Effect of amine length in the interference of the multipoint covalent immobilization of enzymes on glyoxyl agarose beads

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A R T I C L E   I N F O

A B S T R A C T

Trypsin, chymotrypsin, penicillin G acylase and ficin extract have been stabilized by immobilization on glyoxyl agarose, adding different aliphatic compounds bearing a primary amine group during the immobilization: ethyl amine, butyl amine, hexyl amine (at concentrations ranging from 0 to 20 mM) and octyl amine (from 0 to 10 mM) to analyze their effects on the immobilized enzyme stability. As expected, the presence of amines reduced the intensity of the enzyme-support multipoint covalent attachment, and therefore the enzyme stability. However, it is clear that this effect is higher using octyl amine for all enzymes (in some cases the enzyme immobilized in the presence of 10 mM octyl amine was almost inactivated while the reference kept over 50 % of the initial activity). This way, it seems that the most important effect of the presence of aminated compounds came from the generation of steric hindrances to the enzyme/support multi-reaction promoted by the ammines that are interacting with the aldehyde groups. In some instances, just 1 mM of aminated compounds is enough to greatly decrease enzyme stability. The results suggested that, if the composition of the enzyme extract is unknown, to eliminate small aminated compounds may be necessary to maximize the enzyme-support reaction.

1. Introduction

Enzymes are very interesting alternatives to industrial catalysts (Schmid et al., 2001; Sheldon and Brady, 2019; Woodley, 2020, 2019). Their high selectivity, specificity and activity under mild conditions make them ideal biocatalysts from the point of view of green chemistry (Schmid et al., 2001; Sheldon and Brady, 2019; Woodley, 2020, 2019). However, they have been designed by Nature to fulfill the physiological requirements, and that makes that many enzyme features need to be improved before utilization (Schoemaker, 2003). In this sense, many different tools are available to the researcher to solve the enzyme limitations, many of them relatively recent, such as metagenomics (Fernández-Arrojo et al., 2010; Ferrer et al., 2009; Vieites et al., 2009), directed evolution (Cao et al., 2020; Markel et al., 2020; Qu et al., 2019), chemical modification (Boutureira and Bernardes, 2015; Reddy et al., 2020; Sakamoto and Hamachi, 2019; Spicer and Davis, 2014) or

immobilization (Arana-Peña et al., 2021; Bilal et al., 2019; Garcia-Galan et al., 2011; Iyer and Ananthanarayan, 2008; Mateo et al., 2007b; Wahab et al., 2020). In many instances, some strategies are used in a simultaneous, even synergic way (Fernandez-Lafuente, 2009; Fernandez-Lorente et al., 2008; Hernandez and Fernandez-Lafuente, 2011; Rodrigues et al., 2014, 2011, 2009; Tacias-Pascacio et al., 2019b, 2019a). Advances have permitted to create enzymes bearing two active centers (plurizymes) (Santiago et al., 2018) and, after modifying only one of them with an organometallic catalysts, cascade reactions were catalyzed by the modified plurizyme (Alonso et al., 2020).

However, immobilization remains as a key step in many biocatalysts developments. Initially developed for facilitating the recovering of the enzymes (Di Cosimo et al., 2013; Liese and Hilterhaus, 2013; Sheldon and van Pelt, 2013), nowadays, a proper enzyme immobilization protocol must permit the enzyme stabilization and hyperactivation (Garcia-Galan et al., 2011; Mateo et al., 2007b; Rodrigues et al., 2013),

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tailoring its selectivity or specificity (Abreu Silveira et al., 2019; Rodrigues et al., 2013), reducing its inhibition (Mateo et al., 2007b), or even purifying the enzyme (Barbosa et al., 2015).

Enzyme stabilization by immobilization may be achieved if an intense and controlled multipoint covalent attachment may be established between enzyme and support, as all points of the enzyme surface attached to the support must maintain the relative distance except by the length of the spacer arm (Mateo et al., 2007b). To reach this, the support, the reactive groups in enzyme and support and the immobilization protocol must be carefully selected and designed (Barbosa et al., 2013; J. C. S. D. Dos Santos et al., 2015a, 2015b, 2015c, 2015d). Among the most efficient active groups on the support to get this multipoint covalent attachment, glutaraldehyde (Barbosa et al., 2014; Migneault et al., 2004), epoxide (Hilterhaus et al., 2008; Katchalski-Katzir and Kraemer, 2000; Mateo et al., 2007a), vinyl sulfone (Dos Santos et al., 2015a, 2015b, 2015c, 2015d; Ortega-Muñoz et al., 2010; Santos-Moriano et al., 2016) and glyoxyl (Mateo et al., 2006) stand up.

Glyoxyl supports have some special characteristics that determine its great potential, even when it only involves primary amino groups in the immobilization (Mateo et al., 2005). The short spacer arm, the lack of steric hindrances for the reaction between the groups in the enzyme and support and its high stability are properties that favor an intense enzyme support multi-interaction (Mateo et al., 2006). The fact that one glyoxyl-amino bond is very weak and unable to fix the enzyme to the support, making the establishment of several enzyme-support bonds necessary to fix the enzyme on the support, is apparently a bad feature of this support activation method. Because of this feature, only supports with good geometric congruence with the enzyme (e.g., offering almost flat surfaces) and with high activation levels, and the immobilization at alkaline pH value, can even immobilize the enzymes. However, this is the feature that makes glyoxyl-supports so suitable to stabilize enzymes: the low energy and reversibility of the imino bond does not introduce strong distortions of the enzyme structure and, even more importantly, directs the immobilization towards the area where there are more reactive groups on the enzyme surface (Mateo et al., 2005). This means that the enzyme is immobilized by the area where it is the easiest to get a very intense multipoint covalent attachment (Mateo et al., 2006), although, in some instances, it may be not the area where a highest stabilization may be obtained if it is not very relevant for enzyme stability (Grazu et al., 2012; Grazú et al., 2010; Mansfeld et al., 1999; Mansfeld and Ulbrich-Hofmann, 2000). This also makes the immobilization rate very dependent on the activation degree of the support and on the immobilization pH value (in many instances, enzyme immobilization is not observed at pH under 9) (Mateo et al., 2005). However, even this immobilization pH limitation may be transformed into an advantage, as this make that glyoxyl support may be utilized to immobilize/stabilize/purify multimeric enzymes using a neutral pH value in the immobilization (Bolivar et al., 2009; Rocha-Martin et al., 2009).

This multipoint covalent immobilization may be hindered by the presence of some compounds during multipoint immobilization. Borate is able to block the aldehyde groups and reduces the immobilization rate and also the intensity of the multipoint covalent attachment (Lopez-Gallego et al., 2015). By observing the mechanism of immobilization (Mateo et al., 2005), the presence of small compounds bearing primary amino compounds should be another problem in the multipoint covalent immobilization. These aminated compounds may be contained in the protein extract without the knowledge of the researcher (amino acids, aminated sugars). However, we have not found any papers where a systematic analysis on the effects of aminated compounds in the multipoint covalent immobilization of enzymes on glyoxyl supports, that way the intensity of this possible effects or the concentration of aminated comunds where it may be to be significxat has not been studied to date.

The presence of compounds bearing primary amino groups will generate a first obvious problem, as part of the glyoxyl groups will be, even reversibly, blocked by them. This may reduce the available groups

for the multipoint covalent attachment. Moreover, we can also visualize a second problem: this blocking may generate some steric hindrances (as the aminated compound interacting with the aldehyde group will form a layer that be over the glyoxyl layer) for the reaction between the enzyme and the support, giving a lower intensity in the multipoint covalent attachment as a result (see Fig. 1). If this steric effect is relevant, the bulkier the compound, the higher the negative effect on the multipoint covalent immobilization will occur. This effect has been never studied before and may become even more relevant than the blocking of the reactive groups located in the support.

In this paper, we have selected 3 enzymes that have been greatly stabilized via multipoint covalent attachment on glyoxyl supports, where the literature indicates that the more intense the multipoint covalent attachment is, the higher enzyme stabilization is obtained. These enzymes are trypsin and chymotrypsin from bovine pancreas (Pedroche et al., 2007) and penicillin G acylase from *Escherichia coli* (Abian et al., 2004). We have also included in the study ficin (Morellon-Sterling et al., 2020), a plant proteases extract where an optimal level of multipoint covalent immobilization has been found and them stability starts to decrease, not only when immobilizing the enzyme on glyoxyl via multipoint covalent attachment but also when intermolecular glutaraldehyde crosslinking is produced (Siar et al., 2018, 2017, 2019). As support, we have used agarose to prevent any unexpected interference with the groups in the matrix (Zucca et al., 2016). As model aminated compounds, we have selected aliphatic amines with different sizes in the chain, i.e.: ethyl amine, butyl amine, hexyl amine and octyl amine. They differ in size (enabling the study of the effect of the staring hindrances in the final immobilized enzyme stability), while the pK of the ethyl amine (10.0 ± 0.1) is the only that is significantly lower than that of the other amines (with pK values of 10.69 +/- 0.10, 10.69 +/- 0.10, 10.75 +/- 0.10, respectively) (Calculated using Advanced Chemistry Development (ACD/Labs) Software V11.02 (© 1994–2020 ACD/Labs)). The pK of the amine group will mark the reactivity with the glyoxyl groups at pH 10, therefore, it will determine the blocking of the support reactive groups by the aminated compounds We have analyzed how different concentrations of each of these compounds bearing a primary amino group affect the final enzyme stability, and for the most relevant cases, the number of Lys involved in the multipoint covalent immobilization has been determined (when the pure enzymes were available).

2. Material and methods

2.1. Materials

Chymotrypsin and trypsin (pure enzymes in lyophilized powder) and PGA (60 mg of protein per mL, no pure enzyme) were purchased from Sigma Aldrich (Spain). Ficin extract (60 mg of protein per mL) was obtained as described elsewhere (Siar et al., 2020). The protein concentration was determined through Bradford's method using bovine serum albumin as standard (Bradford, 1976). 6-nitro-3-(phenyl acetamido) benzoic acid (NIPAB), N-α-benzoyl-DL-arginine *p*-nitroanilide hydrochloride (BAPNA), N-benzoyl-L-tyrosine *p*-nitroanilide (BTPNA), butyl amine, hexyl amine, octyl amine and sodium borohydride were also purchased from Sigma Aldrich (Spain). Ethyl amine was purchased from Fisher chemical (Ukrain). 6% BCL Agarose Beads Standard were purchased from ABT (Spain) and were used to produce glyoxyl-agarose as previously described (Guisán, 1988). All other reagents were of analytical grade.

2.2. Methods

All experiments were performed in triplicate and the data are given as mean values and standard deviation.

2.2.1. Assay of enzymes activity

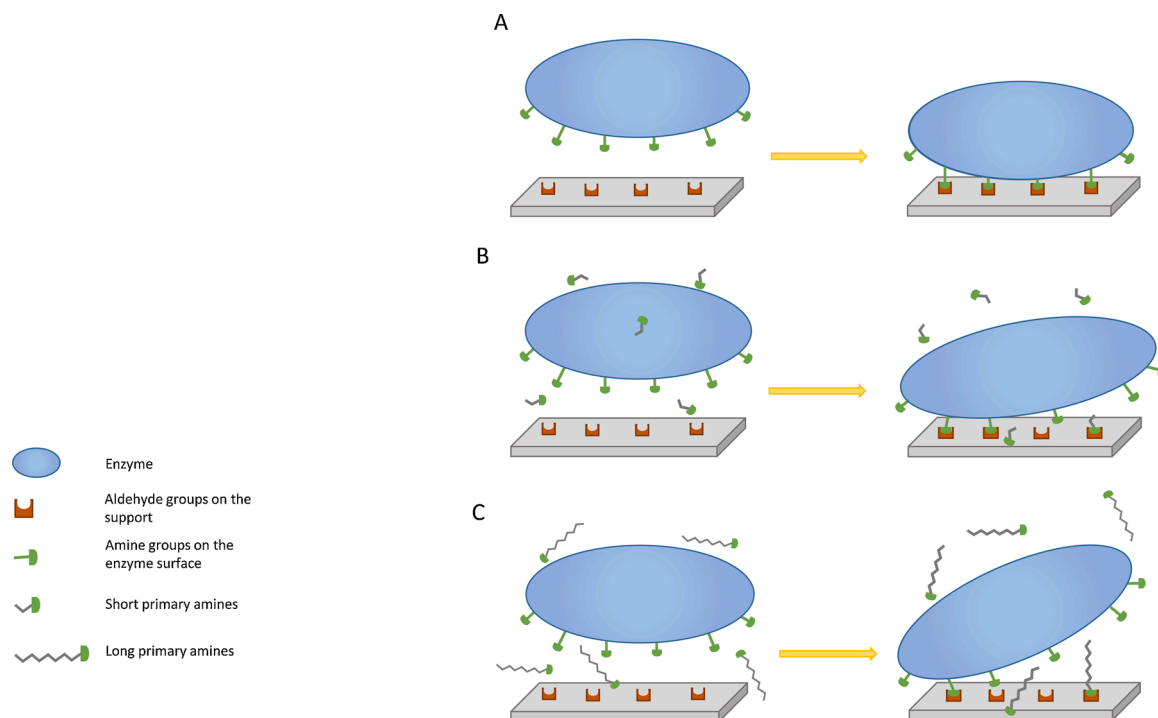Enzyme activities were determined using a spectrophotometer with

Fig. 1. Immobilization of an enzyme on glyoxyl-agarose supports (A) in absence of primary amines in the medium, (B) in presence of short length amines and (C) in presence of long length amines.

magnetic stirring at 200 rpm, with the temperature controlled at 25 °C, except for ficin activity determination, where the activity was determined at 55 °C. One unit (U) of activity was defined as the amount of enzyme that hydrolyses 1 μmol of substrate per minute under the specified conditions.

Ficin and trypsin activities were quantified determining the change in absorbance at 405 nm caused by the release of *p*-nitroaniline (ε is 9960 M^{-1} cm^{-1} under these conditions (Rocha-Martin et al., 2018)), produced by the hydrolysis of BAPNA. For ficin activity determination, BAPNA was prepared at 1 mM in 100 mM phosphate at pH 7.0, containing 5 mM cysteine and 5 mM EDTA. 200 μL of enzyme solution or suspension were added to 2.5 mL of substrate solution and incubated at 55 °C for 15 min (Siar et al., 2017). When it was used as trypsin substrate, BAPNA was prepared at 2 mM in 50 mM sodium phosphate at pH 7.0 containing 30 % ethanol (Pedroche et al., 2007). To initialize the reaction, 200 μL of enzyme solution or suspension were added to 2.5 mL of BAPNA solution.

Chymotrypsin activity was determined by measuring changes in absorbance at 386 nm produced by the release of *p*-nitroaniline following the hydrolysis of BTPNA (ε is 12,500 M^{-1} cm^{-1} under these conditions (Moyano et al., 2013)). A 40 mM BTPNA stock solution was prepared in DMSO, 75 μL BTPNA solution was added to 2.5 mL of 100 mM sodium phosphate at pH 7.0 containing 40 % ethanol. To initialize the reaction, 200 μL of enzyme solution or suspension were added.

Activity of PGA was determined using NIPAB as described by Kutzbach et al. (Kutzbach and Rauenbusch, 1974). The activity was measured following the increase in absorbance at 405 nm caused by the hydrolysis of NIPAB (ε under these conditions is 8730 M^{-1} cm^{-1} (Kutzbach and Rauenbusch, 1974)). The assay was performed using 0.15 mM NIPAB in 50 mM sodium phosphate at pH 7.5. To initialize the reaction 100−200 μl of enzyme suspension or solution were added.

2.2.2. Immobilization of the enzymes on glyoxyl agarose beads

Enzyme immobilizations were performed following the activity of supernatant, suspension and a reference, immobilization yield was in all cases very close to 100 % (Boudrant et al., 2020). The immobilization time was in all cases 3 h.

All immobilizations were performed at pH 10.05, using 50 mM sodium carbonate. Ficin was immobilized using 10 mg of enzyme per g of support, chymotrypsin and trypsin were immobilized using a loading of 3 mg of enzyme per g of support (3 mM of benzamidine was added to prevent autolysis in the case of trypsin (Blanco and Guisán, 1988)) using the protocols previously described (Abian et al., 2004; Guisán et al., 1991; Mateo et al., 2005; Siar et al., 2017).

PGA was immobilized using 3 mg of enzyme per g of support. The enzyme was added to 50 mM carbonate solution containing 100 mM phenyl acetic acid and 20 % glycerol to prevent enzyme inactivation, at pH 10.05 (Abian et al., 2004; Rosell et al., 1995), and the immobilization was performed at a ratio of 1 g of support per 10 mL of enzyme solution.

To analyze the effect of the amines in the immobilization, 1,2,5, 10 or 20 mM of the amines were added to the enzyme immobilization solution, except for octyl amine that only was soluble at pH 10.05 at a maximum concentration of 10 mM.

After enzymes immobilization, 1 mg of solid sodium borohydride was added per ml of immobilization suspension and the immobilization suspensions were stirred for 30 min. Finally, the biocatalysts were washed with water, vacuum dried to eliminate inter-particle water and stored at 6−8 °C.

2.2.3. Thermal inactivations of the different biocatalysts

The different biocatalysts were inactivated at pH 5.0, 7.0 and 9.0, using temperatures where the inactivation courses showed a reliable inactivation rates. The used buffers were 100 mM sodium acetate at pH 5.0, 100 mM sodium carbonate at pH 9.0 and 100 mM sodium phosphate at pH 7.0 (Kornecki et al., 2020; Zaak et al., 2017a). Periodically, samples were withdrawn, and their residual activities were determined.

2.2.4. Determination of amino acid composition

Amino-acid composition of free and immobilized enzymes was determined according to the method of Alaiz et al. (Alaiz et al., 1992) with slight modifications. In this sense, samples were hydrolysed by

incubation in 6 N HCl at 110 °C for 24 h in tubes sealed under nitrogen. Amino acids were determined in the acid hydrolysate by ultra-high-performance liquid chromatography (Acquity Arc, Waters, USA), after derivatization with diethyl ethoxymethylenemalonate, using D,L-α-aminobutyric acid as internal standard, and a 3 mm X 150 mm reversed-phase column (XSelect HSS T3 XP, 2.5 μm; Waters). A binary gradient system with the solvents (A) 25 mM sodium acetate 0.02 % sodium azide (pH 6.0), and (B) acetonitrile was used. Calibration curves for each amino acid were developed using a mix of amino acid standard at the same hydrolysis conditions of the samples (Merck, Spain) and the resultant peaks were analysed with EMPOWER software (Waters, USA). Calculations were performed considering that each trypsin and chymotrypsin molecules have 14 Lys groups (Hunt et al., 1997). The calculations were performed using some reference amino acids and comparing the intensity of their peaks with that of the Lys (. Dos Santos et al., 2015a, 2015b, 2015c, 2015d; Pedroche et al., 2007).

3. Results

3.1. Effect of the presence of aliphatic amines on the results obtained in the immobilization of trypsin on glyoxyl agarose

The effect of the presence of amines on the immobilization of trypsin is the first example that we are going to discuss. Enzyme immobilization after 15 min is complete even using 20 mM of the amines, in all cases the final enzyme activity was over 85 % (not shown results). This shows that trypsin immobilization under optimal conditions on this support is so rapid that the researcher can reach the wrong conclusion that there is nothing interfering with enzyme immobilization. Figs. 2–5 show the inactivation courses at pH 5, 7 and 9 of the biocatalysts prepared using the different amines at different concentrations. The presence of ethyl amine (Fig. 2) does not have a very relevant effect on enzyme stability when inactivated at pH 5 and 9, slightly decreasing the enzyme stability mainly when using 20 mM of ethyl amine. The negative effect of the presence of ethyl amine during the enzyme immobilization was higher at pH 7, with a low impact using 1−5 mM ethyl amine and becoming

clearer using 20 mM. However, the effect of the presence of ethyl amine during the enzyme immobilization on the enzyme stability using this enzyme was not very negative. Similar results were found using butyl amine (Fig. 3). Using hexyl amine during the enzyme immobilization, the effect was not very relevant at any pH value (Fig. 4). Fig. 5 shows the results using the bulkiest amine utilized in this assay, and even though the effect of its incorporation during enzyme immobilization in the inactivations at pH 5 and 9 was not very clear. At pH 7 there is a clear negative effect, much clearer than using the other amines. However the effect, although clear, was not very high: the residual activity of the biocatalyst prepared in absence of octyl amine was 43 % and in the presence of 10 mM was 19 % after 4 h of inactivation at pH 7 and 78 °C.

Therefore, in the case of trypsin, the presence of these amines does not seem to be very relevant on the final performance of the biocatalysts.

3.2. Effect of the presence of aliphatic amines on the results obtained in the immobilization of chymotrypsin on glyoxyl agarose

The next example is the immobilization of chymotrypsin on glyoxyl. As in the case of trypsin, immobilization was completed after only 15 min in all cases, and all enzyme preparations presented a similar expressed activity (around 70 %).

The effect of ethyl amine during immobilization on enzyme stability (Fig. 6) was mainly relevant when the inactivation was performed at pH 7. At pH 7, the biocatalysts prepared using 10 or 20 mM of ethyl amine presented a residual activity after 3 h around 25 %, while in absence of amine, this residual activity was 60 %. The effect was already very relevant using just 1 mM of ethyl amine. When inactivating the enzyme at pH 5 and 9, the effect on the enzyme stability in the presence of ethyl amine during the chymotrypsin immobilization was clearly smaller than at pH 7, but clearer than using trypsin (Fig. 2). Using butyl amine in the immobilization (Fig. 7), the negative effect increased, decreasing the enzyme stability at all the inactivation pH values. At pH 5, using 20 mM of butyl amine during immobilization, the enzyme residual activity after 3 h of inactivation was 15 %, while that value in the biocatalysts prepared in absence of amine was 37 %. When inactivating the biocatalysts
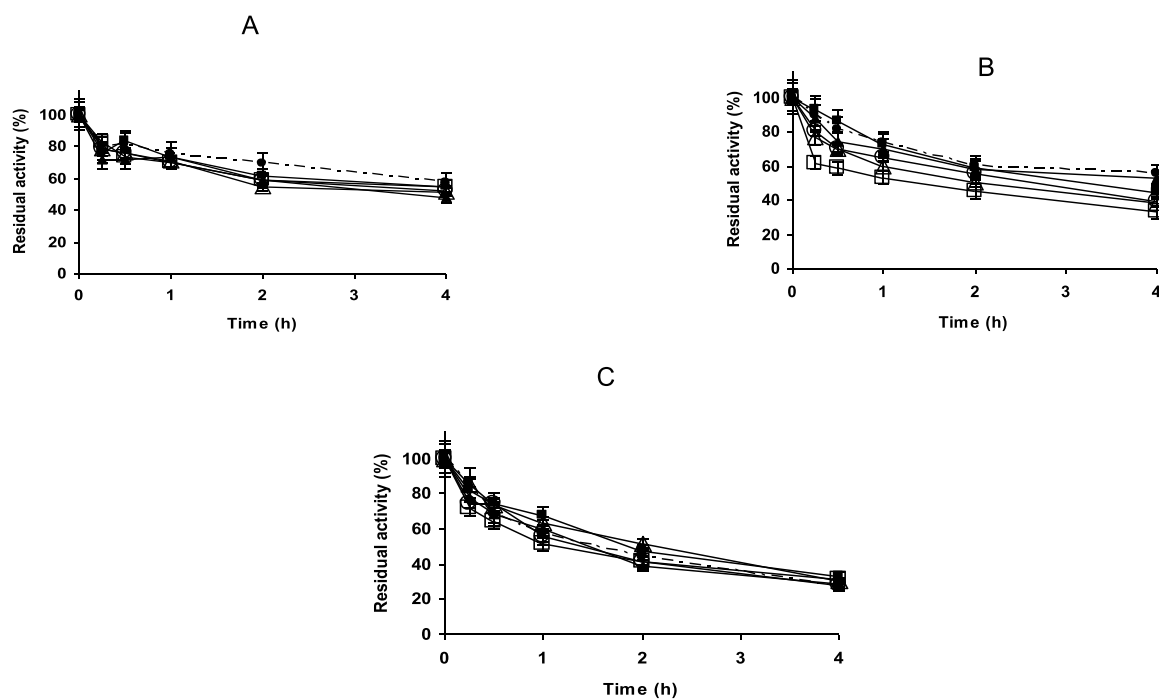

Fig. 2. Effect of the presence of ethyl amine during the immobilization of trypsin on the stability of the immobilized enzyme. Inactivations have been performed at (A) pH 5.0, 83 °C; (B) pH 7.0, 78 °C; (C) pH 9.0, 70 °C. Other specifications are described in Methods section. Full circles: Reference without amine. Full triangles: 1 mM. Full squares: 2 mM. Empty circles: 5 mM. Empty triangles: 10 mM. Empty squares: 20 mM.
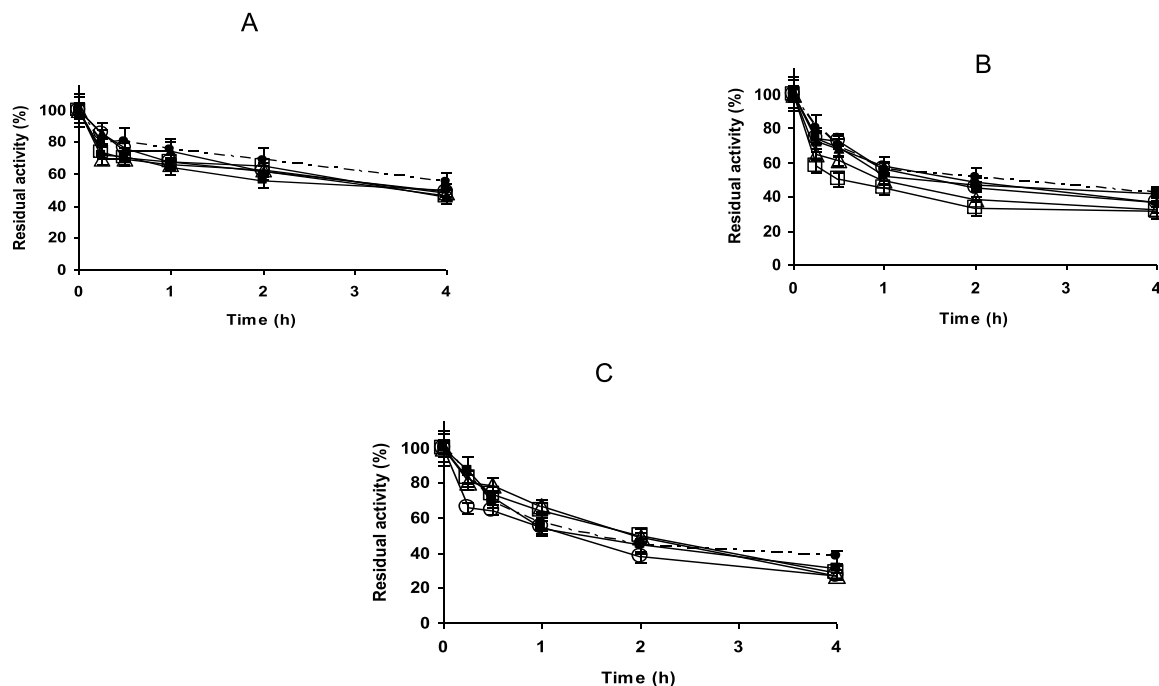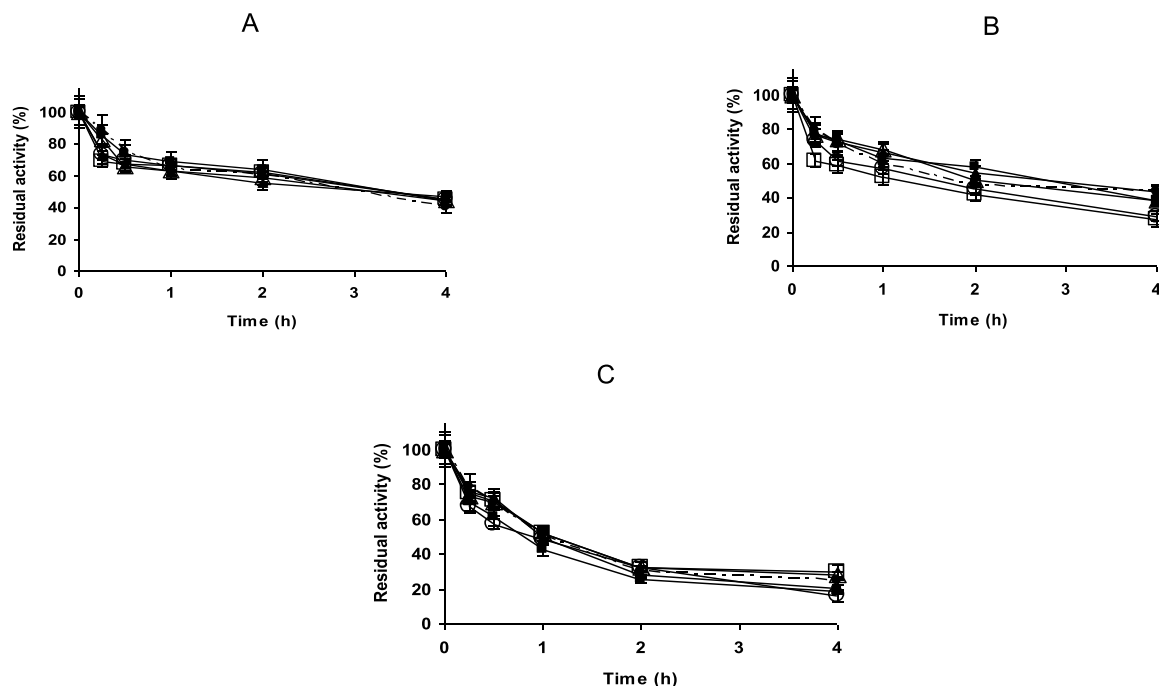
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Fig. 3. Effect of the presence of butyl amine during the immobilization of trypsin on the stability of the immobilized enzyme. Inactivations have been performed at (A) pH 5.0, 83 °C; (B) pH 7.0, 78 °C; (C) pH 9.0, 70 °C. Other specifications are described in Methods section. Full circles: Reference without amine. Full triangles: 1 mM. Full squares: 2 mM. Empty circles: 5 mM. Empty triangles: 10 mM. Empty squares: 20 mM.

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Fig. 4. Effect of the presence of hexyl amine during the immobilization of trypsin on the stability of the immobilized enzyme. Inactivations have been performed at (A) pH 5.0, 83 °C; (B) pH 7.0, 78 °C; (C) pH 9.0, 70 °C. Other specifications are described in Methods section. Full circles: Reference without amine. Full triangles: 1 mM. Full squares: 2 mM. Empty circles: 5 mM. Empty triangles: 10 mM. Empty squares: 20 mM.

at pH 7, the effects were clearer, while at pH 9 the destabilizing effects of the butyl amine presence during the enzyme immobilization were not very relevant. At pH 5 and 7, the effect of just 1 mM butyl amine was very significant, although it increased with amine concentrations. Fig. 8 shows the results using hexyl amine in the chymotrypsin immobilization. Again, the more evident negative effects on enzyme stability were detected in inactivations at pH 7, being this effect at pH 5 and 9 smaller.

At pH 7, even 1 mM of hexyl amine reduced the enzyme stability, and the increase of this value to 20 mM produced a progressive but small decrease in enzyme stability. At pH 9, the effect was only detected using 2 mM or more of the amine, while at pH 5 the effects were more dependent on the amine concentration. Again, the clearest effects of the amines during enzyme immobilization were found using octyl amine during immobilization (Fig. 9). With this enzyme, the effects of octyl
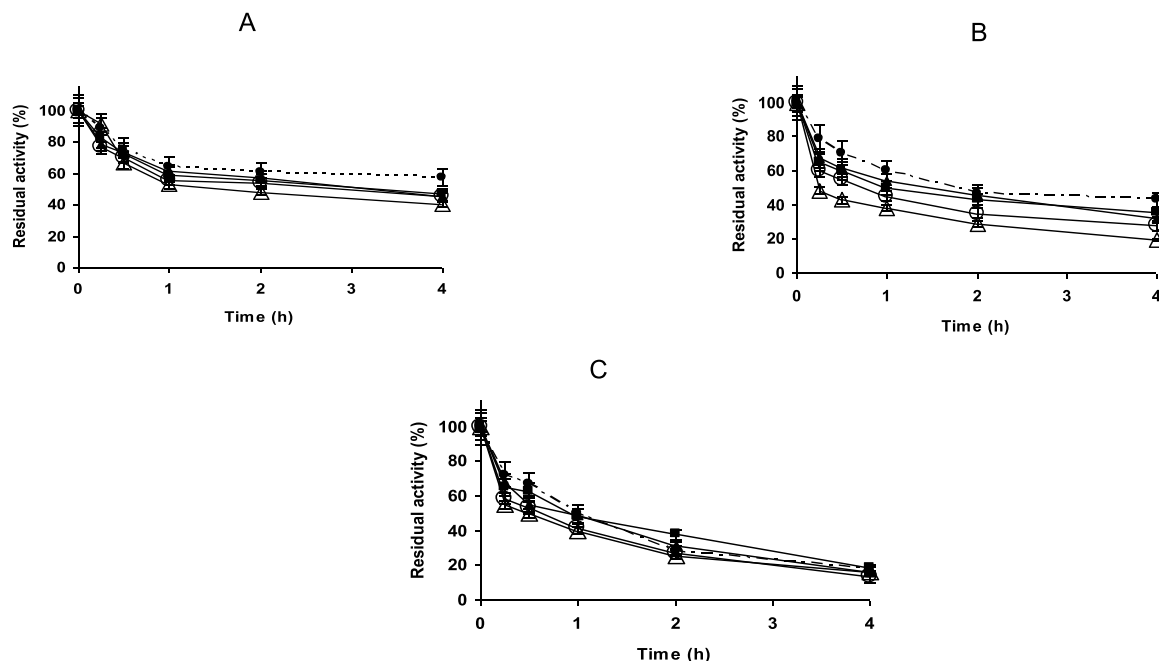
Fig. 5. Effect of the presence of octyl amine during the immobilization of trypsin on the stability of the immobilized enzyme. Inactivations have been performed at (A) pH 5.0, 83 °C; (B) pH 7.0, 78 °C; (C) pH 9.0, 70 °C. Other specifications are described in Methods section. Full circles: Reference without amine. Full triangles: 1 mM. Full squares: 2 mM. Empty circles: 5 mM. Empty triangles: 10 mM.
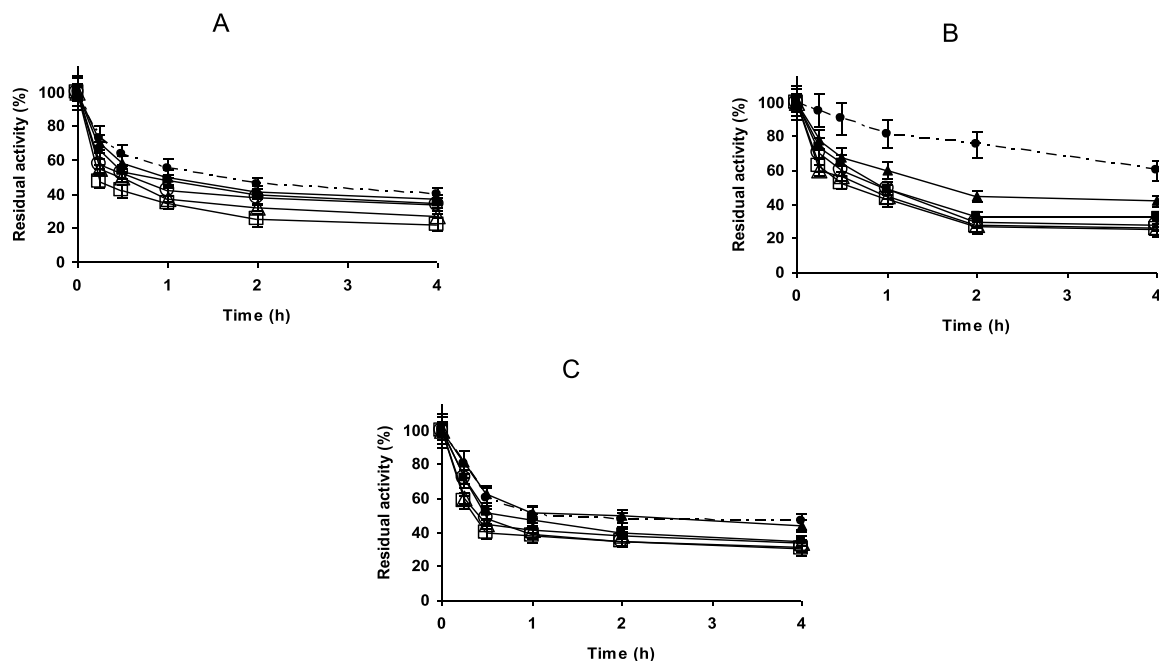

Fig. 6. Effect of the presence of ethyl amine during the immobilization of chymotrypsin on the stability of the immobilized enzyme. Inactivations have been performed at (A) pH 5.0, 83 °C; (B) pH 7.0, 69 °C; (C) pH 9.0, 62 °C. Other specifications are described in Methods section. Full circles: Reference without amine. Full triangles: 1 mM. Full squares: 2 mM. Empty circles: 5 mM. Empty triangles: 10 mM. Empty squares: 20 mM.

amine during immobilization were very relevant at all inactivation pH values, and much higher than those detected using the other amines, even if the maximum concentration of this amine was 10 mM by solubility reasons. At pH 5 and 9, the effect was only important using 5 or 10 mM, while at pH 7, the negative effect of octyl amine was detected using just 1 mM, using 2 mM–20 mM values were similar. The effects of the presence of octyl amine during the enzyme immobilization on the stability of chymotrypsin were more relevant than when studying trypsin.

3.3. Effect of the presence of aliphatic amines on the results obtained in the immobilization of penicillin G acylase on glyoxyl agarose

Penicillin G acylase has been also included in this study. Although the immobilization of the enzyme in absence of amines was complete after only 15 min, the presence of amines in the immobilization slowed down the enzyme immobilization, and this effect was larger when using higher amine concentrations. In all cases, immobilization was completed after 1 h. Final activity was in all cases over 90 % (not shown
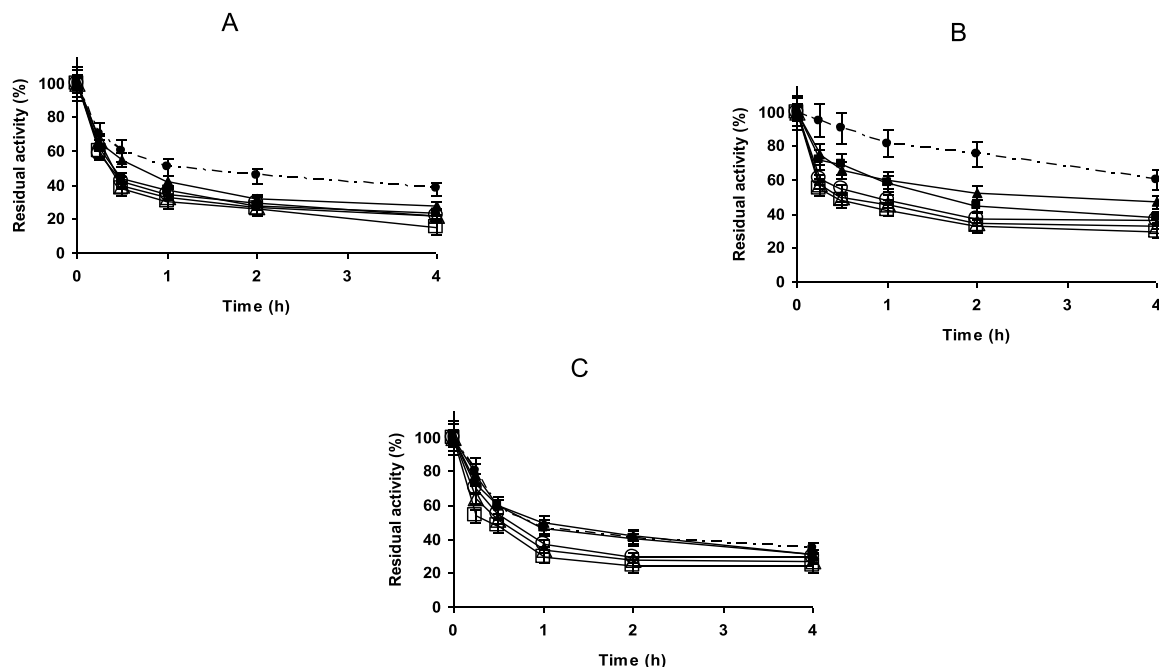
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Fig. 7. Effect of the presence of butyl amine during the immobilization of chymotrypsin on the stability of the immobilized enzyme. Inactivations have been performed at (A) pH 5.0, 83 °C; (B) pH 7.0, 69 °C; (C) pH 9.0, 62 °C. Other specifications are described in Methods section. Full circles: Reference without amine. Full triangles: 1 mM. Full squares: 2 mM. Empty circles: 5 mM. Empty triangles: 10 mM. Empty squares: 20 mM.
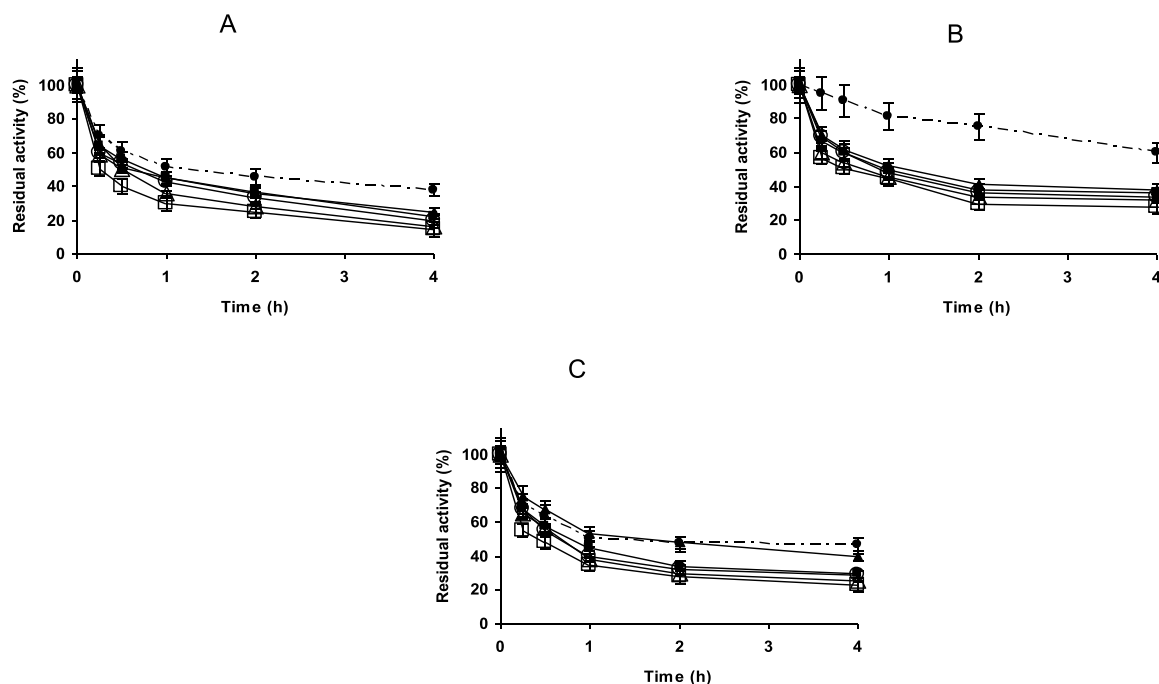
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Fig. 8. Effect of the presence of hexyl amine during the immobilization of chymotrypsin on the stability of the immobilized enzyme. Inactivations have been performed at (A) pH 5.0, 83 °C; (B) pH 7.0, 69 °C; (C) pH 9.0, 62 °C. Other specifications are described in Methods section. Full circles: Reference without amine. Full triangles: 1 mM. Full squares: 2 mM. Empty circles: 5 mM. Empty triangles: 10 mM. Empty squares: 20 mM.

results).

The effects of the amines on the final enzyme stability are shown in Figs. 10–13. Using ethyl amine (Fig. 10), the immobilized enzyme stability decreased in a not very significant way at pH 5, 7 and 9, and this effect increased when the amine concentration increased. The negative effect was increased using butyl amine during the enzyme immobilization (Fig. 11). This negative effect on enzyme stability due to the presence of butyl amine during the enzyme immobilization was more relevant when inactivating the enzyme at pH 7 and the lowest effect was at pH 9. In inactivations at pH 5 and 7, the negative effect of the presence of butyl amine during enzyme immobilization on its stability progressively increased when increasing the concentration of amine, while at pH 9 the effect of 1 mM and 20 mM of the amine was not very different. Using hexyl amine, the effects were not very different (Fig. 12), with an increase on the negative effects of the amine presence during the enzyme immobilization when its concentrations increased at all studied pH
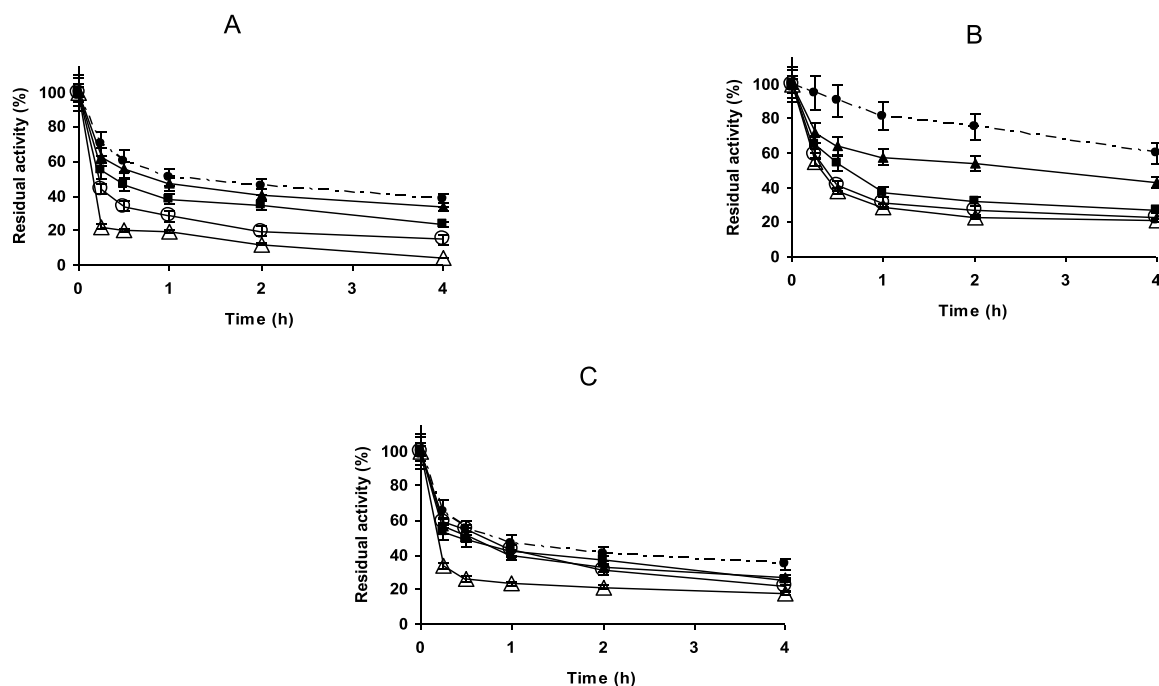
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Fig. 9. Effect of the presence of octyl amine during the immobilization of chymotrypsin on the stability of the immobilized enzyme. Inactivations have been performed at (A) pH 5.0, 83 °C; (B) pH 7.0, 69 °C; (C) pH 9.0, 62 °C. Other specifications are described in Methods section. Full circles: Reference without amine. Full triangles: 1 mM. Full squares: 2 mM. Empty circles: 5 mM. Empty triangles: 10 mM.

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Fig. 10. Effect of the presence of ethyl amine during the immobilization of penicillin G acylase on the stability of the immobilized enzyme. Inactivations have been performed at (A) pH 5.0, 55 °C; (B) pH 7.0, 55 °C; (C) pH 9.0, 42 °C. Other specifications are described in Methods section. Full circles: Reference without amine. Full triangles: 1 mM. Full squares: 2 mM. Empty circles: 5 mM. Empty triangles: 10 mM. Empty squares: 20 mM.
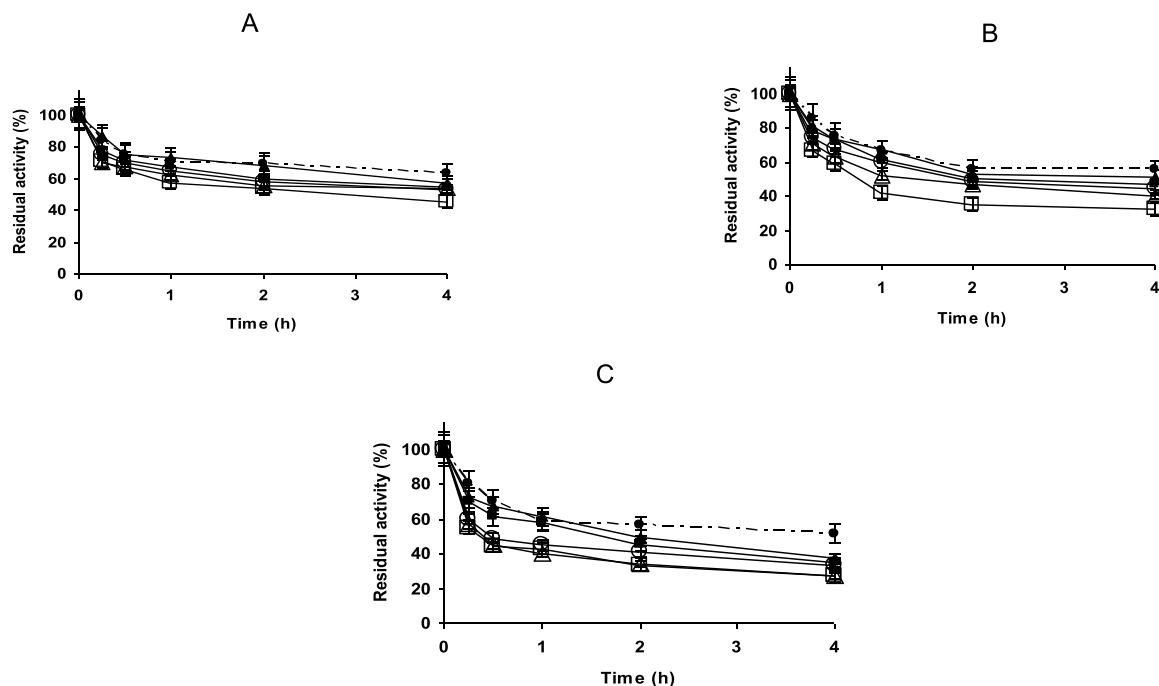
values of inactivation. Once again, the negative effects of octyl amine during the enzyme immobilization were much clearer (Fig. 13) than when using the other amines, at all the pH values, mainly using 5 and 10 mM. The effects were clearer at pH 7 and 9 than at pH 5.

3.4. Effect of the presence of aliphatic amines on the results obtained in the immobilization of ficin extracts on glyoxyl agarose

This enzyme was immobilized in a slower fashion that the other enzymes even in the absence of amines (full immobilization took one hour), and the presence of amines slow down this (to almost 3 h using the highest amines concentration). The activity was in all cases around
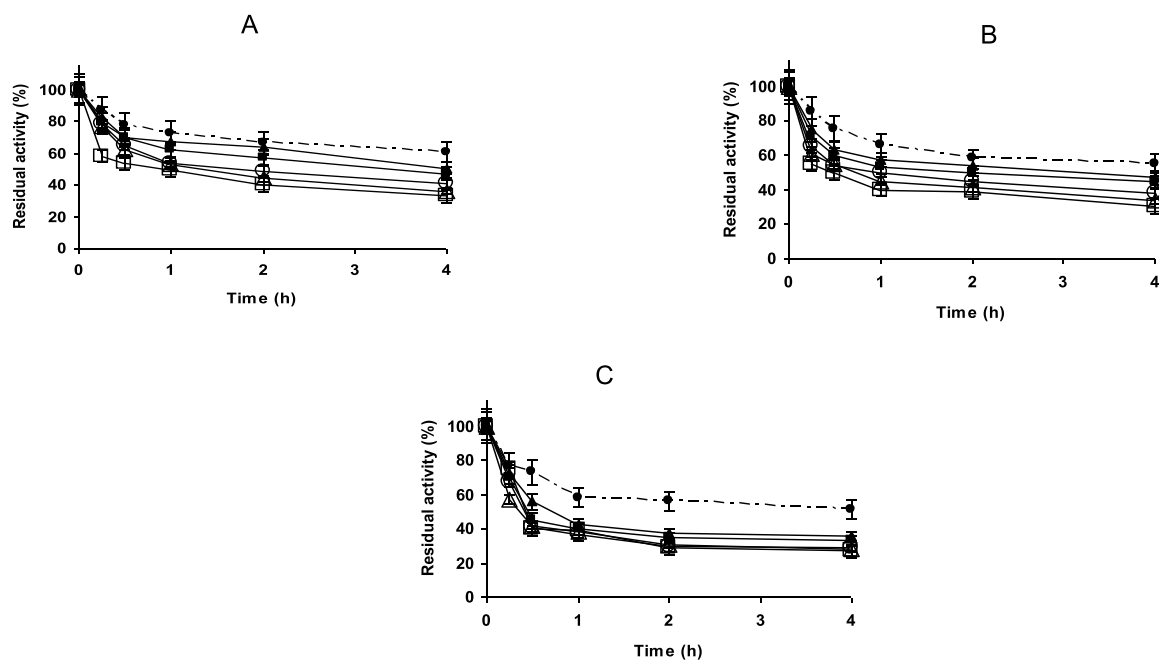
Fig. 11. Effect of the presence of butyl amine during the immobilization of penicillin G acylase on the stability of the immobilized enzyme. Inactivations have been performed at (A) pH 5.0, 55 °C; (B) pH 7.0, 55 °C; (C) pH 9.0, 42 °C. Other specifications are described in Methods section. Full circles: Reference without amine. Full triangles: 1 mM. Full squares: 2 mM. Empty circles: 5 mM. Empty triangles: 10 mM. Empty squares: 20 mM.

Fig. 12. Effect of the presence of hexyl amine during the immobilization of penicillin G acylase on the stability of the immobilized enzyme. Inactivations have been performed at (A) pH 5.0, 55 °C; (B) pH 7.0, 55 °C; (C) pH 9.0, 42 °C. Other specifications are described in Methods section. Full circles: Reference without amine. Full triangles: 1 mM. Full squares: 2 mM. Empty circles: 5 mM. Empty triangles: 10 mM. Empty squares: 20 mM.
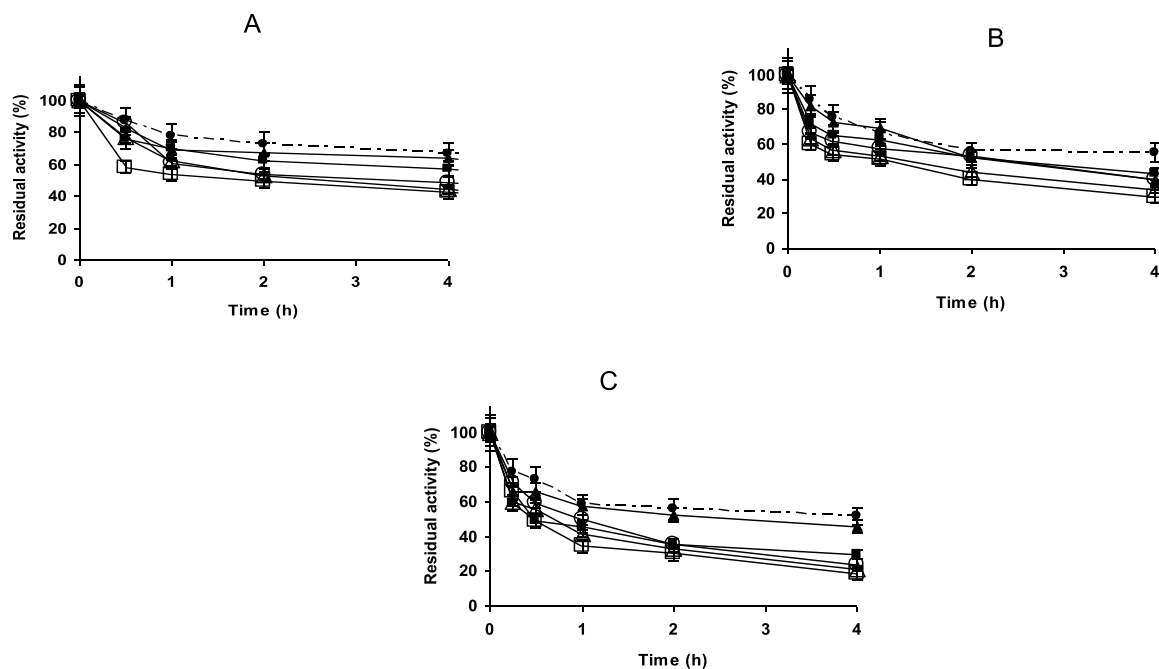
80 % (results not shown).

Although this enzyme reached a maximum of stability after 3 h of immobilization, suggesting that too intense an enzyme-support interaction may distort the enzyme and exposes some sensible groups to the medium, Figs. 14–17 show again some negative effects of the amines on the stability of immobilized ficin biocatalyst. Using ethyl amine (Fig. 14), its negative effect on the final enzyme stability progressively increased at all pH values. This effect was more intense when the enzyme was inactivated at pH 9 and least significant at pH 7. These negative effects of the presence of amines during enzyme immobilization were similar using butyl amine (Fig. 15) and hexyl amine (Fig. 16). Octyl amine presence during the enzyme immobilization presented again the clearest negative effect on enzyme stability, being the effect progressive as the amine concentration increased (Fig. 17).
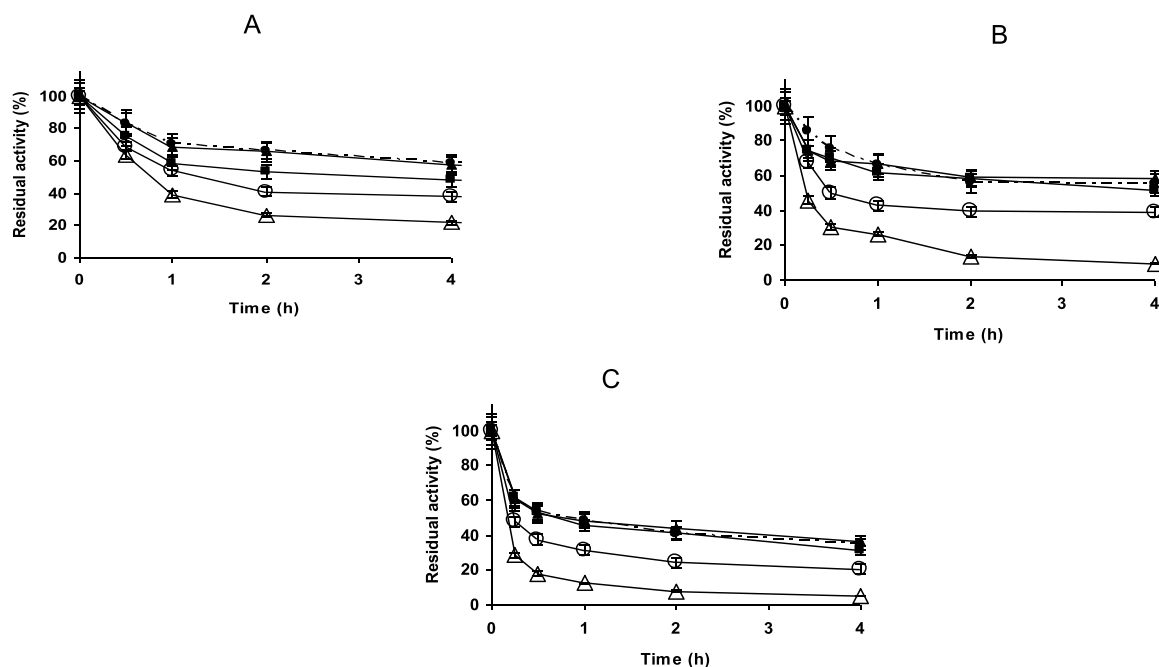
Fig. 13. Effect of the presence of octyl amine during the immobilization of penicillin G acylase on the stability of the immobilized enzyme. Inactivations have been performed at (A) pH 5.0, 55 °C; (B) pH 7.0, 55 °C; (C) pH 9.0, 42 °C. Other specifications are described in Methods section. Full circles: Reference without amine. Full triangles: 1 mM. Full squares: 2 mM. Empty circles: 5 mM. Empty triangles: 10 mM.

Fig. 14. Effect of the presence of ethyl amine during the immobilization of ficin extract on the stability of the immobilized enzyme. Inactivations have been performed at (A) pH 5.0, 65 °C; (B) pH 7.0, 65 °C; (C) pH 9.0, 65 °C. Other specifications are described in Methods section. Full circles: Reference without amine. Full triangles: 1 mM. Full squares: 2 mM. Empty circles: 5 mM. Empty triangles: 10 mM. Empty squares: 20 mM.
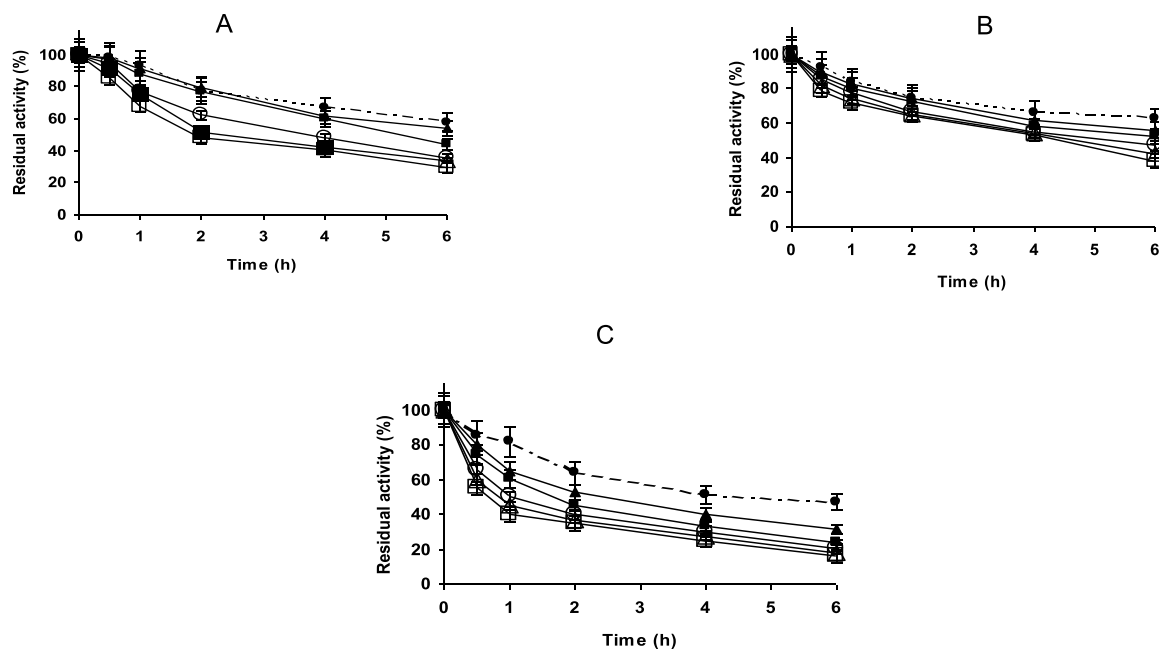
3.5. Determination of the multipoint covalent immobilization in trypsin and chymotrypsin glyoxyl agarose

Table 1 shows the number of Lys, which may be found in the trypsin and chymotrypsin, free or immobilized, calculated by amino acids analysis after acid hydrolysis as described in methods. The table compares the results using the biocatalysts prepared in the absence of amines, and that prepared in the presence of 10 mM octyl amine, that is the one that more significantly reduce the enzyme stability. Both

enzymes have an similar number of Lys involved in the multipoint covalent immobilization in absence of aminated compounds, around 7 groups, and a similar molecular size. The decrease in the Lys involved in the immobilization is clear when using 10 mM octyl amine. The number of Lys involved in the immobilization decreased bonds decreased from 7 to 5 in presence of 10 mM ocyl amine for trypsin, and from 6.6 to 4.6 using chymotrypsin. This way, we can confirm that the effects found in this paper of the presence of amines in the immobilized enzyme stabilities were related, at least in part, to the intensity of the multipoint
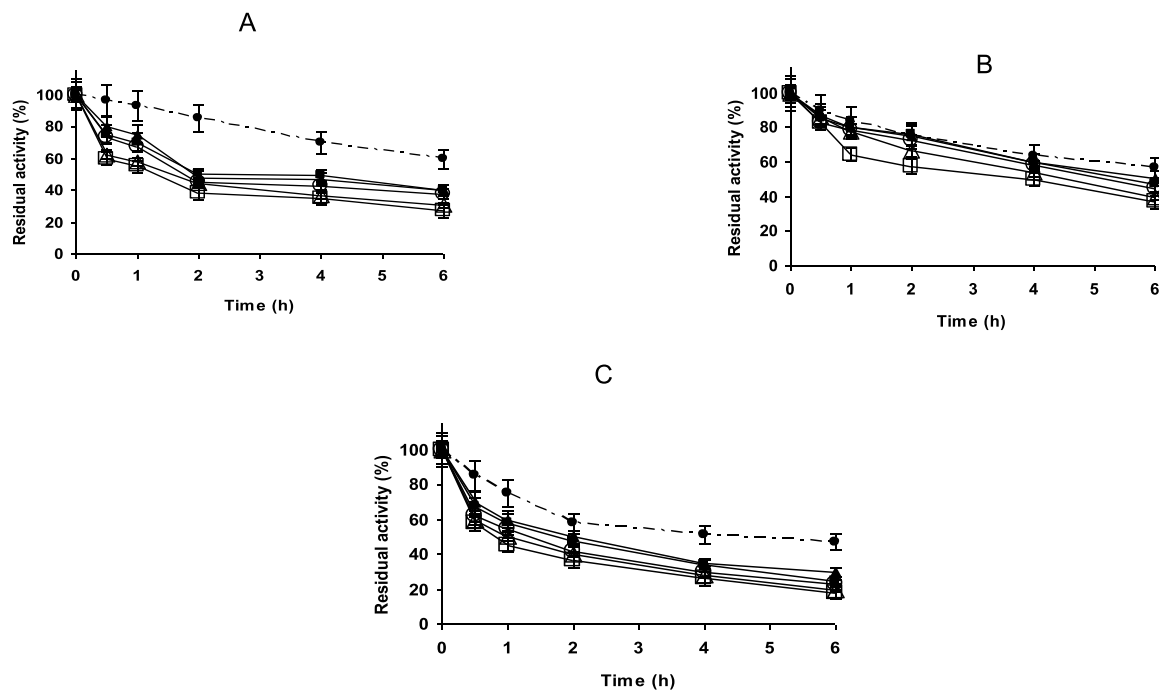
Fig. 15. Effect of the presence of butyl amine during the immobilization of ficin extract on the stability of the immobilized enzyme. Inactivations have been performed at (A) pH 5.0, 65 °C; (B) pH 7.0, 65 °C; (C) pH 9.0, 65 °C. Other specifications are described in Methods section. Full circles: Reference without amine. Full triangles: 1 mM. Full squares: 2 mM. Empty circles: 5 mM. Empty triangles: 10 mM. Empty squares: 20 mM.

Fig. 16. Effect of the presence of hexyl amine during the immobilization of ficin extract on the stability of the immobilized enzyme. Inactivations have been performed at (A) pH 5.0, 65 °C; (B) pH 7.0, 65 °C; (C) pH 9.0, 65 °C. Other specifications are described in Methods section. Full circles: Reference without amine. Full triangles: 1 mM. Full squares: 2 mM. Empty circles: 5 mM. Empty triangles: 10 mM. Empty squares: 20 mM.
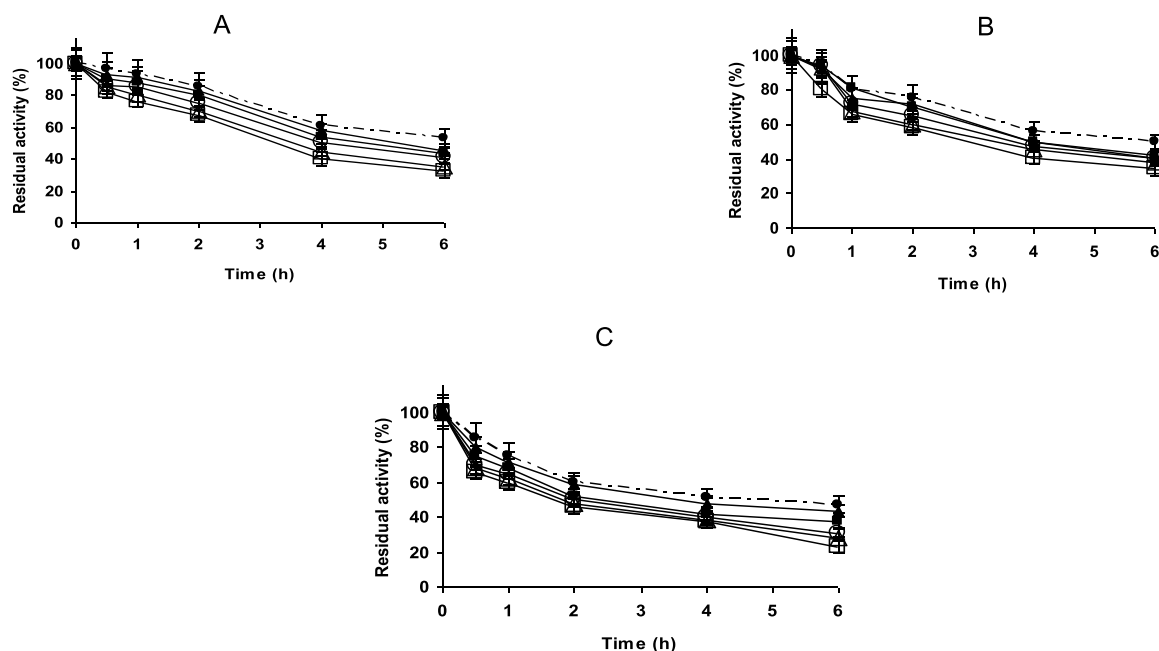
covalent attachment.

4. Discussion

The results presented in this paper show that the presence of compounds bearing primary amino groups during the multipoint covalent immobilization of enzymes in glyoxyl agarose alters the final biocatalyst stability. Although considering that this is an expected result, it has not

been reported before; neither the impact on the enzyme stability nor the concentration of aminated compounds that produce this effect has been previously reported in any paper. Moreover, the main effect is fully unexpected. In all cases, octyl amine was the amine that produced the highest decrease in enzyme stabilization via multipoint covalent attachment. Considering that it was the amine with the highest pK, and this makes that a lower modification of the glyoxyl support at pH 10 is produced, this higher effect on enzyme-support multipoint covalent
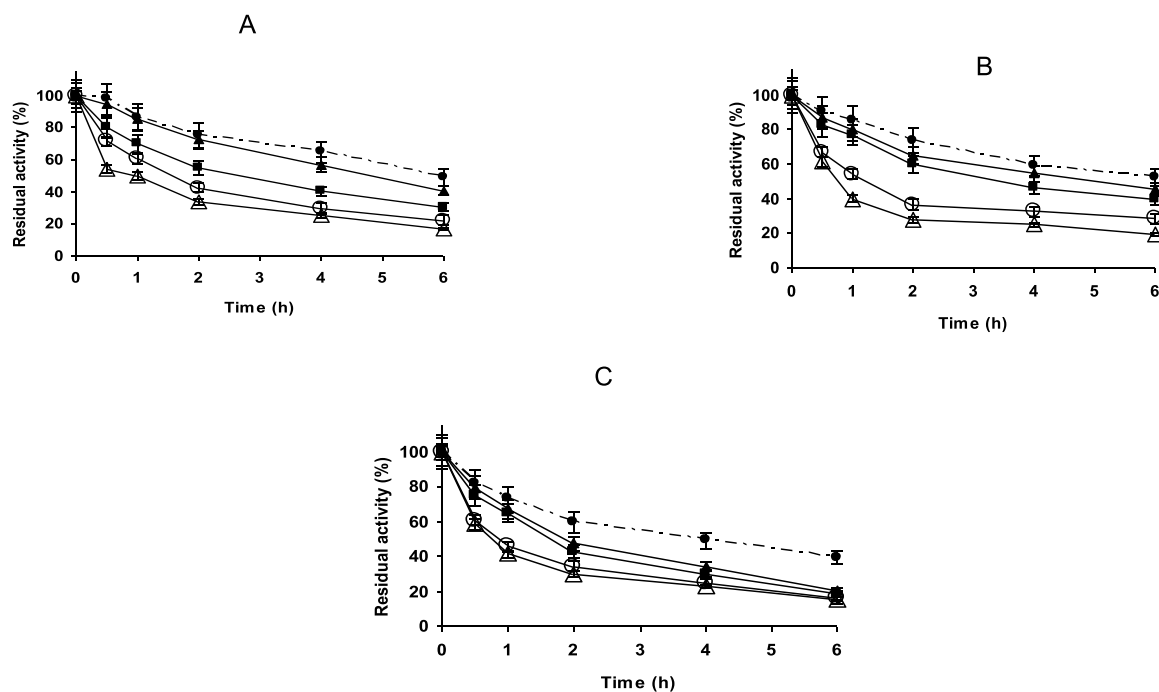
Fig. 17. Effect of the presence of octyl amine during the immobilization of ficin extract on the stability of the immobilized enzyme. Inactivations have been performed at (A) pH 5.0, 65 °C; (B) pH 7.0, 65 °C; (C) pH 9.0, 65 °C. Other specifications are described in Methods section. Full circles: Reference without amine. Full triangles: 1 mM. Full squares: 2 mM. Empty circles: 5 mM. Empty triangles: 10 mM.

Table 1

Determination of the number of free Lys per enzyme molecule in different enzyme preparations. Experiments were performed as described in Methods.

NUMBER OF FREE LYS PER PROTEIN MOLECULE			
ENZYME	FREE ENZYME	REFERENCE IMMOBILIZED ENZYME	ENZYME IMMOBILIZED IN THE PRESENCE OF 10 mM OCTYL AMINE
TRYPSIN	14.0 ± 0.5	7 ± 0.3	9.0 ± 0.3
CHYMOTRYPSIN	14.0 ± 0.4	7.4 ± 0.2	9.4 ± 0.3

attachment should be caused by the larger size of the compound, which promoted larger steric hindrances of the protein surface to reach the surface of the support, that way reducing the intensity of the multipoint covalent immobilization. Ethyl amine, with the lowest pK should be the compound that produced the highest blocking of the support, while octyl amine should be the one that leaves more free aldehydes. However, the latter was, for all enzymes, the amine with more negative effects on enzyme stability. These steric hindrances effects of the presence of aminated compounds during enzyme immobilization on the intensity of the enzyme-support multipoint covalent attachment have been never described, nor even suggested, before. Some papers propose that groups whose reactive groups suffer steric hindrances should be unsuitable for multipoint covalent attachment (Mateo et al., 2005; J. C. S.. Dos Santos et al., 2015a, 2015b, 2015c, 2015d). Using heterofunctional supports, where the adsorbent group layer was over the chemically reactive groups in the support (Barbosa et al., 2013), a low stabilization of the enzyme after immobilization was explained (as an hypothesis) on the generation of steric hindrances by this adsorbed groups layer. The results reported in this paper seem to reinforce that hypothesis.

The final effect of the presence of aminated compounds on the immobilized enzyme stability depended on the enzyme and the inactivation conditions. In some cases the presence of only 1 mM of the aminated compound during enzyme immobilization was enough to strongly decrease the enzyme stability, without a very significant increase of the effect when increasing the amine concentrations, while in

other cases a progressive increase of the effect of the amine concentration could be found. Curiously, this negative effect depended on the inactivation pH. At pH 9, except for ficin, the decrease in stability was lower when the enzyme was immobilized in the presence of amines, perhaps because at this pH, enzyme chemical modification started to have relevance. And the enzyme chemical modification may not be prevented as efficiently as enzyme distortion by an increase in enzyme rigidity. Using octyl amine during the enzyme immobilization, the negative effect on enzyme stability was more homogenous and clear at all inactivation pH values. It should be considered that the inactivation pH may alter the pathway of the enzyme inactivation reducing or increasing the relevance of a higher increment on the enzyme rigidity in one specific area of the enzyme surface (Sanchez et al., 2016).

The final stability of the immobilized enzyme depended on the amine concentration that was presented during the enzyme immobilization, but this effect depends on the enzyme and the inactivation pH value. In some cases the presence of only 1 mM of the aminated compound was enough to strongly decrease the enzyme stability, with not very significant increases of the effects when increasing the amine concentration to 20 mM, while in other cases a progressive increase of the effect could be found, with scarce effect using 1 or 2 mM of the amine. This suggests the complexity of the different phenomena that are occurring during the enzyme-support multipoint immobilization and the further inactivation of the immobilized enzymes.

In fact, the effect of the presence of amines was dissimilar, depending on the enzymes. The least significant effect was found using trypsin, where stability was not very different even using the highest concentrations of all assayed amines, while for chymotrypsin (enzyme with similar size and similar intensity of multipoint covalent attachment (Table 1)) and penicillin G acylase, they were very significant in certain cases, mainly using octyl amine.

Using this very low concentration of aminated compounds, and for only 3 h, it is unlikely that the support may suffer a large modification by them (using ethylenediamine, it is necessary to use 2 M and 24 h to fully modify the glyoxyl support) (Zaak et al., 2017b), and in any case the degree of support modification will be independent on the use of

butyl or octyl amine, as their pKs are almost identical. However, to check if the modification of the support by the aminated compounds could be, at least partially, responsible for the changes in enzyme stability, the biocatalysts of chymotrypsin and penicillin G acylase prepared in the presence of 10 mM octyl amine were washed for 1 min with 10 volumes of immobilization buffer, without the aminated compound, and the washing was performed 10 folds. This should release most of the possible immobilized amino groups, as the imino bond is not very stable (Mateo et al., 2005). The stabilities of both preparations resulted almost identical to that of the biocatalysts not washed before reduction (not shown results), confirming that the main effect of the aminated compounds was to decrease the number of enzyme-support bonds.

5. Conclusion

The multipoint covalent immobilization of enzymes on glyoxyl support may be greatly hampered by the presence of aminated compounds; even only 1 mM of these compounds may reduce the final enzyme stability in some instances. The size of the aminated compound has shown to be very important, the difference in the pKs of the aminated compounds may also play a role in these negative effects, but in this study, the change from pK 10.0–10.7 did not seem to be very relevant. This should be related to the generation of steric hindrances to the enzyme-support multipoint covalent attachment, not only to the marginal blocking of aldehyde groups in the support.

Glyoxyl agarose is able to immobilize enzymes in an extremely quick fashion at alkaline pH values, and the fact that after a short time all the enzyme is immobilized does not mean that there are no aminated compounds interfering on the enzyme-support reaction. Moreover, in some instances, just 1 mM of aminated compound has a negative effect on immobilized enzyme stability that is just slightly lower than 20 mM. For this reason, following the results in this paper, it seems recommendable to dialyze all enzyme preparations that are going to be immobilized/stabilized on glyoxyl agarose via multipoint covalent attachment, mainly if the researcher does not know the other components of the enzyme extract (e.g., amino acids, aminated sugars, buffers like Tris, etc.). These components may have also a very negative effect on the enzyme-support multi-reaction, reducing the final enzyme stabilization. In fact, if the enzyme extract has some of these compounds, the final properties of the immobilized enzyme will depend on the concentrations of the enzyme extract employed during the enzyme immobilization, and this will produce a false effect of the enzyme concentration during the enzyme immobilization on the immobilized enzyme properties. This is important considering that this effect of the enzyme loading in the support on the final immobilized enzyme features may be real in certain cases, as it has been reported in the case of the immobilization of lipases on octyl agarose beads) (Fernandez-Lopez et al., 2017; Zaak et al., 2017c).

Author statement

In this paper, Roberto Morellon-Sterling, El-Hocine Siar, Sabrina Ait Braham and Diandra de Andrades prepared the biocatalysts and analyzed their performance, Justo Pedroche and Mª del Carmen Millán analyzed the aminoacid composition and Roberto Fernandez-Lafuente designed and supervised the experiments. All authors contributed to the writing of the paper.

Declaration of Competing Interest

The authors report no declarations of interest.

Acknowledgments

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