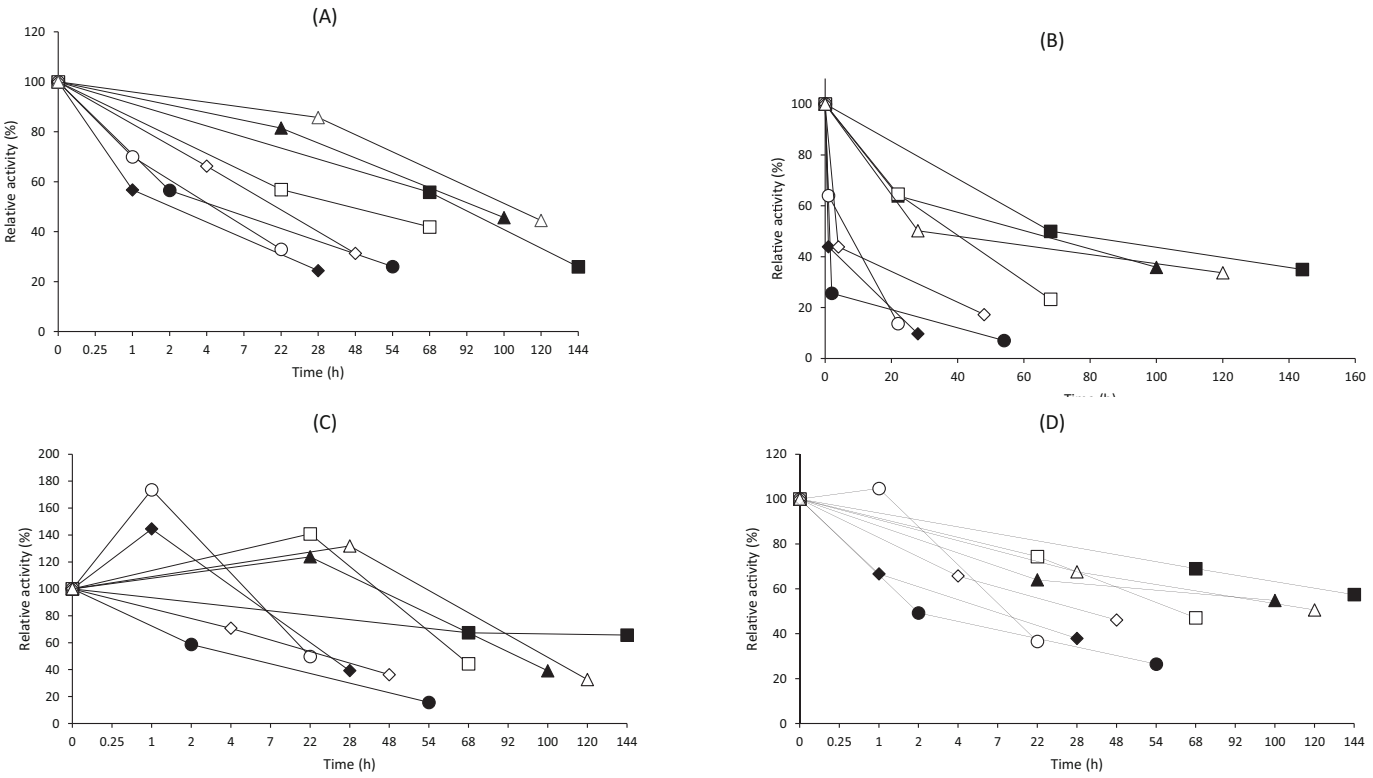

Fig. 6. Relative activity of lowly loaded CALB biocatalysts during inactivation courses at pH 5.0 and 70 °C, using different substrates: (A) pNPB; (B) triacetin; (C) (*R*)-methyl mandelate and (D) (*S*)-methyl mandelate. Symbols: Full square: octyl-CALB. Full circle: octyl-CALB-EDA. Full diamond: octyl-CALB-EDA-Glu. Full triangle: octyl-CALB-Glu. Empty square: octyl-VS-EDA-CALB. Empty circle: octyl-VS-EDA-CALB-EDA. Empty diamond: octyl-VS-EDA-CALB-EDA-Glu. Empty triangle: octyl-VS-EDA-CALB-Glu.

residual activity, the most stable preparation was again octyl-CALB, followed by both biocatalyst modified with Glu, although initially octyl-VS-EDA-CALB-EDA showed a certain increase in the activity, it becomes the second with the lowest recovered activity in the second point, the least stable preparation with this substrate was octyl-CALB-EDA.

At pH 7 the picture is different. Triacetin provided the lowest stability in all cases (Fig. 2S), although the use of *S*-methyl mandelate to determine the activity gave similar profiles using both covalent preparations modified with glutaraldehyde to those using triacetin. *p*NPB gave the highest stability using octyl-CALB, while gave the second worst inactivation courses using octyl-VS-EDA-CALB, octyl-VS-EDA-CALB-Glu and octyl-VS-EDA-CALB-EDA-Glu, in other instances giving very similar inactivation courses to the use of *S*-methyl mandelate. *R*-methyl mandelate gave some hyperactivation in the first time using octyl-CALB-EDA-Glu, octyl-VS-EDA-CALB and octyl-VS-EDA-CALB-EDA, generally giving the highest stabilities. That way, using *p*NPB, octyl-CALB-EDA and octyl-CALB-EDA-Glu are the least stable biocatalyst (Fig. 7), being octyl-VS-EDA-CALB –Glu and octyl-VS-EDA-CALB the most stable ones. Using triacetin, the biocatalysts with a most different (and lower) stability were octyl-CALB-EDA and octyl-CALB-EDA-Glu, being again octyl-VS-EDA-CALB-Glu and octyl-VS-EDA-CALB the most stable ones. Using *R*-methyl mandelate, a certain hyperactivation is observed in the first time except for octyl-CALB-EDA, octyl-CALB-EDA and octyl-CALB-EDA-Glu remained the least stable preparations, but using this substrate, octyl-VS-EDA-CALB-Glu was the least stable among the covalent preparations, octyl-VS-EDA-CALB became the most stable biocatalyst (Fig. 7).

At pH 9, again there are some differences. *p*NPB is the substrate that gave the highest stability using octyl-CALB (Fig. 3S), while *R*-methyl mandelate (that permitted to observe some enzyme hyperactivation in the first inactivation point) gave the highest stability for all covalently immobilized enzyme formulations, and gave similar inactivation courses to the ones obtained using *p*NPB when studying octyl-CALB-EDA and octyl-CALB-Glu. Triacetin was the substrate that promoted the highest inactivation rates for all biocatalysts except both covalent biocatalyst modified with Glu, which gave inactivation curses similar to those obtained using triacetin or *S*-methyl mandelate. That way, Fig. 8 shows that the covalent biocatalysts remained more stable than the only physically immobilized enzymes using all substrates. The least stable biocatalyst always is octyl-CALB-EDA, while the most stable one depends on the substrate, octyl-VS-EDA-CALB-Glu is the most stable using triacetin and *p*NPB (shortly followed by octyl-VS-EDA-CALB), using both isomers of methyl mandelate the most stable preparation was octyl-VS-EDA-CALB, with octyl-VS-EDA-CALB-Glu and octyl-VS-EDA-CALB-EDA showing very similar inactivation courses.

That way, the comparison between the different biocatalysts stability depends on the used substrate and the inactivation conditions, suggesting that only a comparison under "real" conditions of the different biocatalysts permits to really define the optimal biocatalyst, as occurred when analyzing the enzyme activity.

However, the main objective of this paper is not to prepare industrial biocatalysts of CALB (although some of the new biocatalysts offer increased activity and stabilities), but to analyze if the catalytic parameters of the enzyme can be correlated with the loses in activity. As Kcat and Km cannot be determined, we have focused in the Ea of the hydrolysis of triacetin catalyzed by the partially inactivated biocatalysts. Triacetin was selected due to its higher stability compared to methyl mandelate.

3.8. Changes of the Ea of the reaction of hydrolysis of triacetin catalyzed by different immobilized biocatalyst at different inactivation values

Figs. 9–11 show the variation in the apparent activation energy for

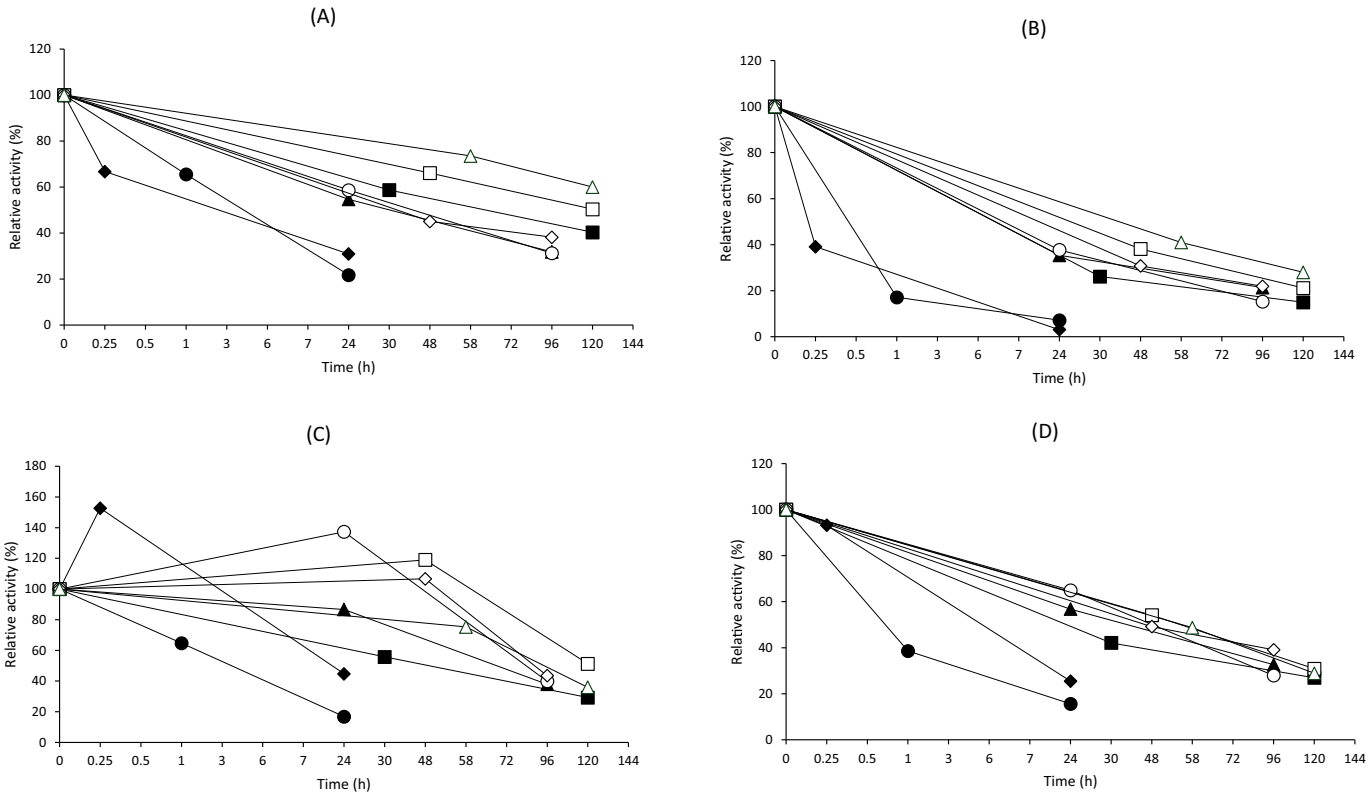

Fig. 7. Relative activity of lowly loaded CALB biocatalysts during inactivation courses at pH 7.0 and 62 °C, using different substrates: (A) pNPB; (B) triacetin; (C) (*R*)-methyl mandelate and (D) (*S*)-methyl mandelate. Symbols: Full square: octyl-CALB. Full circle: octyl-CALB-EDA. Full diamond: octyl-CALB-EDA-Glu. Full triangle: octyl-CALB-Glu. Empty square: octyl-VS-EDA-CALB. Empty circle: octyl-VS-EDA-CALB-EDA. Empty diamond: octyl-VS-EDA-CALB-EDA-Glu. Empty triangle: octyl-VS-EDA-CALB-Glu.

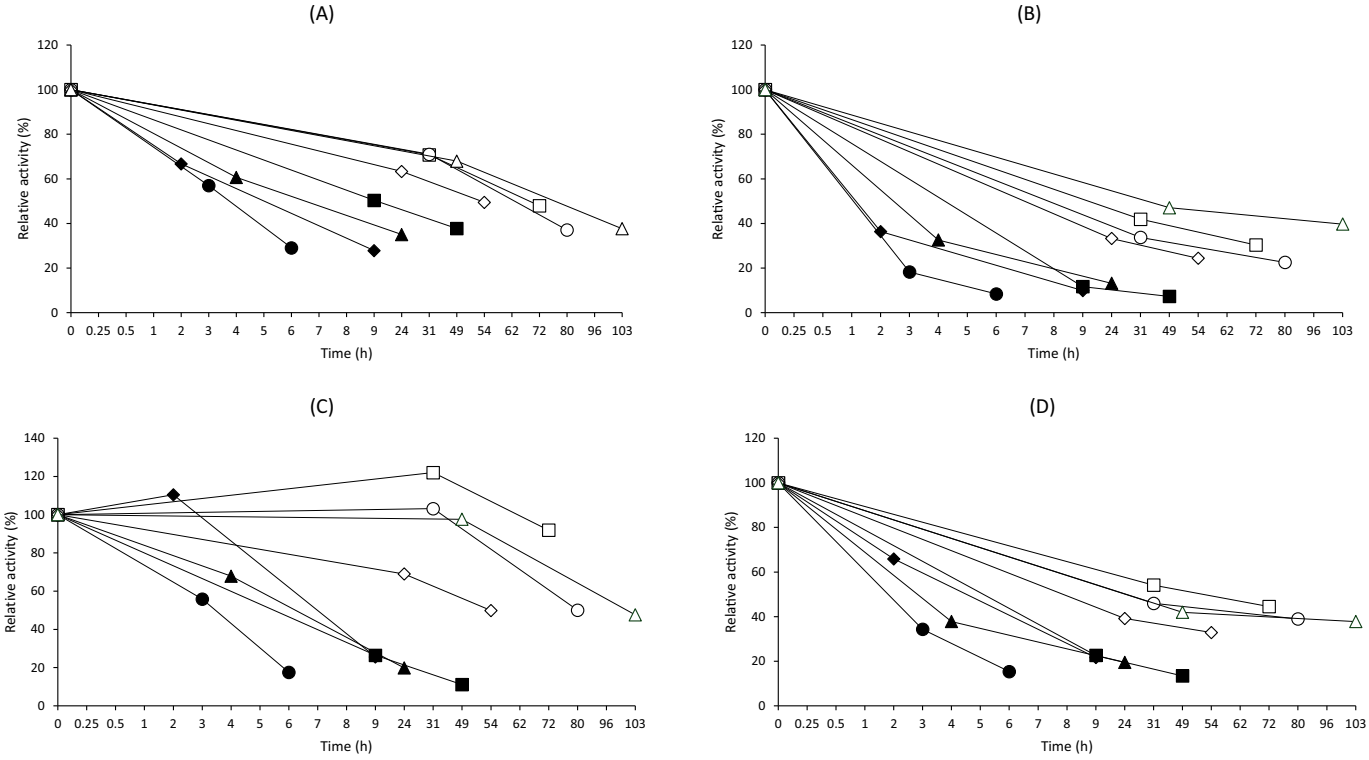

Fig. 8. Relative activity of lowly loaded CALB biocatalysts during inactivation courses at pH 9.0 and 55 °C, using different substrates: (A) pNPB; (B) triacetin; (C) (*R*)-methyl mandelate and (D) (*S*)-methyl mandelate. Symbols: Full square: octyl-CALB. Full circle: octyl-CALB-EDA. Full diamond: octyl-CALB-EDA-Glu. Full triangle: octyl-CALB-Glu. Empty square: octyl-VS-EDA-CALB. Empty circle: octyl-VS-EDA-CALB-EDA. Empty diamond: octyl-VS-EDA-CALB-EDA-Glu. Empty triangle: octyl-VS-EDA-CALB-Glu.

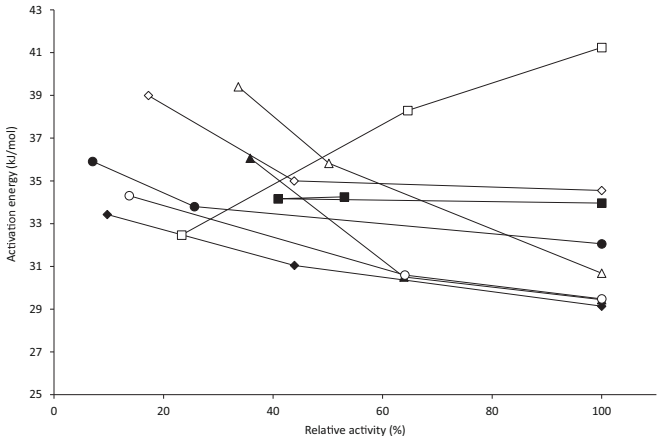

Fig. 9. Activation energy (Ea) values for different immobilized CALB biocatalysts in the hydrolysis of triacetin after the inactivation courses at pH 5.0 and 70 °C. Symbols: Full square: octyl-CALB. Full circle: octyl-CALB-EDA. Full diamond: octyl-CALB-EDA-Glu. Full triangle: octyl-CALB-Glu. Empty square: octyl-VS-EDA-CALB. Empty circle: octyl-VS-EDA-CALB-EDA. Empty diamond: octyl-VS-EDA-CALB-EDA-Glu. Empty triangle: octyl-VS-EDA-CALB-Glu.

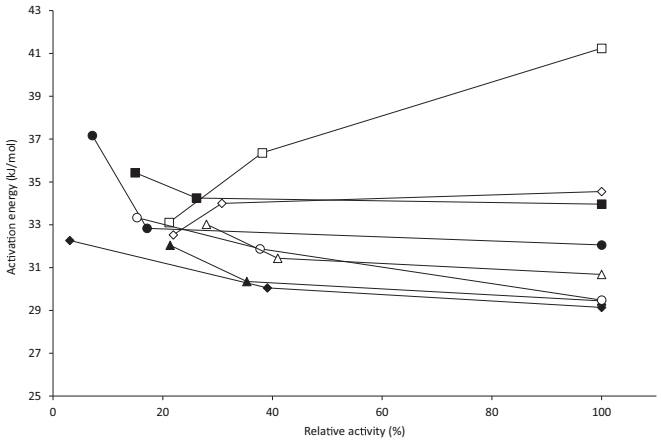

Fig. 10. Activation energy (Ea) values for different immobilized CALB biocatalysts in the hydrolysis of triacetin after the inactivation courses at pH 7.0 and 62 °C. Symbols: Full square: octyl-CALB. Full circle: octyl-CALB-EDA. Full diamond: octyl-CALB-EDA-Glu. Full triangle: octyl-CALB-Glu. Empty square: octyl-VS-EDA-CALB. Empty circle: octyl-VS-EDA-CALB-EDA. Empty diamond: octyl-VS-EDA-CALB-EDA-Glu. Empty triangle: octyl-VS-EDA-CALB-Glu.

the hydrolysis of triacetin catalyzed by different biocatalysts during progressive inactivation at pH 5, 7 and 9. We have not found similar studies in the literature. In most cases, a decrease in residual activity was accompanied by an increase in Ea, as expected. However, some exceptions were observed.

When the inactivation was performed at pH 5 (Fig. 9), Ea changes varied. The Ea of the reaction catalyzed using octyl-CALB at different inactivation levels increased very slightly during the inactivation (it moves from 33.96 kJ/mol, 34.16 kJ/mol and 34.35 kJ/mol). However, the final activity is around 10 % of the initial one (see Fig. 3), indicating a minimal impact on activation energy of the conformational changes promoting the enzyme inactivation. Octyl-CALB-EDA showed a more pronounced increase in Ea, from 29.56 to 31.30 and finally to 33.41 kJ/mol. Similarly, octyl-CALB-EDA-Glu increased from 29.14 to 31.05 and then to 33.43 kJ/mol. Octyl-CALB-Glu also followed the trend, with Ea rising from 29.14 to 30.51 and then to 36.00 kJ/mol. Thus, although the comparison of the Ea among different biocatalysts (intact or partially inactivated) did not fit the expected increase of the Ea of the reaction when the enzyme activity decreases in many instances, all biocatalysts

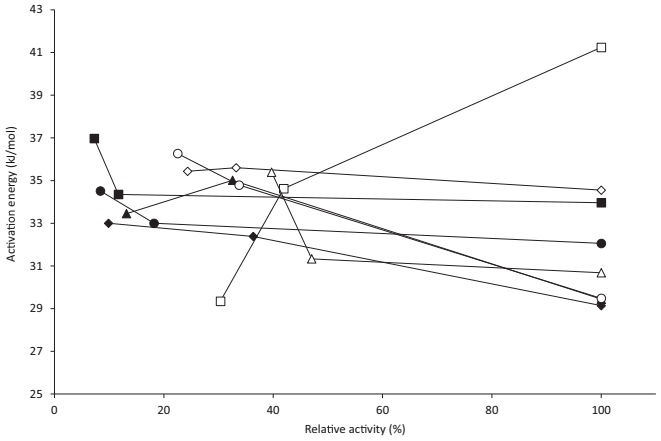

Fig. 11. Activation energy (Ea) values for different immobilized CALB biocatalysts in the hydrolysis of triacetin after the inactivation courses at pH 9.0 and 55 °C. Symbols: Full square: octyl-CALB. Full circle: octyl-CALB-EDA. Full diamond: octyl-CALB-EDA-Glu. Full triangle: octyl-CALB-Glu. Empty square: octyl-VS-EDA-CALB. Empty circle: octyl-VS-EDA-CALB-EDA. Empty diamond: octyl-VS-EDA-CALB-EDA-Glu. Empty triangle: octyl-VS-EDA-CALB-Glu.

based on physical adsorption showed an increase in Ea with increasing the decrease in enzyme activity during the inactivation.

However, the covalently bound biocatalyst octyl-VS-EDA-CALB behaved differently. Despite a decrease in activity, Ea decreased during inactivation, from 41.24 to 38.29 and then to 32.47 kJ/mol—opposite to the expected trend. In contrast, octyl-VS-EDA-CALB–EDA followed the anticipated pattern, with Ea increasing from 29.48 to 30.60 and then to 34.31 kJ/mol. Octyl-VS-EDA-CALB–EDA-Glu also showed a steady increase in Ea, from 34.55 to nearly 39.00 kJ/mol, and octyl-VS-EDA-CALB–Glu increased from 30.68 to 39.40 kJ/mol. Thus, at pH 5, only the parent octyl-VS-EDA-CALB deviated from the expected trend, and notably, it also had the highest initial Ea of all biocatalysts (Table 2).

At pH 7 (Fig. 10), the overall trend was similar: Ea generally increased as residual activity decreased, except again for octyl-VS-EDA-CALB, which showed a progressive decrease in Ea from 41.24 to 36.36 and then to 33.11 kJ/mol. Additionally, octyl-VS-EDA-CALB–EDA-Glu also deviated from the expected behavior, with Ea decreasing slightly from 34 to 32 kJ/mol.

At pH 9 (Fig. 11), the trend was maintained across most biocatalysts, with increasing Ea upon inactivation, except once again for octyl-VS-EDA-CALB, which decreased from 41 to 34 and then to 29 kJ/mol during inactivation.

In summary, across all inactivation pH values, most biocatalysts exhibited an increase in Ea as activity declined, supporting the general assumption that partial inactivation hinders transition-state formation and increases the energy barrier for catalysis. The main exception was octyl-VS-EDA-CALB, which consistently showed a decrease in Ea despite reduced activity. A second exception was observed for octyl-VS-EDA-CALB–EDA-Glu inactivated at pH 7. A plausive explanation for these anomalous results may be explained by the different conformational changes that the different biocatalyst can experience on the different conditions, perhaps leading to enzyme configurations with a lower Kcat (the most related parameter to Ea) but with an even higher increment in the Km, giving as a final result a decrease in the observed activity while also decreasing the Ea for this reaction. This possibility may be supported by the higher activity of the covalently immobilized biocatalysts compared to the activity of the just physically adsorbed enzyme, that is, some conformational CALB changes can lead to increase in enzyme activity, well by lowering Km or by increasing Kcat. As we cannot determine the values of Kcat and Km due to the lack of substrate saturation of the immobilized enzyme, to ensure the real reason for this discrepancy

between decreases of enzyme activity caused by the incubation at high temperature while Ea also decreased, is no currently possible. It is important to note, however, that while the trend of increasing Ea with decreasing activity was mostly preserved during progressive inactivation within a given biocatalyst, comparisons of Ea across different biocatalysts—either intact or inactivated—did not follow a consistent pattern. Some partially inactivated biocatalysts exhibited both lower activity and lower Ea, while others with higher activity showed higher Ea values. This observation indicates that changes in Ea alone cannot fully explain the differences in catalytic performance among biocatalysts.

As previously discussed, these inconsistencies may reflect changes in enzyme–substrate affinity or altered acyl-enzyme formation. For instance, if the acyl-enzyme intermediate is not efficiently formed due to structural changes, then even a lower transition-state energy (i.e., lower Ea) would not translate to improved catalytic performance. To better dissect these effects, further analyses such as Eyring plots or separate estimation of entropy and enthalpy contributions may provide more detailed insight into how inactivation affects catalytic efficiency versus substrate binding [62–69]. Unfortunately, the extremely high Km of the utilized biocatalysts make no possible to analyze these parameters.

4. Conclusion

This study demonstrates how chemical modification of immobilized enzymes significantly alters their catalytic properties and stability, with outcomes strongly dependent on the substrate used, the immobilization strategy, and the conditions under which inactivation occurs. The same chemical treatment can enhance performance under certain conditions while impairing it under others. These findings reinforce the concept that immobilization and post-immobilization modifications generate "new enzymes" with distinct structural and functional characteristics.

Thermal inactivation, commonly used to assess enzyme stability, was shown to yield different inactivation profiles depending on the substrate used to monitor residual activity. This variability not only affects the absolute stability of each biocatalyst but also shifts the relative stability ranking among them. In some cases, after some inactivation time, some CALB biocatalysts retained or even improved activity with certain substrates, such as *R*-methyl mandelate, while becomes almost full inactive versus other ones. This highlights the complex interplay between structural changes and catalytic performance.

Kinetic analysis revealed that interfacially adsorbed CALB exhibits unusually high Km values, potentially due to substrate partitioning within the hydrophobic octyl layer, toward the active center is oriented. This results in a pseudo–first-order kinetic regime, where activity increases linearly with substrate concentration, complicating the estimation of traditional kinetic parameters but potentially offering operational advantages.

Another key insight from this work is that apparent activation energy (Ea), often derived from Arrhenius plots under first-order assumptions, does not consistently correlate with enzyme activity across different biocatalyst formulations. While an increase in Ea was frequently observed upon enzyme inactivation—consistent with reduced catalytic efficiency—there were notable exceptions. This reinforces that Ea alone is insufficient to explain biocatalyst performance. Contributing factors include changes in substrate affinity (Km), turnover number (kcat), enzyme microenvironment, and conformational integrity, as well as the presence of partially active or inactive subpopulations.

Overall, these results align with previous literature indicating that enzyme activity is a multifactorial phenomenon. While Ea provides insight into catalytic barriers, it must be interpreted in conjunction with other kinetic parameters to fully understand enzyme performance. Selecting or optimizing a biocatalyst requires testing under conditions that closely match the intended process, as conclusions drawn from unrelated assays may lead to misleading decisions. Future studies should aim to further dissect the interplay between Ea, Km, and kcat—particularly in the context of immobilized and chemically modified enzymes—to guide the rational design of robust biocatalytic systems.

From a broader perspective in biocatalysis, this study highlights the persistent challenges in the rational design of immobilized and modified enzymes with predictable performance characteristics. The observed complexity necessitates thorough empirical characterization tailored to the specific application and substrate(s) of interest. It also suggests the value of employing complementary biophysical and structural techniques, where feasible, to gain deeper insights into the conformational and dynamic changes underlying the observed functional effects, moving beyond reliance on simple kinetic and thermodynamic parameters alone.

CRediT authorship contribution statement

Camila R. Hackenhaar: Writing – review & editing, Writing – original draft, Investigation, Formal analysis, Data curation. **Pedro Abellanas-Perez:** Writing – review & editing, Writing – original draft, Investigation, Formal analysis, Data curation. **Diego Carballares:** Writing – original draft, Investigation. **Juan M. Bolivar:** Writing – review & editing, Supervision, Formal analysis, Data curation, Conceptualization. **Rafael C. Rodrigues:** Writing – review & editing, Writing – original draft, Supervision, Funding acquisition, Formal analysis, Data curation, Conceptualization. **Roberto Fernandez-Lafuente:** Writing – review & editing, Writing – original draft, Supervision, Methodology, Funding acquisition, 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.2025.147310.

Data availability

No data was used for the research described in the article.

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