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Exploiting the Versatility of Aminated Supports Activated with Glutaraldehyde to Immobilize β-galactosidase from *Aspergillus oryzae*

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Abstract: The enzyme β-galactosidase from *Aspergillus oryzae* has been immobilized in aminated (MANAE)-agarose beads via glutaraldehyde chemistry using different strategies. The immobilization on MANAE-supports was first assayed at different pH values (this gave different stabilities to the immobilized enzymes) and further modified with glutaraldehyde. Dramatic drops in activity were found, even using 0.1% (*v*/*v*) glutaraldehyde. The use of a support with lower activation permitted to get a final activity of 30%, but stability was almost identical to that of the just adsorbed enzyme. Next, the immobilization on pre-activated glutaraldehyde beads was assayed at pH 5, 7 and 9. At pH 7, full, rapid immobilization and a high expressed enzyme activity were accomplished. At pH 9, some decrease in enzyme activity was observed. Direct covalent immobilization of the enzyme was very slow; even reducing the volume of enzyme/support ratio, the yield was not complete after 24 h. The stability of the biocatalyst using pre-activated supports was about 4–6 folds more stable than that of the enzyme immobilized via ion exchange at pH 5, with small differences among them. Thus, the immobilization of the enzyme at pH 7 at low ionic strength on pre-activated glutaraldehyde supports seems to be the most adequate in terms of activity, stability and immobilization rate.

Keywords: glutaraldehyde; enzyme immobilization; enzyme stabilization; enzyme inactivation

1. Introduction

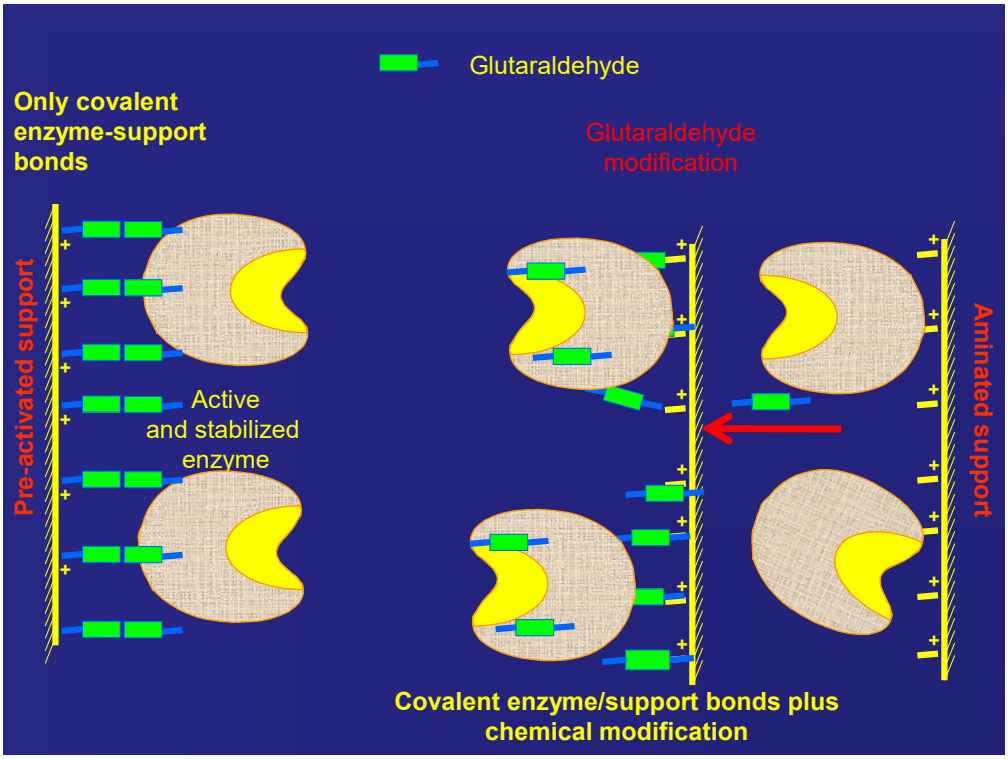
Immobilization of enzymes is usually required for their industrial implementation to solve the problem of enzyme solubility and the difficulties of enzyme reuse [1–4]. However, a proper immobilization protocol may also improve some enzyme features that may become a drawback in an industrial reactor, such as their moderate stability, inhibition by substrates or products, and moderate selectivity or specificity versus industrially relevant substrates (in many instances far from the physiological ones) [5–10].

Enzyme covalent immobilization may have some advantages compared to physical adsorption, such as prevention of enzyme desorption during operation and increased stability via multipoint covalent attachment [8,11]. However, it also has a problem: both enzyme and support must be discarded after enzyme inactivation [8].

The enzyme β-galactosidase (β-gal) from *Aspergillus oryzae* is a glycosylated enzyme whose structure has been recently resolved [12]. The pH/activity profile (with an optimal activity at acidic pH value) makes this enzyme not recommended to hydrolyze lactose in milk [13]. However, the enzyme is interesting for the hydrolysis of lactose in acid milk whey, and also for the production of galacto-oligosaccharides (prebiotics with great interest) or modification of galactose with other alcohols [14–18].

Among the strategies to covalently immobilize proteins, the use of glutaraldehyde may be of outstanding interest because of its high versatility [19–22] (Scheme 1), even though the exact glutaraldehyde chemistry is still under debate [23–25]. One accepted point is that glutaraldehyde forms some kind of cycles including the amino groups that make the amino-glutaraldehyde groups very stable [21,23,24]. This reagent is used to pre-activate supports having primary amino groups [24], and causes the support to become a heterofunctional one [26]. The activation under certain conditions permit to modify each primary amino group in the support with two molecules of glutaraldehyde, this amino-glutaraldehyde-glutaraldehyde group being very reactive with not ionized primary amino groups. Another alternative is the use of glutaraldehyde to crosslink enzyme and support after enzyme ion exchange immobilization on the aminated support [24]. In this strategy, both enzyme and support are modified under mild conditions to ensure that a maximum of one glutaraldehyde molecule react with each primary amino group. The high reactivity of the amino-glutaraldehyde moieties is introduced in both support and enzyme to get an intense multipoint covalent attachment, but also modifies the Lys groups not involved in the immobilization [24,27]. This may introduce a one point chemical modification of the primary amino groups of the enzyme (with positive or negative results on enzyme stability) or intra- or intermolecular crosslinking of the enzyme molecules, which should have a positive effect on enzyme stability [24]. The use of pre-activated support avoids this protein modification, but may have problems to give many enzyme support bonds, as amino-glutaraldehyde-glutaraldehyde groups are not very reactive with the ε-amino group of Lys (pK 10.7) at neutral pH value. This is a problem because the glutaraldehyde groups are not stable at alkaline pH values and therefore may not be used at these pH values [24].

In both cases, enzymes tend to become ionically immobilized before the enzyme reacts covalently with the support, because ion exchange is far more rapid than the covalent reaction [19,22,24]. Thus, permitting or not (e.g., using high ionic strength) the immobilization of β-gal via ion exchange on the support before the covalent immobilization, the results may become different [19,22]. Using this enzyme, the versatility using glutaraldehyde becomes even higher. β-gal may be immobilized via ion exchange at pH values ranging from 5 to 9 and this immobilization pH altered their final features, e.g., stability [28]. This was explained by the different orientation of the β-gal on the support surface. If this is the case, the pH of the first immobilization may also alter the number of enzyme-support linkages that may be achieved using glutaraldehyde, as the amount of nucleophiles may be different in different areas of the protein or the multipoint attachment may have different effects on enzyme stability depending on the protein area involved in the immobilization [29–32]. It should be considered that the most stable ionically immobilized enzyme was even less stable than the free enzyme under conditions where the enzyme had no tendency to aggregate, while, at pH 5 (near the *ip* of the protein), there is clear stabilization [28]. This glycosylated enzyme is difficult to be stabilized via multipoint immobilization because the sugar chains generate some hindrances to the enzyme/support reaction. Thus, the best results using this enzyme in terms of stabilization after immobilization have been reported using amino-epoxy supports, where a stabilization factor of 12 was obtained [33]. Now, we are going to try the immobilization of the enzyme on MANAE-agarose via glutaraldehyde chemistry using the different possibilities presented in this introduction. Agarose is a good support for enzyme immobilization, one of the reasons being that the only groups in the support are those that the researcher introduces, making it easier to understand the results [34].



Scheme 1. Schematic representation of the versatility of glutaraldehyde.

Thus, in this paper, we have intended the comparison of the different strategies that the versatility of glutaraldehyde chemistry and aminated supports enabled: the comparison between the just ionically immobilized enzyme with the ionically immobilized *plus* glutaraldehyde treated biocatalysts, the immobilization via ion exchange in glutaraldehyde pre-activated supports, and the direct covalent immobilization. All ion exchanges were performed at different pH values to permit the adsorption of the enzyme via different areas [28]. To our knowledge, this is the first paper where all these variables are considered in immobilization of any enzyme using glutaraldehyde chemistry.

2. Results and Discussion

2.1. Immobilization of β-gal on MANAE-Agarose at Different pH Values

Figure 1 shows the immobilization of β-gal at pH 5, 7 and 9. It is very rapid at all pH values; so rapid that, in the shortest time the first supernatant sample was taken, immobilization was almost total, with scarce differences among the three pH values. This is thus even though the ionization state of the support (pK of 6.8 and 9.8) and the enzyme will be very different at pH 5, 7 or 9. After immobilization, the activity recovered in all cases was almost 90% of the offered activity, in agreement with the immobilization courses. Figure 2 shows how the immobilization pH value affected the β-gal stability, showing the most and least stabilized enzymes at pH 5 and 9, respectively. This agrees with previous reports [28], although the new support is richer in amino groups.

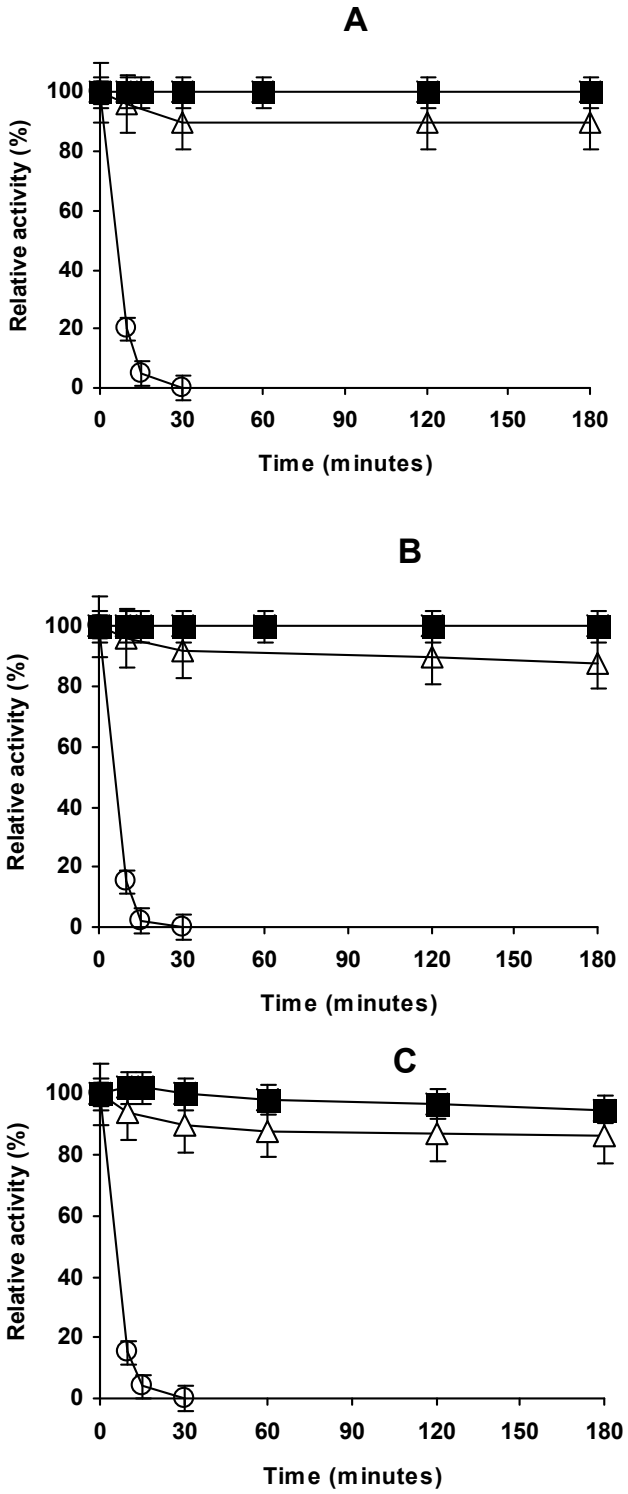


Figure 1. Immobilization courses of β-gal on MANAE-agarose beads at: (**A**) pH 5; (**B**) pH 7; and (**C**) pH 9. Immobilization was performed at 25 °C using 5 mM sodium acetate, sodium phosphate or sodium bicarbonate, respectively, as described in Methods Section. A support having 60 μmol MANAE groups/g of support was utilized. All experiments were performed in triplicate and the values are given as mean value ± the experimental error. Other specifications are described in Methods Section. Squares: Reference; Triangles: immobilization suspension; and Circles: immobilization supernatant.

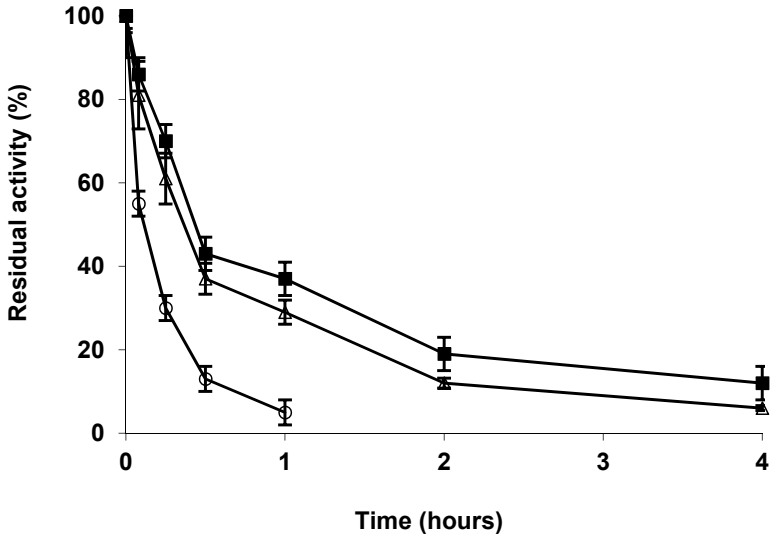


Figure 2. Effect of immobilization pH in the inactivation courses of the different β-gal-MANAE preparations. The inactivation was performed in 25 mM sodium phosphate at pH 7 and 50 °C. The support presented 60 μmol MANAE groups/g of support. All experiments were performed in triplicate and the values are given as mean value ± the experimental error. Other specifications are described in Methods Section. Squares: β-gal was immobilized at pH 5; Triangles: β-gal was immobilized at pH 7; and Circles: β-gal was immobilized at pH 9.

2.2. Treatment of the MANAE-Agarose Immobilized Enzyme with Glutaraldehyde

This strategy is the easiest to transform the enzyme immobilized via ionic exchange in a covalent immobilization. Using 1% (*v*/*v*) glutaraldehyde, the enzyme activity fully disappeared after a few minutes (results not shown). The treatment of the immobilized enzyme with 0.1% (*v*/*v*) glutaraldehyde promoted the rapid inactivation of the enzymes immobilized at the three pH values (Figure 3A). Although the glutaraldehyde chemical modification pH was now 7 for all enzyme preparations, some differences in the inactivation courses could be found depending on the immobilization pH values, reinforcing that each preparation presented a different stability. The enzyme immobilized at pH 9 lost the activity more rapidly than when the enzyme was immobilized at pH 7 or 5 (here, the inactivation was the slower). However, in the best case, only 10% of the initial activity was maintained.

The results could be derived from an intense enzyme-support reaction that leads to enzyme inactivation, or to a very negative effect of the glutaraldehyde modification in the enzyme properties. Thus, the effects of the modification with 0.1% (*v*/*v*) glutaraldehyde on the activity of the free enzyme have been checked (Figure 3B). The free enzyme became partially inactivated in the presence of glutaraldehyde (Figure 3B), but to a much lower extent (just around 10%) than using the ionically absorbed enzyme. Even using 1% glutaraldehyde, a very high percentage of enzyme activity was maintained.

Thus, it seems that the results were a consequence of the reaction of the enzyme and the support, or of a more drastic modification of the immobilized enzyme by glutaraldehyde due to some distortion of the ionically exchanged enzyme. Trying to avoid the activity losses, we added 100 mM glucose to protect the active center [35], but this did not improve the activity recovery (results not shown).

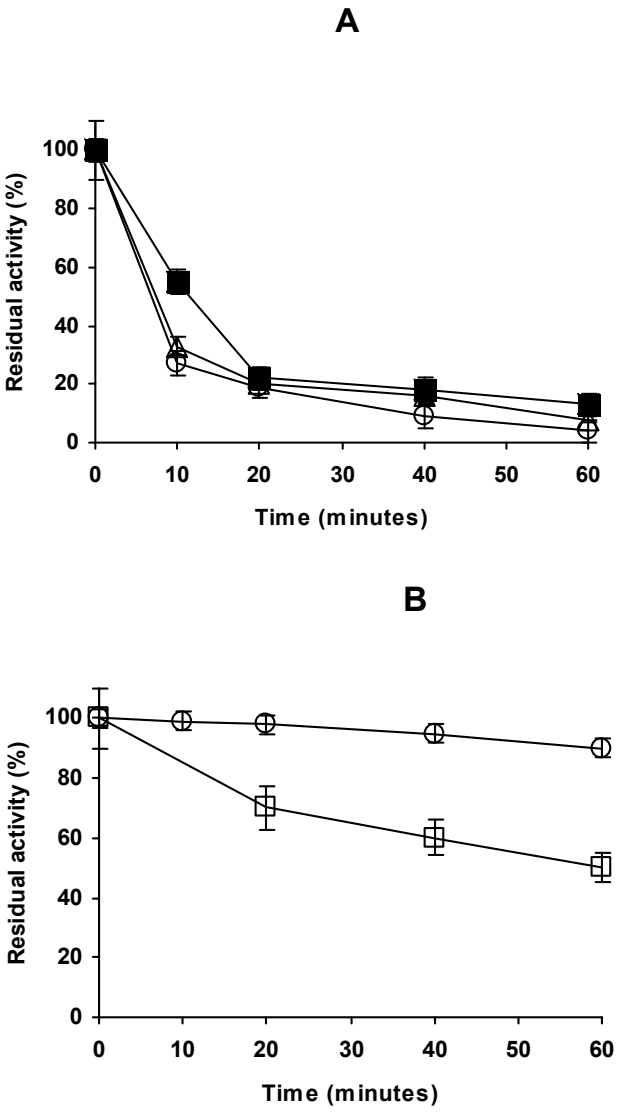


Figure 3. Effect on the enzyme activity of the incubation of: (**A**) β-gal-MANAE; or (**B**) free β-gal in the presence of glutaraldehyde. The experiments were carried out in 25 mM sodium phosphate at pH 7 and 25 °C. Other specifications are described in methods. A: (0.1% (*v*/*v*) glutaraldehyde). The support had 60 μmol MANAE groups/g of support. All experiments were performed in triplicate and the values are given as mean value ± the experimental error. (**A**) Squares: β-gal was immobilized on MANAE-agarose at pH 5; Triangles: β-gal was immobilized on MANAE-agarose at pH 7; and Circles: β-gal was immobilized on MANAE-agarose at pH 9. (**B**) Squares: 1% glutaraldehyde; and Circles: 0.1% glutaraldehyde.

As a last option, a support having a lower density of amino groups was prepared (having 60% of the amino groups of the maximum activated support that has around 60 μmol of MANAE groups per gram of support). In this instance, the ionically exchanged enzyme inactivation was slower, but still very significant; only 40% of the enzyme activity was maintained after 24 h of incubation (Figure 4A). To prevent further enzyme inactivation, the biocatalyst was reduced with 1 mg/mL of sodium borohydride [36], which caused the final activity to be just around 30% of the initial one. Figure 4B shows that the stability of this preparation was very similar to that of the just adsorbed enzyme. In other words, this method did not seem to be very suitable to immobilize the β-gal.

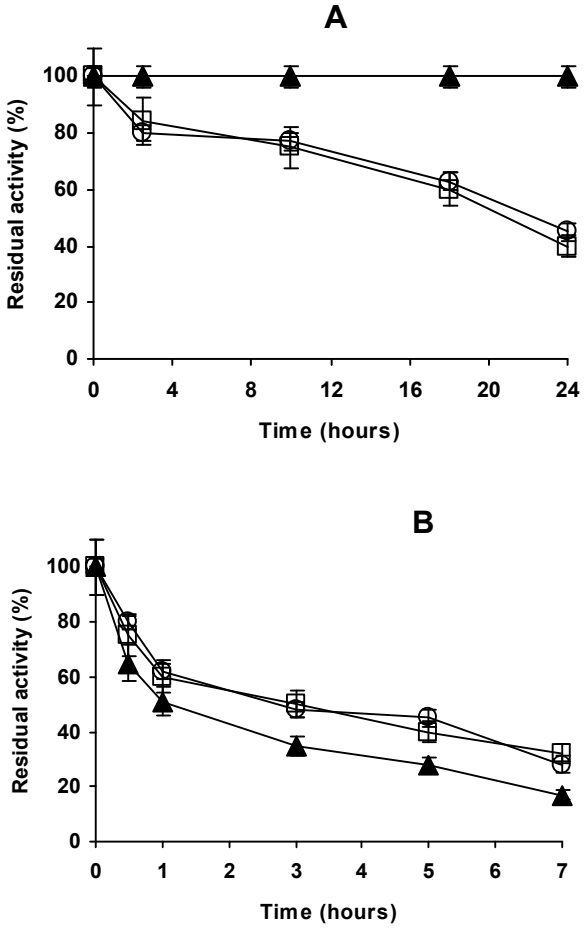


Figure 4. Preparation and inactivation of β-gal-MANAE treated with 0.1% glutaraldehyde: (**A**) Activity evolution of β-gal-MANAE immobilized at pH 5 and treated with 0.1% glutaraldehyde at pH 7 and 25 °C using a support having 40 μmol MANAE groups/g of support. Other specifications are described in methods. (**B**) Inactivation of different β-gal-MANAE preparations at pH 5 and 55 °C. Circles: β-gal-MANAE treated with glutaraldehyde in the presence of 100 mM glucose. All experiments were performed in triplicate and the values are given as mean value ± the experimental error. Squares: β-gal-MANAE treated with glutaraldehyde in the absence of glucose. Triangles: β-gal-MANAE no treated with glutaraldehyde.

Therefore, it seems that glutaraldehyde is able to promote β-gal rapid inactivation. However, glutaraldehyde fixed to a surface has not the mobility of free glutaraldehyde and cannot reach internal pockets of the enzyme. That way, it may be likely that the enzyme may remain active using glutaraldehyde pre-activate supports.

2.3. Immobilization of β-gal on Glutaraldehyde Pre-Activated Supports

First, we have performed the immobilization at pH 5, 7 and 9 using the same conditions utilized in the immobilization on MANAE-agarose via ion exchange, to permit this first step in the immobilization. Figure 5A shows that in this case the enzyme remained active. However, the situation was quite different at the different pH values. At pH 5, the enzyme was almost not immobilized. At this pH, the main mechanism of immobilization should be ion exchange due to the poor reactivity of all amino groups of the enzyme. Furthermore, the presence of glutaraldehyde dimers over the amino groups seems to hinder the capacity of the enzyme to establish several ionic bridges with the support (the enzyme was readily immobilized at this pH value on naked MANAE supports). Steric hindrances are reported as an important problem on the enzyme-support multi-interaction, due to the complexity

of both structures [26]. Thus, the enzyme remained fully active but mostly in the supernatant. At pH 7 and 9, a rapid immobilization took place, but, at pH 9, the immobilization was accompanied by a certain enzyme inactivation, while, at pH 7, the enzyme remained almost fully active.

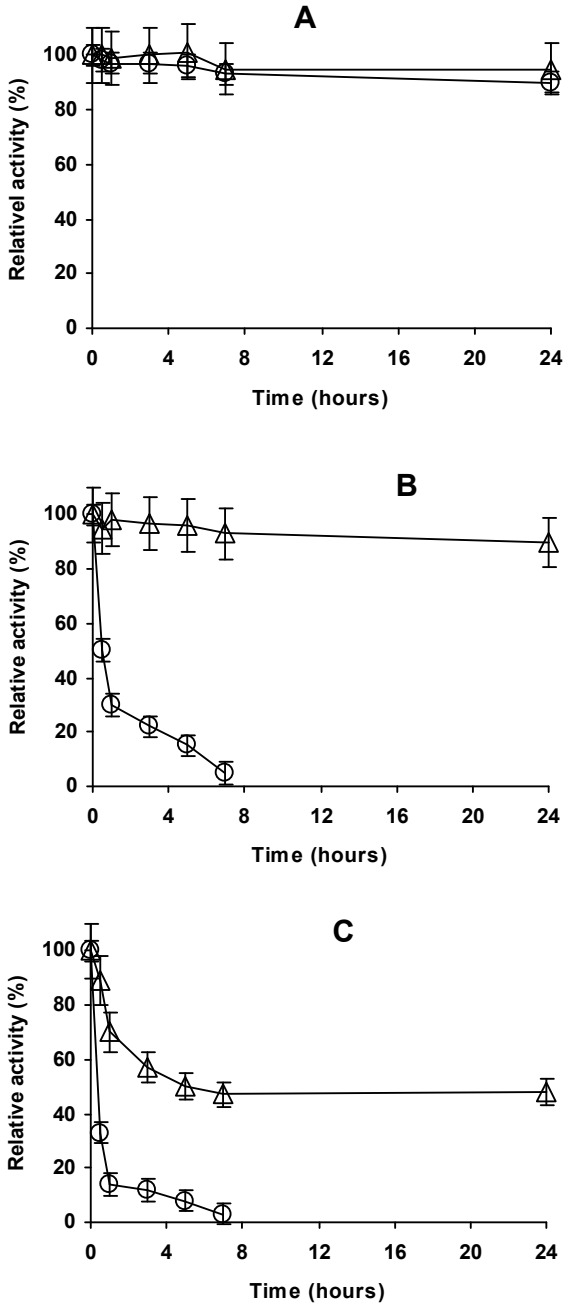


Figure 5. Effect of the pH value on the immobilization courses of β-gal on pre-activated MANAE-glutaraldehyde supports. The immobilization was performed at: (**A**) pH 5; (**B**) pH 7; or (**C**) pH 9 at 25 °C using 5 mM sodium acetate, sodium phosphate or sodium bicarbonate, respectively, as described in Methods Section. A support having 60 μmol MANAE groups/g of support was utilized. All experiments were performed in triplicate and the values are given as mean value ± the experimental error. Other specifications are described in Methods Section. Triangles: immobilization suspension; and Circles: immobilization supernatant. Incubation of the immobilized enzymes at pH 7 or 9 after 24 h in 500 mM NaCl/25 mM sodium phosphate produced the full release of the enzyme immobilized on MANAE-agarose [37,38], but all enzyme molecules remained immobilized on the glutaraldehyde preparations, showing the success of getting a covalent immobilization.

Finally, we tried to get the direct covalent immobilization of the enzyme on the support [19,22]. In this case, the immobilization will proceed by the most reactive primary amino group, and the main effect of the pH is to control the protein reactivity, which should increase using a higher pH value, but at pH over 8 this effect is detrimental for the glutaraldehyde groups stability [24]. Thus, to this goal we used pH 8 and 250 mM sodium phosphate. Figure 6 shows that while the enzyme was almost not immobilized on MANAE support, some immobilization was achieved using the glutaraldehyde MANAE support. However, the immobilization was much slower than when the ion exchange was permitted and after 24 h no more than 20% of the enzyme was immobilized. A lower immobilization rate is expected due to the low reactivity of glutaraldehyde, but in this case the difference became very significant. To improve the immobilization rate, we reduced the volume of enzyme (increasing the enzyme concentration to keep the total amount of protein) from 1:10 to 1:3. This permitted to immobilize 80% of the enzyme after 48 h (Figure 5B).

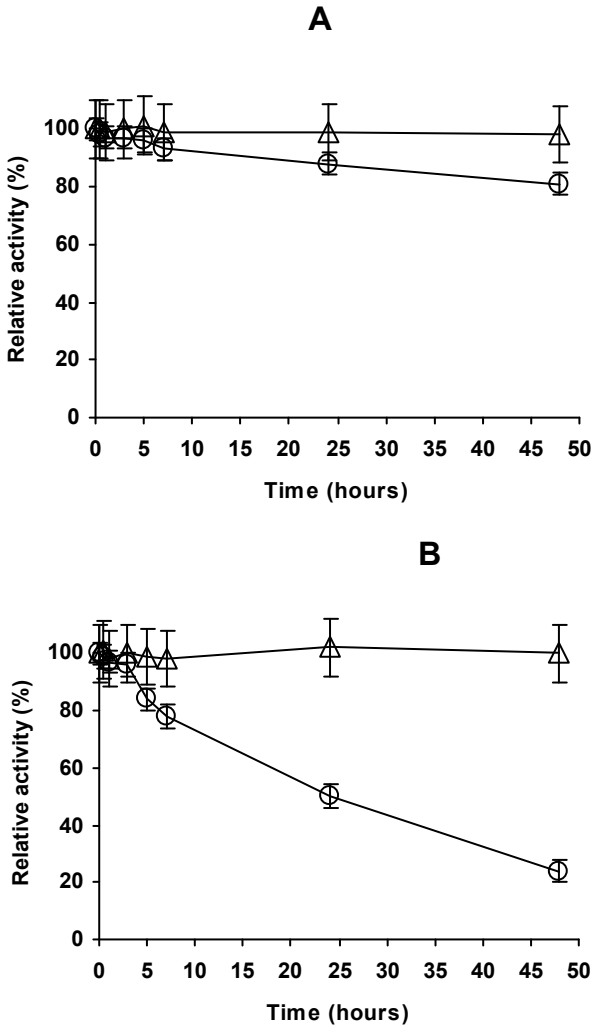


Figure 6. Immobilization courses of β-gal on pre-activated MANAE-glutaraldehyde supports at pH 8 and 250 mM sodium phosphate. The immobilization was performed using a: (**A**) 1/10; or (**B**) 1/3 support/enzyme suspension ratio at 25 °C. A support having 60 μmol MANAE groups/g of support was utilized. All experiments were performed in triplicate and the values are given as mean value ± the experimental error. Other conditions are described in methods Section. Triangles: immobilization suspension; and Circles: immobilization supernatant.

2.4. Stability of the Different Preparations

Figure 7 shows the inactivation courses of the three glutaraldehyde preparations, compared to that of the enzyme just ionically adsorbed at pH 5. Both covalent enzyme preparations where the enzyme was previously ionically exchanged presented observed half-lives about six-fold higher than that of the enzyme just ionically adsorbed at pH 5, while the directly covalently immobilized enzyme was the least stable one, showing a lower stability than even the just ionically immobilized enzyme.

Considering activity, stability and immobilization rate, it seems that immobilization at pH 7 and low ionic strength is the recommended method to immobilize β-gal using the glutaraldehyde chemistry.

It should be remarked that the glutaraldehyde biocatalysts prepared at pH 7 and 9 were now almost identical in stability, while the ion exchange gave much lower stability for the enzyme immobilized at pH 9. That means that the stabilization achieved when the enzyme was immobilized at pH 9 by the glutaraldehyde reaction was higher that immobilizing the enzyme at pH 7. However, the final stability became fairly similar because the starting stability was lower when the immobilization was performed at pH 9 [28]. However, perhaps more covalent bonds could be formed when the enzyme was immobilized at pH 9.

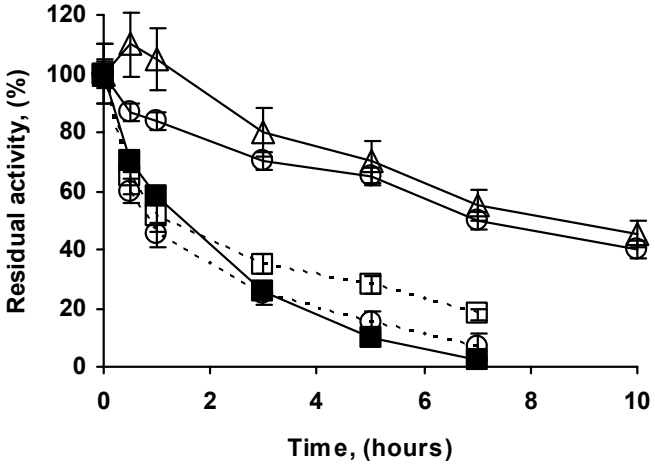


Figure 7. Inactivation courses of different MANAE-glutaraldehyde-β-gal preparations at pH 5 and 55 °C. Experiments were performed as described in Methods Section; the enzymes were immobilized on a support having 60 μmol MANAE groups/g of support. All experiments were performed in triplicate and the values are given as mean value ± the experimental error. Dashed line, Open circles: Free β-gal; Dashed line, Open Squares: β-gal-MANAE immobilized at pH 5; Open Triangles. Solid line: MANAE-glutaraldehyde-β-gal immobilized at pH 7 at low ionic strength; Open Circles, Solid line: MANAE-glutaraldehyde-β-gal immobilized at pH 9 at low ionic strength; and Solid Squares, Solid line: MANAE-glutaraldehyde-β-gal immobilized at pH 8 at high ionic strength.

3. Materials and Methods

3.1. Materials

β-Galactosidase from *A. oryzae* (20 Units oNPG/mg of protein), o-nitrophenyl-β-galactopyranoside (oNPG), glycidol, glutaraldehyde (25% (*v*/*v*), stabilized with methanol) sodium borohydride, sodium periodate and ethylendiamine were purchased from Sigma-Aldrich (St. Louis, MO, USA). Four-percent CL agarose beads were from GE Healthcare. MANAE supports were prepared from glyoxyl supports [39] with a modification of the protocol previously described [40]; reaction time was 24 h before reduction. All other reagents were of analytical grade.

3.2. Preparation of Glutaraldehyde Agarose Beads

Ten grams of MANAE agarose was suspended in 20 mL of 15% (*v*/*v*) glutaraldehyde in 200 mM phosphate buffer pH 7.0. The suspension was kept under mild stirring overnight at 4 °C. After that, the support was filtered and washed with an excess of distilled water. The activated support was used immediately after preparation. This ensures that each primary amino in the support has been modified with two glutaraldehyde molecules [21,41].

3.3. Standard Determination of Enzyme Activity

This assay was performed by measuring the increase in absorbance at 380 nm produced by the release of o-nitrophenol in the hydrolysis of 10 mM oNPG in 100 mM sodium acetate at pH 4.5 and 25 °C (ε was 10493 $M^{-1}cm^{-1}$ under these conditions) [28]. This wavelength was chosen because this enzyme is active at acid pH values [12–14], and at these pH conditions, the ε of oNP is negligible at 410 nm. To start the reaction, 50–100 μL of the enzyme solution or suspension were added to 2.5 mL of substrate solution. One unit of activity (U) was defined as the amount of enzyme that hydrolyzes 1 μmoL of oNPG per minute under the conditions described previously. Protein concentration was determined using Bradford's method [42].

3.4. Immobilization of β-Galactosidase on Different Supports

The immobilizations were carried out employing 20 oNPG units of beta-galactosidase per g of wet support (1 mg of enzyme per gram of support) and performed at 25 °C. This low loading was used to prevent diffusion limitations that could distort the results. Thus, the commercial enzyme samples were dissolved in the corresponding volume of 5 mM sodium acetate at pH 5, sodium phosphate at pH 7 or sodium carbonate at pH 9. Then, ten grams of different supports (MANAE or glutaraldehyde agarose) were added. Reference suspensions were prepared in identical conditions, but using no activated agarose as matrix. In this matrix immobilization was negligible.

An exception was made when ion exchange was not desired. In this case, 10 g of glutaraldehyde agarose was suspended in 30 mL of enzymatic solution prepared in 250 mM sodium phosphate at pH 8, maintaining the amount of enzyme per gram of support. The suspension was kept under stirring and samples of the suspension and the supernatant were withdrawn and enzyme activity was analyzed as described above after the desired immobilization time. When the different immobilizations were completed, the suspensions were washed, filtered and stored at 4 °C for further characterization.

In some instances, the enzyme immobilized on MANAE supports at the different pH values was washed and re-suspended, 5 mM sodium phosphate at pH 7 was used in both steps to ensure the pH of the final suspension. Then, 0.1% or 1% (*v*/*v*) glutaraldehyde was added to achieve some covalent enzyme-supports bonds [20]. After 1 h, the immobilized enzyme was washed and left at pH 7 for 24 h before storage it at 4 °C. All experiments were performed in triplicate and the values are given as mean value ±the experimental error.

3.5. Thermal Stability of the Enzyme Preparations

The enzyme was incubated at different pH values (5.0 and 7.0) in 25 mM of the sodium acetate or sodium phosphate respectively and a temperature that permitted a reasonable inactivation rate was selected. The objective was to compare the different immobilized enzyme stabilities under different conditions, in this case pH value and buffer nature. This may affect the ionization of the support/enzyme and that way, positive or negative interactions may be presented under certain pH values and not under other ones. At the desired times, samples of the inactivation suspension were withdrawn and the enzyme activity was measured using oNPG. All experiments were performed in triplicate and the values are given as mean ± the experimental error. Half-lives are given from the observed values from the inactivation courses, which follow a not first order inactivation showing some heterogeneity in the immobilized enzyme biocatalysts.

4. Conclusions

The versatility of glutaraldehyde has enabled to prepare β-gal biocatalysts with very good activity retention and a moderate stabilization. The glutaraldehyde molecules fixed on the support are unable to inactivate the enzyme. However, as described above, if free glutaraldehyde was added to the ionically exchanged enzyme, this became inactivated. Using free enzyme, this negative effect of glutaraldehyde modification is decreased. The effect was also decreased using a support with a lower activation degree. In any case, the stability of the enzyme did not improve and this immobilization strategy could be discarded. Considering activity, stability and immobilization rate, the proposed optimal protocol of β-gal immobilization from the seven studied possibilities is the immobilization on pre-activated supports at pH 7 and permitting a previous ion exchange of the enzyme on the support. The enzyme activity is almost fully preserved and a stabilization of six folds may be accomplished, in a very rapid and simple way.

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Author Contributions: R.F.-L. and M.S. conceived and designed the experiments; R.F.-L. analyzed the data and wrote the paper; and H.Z., S.P., T.L.d.A. performed the experiments.

Conflicts of Interest: The authors declare no conflict of interest.

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