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ORIGINAL ARTICLE

Effect of Tris Buffer in the Intensity of the Multipoint Covalent Immobilization of Enzymes in Glyoxyl-Agarose Beads

Sabrina Ait Braham[1] · Roberto Morellon-Sterling · Diandra de Andrades[2,3] · Rafael C. Rodrigues[3] · El-Hocine Siar[2,4] · Ali Aksas[1] · Justo Pedroche[5] · Maria del Carmen Millán[5] · Roberto Fernandez-Lafuente[2,6]

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Abstract

Tris is an extensively used buffer that presents a primary amine group on its structure. In the present work trypsin, chymotrypsin and penicillin G acylase (PGA) were immobilized/stabilized on glyoxyl agarose in presence of different concentrations of Tris (from 0 to 20 mM). The effects of the presence of Tris during immobilization were studied analyzing the thermal stability of the obtained immobilized biocatalysts. The results indicate a reduction of the enzyme stability when immobilized in the presence of Tris. This effect can be observed in inactivations carried out at pH 5, 7, and 9 with all the enzymes assayed. The reduction of enzyme stability increased with the Tris concentration. Another interesting result is that the stability reduction was more noticeable for immobilized PGA than in the other immobilized enzymes, the biocatalysts prepared in presence of 20 mM Tris lost totally the activity at pH 7 just after 1 h of inactivation, while the reference at this time still kept around 61 % of the residual activity. These differences are most likely due to the homogeneous distribution of the Lys groups in PGA compared to trypsin and chymotrypsin (where almost 50% of Lys group are in a small percentage of the protein surface). The results suggest that Tris could be affecting the multipoint covalent immobilization in two different ways, on one hand, reducing the number of available glyoxyl groups of the support during immobilization, and on the other hand, generating some steric hindrances that difficult the formation of covalent bonds.

Keywords Enzyme stabilization · Enzyme immobilization · Glyoxyl agarose · Tris buffer · Multipoint covalent attachment

Sabrina Ait Braham and Roberto Morellon-Sterling contributed equally to this work.

✉ Roberto Fernandez-Lafuente
rfl@icp.csic.es

Extended author information available on the last page of the article

Introduction

Multipoint covalent immobilization (MCI) of enzymes on glyoxyl supports is one of the most powerful strategies to improve enzyme stability via immobilization, and many papers use it to improve enzyme performance in different processes [1–47]. This immobilization/stabilization method was proposed by Prof Guisán in 1988 [42], but it still maintains its utility nowadays [27].

This immobilization protocol has some peculiarities that make it very suitable for enzyme stabilization via MCI. The most relevant one is the low energy of the imine bond formed between the amine groups of the protein and the glyoxyl groups of the support: enzyme immobilization only occurs if several imino bonds are simultaneously formed [30]. This, at first glance, is a drawback of the method: it is a necessity to use alkaline pH for enzyme immobilization (or add some imino stabilizer agent [1, 5], but in that case, we lost this particular advantage of the method), the immobilization rate exponentially depends on the enzyme-Lys superficial density [36] and the glyoxyl groups in the support [30], and it is necessary to finally reduce the biocatalysts with sodium borohydride [48]. However, this characteristic causes enzyme immobilization to occur directly by the area of the protein that is the richest one in reactive groups, that is, the area where the highest enzyme-support reaction intensity may be achieved [30]. Although this may not be the most relevant area for enzyme stabilization [49–51], this intense multipoint covalent attachment usually promotes high stabilization factors [52, 53].

The efficacy of this protocol for immobilization/stabilization of enzymes may be diminished if some compounds are present in the immobilization suspension, for example, borate can block the aldehyde groups decreasing the potential of this support for an intense MCI of the enzymes [54].

Similarly, compounds bearing primary amino groups can establish a competition with the Lys groups in the protein by the glyoxyl groups in the support. As they can establish just a single-point interaction, it should be reversible, and the intensity of the effects that the presence of this kind of compounds can promote in multipoint immobilization is unclear. Immobilizing crude protein extracts, it is possible that some compounds having primary amino groups may be present (e.g., amino acids) in the protein extract. Even using pure enzyme solutions, one of the most utilized buffers in biochemistry is tris(hydroxymethyl)aminomethane or 2-amino-2-(hydroxymethyl)propane-1,3-diol (Tris). This compound bears a primary amino group, with a pK of around 8 [55], although it is very *T*-dependent (decreasing 0.03 unit per degree of temperature increase). This makes that Tris may be significantly more reactive than the ε-amines in the Lys (pK over 10.5) of proteins versus glyoxyl support at pH 10.05, reducing the availability of aldehyde groups for the enzyme-support reaction, even if this blocking is reversible. However, we have been unable to find any publication where a systematic analysis of these likely Tris effects on MCI of enzymes had been studied.

For this reason, we have analyzed in this paper the effect of the Tris concentration on the MCI on glyoxyl-agarose of several enzymes. These enzymes have been selected because they become more stabilized when the multipoint covalent is more intense: trypsin and chymotrypsin from bovine pancreas [21] and penicillin G acylase (PGA) from *Escherichia coli* [36]. Agarose beads have been used as support, due to their inertness, that prevents any uncontrolled enzyme-support interaction [53]. To confirm the effect of Tris on the intensity of MCI, the number of Lys involved in the MCI has been analyzed in some instances, when the enzymes were immobilized in the absence and in the presence of Tris.

Materials and Methods

Materials

Chymotrypsin and trypsin (pure lyophilized solid powder), PGA solution (60 mg of protein/mL), 6-nitro-3-(phenyl acetamido) benzoic acid (NIPAB), *N*-α-benzoyl-DL-arginine p-nitroanilide hydrochloride (BAPNA), *N*-benzoyl-L-tyrosine *p*-nitroanilide (BTPNA), and sodium borohydride were purchased from Sigma-Aldrich (Spain). Bradford's method was used for the determination of protein concentration, using bovine serum albumin as standard [56]. Six percent of BCL Agarose Bead Standard was purchased from ABT (Spain). Agarose beads were used to produce glyoxyl agarose as previously described [30, 57]. All other reagents were of analytical grade.

Methods

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

Enzyme Activity Assay

One activity unit (U) of activity was defined as the amount of enzyme that hydrolyzes 1 μmol of substrate per minute under the specified conditions. Enzyme activity was determined at 25 °C using a spectrophotometer with magnetic stirring at 200 rpm.

Trypsin activity was determined by measuring the change in absorbance at 405 nm caused by the release of p-nitroaniline (ε under these conditions is 9.96 mM^{-1} cm^{-1}) [58], produced by the hydrolysis of 2 mM BAPNA in 50 mM sodium phosphate at pH 7.0 containing 30% ethanol [21]. To start the reaction, we added 50–200 μL of enzyme solution or suspension to 2.5 mL of BAPNA solution.

Chymotrypsin activity was determined by measuring the variation in absorbance at 386 nm produced by the release of *p*-nitroaniline (ε under these conditions is 12,500 M^{-1} cm^{-1}) [59], generated in the hydrolysis of BTPNA. A 40 mM BTPNA stock solution was prepared in DMSO. The reaction was started by adding 200 μL of enzyme solution or suspension to 75 μL of BTPNA solution in 2.5 mL of 100 mM sodium phosphate at pH 7.0 containing 40% ethanol.

Activity of PGA was measured using NIPAB as described by Kutzbach et al. [60] Using 2.5 mL of 0.15 mM NIPAB in 50 mM sodium phosphate at pH 7.5, the reaction was started by adding 100–200 μL of enzyme suspension or solution, and the assay was performed continuously following the increase of absorbance at 405 nm (ε under these conditions is 8730 M^{-1} cm^{-1}) [60].

Enzyme Immobilizations

All enzyme immobilizations were performed following the activity of supernatant and suspension of the immobilization suspension and a reference solution of the enzyme under identical conditions to the immobilization suspension but in absence of the glyoxyl-agarose support [61]. First, the enzyme solutions at pH 10.05, containing the buffers, additives, and finally the enzyme, after the support were added.

Immobilization of the Enzyme on Glyoxyl-Agarose Beads

Chymotrypsin and trypsin (in the presence of 3 mM benzamidine [62]) were immobilized using a loading of 3 mg enzyme/g of support, in 100 mM sodium bicarbonate at pH 10.05 and 25 °C, using the protocols previously described [30, 63–65]. When trypsin was immobilized, the buffer contained 5 mM benzamidine.

PGA was immobilized using 3 mg of enzyme/g of support. The enzyme was added to a solution composed of 100 mM sodium carbonate containing 100 mM phenyl acetic acid and 20% glycerol (to prevent enzyme inactivation) at pH 10.05 [36, 66].

In some instances, Tris was added to the immobilization suspension using different concentrations (from 0 to 20 mM) before adding the enzyme or the support, adjusting the pH to 10.05 after this

As an end point to the enzyme immobilizations, 1 mg/mL of solid sodium borohydride was added, and the immobilization suspensions were stirred for 30 min [48]. Finally, the biocatalysts were washed with water, vacuum dried to eliminate interparticle water, and stored at 6–8 °C.

Thermal Inactivation of the Different Biocatalysts

The different biocatalysts were incubated in 100 mM sodium acetate at pH 5.0, 100 mM sodium phosphate at pH 7.0, or 100 mM sodium carbonate at pH 9.0. As the stabilities of the different enzymes included in this study and the effect of the pH on their stability is very diverse, the temperatures were different in each instance to have reliable and not too long inactivation courses [67, 68].

Determination of Amino Acids

Amino-acid composition of free and immobilized enzymes was determined according to the method of Alaiz et al. [69] with slight modifications. Protein samples were hydrolyzed by incubation in 6 N HCl at 110 °C for 24 h in tubes sealed under nitrogen atmosphere. 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 × 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 analyzed with Empower software (Waters, USA). Calculations were performed considering that each trypsin and chymotrypsin molecules have 14 Lys groups [70]. The number of Lys involved in the immobilization was calculated using some reference amino acids and comparing the intensity of their peaks with that of the Lys [21, 71].

Results and Discussion

Effect of the Presence of Tris Buffer in the Immobilization/Stabilization of Trypsin on Glyoxyl Agarose

The presence of even 20 mM Tris buffer during the immobilization of the enzyme did not reduce the immobilization rate enough to detect some activity in the supernatant in the first

time of enzyme activity determination (10 min) while the final activity of the immobilized enzymes was in all cases fairly similar (70–80%) (results not shown). This does not mean that the immobilization rate is not slower; it just indicates that it is so quick that the immobilization is complete in our first activity determination. After reduction of the biocatalysts, the stabilities of the different trypsin biocatalysts were analyzed at pH 5, 7, and 9 (Fig. 1). At pH 5, the presence of 1 mM of Tris during the enzyme immobilization has no relevant effect on enzyme stability, but 2 mM Tris already has some negative impact on the immobilized enzyme stability. The negative effect of the presence of Tris during the enzyme immobilization on the enzyme stability progressively increased when the Tris concentration did, reaching the maximal reduction on enzyme stability at the highest used concentration of Tris, 20 mM. In inactivations at pH 7 and 9, the effect of Tris during the enzyme immobilization is clear even using 1 mM, and in both cases, the effect increases with the Tris concentration. The growing effect of the presence of Tris in the enzyme stability during the enzyme immobilization with its concentration should be related to a higher degree of aldehyde groups in the support blocked by this compound that will make the enzyme-support reaction more complex (Fig. 2). Together with the decrease of aldehyde available for the enzyme-support reaction, it can be considered the promotion of some, even minimal, steric hindrances for the enzyme-support reaction. The Tris molecules interacting with the glyoxyl groups will be on the aldehyde layer, and that can produce steric hindrances, similar to those described using heterofunctional-epoxide supports [49, 72–74].

The effect is intense at all studied inactivation pH values, a little smaller at pH 5, where after 4 h, the biocatalysts prepared in the absence of Tris maintained 55% of the activity and that prepared using 20 mM Tris only maintained 19% of the initial activity. The highest effect

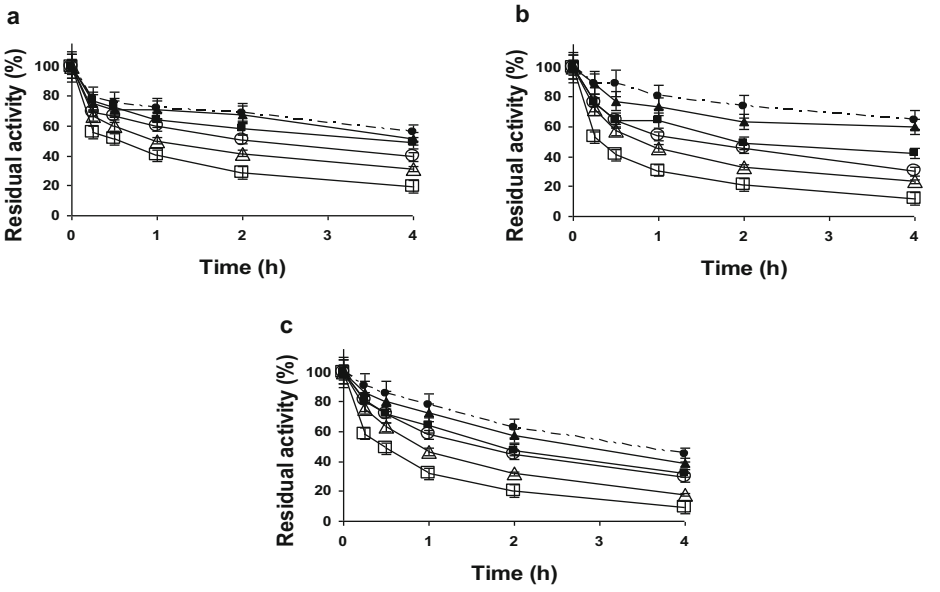

Fig. 1 Effect of the presence of Tris during the immobilization on the stability of trypsin immobilized on glyoxyl-agarose. Inactivations have been performed at (**A**) pH 5.0, 77 °C; (**B**) pH 7.0, 72 °C; (**C**) pH 9.0, 65 °C. The measurements were conducted as described in the "Materials and methods" section. Full circles: reference without amine. Full circles: reference without Tris. Full triangles: Tris 1 mM. Full squares: 2 mM Tris. Empty circles: Tris 5 mM. Empty triangles: Tris 10 mM. Empty squares: Tris 20 mM

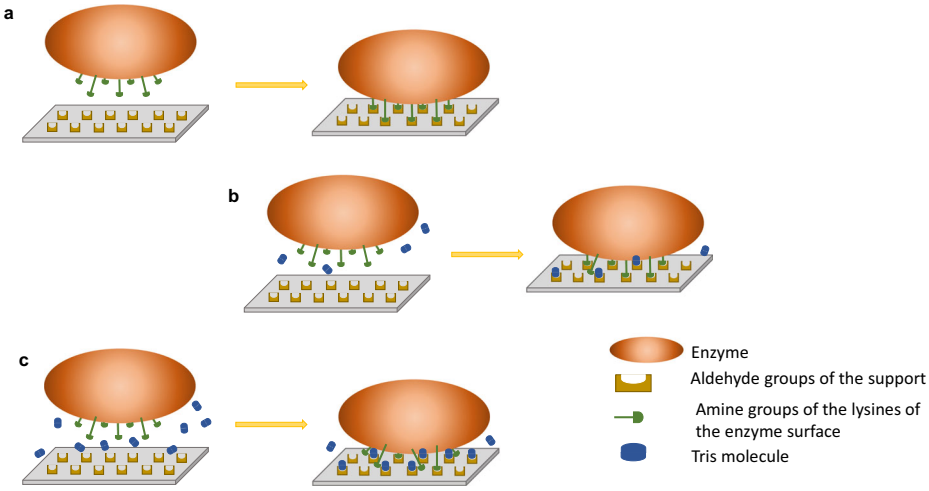

Fig. 2 Immobilization of an enzyme on glyoxyl-agarose supports (**A**) in absence of Tris in the medium, (**B**) in presence of low molarity Tris, and (**C**) in presence of high molarity Tris

could be visualized at pH 7, where the residual activity values are 64% and 11%, while at pH 9, they are in between 44 and 9%. These differences in the negative impact of Tris on the final stability of the immobilized enzyme could be caused by different pathways of the enzyme inactivation at the different inactivating conditions, that can alter the stabilization achieved by the multipoint covalent immobilization [75].

These results agree with previous reports that suggested that, to get an intense multipoint covalent immobilization, the immobilization protocol should be designed to not make the support-enzyme interaction difficult. That way, together to the immobilization temperature and pH, activation of the support, or geometrical congruence of the enzyme and the support, the nature of the buffer becomes a critical point [21, 76].

Effect of the Presence of Tris Buffer in the Immobilization/Stabilization of Chymotrypsin on Glyoxyl Agarose

As in the previous case, even in the presence of 20 mM Tris, enzyme activity was immobilized after 10 min. The effect of Tris during enzyme immobilization on the stability of the final biocatalysts is also clear using this enzyme at pH 5 and 7; at pH 9, it shows a different pattern (Fig. 3). At pH 5, the negative effect on the immobilized enzyme stability of the presence of Tris during the immobilization is evident even using just 1 mM and increased with its concentration. That way, at pH 5, using 20 mM Tris during enzyme immobilization, the biocatalysts residual activity during the inactivation were 12% while the activity of the reference was 45%. At pH 7, the trend was similar, but the effect was slightly higher (10% versus 47%). At pH 9, the effect was not very significant using 1–10 mM Tris but resulted evident using 20 mM; the destabilization effect of 20 mM during enzyme immobilization was similar at this pH to that found at pH 5 (11% versus 42%). The differences found when inactivating the enzyme at different pH values could be explained also by the different pathways followed by the enzyme during immobilization under the different pH values [75].

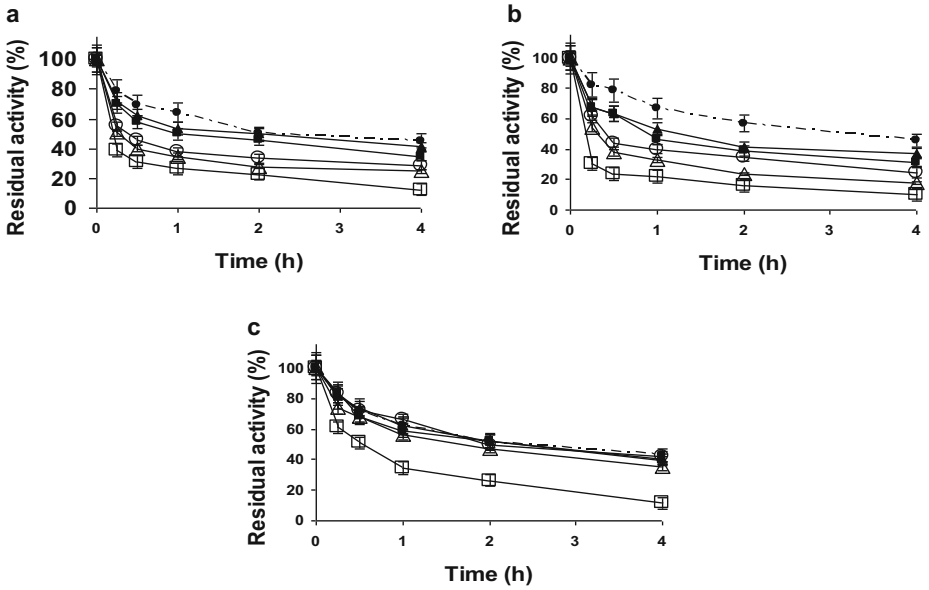

Fig. 3 Effect of the presence of Tris during the immobilization on the stability of chymotrypsin immobilized on glyoxyl-agarose. Inactivations have been performed at (**A**) pH 5.0, 73 °C; (**B**) pH 7.0, 68 °C; (**C**) pH 9.0, 58 °C. The measurements were conducted as described in the "Materials and methods" section. Full circles: reference without amine. Full circles: reference without Tris. Full triangles: Tris 1 mM. Full squares: 2 mM Tris. Empty circles: Tris 5 mM. Empty triangles: Tris 10 mM. Empty squares: Tris 20 mM

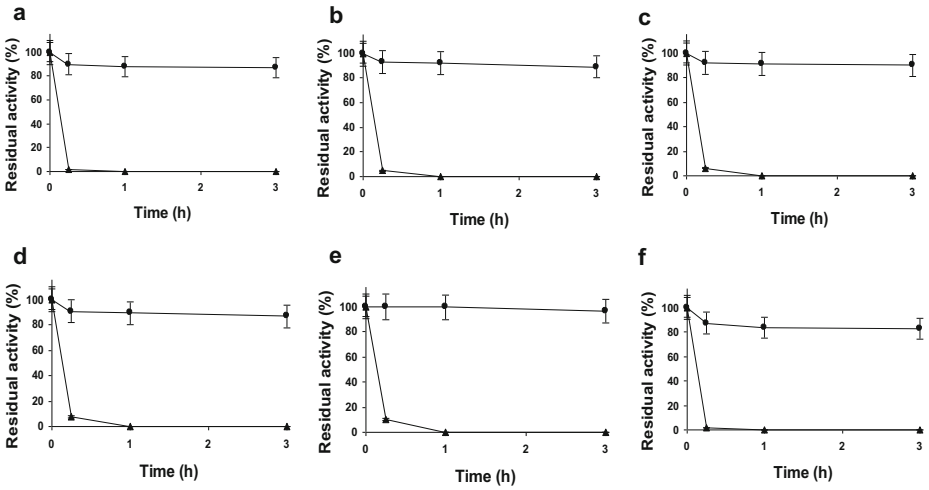

Fig. 4 Effect of the presence of Tris on the immobilization rate of PGA in glyoxyl-agarose at pH 10.05 with (**A**) 1 mM Tris, (**B**) 2 mM Tris, (**C**) 5 mM Tris, (**D**) 10 mM Tris, (**E**) 20 mM Tris, and (**F**) reference without Tris. The measurements were conducted as described in the "Materials and methods" section. Full circles: suspension. Full triangles: supernatant

Effect of the Presence of Tris Buffer in the Immobilization/Stabilization of PGA on Glyoxyl Agarose

Although in the absence of Tris, enzyme activity was immobilized in the first activity determination, Fig. 4 shows how the growing concentration of Tris produced a reduction on the PGA immobilization rate (not very significant, but clear), and this effect increased when the concentration of Tris did, although in all cases, an immobilization yield of 100% was observed after 1 h. However, the final expressed activity was similar in all cases (around 90%). Considering the multipoint immobilization mechanism of glyoxyl agarose [30], this suggested that Tris is making the first establishment even of the first several PGA support bonds more difficult.

The immobilized enzyme inactivations at pH 5, 7, and 9 (Fig. 5) show that this is also reflected in a serious decrease in enzyme stability. This was much more serious than with both smaller proteins. When inactivating the enzyme at pH 5, this negative effect of Tris was progressive, and the residual activity of the reference after 4 h was 47% while the biocatalysts prepared using 20 mM Tris and maintained less than 1% of the initial activity. In inactivations at pH 7, the negative progressive effect of Tris was again observed, and using 20 mM Tris during the immobilization, the residual activity was 0 after just 2 h of inactivation, while the reference maintained 59% of the initial activity and 53% after 4 h of inactivation. At pH 9, the negative effect of the presence of Tris during the enzyme immobilization was again progressive with its concentration; the biocatalyst prepared in 20 mM Tris suffered a drastic drop in enzyme activity in the first minutes of the inactivation experiment, and then, the small residual activity was maintained a long time.

This higher impact of Tris on the stability of immobilized PGA compared to the results found using trypsin and chymotrypsin could be based on the larger size of the first, that

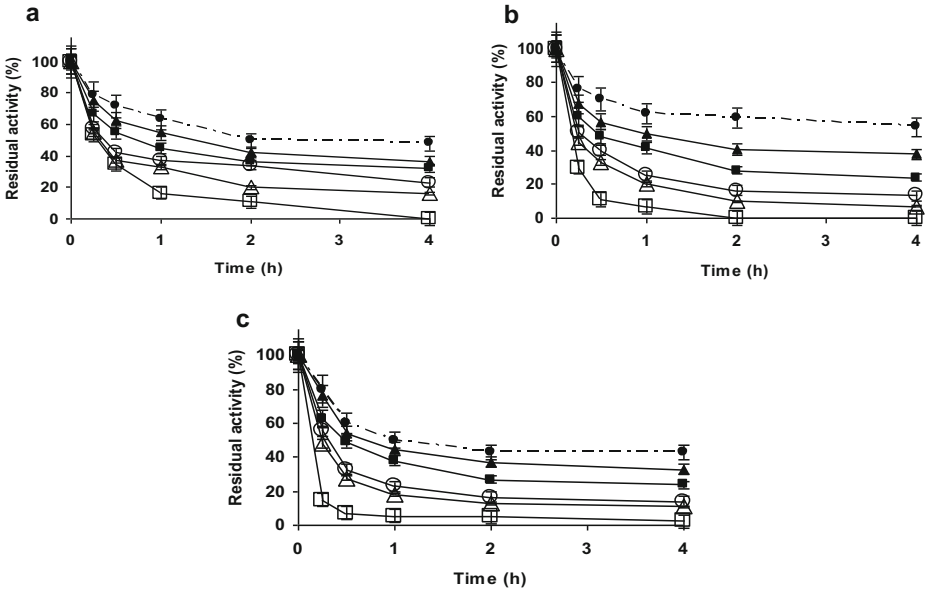

Fig. 5 Effect of the presence of Tris during the immobilization on the stability of PGA immobilized on glyoxyl-agarose. Inactivations have been performed at (**A**) pH 5.0, 77 °C; (**B**) pH 7.0, 72 °C; (**C**) pH 9.0, 65 °C. The measurements were conducted as described in the "Materials and methods" section. Full circles: reference without amine. Full circles: reference without Tris. Full triangles: Tris 1 mM. Full squares: 2 mM Tris. Empty circles: Tris 5 mM. Empty triangles: Tris 10 mM. Empty squares: Tris 20 mM

Table 1 Determination of the number of free Lys per enzyme molecule in different enzyme preparations. Experiments were performed as described in sect. "Methods."

Number of free Lys per protein molecule			
Enzyme	Free enzyme	Reference immobilized enzyme	Enzyme immobilized in the presence of 20 mM Tris
Trypsin	14.0 ± 0.5	7 ± 0.3	9.8 ± 0.2
Chymotrypsin	14.0 ± 0.4	7.4 ± 0.2	9.5 ± 0.3

involved a larger area of the enzyme in the enzyme-support reaction making a higher number of enzyme-support linkages feasible. This could enable a larger number of enzyme-support bonds but can be more hindered by the partial blocking of the support by Tris (e.g., the likely steric hindrances generated). Other possibility if the fact that, while trypsin and chymotrypsin have an area of the protein surface where a high number of Lys groups is accumulated (enabling to involve in the immobilization around 50% of the total Lys of the enzyme surface) [21], in the case of PGA, the Lys, although very abundant, are homogenously distributed along the enzyme surface [36], making more difficult the competition of the enzyme with small aminated compounds in the immobilization media.

We have washed the biocatalyst prepared in 20 mM Tris 5-folds before reduction with 10 volumes of immobilization buffer before reducing the biocatalyst, to eliminate Tris from the support. No significant difference in enzyme stability was found compared to the biocatalyst continuously prepared in the presence of 20 mM Tris (results not shown). When adding 20 mM Tris after 2.5 h of immobilization and then reducing the biocatalysts, the stability was only slightly lower than when the biocatalysts were 3 h in absence of Tris (results not shown).

Determination of the Number of Enzyme-Support Bonds

Using trypsin and chymotrypsin, that were fully pure, we have analyzed the number of enzyme-Lys support bonds of the biocatalysts prepared in the presence of 20 mM of Tris or in its absence (Table 1). In the absence of Tris, both enzymes involve in the immobilization a similar number of Lys (7 from 14 using trypsin, 6.6 from 14 using chymotrypsin), as a result of the fact that they have an area of the protein surface where a high percentage of their Lys is concentrated, facilitating an intense multipoint covalent immobilization, as can be confirmed from the enzyme structure [21]. The figures are very different in the presence of 20 mM of Tris: 4.2 Lys residues are involved in the immobilization for trypsin and 4.5 for chymotrypsin. That way, we can confirm that the presence of Tris produces a reduction in the number of enzyme-support linkages, which can explain the reduction of the enzyme stability after enzyme immobilization [21].

Conclusion

The presence of an aminated buffer, such as Tris, produces a severe decrease on the number of enzyme-support bonds when the enzymes are immobilized on glyoxyl supports. This effect grows with the buffer concentration, and it is translated in a decrease on immobilized enzyme stability. The effect is higher with PGA, and although it is noted at all inactivation pH values, it did not have the same intensity in all instances, perhaps due to different inactivation ways when the enzyme is inactivated under different conditions. This occurred even when the one point imino bonds are

reversible; therefore, a similar negative effect in the intensity of the multipoint covalent attachment may be expected using any other supports able to react with primary amino bonds (e.g., epoxide [77] or vinyl sulfone [71]). The negative effect on the final stability increases with the Tris concentration, but in most cases, it is relevant even using only 1 mM.

Likely, this effect may be extrapolated to other aminated compounds, although the low pK of Tris can make it especially reactive with the glyoxyl support. That way, it is necessary for the researcher to ensure the composition of the medium where the enzyme to be immobilized is stored, and if there are doubts on the presence of some small compounds bearing primary amino groups, it should be dialyzed. If the occurrence of steric hindrances in the reaction between enzyme and support reactions may play a relevant role for this decrease in the number of enzyme-support bonds, this can have special relevance using crude protein extracts, as some of them may be large compounds (e.g., aminated sugars, small peptides).

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Code Availability Not applicable.

Author Contributions In this paper, Roberto Morellon-Sterling, El-Hocine Siar, Sabrina Ait Braham, and Diandra de Andrades prepared the biocatalysts and analyzed their performance, under the supervision of Rafa C. Rodrigues, Ali Aksas, and Roberto Fernandez-Lafuente. Justo Pedroche and Ma del Carmen Millán analyzed the amino-acid composition. Roberto Fernandez-Lafuente designed the experiments. All authors contributed to the writing of the paper.

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Data Availability Data are available from the authors.

Declarations

Ethics Approval Not applicable.

Consent to Participate The authors declare that they consent to participate.

Consent for Publication The authors declare that they consent for publication.

Conflict of Interest The authors declare no competing interests.

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Affiliations

Sabrina Ait Braham[1] · Roberto Morellon-Sterling · Diandra de Andrades[2,3] · Rafael C. Rodrigues[3] · El-Hocine Siar[2,4] · Ali Aksas[1] · Justo Pedroche[5] · Maria del Carmen Millán[5] · Roberto Fernandez-Lafuente[2,6]

[1] Laboratoire de Biotechnologies Végétales et Ethnobotanique, Faculté des Sciences de la Nature et de la Vie, Université de Bejaia, 06000 Bejaia, Algeria

[2] Departamento de Biocatálisis, Instituto de Catálisis-CSIC, C/ Marie Curie 2, Campus UAM-CSI, Cantoblanco, 28049 Madrid, Spain

[3] Biotechnology, Bioprocess, and Biocatalysis Group, Food Science and Technology Institute, Federal University of Rio Grande do Sul, Porto Alegre, RS, Brazil

[4] Transformation and Food Product Elaboration Laboratory, Nutrition and Food Technology Institute (INATAA), University of Brothers Mentouri Constantine 1, Constantine, Algeria

[5] Group of Plant Proteins, Department of Food and Health, Instituto de la Grasa-CSIC, Seville, Spain

[6] Center of Excellence in Bionanoscience Research, External Scientific Advisory Academics, King Abdulaziz University, Jeddah 21589, Saudi Arabia