Journal of Biotechnology 391 (2024) 72–80

Contents lists available at ScienceDirect

Journal of Biotechnology

journal homepage: www.elsevier.com/locate/jbiotec

Tuning almond lipase features by the buffer used during immobilization: The apparent biocatalysts stability depends on the immobilization and inactivation buffers and the substrate utilized

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ARTICLE INFO

Keywords:
Immobilization buffers
Inactivation buffers
Enzyme activity/stability
Enzyme specificity

ABSTRACT

The lipase from *Prunus dulcis* almonds was inactivated under different conditions. At pH 5 and 9, enzyme stability remained similar under the different studied buffers. However, when the inactivation was performed at pH 7, there were some clear differences on enzyme stability depending on the buffer used. The enzyme was more stable in Gly than when Tris was employed for inactivation. Then, the enzyme was immobilized on methacrylate beads coated with octadecyl groups at pH 7 in the presence of Gly, Tris, phosphate and HEPES. Its activity was assayed versus triacetin and *S*-methyl mandelate. The biocatalyst prepared in phosphate was more active versus *S*-methyl mandelate, while the other ones were more active versus triacetin. The immobilized enzyme stability at pH 7 depends on the buffer used for enzyme immobilization. The buffer used in the inactivation and the substrate used determined the activity. For example, glycine was the buffer that promoted the lowest or the highest stabilities depending on the substrate used to quantify the activities.

1. Introduction

Lipases have many potential applications. They can be employed to hydrolyze glycerides to produce free fatty acids and glycerol (Goswami et al., 2013; Murty et al., 2002); it may also be utilized to produce structured lipids by esterification, acidolysis, transesterification or interesterification (Akil et al., 2020; Kind and Kursula, 2019; Okulus et al., 2022; Soumanou et al., 2013; Utama et al., 2019; Yoshioka et al., 2023), in the production of biolubricants (Bolina et al., 2021; Mendes et al., 2023; Monteiro et al., 2023) or biodiesel (Abdulmalek and Yan, 2022; Wancura et al., 2023; Zambare et al., 2021), in the resolution of racemic mixtures (Ferraccioli, 2021; Gupta et al., 2021; Serra and De Simeis, 2020; Tarczykowska et al., 2017), etc. These reactions can be carried out in a wide variety of media, from organic solvents (Ghanem and Aboul-Enein, 2004; Kobayashi and Adachi, 2004; Kumar et al., 2016), to ionic liquids (Elgharbawy et al., 2018; Šibalić et al., 2023), from supercritical fluids (Dias et al., 2018; Fan and Qian, 2010) to eutectic solvents (Durand et al., 2013; Tan and Dou, 2020), or even in solvent-free systems (Chang and Wu, 2009; Chen et al., 2024; Fernandes et al., 2012; Martinez-Garcia et al., 2024; Roque et al., 2024; Sousa et al., 2021). Due to this diversity of applications, there is an increasing interest in the search of new lipases from different origins and vegetable lipases are in a prime position (Ding et al., 2019; Mishra and Kandali, 2019; Moussavou Mounguengui et al., 2013; Mukherjee, 1994; Seth et al., 2014; Villeneuve, 2003). In this context, oleaginous organisms stand out, with many reports in the literature (Kotogán et al., 2018;

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https://doi.org/10.1016/j.jbiotec.2024.06.009
Received 8 April 2024; Received in revised form 24 May 2024; Accepted 11 June 2024
Available online 13 June 2024

Ktata et al., 2020; Nomaguchi et al., 2018; Polizelli et al., 2008; Vyas and Chhabra, 2017).

However, the lipase from *Prunus dulcis* (or *Amygdalus communis*) almonds has been the object of a few published studies. We have been able to find just two papers related to this lipase. The first paper describes the enzyme as a very stable one, with 65 ºC as optimal temperature in the hydrolysis of soybean oil, and with the activity altered by some divalent cations and detergents (Yeşiloğlu and Başkurt, 2008). The second paper is the first attempt to immobilize this lipase, showing the modulation of the enzyme features by immobilization (Cherni et al., 2024).

For the industrial application of this lipase, its use as an immobilized lipase may have some advantages. Its immobilization facilitates its recovery and reuse, as long as the enzyme remains active (Bolivar et al., 2022; Di Cosimo et al., 2013; Liese and Hilterhaus, 2013; Sheldon and van Pelt, 2013). It can also simplify the downstream product and enable the use of a diversity of reactors, with a more accurate reaction control (Bolivar et al., 2022). Moreover, a proper immobilization protocol may improve many enzyme features (Garcia-Galan et al., 2011; Homaei et al., 2013; Iyer and Ananthanarayan, 2008; Mateo et al., 2007) Enzyme stabilization is the usual target pursued by immobilizing an enzyme (Sheldon et al., 2021), but these positive effects should not be taken for granted; a bad selection of the support, active group in the support, or immobilization protocol can lead to a decrease in enzyme stability after its immobilization (Bolivar et al., 2022; Rodrigues et al., 2021). Moreover, enzyme purification from a crude extract coupled to the enzyme immobilization can be achieved if the immobilization protocol is designed for this objective (Barbosa et al., 2015), mainly using heterofunctional supports (Barbosa et al., 2013). Enzyme activity may be enhanced, mainly if the enzyme is utilized under drastic conditions, as a consequence of enzyme stabilization (Garcia-Galan et al., 2011). If the activity determination conditions are able to partially distort the free enzyme (Bolivar et al., 2022), its activity may decrease and that may not occur with the immobilized-stabilized enzyme (Boudrant et al., 2020). In some instances, a truly more active enzyme conformation may be achieved (*e.g.*, lipases immobilized via interfacial activation on hydrophobic supports) (Rodrigues et al., 2019). Due to enzyme distortions caused by the enzyme-support interactions, or to the fact that the enzyme is now in a confined space, enzyme selectivity, specificity or even inhibition may be altered (Rodrigues et al., 2013; Secundo, 2013). That way, enzyme immobilization should be considered an important tool in the design of an industrial biocatalyst (Bolivar et al., 2022). Against these advantages, enzyme immobilization presents its costs as main drawback; that includes the price of the support (or the elements required for producing the solid, in cases where there is not a pre-existing support), the storage of these materials, the process itself, the possible losses of enzyme activity and the final disposal of the inactivated biocatalyst (Bolivar et al., 2022).

Lipase immobilization on hydrophobic supports is one of the most successful strategies for preparing immobilized biocatalysts from these enzymes (Rodrigues et al., 2019). It is a very simple immobilization method, (Manoel et al., 2015), enabling the one step immobilization, purification, and stabilization of the lipase (Rodrigues et al., 2019). As the open form of the lipase is the one adsorbed on these supports, maintaining and stabilizing this open form, in many instances a significant increase in enzyme activity may be obtained upon immobilization, mainly using fully soluble substrates (Rodrigues et al., 2019). The reversibility of this immobilization protocol saves support costs, as it can be reused after enzyme inactivation (after releasing the inactive enzyme) to build a new biocatalyst (if the support particle has not been broken during use). As the lipase activity can increase upon immobilization, this activity gain should be considered when evaluating the immobilization costs (Bolivar et al., 2022). This immobilization protocol also ensured having the lipase molecules in monomeric form, as lipases have a strong tendency to form dimers involving two lipase molecules in its open form (Fernández-Lorente et al., 2003; Jose M. Palomo et al., 2003; José M. Palomo et al., 2003; Palomo et al., 2005, 2004; Wilson et al., 2006). This alters the lipase properties (activity and stability), but it has been used to produce matrices of immobilized lipases useful to purify and even immobilize lipases (Fernández-Lorente et al., 2003; Jose M. Palomo et al., 2003; José M. Palomo et al., 2003; Palomo et al., 2005, 2004; Wilson et al., 2006)

Enzyme stability is an important factor when selecting a lipase as a candidate for a specific application. Some recent papers have shown the effect of the buffer nature on the stability of some immobilized lipases (in some cases a very drastic one) and how these effects may depend on the immobilization protocol (Abellanas-Perez et al., 2023a, 2023b; Carballares et al., 2022; Fernandez-Lopez et al., 2016, 2015; Kornecki et al., 2020; Zaak et al., 2017). Moreover, it has been shown how the immobilization of lipases on hydrophobic supports under different conditions can produce biocatalysts with very different features (including different activity and stability) (Arana-Peña et al., 2021, 2020; Lokha et al., 2020). It has also been shown that the enzyme stability may depend on the substrate utilized to determine the enzyme (da Rocha et al., 2022; Paiva Souza et al., 2021; Souza et al., 2022). It may be assumed that during inactivation, all enzyme molecules suffer some conformational changes, and these new enzyme structures can exhibit different activities with different substrates (da Rocha et al., 2022).

In this paper, we have immobilized the lipase from *Prunus dulcis* almonds on methacrylate beads coated with octadecyl groups (Purolite Lifetech™ ECR8804M) (Tacias-Pascacio et al., 2016). This support has been successfully employed in the immobilization of some lipases, in some instances producing biocatalysts with much improved features (*e.g.*, in biodiesel production (Ching-Velasquez et al., 2020; Tacias-Pascacio et al., 2019, 2017) or acidolysis reactions (Verdasco-Martín et al., 2019, 2018)). The objective of this paper was to study the effect of the use of different buffers (both, during enzyme immobilization and immobilized enzyme inactivation), using triacetin and *S*-methyl mandelate as substrates to study if the inactivation courses are equivalent when using substrates with very different structures.

2. Material and methods

2.1. Materials

Triacetin and *p*-nitrophenyl butyrate (*p*NPB) were purchased from GE Healthcare (Spain). *S*- methyl mandelate was from Alfa Aesar (Fisher scientific, Spain). 2-[4-(2-hydroxyethyl)piperazin-1-yl]ethane-1-sulfonic acid (HEPES), glycine (Gly), and trizma® hydrochloride (Tris) were purchased from Sigma-Aldrich. All reagents and solvents were of analytical grade. Purolite Lifetech™ ECR8804M (octadecyl methacrylate) was a kind present from Purolite Ltd. (Wales, UK). Purolite was treated as described by Tacias-Pascacio et al., (Tacias-Pascacio et al., 2017) to fill the pores with water. 10 g of Purolite beads were suspended in 100 mL of methanol and kept under gentle continuous agitation for 1 h to remove the air inside the particles. Next, 100 mL of distilled water was added to this suspension, maintaining the agitation for 15 min. Afterwards, the suspension was vacuum filtered, washed with excess of distilled water, and stored at 4 – 6 °C.

2.2. Methods

2.2.1. Preparation of lipase extracts

Almonds were collected in Sfax (Tunisia) in July 2023. The almonds were germinated for 3 days. In that moment, the cotyledons of the almond seeds were picked up and employed to produce the enzymatic extract. This process was performed according to Bahri (BAHRI, 2012) with minor modifications. A 30 mL solution of 50 mM Tris/HCl, containing 1 mM benzamidine and 1 mM dithiothriol at pH 7 was cooled to 4 ºC. Then, 10 g of sprouted seeds were added, and they were ground using a porcelain mortar. The temperature was maintained at 4°C during the whole process by using an ice-bath. This almond-crushed suspension was vacuum filtered using a number 3 sintered glass filter, keeping the

filtered suspension. This suspension was centrifuged at 10,000 g for 20 min at 4°C to eliminate the solids. The obtained supernatant was stored at 4–6 °C, and it was employed as the lipase crude extract for any further experiment. The protein concentration was quantified by the Bradford method; as standard protein for the calibration curve, albumin from bovine serum was utilized (Bradford, 1976). The protein extract presented a protein concentration of 25 mg /mL. The extract contains not only the lipase, but other proteins, and it was utilized without any additional treatment.

2.2.2. Determination of the enzyme activity using different substrates

All experiments were performed by triplicate and values are given as mean values and experimental error.

2.2.2.1. Hydrolysis of pNPB. The increase in absorbance at 348 nm produced by the release of *p*NP in the hydrolysis of 0.4 mM pNPB in 25 mM sodium phosphate at 25 ºC and pH 7.0 was utilized to quantify the enzyme activity. A wavelength of 348 nm was selected because it is the isosbestic point of *p*NP (Wood et al., 1995). In the reaction, samples of 200 μL of lipase solution or suspension were utilized, adding this amount of lipase to 2.3 mL of substrate solution. The activity (U) is given as μmol of *p*NPB hydrolyzed per minute under the conditions given above.

2.2.2.2. Hydrolysis of triacetin. After preparing 2 mL of 50 mM of triacetin in 50 mM sodium phosphate solution at 25 °C and pH 7.0, 0.2 g of wet biocatalysts were added under continuous stirring. At pH 7, a fast acyl migration occurs to the enzyme product, producing a mixture of 1,2 diacetin and 1,3 diacetin (Hernandez et al., 2011). The enzyme activity was determined by quantifying the hydrolysis percentage at different times. To reach this goal, an HPLC equipped with a Kromasil C18 column of (15 cm × 0.46 cm) coupled to a UV detector at 230 nm were employed. As mobile phase, a 15 % acetonitrile-85 % Milli-Q water (v/v) solution was utilized, fixing the flow at 1 mL/min. The activities of the biocatalysts were determined by the production of diacetins (1,2 and 1,3 diacetins), using conditions were both compounds eluted at the same volume. Only values showing hydrolysis between 15–20 % were utilized to calculate the enzyme activity. The retention times were 18 min for triacetin and 4 min for the diacetins.

2.2.2.3. Hydrolysis of S-methyl mandelate. After preparing 3 mL of solution of 10 mM of *S*-methyl mandelate in 50 mM sodium phosphate at 25 °C and pH 7.0, a certain amount of wet biocatalyst (0.2–0.3 g) was added and the reaction suspension was submitted to continuous stirring. Mandelic acid and its ester were detected employing an HPLC with a UV/VIS detector at 230 nm utilizing as mobile phase 35 % acetonitrile-65 % an aqueous solution of 10 mM ammonium acetate (v/v) employing a Kromasil C18 column (15 cm × 0.46 cm). The utilized flow was 1 mL/min. The retention times were 4.2 min for *S*-methyl mandelate and 2.4 min for mandelic acid. Conversions between 15–20 % were used to calculate the initial reaction rates.

2.2.3. Immobilization of lipase on Purolite Lifetech™ ECR8804M (octadecyl methacrylate)

A protein loading of 25 mg of enzyme/g of wet support was utilized; the immobilization was performed at 4 ºC and pH 7, using 1 mL of enzyme solution (diluted in 9 mL of 50 mM of sodium phosphate, Tris, HEPES or Gly) per g of support.

The activities of supernatant and a solution of the enzyme under identical conditions (used as reference) (Boudrant et al., 2020) were determined utilizing *p*-NPB. After immobilization, the biocatalysts were washed with an excess of distilled water, vacuum dried and stored at 4 °C. The suspension activity was not determined using this assay due to the adsorption of the substrate on this support (Cherni et al., 2024).

2.2.4. Thermal biocatalysts inactivations

The stabilities of the free enzyme extract were determined using *p*NPB as substrate, incubating the enzyme in 50 mM of acetate or citrate at pH 5, 50 mM of phosphate, HEPES, Gly or Tris at pH 7 and 50 mM of carbonate, phosphate, or borate (always the sodium salts) at pH 9 and 35 ºC. Periodically, samples were taken, and their residual activities were measured using *p*NPB as substrate.

Using immobilized enzymes, samples of 0.5 g of the different immobilized biocatalysts were incubated in 5 mL of 50 mM Tris, sodium phosphate, HEPES or glycine at pH 7.0 and 35°C. Residual activities were determined utilizing the triacetin and S-mandelate assays described above.

The inactivation courses represented the biocatalysts residual activities, calculated as the percentage of the initial activities.

3. Results and discussion

The main objective of this paper is to analyze whether it is possible to prepare immobilized biocatalysts of the selected lipase almonds using different immobilization conditions, able to induce different enzyme features. That way, before immobilizing the enzyme, it was necessary to check conditions where the enzyme may exhibit different activity/stability features, focusing on the likely effect of different buffers.

3.1. Activity of the free almond lipase extract under different conditions

First, we checked the effect of the buffers on the enzyme activity versus pNPB at pH 5, 7, and 9 (Table 1). The lowest activity was detected at pH 5, but the values were similar using citrate or acetate. At pH 9, an intermediate activity was observed, and the values were lower using borate and quite similar using phosphate or bicarbonate. At pH 7, the effect of the buffer was the greatest one, and in some instances, it permitted to reach the highest activities versus *p*NPB using the free enzyme. The use of Tris during the activity determination measurement allowed us to obtain the highest activity, followed by phosphate; while glycine and HEPES gave the lowest activities, similar to the ones observed at pH 9 using phosphate or bicarbonate. That way, at pH 7 and using these four buffers, it seemed to be a good option to prepare different immobilized biocatalysts of this enzyme, although at pH 9 some effect concerning the buffer could also be identified.

3.2. Stability of the free almond extract under different conditions

After detecting that the buffer nature altered the activity of the enzyme, we analyzed if they may also affect the stability of the enzyme, as it has occurred in other instances (Abellanas-Perez et al., 2023a, 2023b; Carballares et al., 2022; Fernandez-Lopez et al., 2016, 2015; Kornecki et al., 2020; Zaak et al., 2017). For this purpose, the free enzyme was incubated at pH 5, 7, and 9 using different buffers, following the evolution of the activity over time. The results are shown on Fig. 1. The stability of the enzyme was the highest one at pH 5, again

Table 1
Activity of the free lipase from almond extract versus *p*NPB at 25 ºC under different medium conditions. Other specifications are described in Section 2.1. Specific activity is given as micromoles of substrate hydrolyzed per minute.

pH	Buffer	Specific activity
5	Sodium citrate	0.41±0.02
	Sodium acetate	0.43±0.03
7	Tris-HCl	1.12±0.03
	Glycine	0.61±0.02
	Sodium phosphate	0.98±0.03
	HEPES	0.62±0.02
9	Sodium phosphate	0.61±0.03
	Sodium carbonate	0.62±0.02
	Sodium borate	0.42±0.03

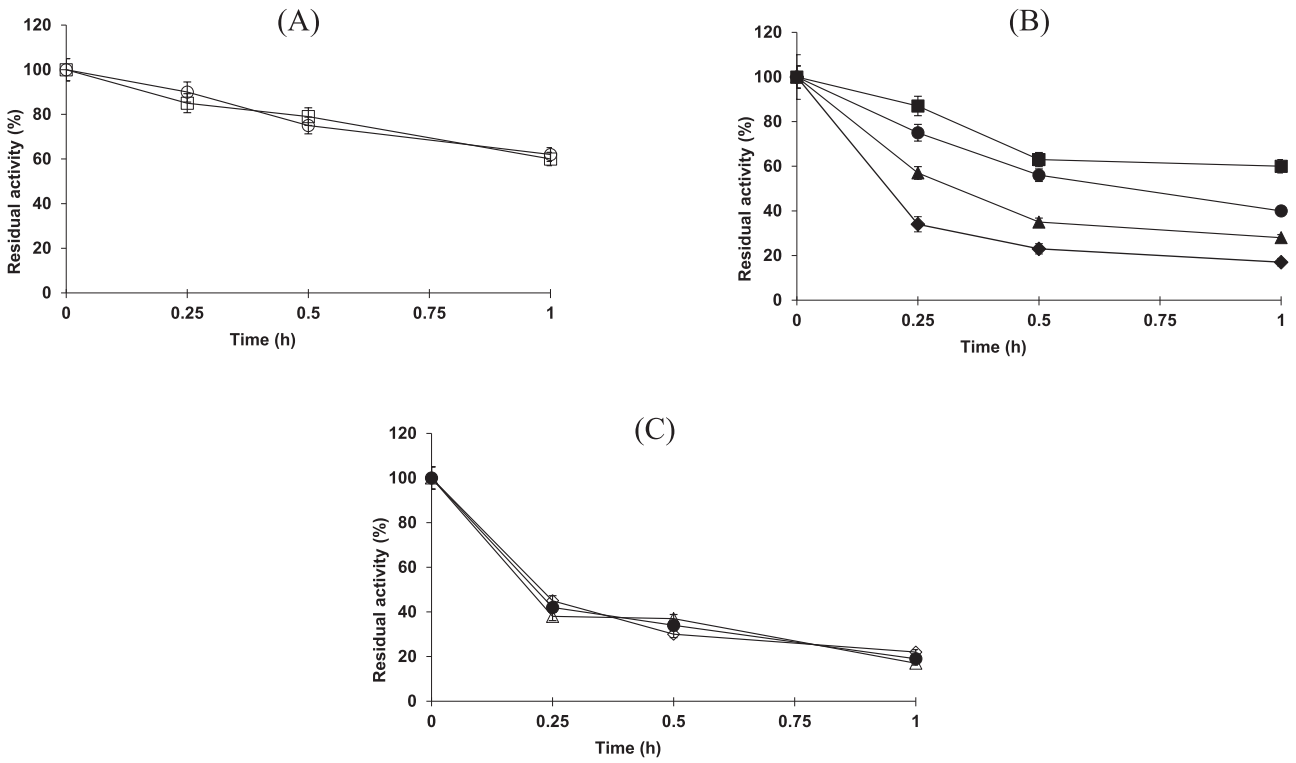

Fig. 1. Effect of different buffers on the thermal inactivation courses of the free lipase from *Prunus dulcis* almond. pH 5 (A): Empty Squares: sodium citrate; empty circles: sodium acetate; pH 7 (B): solid circles: sodium phosphate; solid squares: glycine; solid triangles: HEPES; solid diamonds: tris-HCl; pH 9 (C): empty diamonds: sodium borate; empty triangles: sodium bicarbonate. solid circles: sodium phosphate. The inactivations were carried out at 35 ºC. Other specifications are described in the Methods section.

without differences using the two studied buffers. At pH 9, the stability was lower, and it did not depend on the used buffer (although the activity seemed to depend on the buffer, as shown above). At pH 7, enzyme stability depended on the buffer, becoming higher or lower than that at pH 9 depending on the utilized buffer.

That is, at pH 5 and 9, the studied buffers (citrate and acetate or phosphate, borate, and bicarbonate, respectively) did not affect the stability of the lipase in a relevant way. However, when the inactivation was performed at pH 7, there were some clear differences on the enzyme stability depending on the used buffer. Using Gly, the inactivation is the slowest one, while using Tris, the inactivation is the fastest one, phosphate and HEPES giving intermedium stabilities.

A certain correlation between activity and stability seems to exist at pH 7. It could be expected that a buffer that favors enzyme flexibility can improve enzyme activity and become negative for enzyme stability, and *vice versa*. That way, Gly is the buffer where the highest enzyme stability is found while the lowest activity is detected, and the opposite occurred with Tris. However, HEPES and phosphate did not follow this rule, suggesting that more phenomena may be occurring.

Using free lipases, one additional complexity to consider is how the medium can affect the conformational equilibrium between the open and closed form. Perhaps the change in the ratio of open/closed forms of the lipase in the presence of different buffers may be the explanation for these results, at least in part. Furthermore, the use of free lipase may lead to too complex a situation, as the tendency of these enzymes to form aggregates confronting two lipase open forms produces composites with altered properties. That way, using the free enzyme it may not be ruled out that the changes are associated to changes in the percentages of monomeric/dimeric forms of the enzyme (Fernández-Lorente et al., 2003; Jose M. Palomo et al., 2003; José M. Palomo et al., 2003; Palomo et al., 2005, 2004; Wilson et al., 2006). In any case, the objective was to find conditions where the buffer can alter the enzyme features, and it appears that the use of Tris, phosphate, HEPES and Gly at pH 7 may be the indicated conditions.

3.3. Immobilization of the free almond extract on hydrophobic Purolite under different conditions

As it has been discussed in the introduction section, it has been shown that if the buffer is able to alter the enzyme features (and it appears that it is the case from Table 1 and Fig. 1, mainly at pH 7), and this is the effect of a change in enzyme conformation, the features of the immobilized enzymes may be different when immobilized in the presence of the different buffers, as these changes can be maintained when all the biocatalysts are used under the same conditions (Arana-Peña et al., 2021, 2020; Lokha et al., 2020). That way, the enzyme was immobilized on hydrophobic Purolite at pH 7 using the 4 buffers (Fig. 2). The immobilization rates were quite similar in all cases, slightly slower using Gly, with almost full immobilization after 2 h in all cases. The activity of the suspension using *p*NPB could not be determined, due to the capability of the support of adsorbing the substrate (Cherni et al., 2024). Although the enzyme was not pure, immobilization yields were in all cases next to 100 %, that way the purity of the samples seemed not to affect the immobilization capability of the enzyme on the used support (we are using an enzyme loading lower that the maximum one).

3.4. Activity of the different biocatalysts versus triacetin and S-methyl mandelate

Table 2 shows the activity of the different biocatalysts, measured with triacetin and *S*-methyl mandelate (the preferred isomer for this enzyme) (Cherni et al., 2024), in phosphate at pH 7. The biocatalyst prepared in phosphate was more active versus *S*-methyl mandelate, while the three other biocatalysts were more active versus triacetin than versus *S*-methyl mandelate. That way, the ratio between activities observed using triacetin and methyl mandelate was 1.82 using the

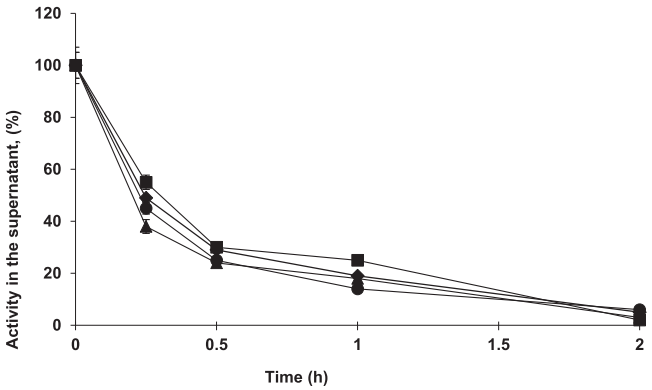

Fig. 2. Immobilization course of the lipase from *Prunus dulcis* almond on hydrophobic Purolite beads, using 25 mg of enzyme per gram of support. The immobilization was carried out by diluting the enzyme crude in 50 mM of different buffers at pH 7.0 and 25 ºC such as it is described in Method section. The reference suspension remained at 100 % of initial activity throughout the whole process. Solid circles: sodium phosphate; solid squares: glycine; solid triangles: HEPES; solid diamonds: Tris-HCl. Other specifications are described in the Section 2.2.

Table 2
Activities versus triacetin and *S*-methyl mandelate of the almond lipase biocatalysts immobilized in the presence of different buffers on hydrophobic Purolite. The experiments were performed using 50 mM sodium phosphate at 25 ºC and pH 7.0. Other specifications are described in Methods section. Activity is given as micromoles of substrate hydrolyzed per minute and per g of biocatalyst.

	Substrate		
Buffer used in the immobilization	Triacetin	*S*- methyl mandelate	$V_{triacetin}/V_{Lmetyl\ mandelate}$
Tris-HCl	7.06 ±0.25	3.87±0.24	1.82
Phosphate	4.71 ±0.14	23.17±0.86	0.20
Glycine	4.89 ±0.18	4.53±0.12	1.08
HEPES	4.26 ±0.21	3.31±0.15	1.29

biocatalyst prepared in Tris and become 0.2 using the enzyme immobilized in the presence of phosphate. This shows the great effect of immobilizing the enzyme under different conditions on the enzyme activity and specificity. The most active biocatalyst using *S*-methyl mandelate was the one prepared using phosphate as buffer, more than 5-fold more active than the second one (the enzyme immobilized in the presence of Gly). The results were fairly different using triacetin, although with shorter differences (a factor of 1.45 between the enzyme immobilized in Tris (the most active one), and the enzyme immobilized in the presence of phosphate or Gly). The least active biocatalyst was, using both substrates, the biocatalyst prepared in the presence of HEPES. That way, the enzyme activity and specificity strongly depended on the buffer used in the immobilization, even when assaying the biocatalysts activities under the same conditions.

3.5. Stability of the different biocatalysts under different conditions using triacetin and S-methyl mandelate to determinate the residual activity

Next, the different biocatalysts were inactivated at pH 7 in the presence of the different buffers used in this paper. As discussed in the introduction section, the inactivation was followed using both, *S*-methyl mandelate and triacetin to determine the residual enzyme activity.

Generally, the activity versus triacetin decreases faster than using *S*-methyl mandelate for the different biocatalysts (Fig. 1S, Figs. 3 and 4), as it occurred in other cases using different substrates in order to follow the thermal inactivation of some immobilized lipases (da Rocha et al., 2022; Paiva Souza et al., 2021; Souza et al., 2022). Focusing on the results obtained using the mandelic ester as substrate to determine the decrease on enzyme activity (Fig. 3), the stability depends on both the buffer used on the enzyme immobilization and the buffer used for the biocatalyst inactivation. If the inactivation was performed in the presence of HEPES, the biocatalyst prepared in the presence of Gly resulted the most stable one while all the other biocatalysts exhibited similar stabilities (although slightly lower for the biocatalysts prepared in Tris). Using Tris during inactivation, again the biocatalyst prepared deactivated in the presence of Gly resulted to be the most stable one, being the enzyme immobilized in the presence of Tris the least stable lipase preparation. If phosphate was the buffer used during inactivation, the differences were very small, although again the biocatalyst prepared in the presence of Gly was slightly more stable than the other 3 biocatalysts. However, if the inactivation was performed in the presence of Gly, the situation was curiously very different: the only biocatalyst that clearly presented a different stability was the one prepared in the presence of Gly, and that was the least stable one, in contraposition with the results found when the biocatalysts were inactivated in the presence of the other 3 buffers.

Using triacetin (Fig. 4) to determine the residual activity of the biocatalysts, the differences on the enzyme stabilities were shorter than when using the mandelic ester. If the inactivation was performed in HEPES, the biocatalyst prepared in Tris was the most stable, however using Tris in the inactivation suspension, the biocatalyst prepared in Gly showed the highest stability. Using phosphate in the inactivation suspension, only the biocatalyst prepared using Tris was slightly less stable than the other biocatalysts, while when using Gly in the inactivation suspension, the biocatalysts prepared in Tris and phosphate were the most stable ones. That is, there is a clear interaction in the effects of inactivating and immobilization buffers in the enzyme stability.

Next, we have compared the stabilities of each biocatalyst in different buffers using both substrates. To facilitate the analysis, Fig. 1S shows the inactivations coupling the same biocatalyst using the 4 buffers of the inactivating solutions and the two substrates used to determine the residual activities.

Starting with the biocatalyst prepared in the presence of HEPES, when measuring the activities using *S*-methyl mandelate, the stability of the biocatalyst in the presence of Gly was the lowest, followed by the stability in Tris while in the presence of the other two buffers the stabilities were fairly similar. Using triacetin as substrate, the lowest stability was found using HEPES in the inactivation suspension, and the highest ones were those observed using Gly or Tris as inactivating buffers. The biocatalysts stabilities under these buffers using triacetin were similar to those observed when inactivating the biocatalyst in the presence of Gly using the mandelic ester. That is, depending on the substrate used to quantify the activities, Gly was the one that promoted the lowest or the highest stabilities when it was presented in the inactivation suspension, for this biocatalyst. This shows the complexity of the involved processes, assuming that the different stabilities/activities using different substrates are the result of different enzyme conformations; the prediction of the stability of an enzyme using one substrate based on the stability found using another substrate can be misleading.

The biocatalyst prepared in the presence of Tris also presented very different inactivation courses depending on the substrate and the buffer employed in the inactivation suspension. Using methyl mandelate, once again the lowest stabilities were those detected in the presence of Gly and Tris, with phosphate and HEPES allowing reaching the highest stabilities. The stabilities using triacetin were lower than when using the mandelic derivative. The lowest stability was that found using phosphate, followed by the use of Gly and HEPES. Using Tris, the stability was the highest, becoming similar to the stability of this biocatalyst detected in the presence of Gly but using *S*-methyl mandelate. Again, a clear interaction between the inactivation buffer and the substrate used

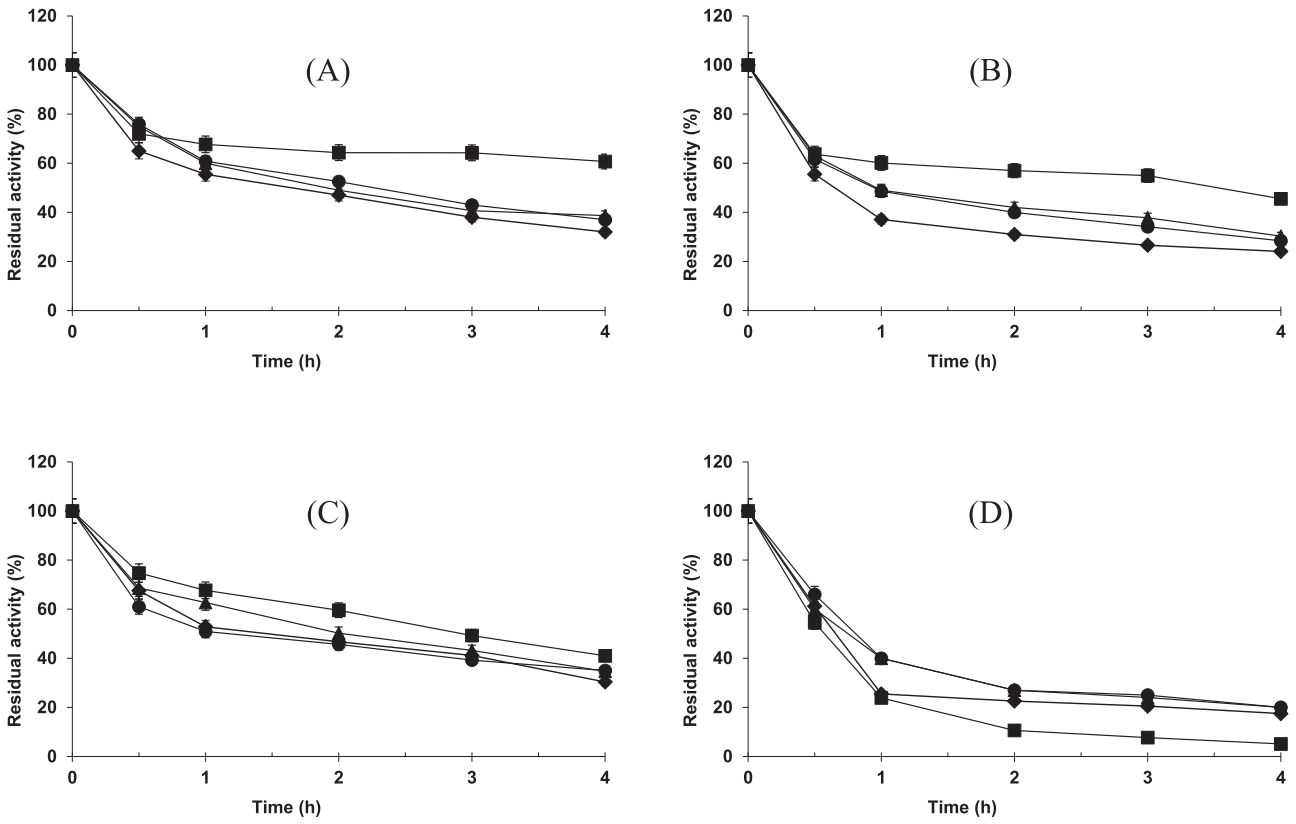

Fig. 3. Effect of the inactivation buffer on the thermal stability at 35ºC of the lipase from *Prunus dulcis* immobilized in the presence of different buffers using *S*-methyl mandelate as substrate to determine their residual activity. The thermal inactivation was performed using different 50 mM buffers at pH 7.0 in the inactivating suspensions. Solid circles: sodium phosphate; solid squares: glycine; solid triangles: HEPES; solid diamonds: tris-HCl. Enzyme immobilized in the presence of HEPES (A); Enzyme immobilized in the presence of Tris-HCl (B); Enzyme immobilized in the presence of sodium phosphate (C) and enzyme immobilized in the presence of Gly (D). Other specifications are described in the Methods section.

to determine the residual activities could be found, and this was unlike the previous biocatalyst, so these effects may also depend on the biocatalysts used.

The biocatalyst prepared in phosphate buffer also exhibited complex interactions between the inactivating buffer and substrate, maintaining in general higher stabilities using *S*-methyl mandelate. Using methyl mandelate, the stability was the lowest when this biocatalyst was inactivated using Gly as buffer during inactivation, and the highest stability was found employing HEPES, shortly followed by the stability found in phosphate. If triacetin was used as substrate to determine the activities, the situation is the opposite one; the stability of the biocatalyst is the highest in the presence of Gly and the lowest in the presence of HEPES.

Finally, we can analyze the effect of the inactivating buffers and substrate utilized to determine the residual activity using the biocatalyst immobilized in the presence of Gly. Again, stability was apparently higher using methyl mandelate than triacetin in most cases. Using *S*-methyl mandelate, the highest stability was that detected using HEPES as inactivating buffer, the use of phosphate and Tris resulted in biocatalysts presenting similar intermedium stabilities, while the lowest stability was that observed using Gly. Using triacetin, the lowest stability was obtained in HEPES (when using methyl mandelate, this was the buffer where the highest stability was found), the biocatalyst immobilized in the presence of Gly and inactivated in the presence of phosphate and Gly displayed intermediate stabilities, (the inactivation courses became similar to those detected using methyl mandelate and Gly). Using Tris as buffer in the inactivating solution, the stability of this biocatalyst was the highest one, and the biocatalyst stability observed using triacetin became much higher than using methyl mandelate in inactivations performed with Gly (but not if the inactivation was performed in any of the other 3 buffers). Explanations should be similar to the ones described above, and these show the complexity of trying to extrapolate one result obtained in a specific condition to another one.

4. Conclusions

The results in this paper show how the stability of an immobilized lipase from almond depends on the immobilization buffer, buffer nature used in the inactivation suspension, and substrate used to determine the residual activity. The interaction between these three parameters suggests that the final immobilization protocol for this enzyme should be determined after studying the enzyme activity/stability using the specific conditions of use and the reaction and substrate that are going to be utilized in the target process. The interactions are not only quantitative, but also qualitative. The immobilization buffer that permits the highest stability in the presence of an inactivating buffer can be the worst for enzyme stability changing the substrate used to determine the enzyme residual activity and/or the inactivation buffer. We can hypothesize that the buffer induces specific conformational changes on the enzyme (during the immobilization in this buffer, as it has been shown previously (Lokha et al., 2020) and favors specific conformational changes during inactivation, which can be more or less adequate to modify a specific substrate (Paiva Souza et al., 2021).

Comparing the results of this paper with previous reports, this is the first lipase immobilized via interfacial activation where phosphate does not present the most negative effect on its stability (Abellanas-Perez et al., 2023a, 2023b; Zaak et al., 2017). It is curious that all previous studies on immobilized lipases where the inactivation had been determined using triacetin or methyl mandelate, usually present a higher stability using the mandelic derivative, which is the least similar substrate to the physiological ones (triglycerides). For this enzyme, the

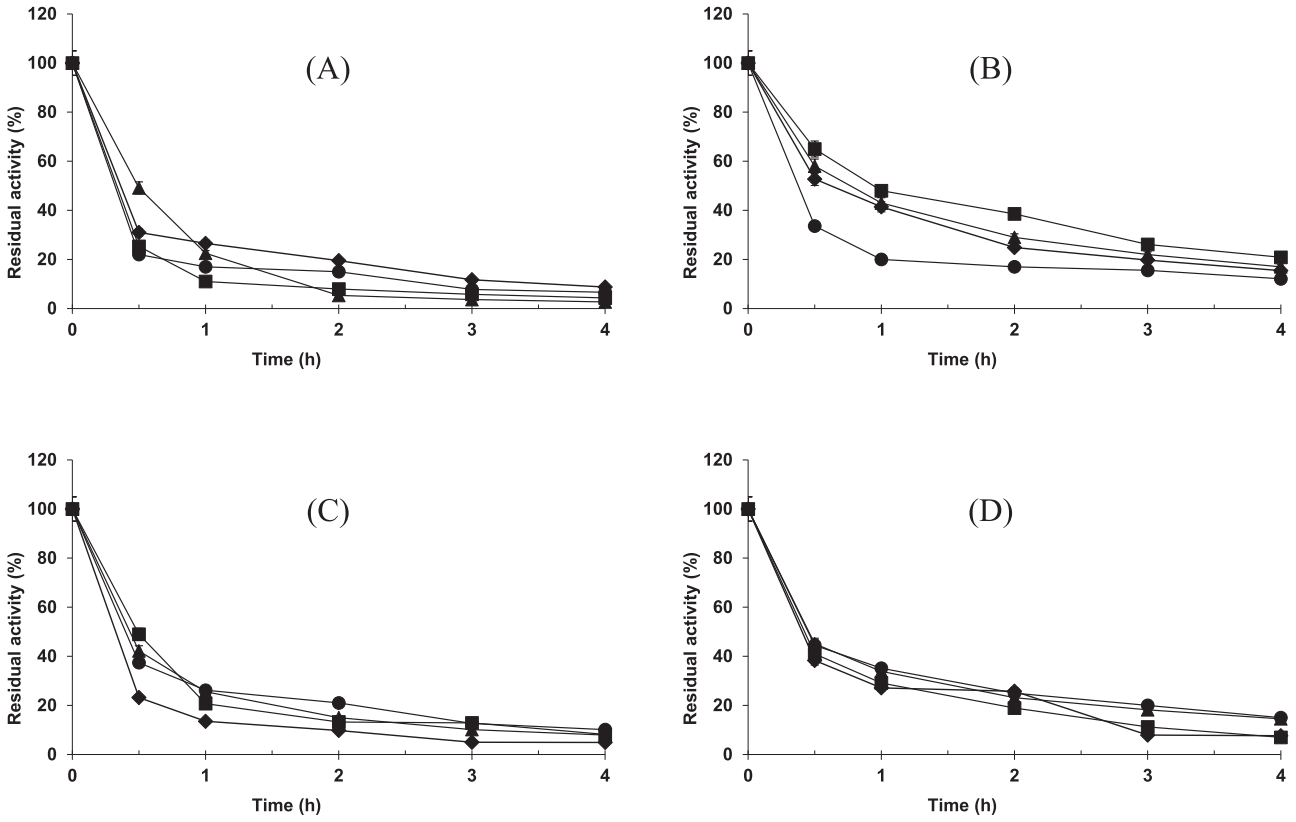

Fig. 4. Effect of the inactivation buffer on the thermal stability at 35 ºC using triacetin as substrate to determine residual activity of the lipase from *Prunus dulcis* almond immobilized in the presence of different buffers. The thermal inactivation experiment was performed using different 50 mM buffers at pH 7.0 in the inactivation suspension. Solid circles: sodium phosphate; solid squares: glycine; solid triangles: HEPES; solid diamonds: tris-HCl. Enzyme immobilized in the presence of HEPES (A); Enzyme immobilized in the presence of Tris-HCl (B); enzyme immobilized in the presence of sodium phosphate (C) and enzyme immobilized in the presence of Glycine (D). Other specifications are described in the Methods section.

differences when using these substrates are shorter than those described using the lipase B from *Candida antarctica* or the lipase from *Thermomyces lanuginosus* (da Rocha et al., 2022; Paiva Souza et al., 2021; Souza et al., 2022) and in certain cases it is even higher using triacetin than methyl mandelate.

The results presented in this paper suggest the complexity of the phenomena occurring in the inactivation of enzymes, and specifically in immobilized lipases. There are multiple interactions between different variables, and we can guess that some variables have not been considered yet. That way, research in this area seems to be fully necessary to advance in the understanding of enzyme stability.

CRediT authorship contribution statement

Oumaima Cherni: Writing – original draft, Investigation, Formal analysis. **Sellema Bahri:** Writing – review & editing, Data curation. **Roberto Fernandez-Lafuente:** Writing – review & editing, Writing – original draft, Supervision, Methodology, Funding acquisition, Formal analysis, Data curation, Conceptualization. **Maria de Lourdes Teixeira de Moraes Polizeli:** Writing – review & editing, Formal analysis. **Javier Rocha-Martin:** Writing – review & editing, Writing – original draft, Formal analysis, Data curation, Conceptualization. **Pedro Abellanas-Perez:** Writing – review & editing, Methodology, Data curation. **Diandra de Andrades:** Writing – review & editing, Investigation, Formal analysis. **Diego Carballares:** Supervision, Investigation, Formal analysis, Data curation. **El Hocine Siar:** Writing – original draft, Investigation, Data curation.

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.

Acknowledgments

We gratefully recognize the financial support from Ministerio de Ciencia e Innovación and Agencia Estatal de Investigación (Spanish Government) (PID2022-136535OB-I00). Oumaima Cherni thanks the Tunisian Ministry of Higher Education and Scientific Research for her fellowship to perform her stay at ICP-CSIC. The authors gratefully acknowledge FAPESP (São Paulo Research Foundation) by research scholarship to DA (Grant No: 2023/01338-7). The help and suggestions from Dr. Ángel Berenguer (Departamento de Química Inorgánica, Universidad de Alicante) are gratefully recognized.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.jbiotec.2024.06.009.

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