Chemical Modification of Protein Surfaces To Improve Their Reversible Enzyme Immobilization on Ionic Exchangers

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The enzyme penicillin G acylase (PGA) is not adsorbed at pH 7 on DEAE- or PEI-coated supports, neither is it adsorbed on carboxymethyl (CM)- or dextran sulfate (DS)-coated supports. The surface of the enzyme was chemically modified under controlled conditions: chemical amination of the protein surface of carboxylic groups (using soluble carbodiimide and ethylendiamine) and chemical succinylation (using succinic anhydride) of amino groups. The full chemical modification produced some negative effects on enzyme stability and activity, although partial modification (mainly succinylation) presented negligible effects on both enzyme features. The chemical amination of the protein surface permitted the immobilization of the enzyme on CM- and DS-coated support, while the chemical succinylation permitted the enzyme immobilization on DEAE- and PEI-coated supports. Immobilization was very strong on these supports, mainly in the polymeric ones, and dependent on the degree of modification, although the enzymes still can be desorbed after inactivation by incubation under drastic conditions. Moreover, the immobilization on ionic polymeric beds allowed a significant increase in enzyme stability against the inactivation and inhibitory effects of organic solvents, very likely by the promotion of a certain partition of the organic solvent out of the enzyme environment. These results suggest that the enrichment of the surface of proteins with ionic groups may be a good strategy to take advantage of the immobilization of industrial enzymes via ionic exchange on ionic polymeric beds.

Introduction

Reversible immobilization of enzymes via ionic adsorption may be a suitable option in the design of industrial biocatalysts.[1,2] The immobilization following this technique implies simple protocols for enzyme immobilization (simply allowing contact of enzyme solution and support) under very mild conditions.[3] Moreover, supports are very stable and may be stored for long time-periods before their use. However, the most relevant advantage of these strategies is the possibility of reusing the supports after enzyme inactivation, just by incubating the immobilized and inactivated enzyme in the presence of acid, under high ionic strength or other compounds.

The main drawbacks of these immobilization strategies are the possible desorption of the proteins during operation[4,5] and the difficulties to improve the enzyme stability using these techniques. The use of ionic polymer to coat the surface of solid supports may solve some of these points, permitting very strong and nondistorting protein adsorptions on a polymeric bed.[6] Moreover, it may stabilize proteins, e.g., multimeric enzymes by preventing subunit dissociation[7,8] or by generating hydrophilic nanoenvironments that stabilize the proteins against the presence of organic solvents.[9−11]

However, in some cases a protein may not be adsorbed strongly enough even on these polymeric ionic beds for a particular application. In these cases, when it is difficult to further increase the strength of the adsorption between the enzyme and the support by improving the support, a likely strategy would be to increase the number of ionic groups on the protein surface which are able to interact with the groups placed on the support. Thus the number of cationic or anionic groups on the enzyme surface may be increased using chemical modification or site-directed mutagenesis (Scheme 1).

Although different techniques, like multipoint immobilization,[12] chemical modification,[13] screening of the most stable enzyme,[14] or molecular biology[15] have been used to improve the enzyme properties, they have been traditionally considered as parallel routes.[16−19] However, it has been reported that the combined use of several of these tools allow great improvement of the final properties of the biocatalyst.[20−27]

In this study, chemical modification will be used as an intermediate tool, modifying the charged groups on the protein surface, to improve the adsorption of these modified proteins on the ionic exchanger. Chemical modification of proteins is a quite well developed technique that has been used in many instances to improve the enzyme performance or to determine groups that are critical for enzyme properties.[28] This technique is very rapid and permits quick evaluation of the prospects of the proposed strategy.

There are two different possibilities of modifying charged groups on the enzyme surface. One of them consists of changing the carboxylic groups to amino groups using a diamine molecule and activating the carboxylic acids with soluble cardodiimide.[21,29,30] This is a simple protocol where the degree of substitution is controlled quite easily, e.g., by controlling the concentration of carbodiimide as previously stated by Koshland.[29] Besides, changes in the isoelectric point and global charge at neutral pH value are very significant because we are

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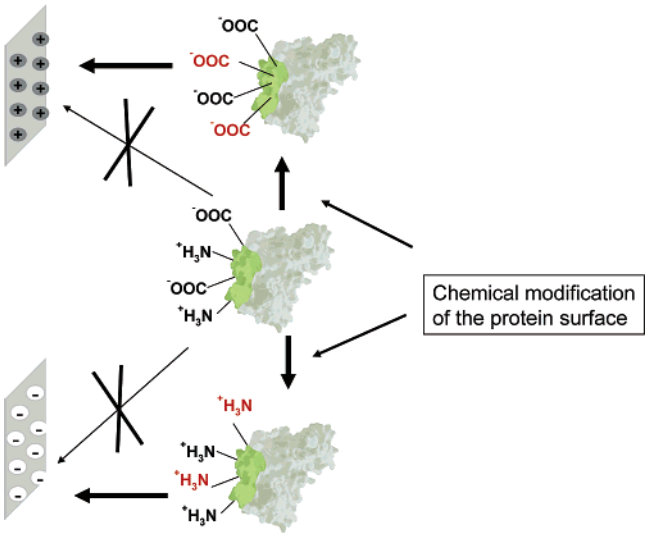
Scheme 1. Proposed Strategy To Improve the Adsorption of Protein on Ionic Exchangers



changing negatively charged groups by positively charged groups, improving the possibilities of adsorption of the enzyme on cationic exchangers. On the other hand, the opposite change may be also quite easily carried out by modifying amino groups with succinic anhydride.[31,32] In this case, control of the degree of modification is also easily done, and modified enzymes should have an easier adsorption on anionic exchangers. It must be stressed that in this case there is not a complete modification of all positive charges, because the Arg residues cannot be activated by succinic anhydride. Therefore, the global change of charge on the protein surface may be lower than in the previous case.

We have chosen penicillin G acylase (PGA) as a model enzyme to study the prospects of this strategy. Despite the in vivo function for this enzyme remaining unidentified, PGA is very relevant in the pharmaceutical industry,[33] because it is currently used in production of 6-APA and 7-ADCA by hydrolysis of penicillin G and cephalosporin G, respectively.[34−35] Moreover, it may be used in many other reactions, such as the synthesis of antibiotics, resolution of racemic mixtures, synthesis of amides, and selective deprotections.[36,37]

Materials and Methods

Penicillin G acylase (PGA) and Penicillin G were kindly provided by Antibióticos S. A. (León, Spain). Succinic anhydride, hydroxylamine, 2,4,6-trinitrobenzenesulfonic acid (TNBS), polyethyleneimine (PEI) 25000 Da, dextran sulfate sodium salt from *Leuconostoc* spp (MW 500000D), 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDAC), ethylenediamine (EDA), methyl (*R*)-(−)-mandelate were supplied by Sigma-Aldrich S.A. (St. Louis, MO). DEAE sepharose, carboxymethyl sepharose, and cyanogen bromide-activated Sepharose 4B (CNBr) was purchased from Amersham Biosciences (Uppsala, Sweden). Cross-linked 4% agarose beads (4BCL) were donated by Hispanagar S. A. (Burgos, Spain). All other reagents were of analytical grade. Dextran sulfate (DS)-coated support was prepared using MANAE agarose[38] following the protocol described elsewhere.[39] Polyethylenimine (PEI) supports were prepared from glyoxyl agarose[40] as previously described.[41] All experiments were performed at least in triplicate, and the results are presented as its mean value. Experimental error was never over 5%.

1. Determination of Penicillin G Acylase (PGA) Activity. Enzyme activity was evaluated using an automatic titrator (DL50 Mettler Toledo) to titrate the release of phenylacetic acid produced by the hydrolysis of 10 mM penicillin G in 0.1 M sodium phosphate/0.5 M NaCl at pH 8 and 25 °C. A 100 mM NaOH solution was used as titrating reagent. One International Unit (IU) of PGA activity was defined as the amount of enzyme that hydrolyzes 1 μmol of penicillin G per minute at pH 8 and 25 °C.

2. Chemical Modification. *2.1. Amination of Soluble and Immobilized Enzymes.* Soluble and immobilized preparations were modified with different concentrations of EDAC in the presence of 1 M EDA at pH 4.75[29,21] to control the modification degree.

2.1.1. Amination of Soluble Enzymes. Five milliliters of soluble PGA (10 mg/mL) was added to 45 mL of EDA 1 M at pH 4.75 Different amounts of solid EDAC were added to the solution to a final concentration of 10^{-2} or 10^{-3} M. After 90 min of gentle stirring at 25 °C, 10 mL of a 0.5 M hydroxylamine solution at pH 7 was added to the enzyme solution. Then, it was dialyzed five times against 50 volumes of a 25 mM potassium phosphate buffer and stored at 4 °C. It has been described that the use of 1 M EDA at pH 4.75 and 10 mM EDAC allows the full modification of the carboxylic groups of the protein surface, while using 1 mM EDAC in 1 M EDA at pH 4.75, the modification degree is only around 40−50%.[42] The aminated soluble enzymes were named as: PGA-EDA 50 (PGA modified with 10^{-3} M EDAC at pH 4.75); PGA-EDA-100 (PGA modified with 10^{-2} M EDAC at pH 4.75). During the amination processes, samples were withdrawn and analyzed for protein concentration[43] and enzyme activity that was determined as described above.

2.2. Succinylation of Soluble and Immobilized Enzymes. Soluble PGA and PGA immobilized on agarose activated with CNBr were incubated in 10 mM phosphate buffer (pH 8.0) with magnetic stirring. Solid succinic anhydride was added in small portions. Different molar ratios of reagent with respect to the sum of lysine and tyrosine were used (1:1; 10:1; 20:1). The reaction was kept at pH 8.0 for 2 h through an automatic titrator (DL50 Mettler Toledo) with 0.25 M NaOH as titrating reagent. Once the reaction was finished, the mixture was dialyzed against 10 mM phosphate buffer pH 7.0 at 4 °C. Succinylated enzyme was treated with 0.5 M hydroxylamine (pH 7.0) for 5 h at 25 °C and dialyzed again. A blank was also performed in the absence of succinic anhydride.

The degree of modification of PGA primary amino groups was determined measuring the decrement in primary amino groups

3. Determination of Primary Amino Groups. The amount of primary amino groups in the different protein preparations was determined by using the 2,4,6-trinitrobenzenesulfonic acid (TNBS) assay described elsewhere.[44] PGA-BrCN agarose derivative (250 mg) was mixed with 3 mL of TNBS 0.1% (aqueous solution), and the mixture was incubated for 30 min at room temperature (from this moment the intensity of the color did not increase). The reference for the assay contained 250 mg of blocked BrCN-agarose and the same TNBS solution. To determine the modification with carbodiimide and EDA of the enzyme, the reference was a PGA-BrCN preparation with the native primary amino blocked by reaction with glutaraldehyde and further reduction with borohydride.[45] Then, the TNBS preparations were packed in a 1 mm cells. The absorbance of modified and unmodified proteins was read at 430 nm.

4. Immobilization of Native and Chemically Modified PGA. *4.1. Immobilization on Sepharose 4B Activated with CNBr.* One gram of washed CNBr-activated Sepharose 4B (prepared according to supplier's instructions) was incubated with 10 mL of PGA solution (0.1 mg/mL) in 25mM sodium phosphate pH 7.5 for 1 h at room temperature with gentle stirring. After that, the gel was filtered and washed with 25 mM sodium phosphate pH 7.5. Then, the gel was blocked with 10 mL of 1 M ethanolamine at pH 8 during 2 h with gentle stirring, and washed with 25 mM potassium phosphate buffer at pH 7 and storage at 4°C.

4.2. Immobilization on Ionic Exchangers Matrixes. As a general method, 1 g of supports was added to 4 mL of enzyme solution containing 10 IU in 5 mM sodium phosphate at pH 7 and 25 °C. During adsorption, samples were withdrawn from the supernatant and the

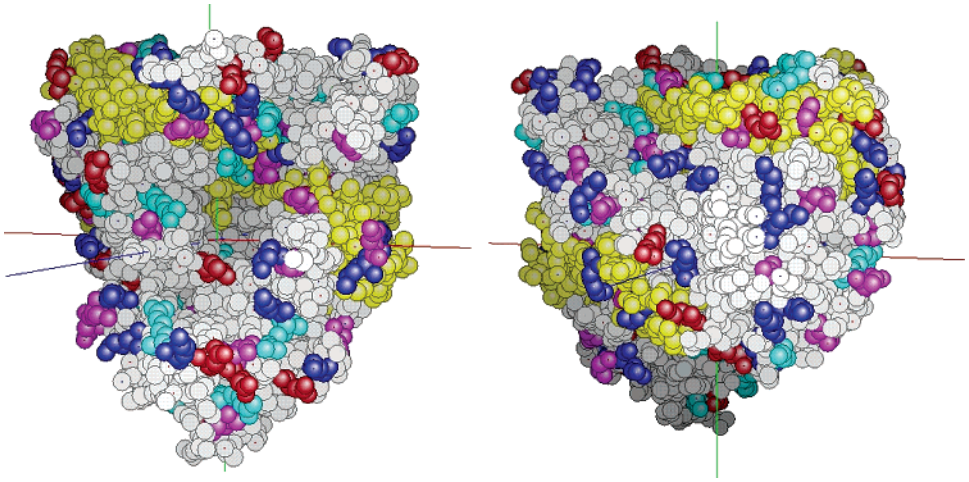


Figure 1. 3D structure of PGA. Space-filled model. Lys are in dark blue, Arg in light blue, Glu in red, and Asp in pink.

suspension to assay enzyme activity as described above. After immobilization, the derivatives were washed with distilled water and stored at 4 °C.

Reference suspensions were prepared having exactly the same enzyme concentration and media conditions (pH, *T*, ionic strength), but adding the corresponding amount of inert agarose instead of the active support. The activity in the supernatant of this reference was fully preserved in all cases; therefore, the decrease in activity in the supernatant of the "immobilization suspension" can be directly correlated to the amount of the enzyme adsorbed on the ionic exchangers.

5. Desorption of the Proteins Adsorbed on the Ionic Exchangers. The different immobilized PGA preparations (10 IU/g of support) were incubated at increasing concentrations of NaCl at 25 °C and pH 7, and after 30 min (longer incubation times did not produce any change in the results) the PGA activity of the suspension and supernatant was assayed as described above.

6. Thermal Stability Assays. Enzyme preparations (soluble or immobilized preparations) were incubated in 25 mM sodium phosphate buffer at 57 °C pH 7.0. Samples of the suspension were withdrawn periodically, and enzyme activity was analyzed as described above. PGA residual activity was expressed as a percentage of initial activity at the given incubation time.

7. Stability Assays in the Presence of Cosolvents. Enzyme preparations were incubated in different percentages of dioxane (v/v) in 25 mM sodium phosphate buffer pH 6.5 at 4 °C. Samples of the suspension were withdrawn periodically, and enzyme activity was analyzed as described above. PGA residual activity was expressed as a percentage of initial activity at the given incubation time.

8. Inhibition of Enzyme Activity in the Presence of Cosolvents. Enzyme preparations were incubated at growing concentrations of dioxane. Enzyme activity was evaluated using an automatic titrator (DL50 Mettler Toledo) to titrate the release of mandelic acid produced by the hydrolysis of 10 mM methyl (R)-(−)-mandelate in 0.1M sodium phosphate and 25 °C. A 100 mM NaOH solution was used as titrating reagent. PGA relative activity was expressed as a percentage of activity referred to the activity in the absence of cosolvent.

Results

1.- Penicillin G Acylase Surface. The crystal structure of PGA is available,[46] and it is therefore possible to evaluate the number of superficial Glu, Asp, Arg, and Lys residues (as the strongest and most abundant ionizable groups on the protein surface). Figure 1 shows the distribution of these groups on PGA surface. PGA has 41 Lys groups (36 of them exposed to the medium), 32 Arg residues (19 of them exposed), 50 Glu residues (41 of them exposed), and 49 Asp residues (36 of them exposed). Only exposed residues can participate in the ionic adsorption of the enzymes on ionic exchangers; moreover, only these groups may be chemically modified under mild conditions.[29] Thus, the enzyme shows 77 carboxylic groups that could be aminated and that could interact with a cationic exchanger. Besides, PGA exhibit 55 cationic exposed groups from Lys and Arg residues and only 36 of them, those corresponding to Lys residues, can be modified as Arg groups cannot be succinylated under the employed conditions. Therefore, amination may be expected to have a higher effect on final charge of enzyme surface properties than succinylation (77 possible new positive charges versus 36 new negative charges).

2.-Amination of PGA To Improve Its Adsorption on Cationic Exchangers. *2.1. Adsorption of Different PGAs on Different Cationic Exchangers.* PGA-EDA-50 should present 37−40 new positively charged groups on the protein surface at pH 7 (p*K* of new amine groups is 9.2).[38] Thus, this modification rendered an enzyme surface with 92−95 Arg+Lys+EDA groups and 40−37 Glu+Asp versus the nonmodified enzyme which showed 55 Lys+Arg residues and 77 Glu+Asp groups on its surface. On the other hand, the PGA-EDA-100 surface presented the transformation of the 77 Glu and Asp to 77 new positively charged groups, achieving a new modified surface without negative charges.

The amination step promoted a slight decrease in enzyme activity (by around 10% in the full modification).[21] Amination of superficial carboxy groups has been reported to decrease the thermostability of PGA, this effect being more relevant in fully modified enzymes than in 50% modified enzymes.[21] However, we have decided to study the adsorption of both modified enzymes on ionic exchangers, to determine the real effect of the modification degree on the adsorption on different supports.

Figure 2 shows that the nonmodified PGA is only partially adsorbed on CM-agarose and DS-activated supports. After 1.5 h only 10% of the offered enzymatic activity was immobilized on both supports, while both modified enzymes are very rapidly adsorbed on the cationic exchangers assayed. The immobilization rates were very similar for both modified enzymes, and both kept their activity unaltered after the immobilization.

Figure 3 shows the desorption of the different PGAs at growing NaCl concentrations. The small fraction of nonmodified PGA adsorbed on the cationic exchangers could be easily desorbed from both, CM (Figure 3A) and DS (Figure 3B) agarose. PGA-EDA-50 required 400 mM NaCl to be fully desorbed from CM-agarose. Using DS, desorption was not detected even using 300 mM NaCl, and full desorption was

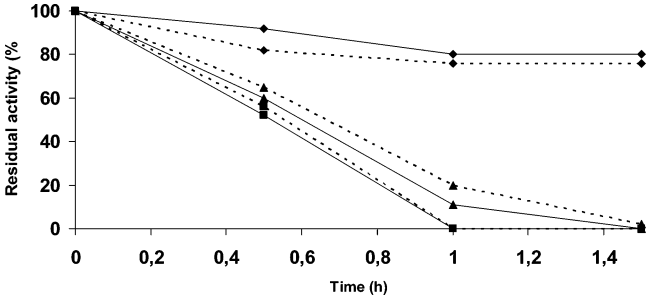


Figure 2. Immobilization course of native PGA and chemically aminated PGA on carboxymethyl agarose (CMA) and dextran sulfate 500 kDa agarose (DSA). (−) Immobilization on CMA, (----) Immobilization on DSA. (◆) Native PGA, (▲) PGA-EDA-50, and (■) PGA-EDA-100. Initial enzyme loading 10 IU/mL support. Immobilizations were performed in 5 mM buffer phosphate at pH 7.0 at room temperature. See Materials and Methods for further details.

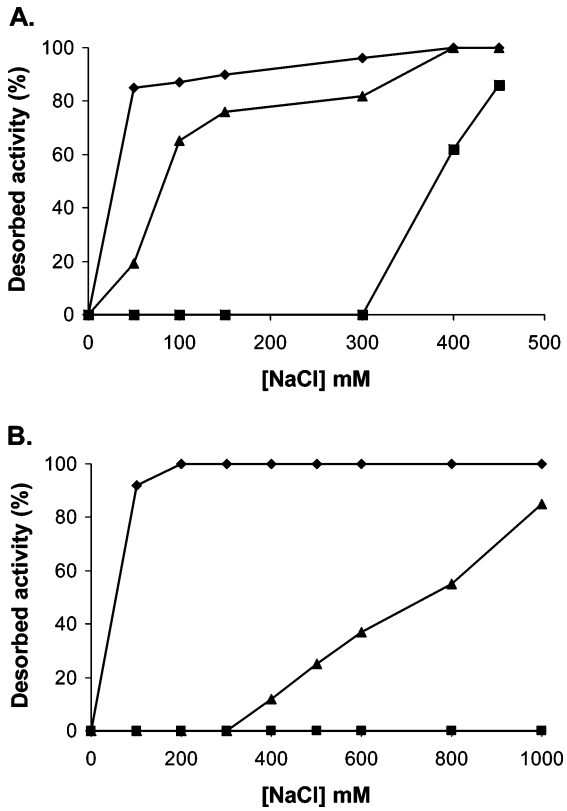


Figure 3. Desorption of aminated PGA adsorbed on different cationic exchanger supports at growing ionic strengths. Activity released from CM-agarose (A) or DS-agarose (B). (◆) Native PGA, (▲) PGA-EDA-50, and (■) PGA-EDA-100 by incubation on growing concentrations of NaCl (see Materials and Methods). Enzyme was immobilized in 5 mM sodium phosphate buffer at pH 7.0, with a protein loading of 10 IU/g support. Further details are described in Materials and Methods.

not observed even using 1 M NaCl. The use of PGA-EDA-100 required a great increase in the amount of salt necessary to desorb PGA from both cationic exchangers: When this enzyme was adsorbed on DS-coated supports, it was not desorbed at all from the support even using 1 M.

Therefore, we have started with an enzyme which was not adsorbed on the support to end with an enzyme which cannot be desorbed even in the presence of 1 M NaCl by just a simple chemical modification of the enzyme surface, where cationic groups were added, decreasing the number of anionic groups. The differences between polymeric-coated supports and standard ones agree with previous reports in the literature.[10,39] Moreover, it is clear that full modification of the enzyme has a much clearer

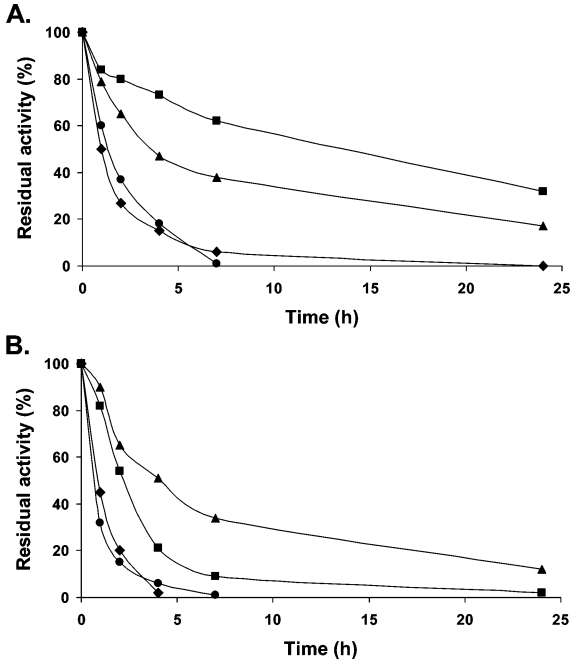


Figure 4. Inactivation courses of different aminated PGA preparations. A. In the presence of cosolvents: (◆) PGA immobilized onto CNBr-sepharose and then modified with PGA modified with 10^{-3} M EDAC; see Materials and Methods for details. (▲) PGA-EDA-50 immobilized onto CM-agarose. (■) PGA-EDA-50 immobilized onto DS-agarose. (●) PGA-Fluka. Experiments were carried out in presence of 60% (v/v) dioxane in 25 mM sodium phosphate buffer (pH 6.5) at 4 °C. B. Thermal inactivation: (◆) PGA immobilized onto CNBr-sepharose and then modified with PGA modified with 10^{-3} M EDAC. (▲) PGA-EDA- 50 immobilized onto CM-agarose. (■) PGA-EDA-50 immobilized onto DS-agarose. (●) Immobilized PGA supplied from Fluka. Experiments were carried out at 57 °C in 25 mM phosphate buffer pH 7.0

effect on enzyme adsorption strength than that of a partial modification. Even though, the partial modification should greatly increase the possibility of an intense multipoint adsorption, the presence of nonmodified Asp and Glu residues (around 40), which have the same charge than the groups placed in the support could promote some repulsions, reducing the adsorption strength. This fact has been previously reported with native enzymes and DS supports, showing that the use of polyvalent cations may reverse this repulsion on ionic bridges, reinforcing the adsorption.[47] However, the enzyme could be desorbed by using 100 mM HCl/1 M NaCl or 9 M gunidine, etc. Therefore, after enzyme inactivation, the support can be recovered and reutilized.

2.2. Stability of Different PGA Preparations. Considering the deleterious effect of chemical modification on the enzyme stability, we have utilized PGA-EDA-50 in further studies. The immobilization of this enzyme on a support activated with CNBr reactive groups showed a stability (both thermal and in the presence of solvents) similar to that of the commercial preparation of Fluka (Figure 4). However, the enzyme adsorbed in the polymeric DS presented a much higher stability in the presence of organic solvents than that of the other preparations (e.g, 15-fold more stable than the BrCN preparation). On the other hand, this derivative presented stability similar to the BrCN in thermal inactivations.

The stabilization against organic solvent was not only due to the immobilization. Therefore, these results could suggest that the immobilization of the aminated PGA on the highly hydrophilic polymeric bed formed by DS could somehow promote a certain partition of the organic solvent in the enzyme environ-

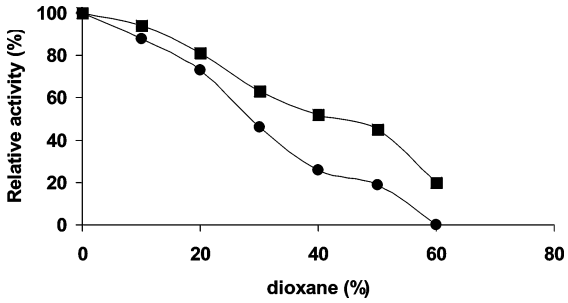


Figure 5. Effect of dioxane on the activity of different PGA preparations. (●) Native PGA immobilized onto CNBr-sepharose. (■) PGA-EDA-50 immobilized onto DS-agarose. Activity was measured in the hydrolysis of 10 mM methyl (*R*)-(−)-mandelate in 50 mM sodium phosphate buffer, adding the corresponding dioxane and adjusting the pH value at 7.0. 100% was considered the activity in full aqueous medium.

Table 1. Determination of Modification Degree of Amino Groups by Anhydride Succinic[a]

enzyme derivative	modified amino groups (%)	residual activity (%)
Agarose- PGA native	0	100
Agarose-PGA 1M*	40	100
Agarose-PGA 10M	95	52
Agarose-PGA 20M	100	52

[a] Native PGA and succinylated PGA were immobilized onto CNBr−Agarose. The name of the preparation refers to the molar ratio of succinic anhydride with respect to the sum of lysine and tyrosine residues. PGA-1M, a molar ratio of 1:1; PGA-10M, a molar ratio of 10; PGA-20M, a molar ratio of 20. The percentage of modification was determined by TNBS. For further details see Materials and Methods.

ment, permitting a much higher stability of the enzyme[39] without really increasing the enzyme rigidity, as showed by the nonrelevant effect of the immobilization on thermal stability.

To confirm this hypothesis, the inhibitory effect of dioxane on the activity of PGA-EDA.-50 immobilized on DS-agarose and native PGA immobilized on BrCN was studied (Figure 5). The first one suffers a lower inhibition effect than that of the covalent preparation. The differences between both covalent derivative and ionically adsorbed derivative were much higher at high concentrations of organic solvent. When the organic solvent concentration was 60%, the covalent derivative was fully inactive while the derivative immobilized on DS retained 20% of activity. This result reinforces the hypothesis that immobilization of the aminated PGA on the highly hydrophilic polymeric support made up by DS, which could somehow promote a certain partition of organic solvent, decreases the organic solvent concentration in the surroundings of enzyme, so this fact would be translated in higher enzyme stability.[9,48,49]

3.-Succinylation of PGA To Improve Its Adsorption on Anionic Exchangers.

3.1. Control of the Modification. Table 1 shows that the modification of the amino groups with an equimolecular relation between theoretical amino groups in the enzyme and succinic anhydride allowed modification of 40% of the amino groups on the PGA surface. The percentage of modification using a 10-fold molar excess reached more than 95% of groups; the modification degree was similar when 20-fold molar excess was used. Thus, we can assume than these last modifications permitted the full modification of all superficial amino groups on the protein surface. We have named PGA-SUCC-40 for PGA succinylated using equimolecular amounts of anhydride succinic, and PGA-SUCC-100 for PGA succinylated using a molar ratio of 10.

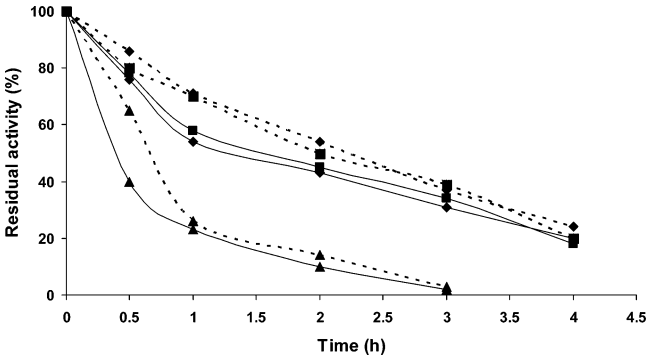


Figure 6. Thermal inactivation courses of soluble modified enzymes and modified CNBr-activated sepharose derivatives. (−) Soluble enzymes; (----) CNBr-sepharosa derivatives. (◆) native PGA; (■) PGA-SUCC-40; (▲) PGA-SUCC-100. Thermal inactivations were carried out at 57 °C and pH 7.0 in 25 mM sodium phosphate buffer.

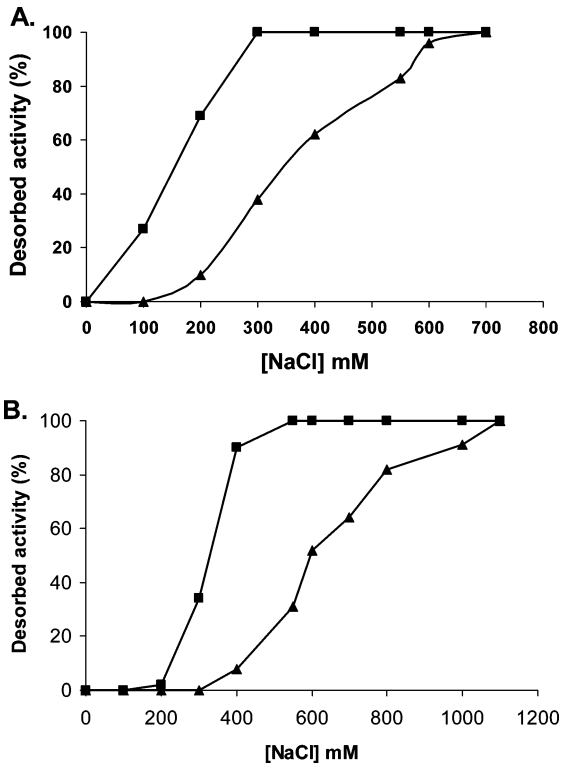


Figure 7. Desorption of different succinylated PGA adsorbed on different anionic exchanger supports at growing ionic strengths. Activity released from DEAE-agarose (A) or PEI-agarose (B): (■) PGA-SUCC-40 and (▲)PGA-SUCC-100 by incubation on growing concentration of NaCl (see Materials and Methods). Enzyme was immobilized at pH 7 as described in Materials and Methods, with a protein loading of 20 IU/mL of support.

PGA-SUCC-40 surface changed from 77 negative charges (Asp+Glu residues) and 55 positive ones (Lys+Arg residues) to 89−91 negative residues (Asp+Glu+Succ) and 41−43 positive residues (Lys+Arg). In the case of the PGA-SUCC-100 surface, the total number of negative charges was increased to 113 (Asp+Glu+Succ) and the number of positive charges was reduced to 19 (Arg).

3.2. Effect of Chemical Modification on the Enzyme Properties. The effect of chemical succinylation on the enzyme activity was almost negligible in the case of PGA-SUCC-40, keeping at 100% after this modification. However, the activity decreased by 50% in the case of PGA-SUCC-100 (Table 1).

The thermal stability of the succinylated enzymes was also compared to that of the unmodified enzyme. Figure 6 shows

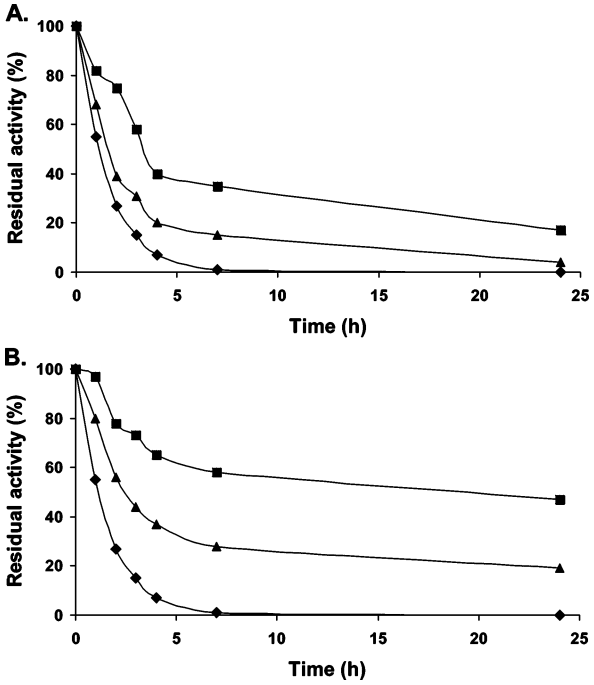


Figure 8. Stability of different succinylated PGA preparations in the presence of 65% (v/v) dioxane in 25 mM sodium phosphate buffer (pH 6.5) derivatives at 4 °C**.** A: (◆) PGA immobilized onto CNBr-sepharose and then modified with succinic anhydride (molar ratio 1:1); see Materials and Methods for details. (▲) PGA-SUCC-40 immobilized onto DEAE-agarose. (■) PGA-SUCC-40 immobilized onto PEI 25 kDa-agarose. B**:** (◆) PGA immobilized onto CNBr-sepharose and then modified with succinic anhydride (molar ratio 10:1). (▲) PGA-SUCC-100 immobilized onto DEAE-agarose. (■) PGA-SUCC-100 immobilized onto PEI 25 kDa-agarose.

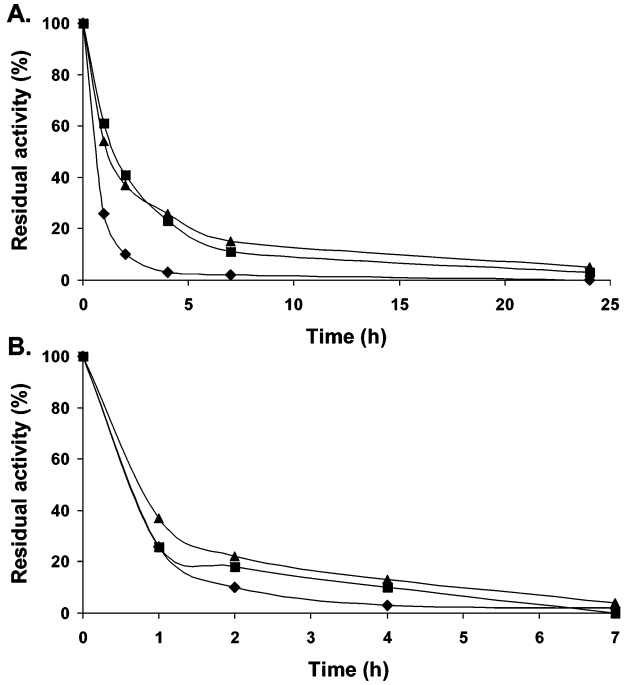


Figure 9. Thermal inactivation courses of different succinylated PGA preparations. A: (◆) PGA immobilized onto CNBr-sepharose and then modified with succinic anhydride (molar ratio 1:1); see Materials and Methods for details. (▲)PGA-SUCC-40 immobilized onto DEAE-agarose. (■) PGA-SUCC-40 immobilized onto PEI 25 kDa-agarose at 4 °C. B: (◆) PGA immobilized onto CNBr-sepharose and then modified with succynic anhydride (molar ratio 10:1) (▲)PGA-SUCC-100 immobilized onto DEAE-agarose. (■) PGA-SUCC-40 immobilized onto PEI 25 kDa-agarose. Experiments were carried out at 57 °C in 25 mM sodium phosphate buffer pH 7.0.

that while PGA-SUCC-40 stability was very similar to the soluble enzyme, both in free or mild covalently immobilized form, PGA-SUCC-100 presented a slightly lower stability (by around a 2−2.5-fold factor).

3.3. Adsorption of Different PGA on Different Anionic Exchangers. The nonmodified enzyme was significantly adsorbed neither on DEAE- nor on PEI-coated supports. However, both succinylated enzymes could be fully adsorbed on both supports in less than 10 min, maintaining their activity during the immobilization process. Figure 7 shows that adsorption was quite stronger using PEI-coated supports than using DEAE, and PGA-SUCC-100 was much more strongly adsorbed than PGA-SUCC-40. The lower adsorption strength in the fully succinylated enzyme, compared to the full aminated one presented above, may be due to the fact that Arg residues remained unmodified: this means a lower global number of negatively charged groups than the positive charged groups obtained in the previous case (132 vesus 113) and the presence of a significant amount of groups[19] with the same charge than those placed in the support, that could promote some repulsions between the enzyme and the support.[47]

3.4. Stability of Different PGA Preparations. Figure 8 shows that both PGA-SUCC preparations adsorbed on PEI were much more stable than the covalent or the protein adsorbed on DEAE in the presence of dioxane, the PGA-SUCC-100 after adsorption being more stable than PGA-SUCC-40, while the stability of both covalently immobilized enzymes become quite similar. In thermal inactivations (Figure 9), all preparations of PGA-SUCC-40 were more stable than PGA-SUCC-100, while the stabilities of the different immobilized preparations were similar. Again, these results suggest that the main effect of immobilization on polymeric ionic exchangers on the enzyme stability in the presence of organic solvents is the partition of organic solvent molecules from the enzyme environment.

4. Discussion

This work presents a successful example on how the combined use of several tools in enzyme technology can offer new and advantageous solutions for problems in the design of industrial biocatalysis. Chemical modification of the protein surface of penicillin G acylase has permitted strong adsorption of the enzyme on ionic exchangers. That way, it is possible to take advantage of reversible immobilization even with enzymes that hardly become adsorbed on these kinds of supports. The use of ionic polymers coating the support surface retained the enzyme activity during immobilization. Moreover, using these supports, the adsorption strength of the enzyme may be strong enough to use the modified and adsorbed enzymes as industrial biocatalyst under many different conditions. This kind of supports permitted to improve the enzyme stability in the presence of organic solvents, very likely by the generation of a hydrophilic micro-environment surrounding the enzyme molecules that promote a partition of the organic solvent molecules.[9,48,49]

Chemical modification of the protein surface is a very fast technique that does not require knowledge of the enzyme structure.[13,50] It is performed with the protein already folded; therefore, it is possible to perform very drastic modifications of the protein surface without considering possible interferences on the protein folding. In fact, it has been found that even full modification of the enzyme ionic groups in the surface by groups with the opposite charge does not have a very great effect on

the enzyme activity. This technique may be used to perform a first evaluation of the potential of a determinate strategy in a relatively rapid fashion. Genetic modification of the enzyme may be the final way of achieving the goal marked in this paper, once the suitability of the idea has been probed using chemically modified enzymes: genetic manipulation may be initially more time-consuming, it should permit the correct folding (and processing in the case of PGA) of the modified protein, but finally, when the mutant protein was available, it may be used without any further treatment.[51]

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