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The nature of the buffer alters the effects of the chemical modification on the stability of immobilized lipases

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ABSTRACT

The objective of this paper was to analyze whether an interaction between the effects of the buffer nature and chemical modification on enzyme stability exists. For this, the lipase B from *Candida antarctica* (CALB) and the lipase from *Thermomyces lanuginosus* (TLL) were immobilized on octyl agarose beads and modified with picryl sulfonic acid (TNBS) and ethylenediamine via the carbodiimide route. The obtained biocatalysts were them inactivated in different buffers (2-amino-2-(hydroxymethyl)propane-1,3-diol (Tris)-HCl, N-(2-hydroxyethyl) piperazine-N′-(2-ethanesulfonic acid) (HEPES) or phosphate). A significant interaction was found between the chemical modification of the enzyme surfaces and their stabilities in these different buffers. While phosphate was the buffer where the lowest enzyme stabilities were found for both unmodified immobilized enzymes, the differences on enzyme stabilities become much smaller after some chemical modifications. In many instances, chemical modification improves enzyme stability when using a buffer and was negative when using the other one (e.g., TNBS modification of TLL was positive using Tris-HCl or negative using the other buffers, amination of CALB decreased its stability when inactivated in Tris-HCl or HEPES while it almost had no effect in phosphate). Thus, clear co-interactions of the effects on enzyme stability between chemical modification and buffer nature were established.

1. Introduction

Although enzymes have many highly adequate features for their applied implementation (high product selectivity, substrate specificity or activity under mild conditions) [1,2], their biological origin results that in many instances some enzyme properties must be improved before their utilization [1–3]. Nowadays, there are many tools to achieve an enzyme with the required features, such as metagenomics (that allows the use of enzymes from non-cultivable or even extinguished microorganisms) [4,5], directed evolution (that permits to improve one specific enzyme feature mimicking natural evolution in an accelerated way) [6,7] or side-directed mutagenesis [8,9] (that even permits to create enzymes bearing multiple different active centers [10], such as plurizymes [11,12]). Enzyme immobilization and chemical modification are other techniques to improve the enzymes features. Enzyme immobilization allows creating heterogeneous biocatalysts, and thus facilitating enzyme recovery and reuse, as well as simplifying the control of the reaction and increasing the range of reactors type that can be used [13]. Nowadays, a properly designed enzyme immobilization is expected to be able to greatly improve enzyme stability, activity, selectivity or specificity, even to be able to purify the target enzyme [14–17]. Global enzyme chemical modification of the enzyme surface permits to alter its physical features and that way alter enzyme properties [18–20]. Among the different chemical modification goals, intramolecular crosslinking allows rigidification of the enzyme structure, and that way, enzyme stabilization versus any distorting agent [21–23]. The modification of previously immobilized enzymes has some advantages: it simplifies the control of the process, it prevents enzyme precipitation of the intermedium or final forms of the enzyme, and if the enzyme is stabilized by immobilization, it may be more resistant to any negative effect of the chemical modification [24,25].

Lipases are among the most used enzymes because of their high stability under different reaction conditions and media, together with their broad specificity (in some instances coupled to a high enantio- and

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/or regio- selectivity or specificity) [26–28]. They are among the enzymes where more promiscuous activities have been detected [29]. These enzymes have a peculiar mechanism of action called interfacial activation, that involves large conformational changes and the strong adsorption of the open form of lipases to drops of hydrophobic substrates [30–35]. This feature has been utilized to immobilize lipases on hydrophobic supports at very low ionic strength [36], enabling the one step immobilization, purification, stabilization and hyperactivation of lipases [37]. These lipases, immobilized via interfacial activation that involves the large hydrophobic pocket surrounding the active center of the lipase, exhibit a response to the medium different to other immobilized lipase biocatalysts, perhaps because they are no longer in the closing/open equilibrium. For example, they may be stabilized by some cations (e.g., Ca^{2+}) [38,39] or destabilized by some anions, (such as phosphate) [40–42] while the effects on the stability of covalently immobilized lipases of these additives are not relevant [68–72]. That way, the use of phosphate as buffer generally reduces stability of lipases immobilized via interfacially activation on hydrophobic supports [40–42].

Considering that the global chemical modification of the surface of immobilized enzymes can be used to alter their physical features, and that way their capabilities to interact with other similarly modified enzyme molecules and the medium, we have decided to analyze how it may alter the effects of the buffers on enzyme stability. For this purpose, we have immobilized the lipase B from *Candida antarctica* (CALB) [43,44] and the lipase from *Themomyces lanuginosus* (TLL) [45] on octyl agarose beads. We have selected these enzymes because they are among the most used lipases at both academic and applied levels [73–75] and because their stabilities have been reported to be strongly modulated by the buffer nature [40–42]. To maximize the enzyme-enzyme interactions, we have used an enzyme loading that exceeds the loading capacity of the support, where enzyme-enzyme interactions have been reported to alter the stability of these enzymes [46,47]. Then, we have transformed all the carboxylic groups of the surface of the immobilized enzymes into amino groups using the carbodiimide route [25] or we have modified all the amine groups of the surface of the enzyme with picryl sulfonic acid (TNBS) [48–52]. The amination of the enzyme surface promotes the transformation of attraction ion interactions between carboxylic and cationic groups of the enzyme surface in repulsion interactions between two cationic groups. The TNBS modification means a decrease of the number of enzyme cationic groups (the bond between the amino groups and the TNBS is via an amide bond) and a relatively hydrophobic group will be pending on these modified groups. That way, the overall hydrophobicity of the enzyme surface should increase [53]. The attraction interactions between cationic and anionic groups of the protein surface (inter or intra molecular ones) will be lost, while repulsion interactions between primary amino groups will be transformed in new hydrophobic interactions. Both modifications mean a deep global change of the enzyme surface physical properties and the possibility of establishing new interactions among the groups of the enzyme surface. The increase in the hydrophobicity could reinforce the adsorption of the enzymes to the hydrophobic surface of the support.

2. Materials and methods

2.1. Materials

Liquid CALB (24.77 mg protein/mL) and TLL (38.49 mg protein/mL) samples were kind gifts from Novozymes (Madrid, Spain). Ethylenediamine (EDA), *p*-nitrophenyl-butyrate (*p*-NPB), picryl sulfonic acid or 2,4,6-trinitrobenzene sulfonic acid solution (TNBS) and ethyl-carbodiimide hydrochloride (ECD) were acquired from Sigma-Aldrich (Madrid, Spain). Octyl-agarose 4BCL beads were procured from GE Healthcare. Protein concentrations were quantified using the BCA dye reagent by measuring the absorbance at 595 nm. [54]. A calibration curve was drawn using bovine serum albumin. All other compounds used in this paper were of analytical grade.

2.2. Methods

All experiments were performed by triplicate as minimum and the results are given as mean value ± standard deviation.

2.2.1. Lipase activity versus p-NPB

The increase in absorbance that occurs due to the release of *p*-nitrophenol, caused by the hydrolysis of *p*-NPB, was measured at 348 nm (isosbestic point of *p*-nitrophenol, ε is 5150 M^{-1} cm^{-1} under these conditions) [55]. A cuvette containing 2.5 mL of 25 mM sodium phosphate at pH 7.0 was prepared. Then, 50 μL of *p*-NPB solution (at a concentration of 50 mM dissolved in acetonitrile) was added. The reaction was started by adding 50 μL of free or immobilized enzyme (solution or suspension, respectively). The system was submitted to continuous magnetic stirring utilizing a Jasco V-730 spectrophotometer (Jasco, Madrid, Spain) with control of temperature (25ºC). A unit of enzyme activity (U) was defined as μmol of hydrolyzed *p*-NPB per minute.

2.2.2. Immobilization of the lipases on octyl-agarose beads

CALB and TLL were immobilized on octyl-agarose beads using an enzyme loading of 24 mg of protein per gram of support, a load than exceeds the capacity of the support [16,36,37]. The immobilization was performed using 1 g of support per 10 mL of enzyme solution in 5 mM of sodium phosphate as buffer at 25 ºC and pH 7.0. When indicated, samples of the reference suspension, and suspension and supernatant of the immobilization suspensions were taken, and their activities were measured employing *p*-NPB as substrate. This permitted to calculate immobilization yield and expressed activity [56]. After an hour, when there were no changes in the supernatant activity, the immobilized biocatalysts were washed with distilled water, vacuum filtered and stored in a fridge at 6 °C.

2.2.3. Amination of the immobilized enzyme using EDA/ECD

Samples of 5 g of the immobilized enzymes were introduced into 50 mL of a 2 M solution of EDA at pH 4.75 and 25 ºC. Then, solid ECD was added to achieve a 10 mM final concentration. The suspension was kept under magnetic stirring for 2 h. Afterwards, the aminated immobilized enzymes were vacuum filtered, washed and stored in a fridge. Using these conditions, it has been described that all the exposed carboxylic groups of proteins become modified [57]. This guaranteed that all enzyme molecules have similar level of modification.

2.2.4. Modification of the primary amino groups of the surface of immobilized lipases with TNBS.

5 g of the lipase biocatalysts were suspended in 50 mL of 100 mM sodium carbonate containing 17.5 mM TNBS at 25ºC and pH 8.0. The suspension was mildly stirred for an hour and afterwards, washed with distilled water, vacuum filtered and stored at 4–6ºC. The biocatalyst took a strong orange color confirming the modification. The further TNBS modification of the already TNBS modified did not provide an increase in the color developed by the biocatalysts, confirming that all reactive and exposed primary amino groups of the enzyme had been modified. That way, all enzymes should have all exposed primary groups modified with a TNBS molecule, and there are not risk of heterogeneity of the modification degree of different enzyme molecules.

2.2.5. Thermal inactivation of different lipase preparations

The CALB and TLL biocatalysts were resuspended in 10 mM of (2-amino-2-(hydroxymethyl)propane-1,3-diol) (Tris)-HCl, sodium phosphate or N-(2-hydroxyethyl)piperazine-N′-(2-ethanesulfonic acid) (HEPES) buffers at pH 7.0 in a ratio 1/30 (g biocatalysts /mL). After analyzing different temperatures of inactivation, those that enabled a reliable determination of the inactivation courses were selected. These

Table 1
Immobilization parameters of lipase B from *Candida antarctica* (CALB) and lipase from *Thermomyces lanuginosus* (TLL)- octyl agarose beads biocatalysts. The immobilization was performed for 2 h and then activities of the supernatant and suspension of the immobilization suspension and that of the reference suspension (that maintained 100% of the initial activity) were determined. Data are given as unit per g of support, 24 mg of lipase extract per g pf enzyme was used in both cases. Other specifications may be found in Methods section.

Biocatalyst	Offered activity, (U/g)	Activity in supernatant, (U/g)	Activity in suspension, (U/g)
Octyl-CALB	140 ± 3	28 ± 1	118 ± 3
Octyl-TLL	260 ± 7	130 ± 4	275 ± 5

inactivation suspensions were incubated in a hot bath at temperatures depending on the enzyme. CALB was incubated at 76ºC for samples suspended in Tris-HCl and HEPES buffers and at 70ºC for samples inactivations in sodium phosphate buffer. TLL was incubated at 72 ºC in all buffers. At defined times, 50 μL of suspension samples were acquired and their activities were determined. Residual activities were calculated as the percentage of the biocatalysts initial activity.

3. Results and discussion

3.1. Preparation of the biocatalysts

CALB and TLL were immobilized on octyl agarose beads offering an amount of enzyme higher than the capacity of the support (24 mg of protein/g of support) [46,47]. In general, a lipase hyperactivation is usually reported when immobilizing the lipases on this support, because of the stabilization of the open form of the lipase [36], but this was not observed for CALB. CALB-octyl permitted an immobilization of 80% of the offered lipase activity. The immobilization suspension decreased the activity to 80% (Table 1). Therefore, the expressed activity of the immobilized enzyme was 75% (20% of the 80% of the immobilized enzyme activity was lost) [56]. The high activity of the biocatalysts inside a porous support fully coated with enzyme molecules, submitted to substrate diffusional limitations, can promote that the activity of the enzyme immobilized in the inner part of the pores did not received the substrate at saturating concentration (at the substrate concentration will be decreasing along the pore due to the high enzyme activity), which could explain this decrease in activity of the immobilized enzyme [13]. This is coupled to the previously described lack of hyperactivation of this enzyme upon immobilization on these supports, which may be explained by the small CALB lid that leaves the active center accessible to the medium in the closed form [58]. Using TLL, immobilization yield was 50% while the activity of the suspension increased to 105%. This means than the activity of the immobilized enzyme increased by 10% after immobilization, a hyperactivation value lower than other reported for this enzyme immobilized in this support at lower loadings [46,47]. The increase of activity (even smaller than that described using lower loadings) should be related to the stabilization of the open form of the lipase [36]. This smaller hyperactivation can be related to the high volumetric activity of this biocatalyst, that very likely promotes that some enzyme molecules located in the core of the porous particles can be exposed to substrate concentrations under the saturating one [13], and that way, this could produce a decrease of the global activity of the biocatalyst. Moreover, some conformational change caused by protein-protein interactions cannot be discarded.

Next, both biocatalysts were modified with TNBS and EDA/EDC. The size of the modifiers is similar to the size of the substrate, that way, it may be expected that all enzyme molecules can be reached by this small reagents, agarose 4BCL pore diameter is very large (in fact, we have been able to create 3–6 (enzyme-polymer)$_n$-enzyme biocatalysts layers fully coating the internal support surface with the enzymes, that way, these problem can be fully discarded) [59–62]. As explained in introduction, the TNBS enzyme modification produces an increase on the hydrophobicity of the enzyme surface, it can change attraction/repulsion ionic interactions by hydrophobic interactions [53]. The amination should produce an increase of repulsive ionic interactions, as carboxylic groups are changed into amino groups [25]. Table 2 shows the effects of this modification on enzymes activity. Both modifications promoted a significant decrease on the activity of octyl-CALB biocatalyst (by 2.2 fold in the case of TNBS modification and 2.6 fold for the aminated biocatalyst). Using TLL the modifications produced smaller enzyme activity decreases, with TNBS by 10% while the amination of the enzyme decreased the activity by 20%. However, our target was to analyze how this can alter the effect of the buffers on the enzyme stability, not really to prepare a biocatalyst of these enzymes. That way we compared the enzyme stabilities in different buffers at pH 7.

Table 2
Effect of the chemical modification on the activity of lipase B from *Candida antarctica* (CALB) and lipase from *Thermomyces* lanuginosus (TLL) immobilized biocatalysts. The chemical modifications were performed as described in Methods section using picryl sulfonic acid (TBBS) or aminated using ethylenediamine/ ethylcarbodiimide hydrochloride (EDA/ ECD). Other specifications may be found in Methods section.

Biocatalysts	Chemical modification	Biocatalyst activity, (%)
Octyl- CALB	None	100
	TNBS	44.8 ± 2.7
	Amination	38.3 ± 2.2
Octyl-TLL	None	100
	TNBS	89.7 ± 3.7
	Amination	81.6 ± 4.9

3.2. Effect of the chemical modifications on enzyme stability

As previously reported, lipases immobilized on octyl agarose beads may be released when submitted at high temperatures [63–65]. The chemical modification of the enzymes could somehow alter the strength of the adsorption of the enzymes to the support. Unfortunately, it could not be established if the enzymes were inactivated and then released from the support, or were released in active form and, as this form was less stable, they were readily inactivated under the used conditions. This is this way because the practical result will be the same: almost no activity will be found in the supernatant of the inactivating suspensions while some protein could be detected [63–65]. The analysis by SDS-PAGE of the biocatalyst could be an alternative, but this could detect only this effect if the differences are very large, we have not detected significant differences among the amount of enzyme still immobilized in the support in the SDS-PAGE of the inactivated enzymes submitted to the different inactivation buffers and modifications, when comparing the biocatalyst after 1 h of inactivation (not shown results). In any case, in this paper we have focused on the study of the interactions between chemical modifications and inactivating buffer. The use of only 10 mM of buffers should reduce their effects to reinforce or decrease the adsorption of the enzymes on the support, although using higher concentrations of these buffers, these effects should be considered.

It should be considered that all enzyme molecules are immobilized via interfacial activation on the support, that way, all should have the same orientation and kind of interactions with the support surface. This is one of the advantages of using this immobilization strategy for these studies, as this will decrease the possibility of heterogeneity of the enzymes in the support, Moreover, all enzyme molecules have all their exposed groups modified in a similar way. This will also prevent the formation of heterogamous enzyme populations (perhaps in terms of an optimal biocatalyst considering activity and stability, partial modifications may be more favorable, but this can generate some heterogeneity in the immobilized enzyme populations).

The comparison of the stabilities of the different preparations on the

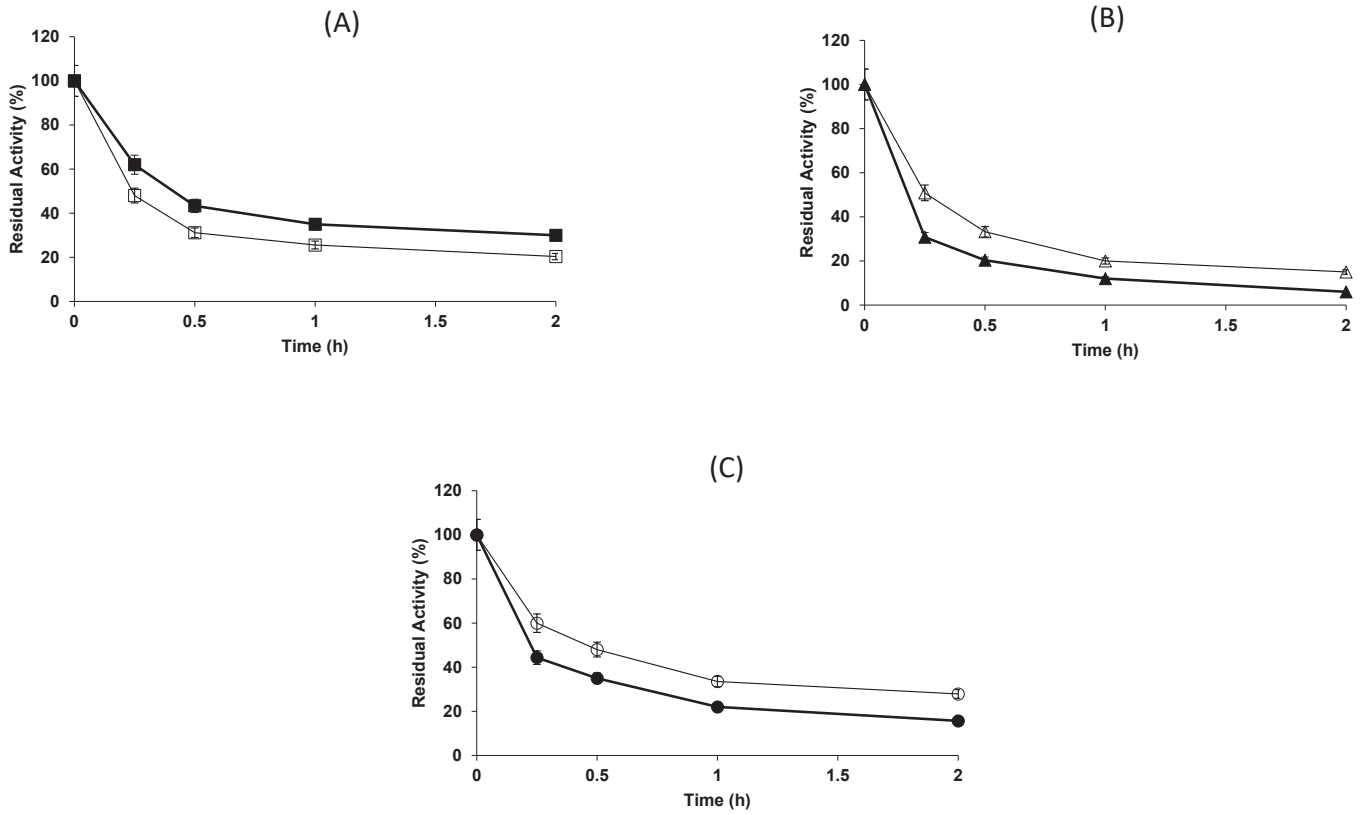

Fig. 1. Effect of the enzyme chemical modification with picryl sulfonic acid (TNBS) on the thermal inactivation courses of highly loaded octyl-lipase from *Thermomyces lanuginosus* (TLL) (24 mg/g). The inactivation was performed using 10 mM of 2-amino-2-(hydroxymethyl)propane-1,3-diol (Tris)-HCl (A); sodium phosphate (B) and N-(2-hydroxyethyl)piperazine-N′-(2-ethanesulfonic acid) (HEPES) (C) at pH 7.0 and 72ºC. Empty squares: No modified biocatalyst; Solid squares: TNBS modified biocatalyst. Other specifications were described in Methods.

different buffers may be found in supporting information (Figs. 1S and 2S), while effects of the chemical modification on the stabilities on the different buffers may be found in the main body of the paper (Figs. 1–4).

Fig. 1SA shows that immobilized TLL presented a slightly higher thermal stability when inactivated in HEPES than when inactivated in Tris-HCl, while, as previously reported, the lowest stability was observed using phosphate as buffer of the inactivation suspension.

The chemical modifications have been performed in a way that all exposed groups of the enzymes become modified [79,82]. However, it is not unlikely that some groups in close contact with the support surface can be partially protected from this modification, but as in all cases the enzymes are immobilized using the same strategy, these effects should be similar and could not be used to explain differences in the performance of the modified enzymes. Fig. 1 shows that the surface hydrophobization obtained by the TNBS chemical modification presented a positive effect on enzyme stability when inactivating the biocatalysts in Tris-HCl and a negative one using HEPES and phosphate. Considering that phosphate is the buffer that has a stronger effect on ionic strength, it seems hard to relate the effects of the buffer (used in any case at low concentration) plus chemical modification with a reinforcement of the enzyme adsorption the support. This way, there is an interaction between the buffers and the modification of the enzyme surface that leads to different effects of chemical modification on enzyme stability when changing the buffer of the inactivating solution. The TNBS modified enzyme became slightly more stable in Tris-HCl than in HEPES (Figure 1SB), being phosphate the buffer where the lowest stability was observed, with a higher difference than when using the unmodified biocatalyst.

When TLL was aminated, enzyme stability increased using Tris-HCl and more using phosphate as buffers of the inactivation suspension, while it had almost no effect using HEPES (Fig. 2). This caused the aminated and immobilized TLL to present a similar stability in HEPES and phosphate, in contraposition to the unmodified enzyme (Figure 1SC). Again, it was clear that the effect of the modification differed when using different buffers in the inactivation (and vice versa).

Next, the interaction between chemical modification and buffer nature was analyzed using CALB. Figure 2SA shows that octyl-CALB was more stable in Tris-HCl than in HEPES, being phosphate the buffer where the lowest biocatalyst stability was found. The negative effect of phosphate on octyl-CALB stability was far more relevant than for TLL biocatalysts, that way, for the comparison of the chemically modified and not modified octyl-CALB biocatalysts, the temperature was reduced to 70ºC when employing phosphate as buffer. Fig. 3 shows the inactivation courses of octyl-CALB-TNBS. Enzyme stability increased in all buffers, more significantly than when using octyl-TLL-TNBS. Figure 2SB shows that after modification, the stability of the immobilized CALB was similar in Tris-HCl and HEPES. On the other hand, although phosphate remained the buffer giving the lowest stability of immobilized CALB, after TNBS modification, its stability in this buffer became nearer to the stabilities in the other buffers than when using the unmodified enzymes. That way, the enzyme was more stabilized by the TNBS modification when inactivated in phosphate, followed by the inactivation in HEPES, which gave a similar inactivation course to that in Tris-HCl.

When octyl-CALB was aminated, its stability decreased using Tris-HCl. This negative effect was smaller using HEPES in the inactivation, and the inactivation course of the aminated enzyme was even slightly better that when inactivating the unmodified enzyme using phosphate (Fig. 4). Figure 2SC shows that although the lowest stability of the aminated enzyme could still be observed in phosphate buffer, the differences with the other two buffers were smaller. The octyl-CALB stabilities in Tris-HCl and HEPES became almost identical. That way, also in the case of CALB there is a clear co-interaction between the effects on

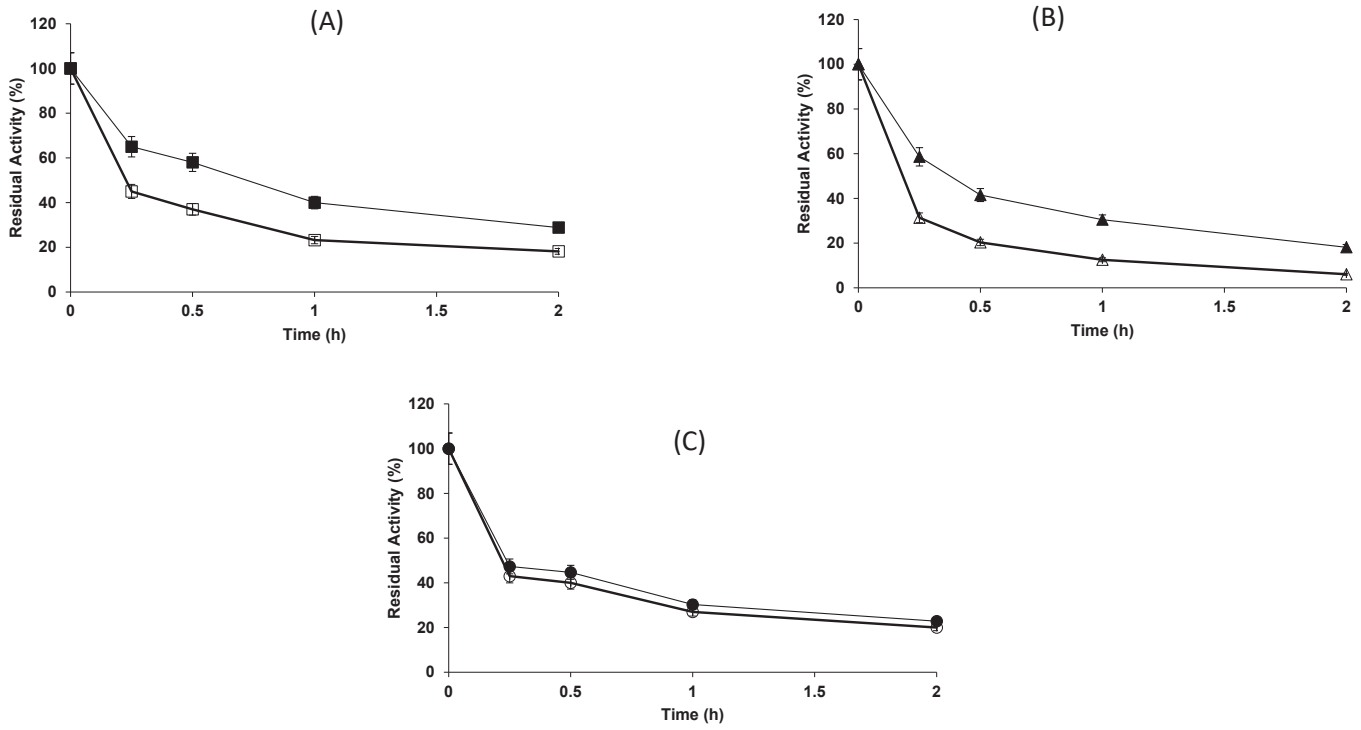

Fig. 2. Effect of the enzyme chemical amination on the thermal inactivation courses of highly loaded octyl-lipase from *Thermomyces lanuginosus* (TLL) (24 mg/g). The inactivation was performed using 10 mM of 2-amino-2-(hydroxymethyl)propane-1,3-diol (Tris)-HCl (A); sodium phosphate (B) and N-(2-hydroxyethyl)piperazine-N′-(2-ethanesulfonic acid) (HEPES) (C) at pH 7.0 and 72ºC. Empty squares: No modified biocatalyst; Solid squares: Chemically aminated biocatalyst. Other specifications were described in Methods.

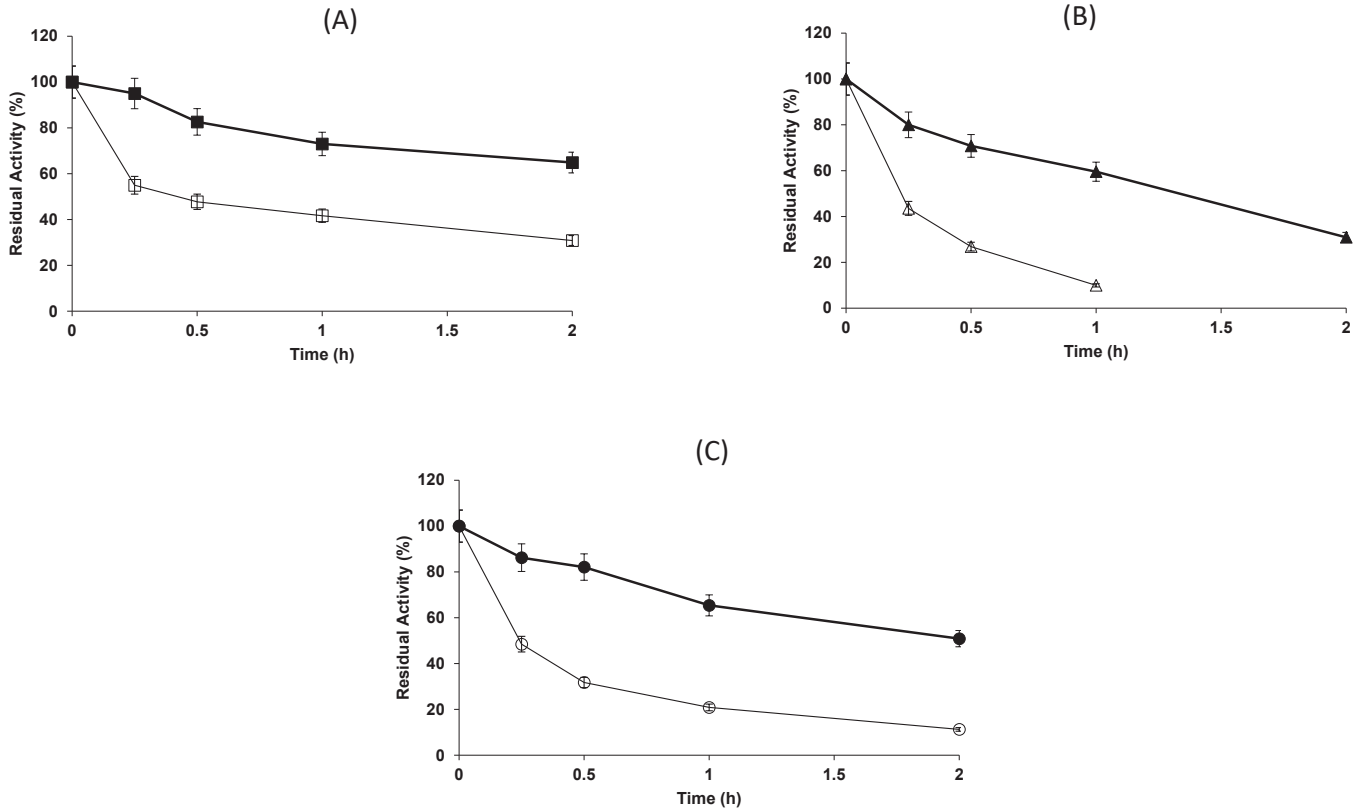

Fig. 3. Effect of the enzyme modification with picryl sulfonic acid (TNBS) on the thermal inactivation courses of highly loaded octyl-lipase B from *Candida antarctica* (CALB) (24 mg/g). The inactivation was performed using: 10 mM of 2-amino-2-(hydroxymethyl)propane-1,3-diol (Tris)-HCl at pH 7.0 and 76 ºC (A); sodium phosphate at pH 7.0 and 70ºC (B) and N-(2-hydroxyethyl)piperazine-N′-(2-ethanesulfonic acid) (HEPES) at pH 7.0 and 76 ºC (C). Empty squares: No modified biocatalyst, Solid squares: TNBS modified biocatalyst. Other specifications were described in Methods.

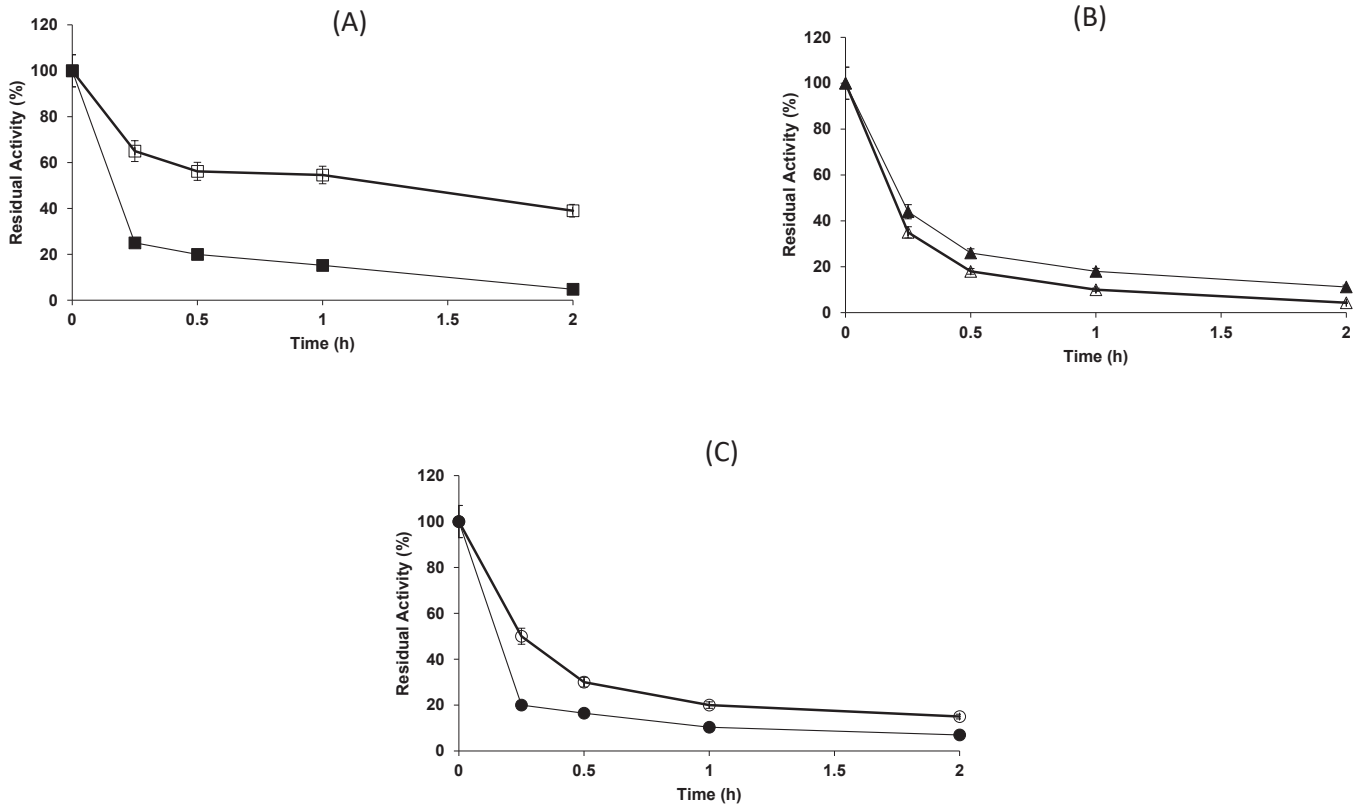

Fig. 4. Effect of the chemical amination on the thermal inactivation courses of highly loaded octyl-lipase B from *Candida antarctica* (CALB) (24 mg/g). The inactivation was performed using: 10 mM of 2-amino-2-(hydroxymethyl)propane-1,3-diol (Tris)-HCl at pH 7.0 and 76 ºC (A); sodium phosphate buffer at pH 7.0 and 70ºC (B) and N-(2-hydroxyethyl)piperazine-N′-(2-ethanesulfonic acid) (HEPES) at pH 7.0 and 76 ºC (C). Empty squares: No modified biocatalyst; Solid squares: Chemically aminated biocatalyst. Other specifications were described in Methods.

the enzyme stability of chemical modification and buffer nature.

4. Conclusion

This paper shows that the effects of chemical modification on immobilized CALB and TLL stabilities can be altered when the buffer is changed. It had been reported that the buffer nature greatly affects the stability of lipases immobilized via interfacial activation and that phosphate usually causes very negative effects in enzyme stability, mainly in the case of CALB. These effects were not so clear using other immobilization protocols, and the same can be extended to the positive effects of some cations (see introduction). That way, it seemed that the conformation and surface interactions of the enzyme with the support and other nearby enzyme molecules strongly modulate the effect of the buffers on immobilized enzyme stability. Now, it has been shown that the chemical modifications of CALB biocatalyst made the differences in stabilities smaller when the enzyme was inactivated in phosphate or the other buffers. Similarly, the chemical amination of TLL biocatalyst caused its stability on phosphate and HEPES to become similar. That way, the effect of immobilized lipase chemical modification can be different depending on the buffer used in the inactivation and a proper selection of the buffer and chemical modification may permit to reach maximum levels of enzyme stability and stabilization.

That way, if an immobilized lipase is chemically modified and its stability is analyzed in a single buffer, stabilization or destabilization effects may be found caused not only by a change on the intrinsic stability of the enzyme molecule, but also in how this enzyme surface interact with the buffer molecules. For example, stabilization detected in this paper were much higher when the immobilized lipases were inactivated in phosphate, as this molecule results in a very negative one for the native enzyme stability, and this negative effect decreased after some chemical modifications. Further investigations are required to fully understand the role of enzyme crowding and buffer nature on the stability of lipases immobilized via interfacial activation.

Moreover, considering that the effects of the buffer nature in enzyme features is a general effect, and that chemical modification may be performed in any enzyme, the relevance of these results may be extrapolate to any other enzyme (immobilized or not) submitted to chemical modification, it seems recommendable to analyze the effects of the chemical modification in a collection of buffers to obtain the optimal chemical modification/buffer pair for a specific application. This could be extended to any change in the enzyme chemical nature, including site-directed mutagenesis, directed evolutions and all other ways to alter the enzyme features. If the buffer can affect the enzyme features, the modifications should be evaluated in different media.

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.

Data availability

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

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.procbio.2023.08.003.

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