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Improved Stabilization of Genetically Modified Penicillin G Acylase in the Presence of Organic Cosolvents by Co-Immobilization of the Enzyme with Polyethyleneimine

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Abstract: Penicillin G acylase (PGA) is an enzyme that hardly interacts with polycationic polymers (e.g., polyethyleneimine, PEI) and thus the enzyme cannot be stabilized against the action of organic solvents by its co-immobilization with the polymer in the same support, neither covalently attached to the support nor adsorbed on the already immobilized enzyme. However, a new mutant PGA bearing eight additional Glu residues homogenously distributed throughout the enzyme surface may interact with the polymer. The co-immobilization of the enzyme and PEI on glyoxyl-agarose allows one to fully take advantage of the stabilization produced by the multipoint covalent attachment and by the protective hydrophilic micro-environment generated by the polycationic polymer, enabling a significant stabilization of the immobilized PGA in the presence of organic solvents.

Keywords: enzyme stability; micro-environments; organic solvents; polyethyleneimine; polymers; solvent partition

Introduction

The enzyme penicillin G acylase (PGA) from *Escherichia coli* is produced as a monomer that is auto-processed into two subunits during its exportation to the periplasmic space.[1] Although its natural function has not been fully identified yet,[2,3] it is currently utilized in the production of 6-amionopenicillanic acid (6-APA) and 7 aminodeacetoxycephaloporanic acid (7-ADCA) by hydrolyzing penicillin G and cephalosporin G, respectively.[4–7] Moreover, it may be employed in many other reactions, like the synthesis of antibiotics, resolution of racemic mixtures, synthesis of amides and selective deprotections.[8–15]

Although PGA has a moderate thermostability which has permitted its rapid implementation in hydrolytic reactions, it is rapidly inactivated by most of the currently used organic cosolvents or in anhydrous media,[16–20] reaction conditions which are required for antibiotic synthesis to shift thermodynamic equilibrium in the synthetic direction or to increase the solubility of hydrophobic compounds.[14,21–26]

One strategy to improve the enzyme stability in organic media may be to generate hydrophilic environments around the enzyme, promoting a certain partition of the organic solvent molecules and a drop in the organic solvent concentration in the enzyme environment. This stabilization has been achieved by different experimental approaches, like co-immobilization of enzymes and polymers on solid supports,[27] adsorption of enzymes in supports coated with polymeric beds,[12,28] or co-aggregation of enzymes and polymers.[29] In these cases, the interactions between the polymers and the enzyme allow one to create a hydrophilic shell that fully covers the enzyme surface, protecting the whole surface. However, the application of these strategies to PGA have not been successful so far, and it has become a necessity to develop more sophisticated strategies to get a significant stabilization of the enzyme.[30,31] Previous failures may be explained by the peculiar surface of PGA where positive and negative charges are equilibrated and,

thus, the polymers hardly become adsorbed avoiding the possibility of protecting PGA by simple immobilization procedures. In fact, native PGA cannot be adsorbed on supports coated with PEI.[32]

Recently, a new PGA mutant has been reported possessing 8 new Glu residues which have been introduced on the protein surface by changing Asn or Gln in different positions homogeneously distributed throughout the PGA surface.[32] When compared with the native enzyme, this mutant PGA presented very similar thermostability and activity/pH profiles while the isoelectric point decreased from 6.4 to 4.3. Therefore, this mutant PGA can be strongly adsorbed on PEI-coated supports.[32]

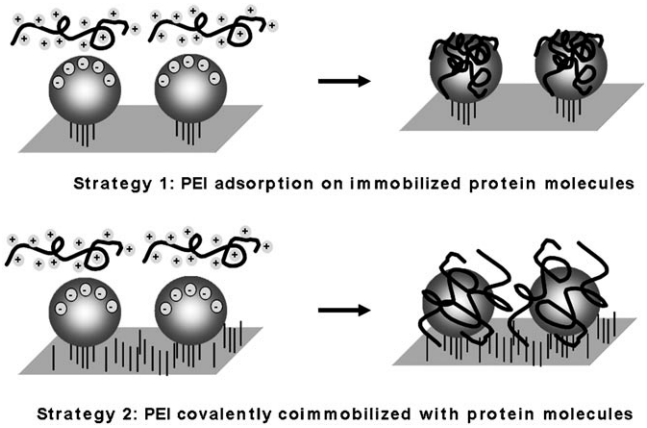
In this manuscript, we have studied the possibilities of stabilizing this mutant PGA against the action of organic solvents by co-immobilizing on glyoxyl agarose the enzyme and polycationic polymers having primary amino groups. This support has been chosen because it has allowed one to stabilize many enzymes *via* multipoint covalent attachment[33] and has been specifically used to stabilize PGA.[34–36] The immobilization of proteins on this support is based on its reaction with primary amino groups of the enzyme.[37] Therefore, the modifications of Asn and Gln on the protein surface may not reduce the reactivity of PGA with glyoxyl-agarose. Moreover, glyoxyl-agarose permits the easy co-immobilization of enzymes and aminated polymers such as PEI.[30,38]

Results and Discussion

Immobilization of the Native and Mutant PGA on Glyoxyl Agarose

Here, we analyzed if these mutations may modify its immobilization on glyoxyl agarose or alter its stabilization due to the multipoint covalent attachment of the enzyme on the activated support.

Interestingly, both native and mutant PGA enzymes were immobilized on glyoxyl agarose in less than 30 min and the enzyme activity was almost fully maintained during the immobilization, since over 90 % of activity was recovered in both cases. Both immobilized enzymes, the mutant and native PGA, presented almost identical stability against thermal or organic solvent inactivation. This result agrees with the fact that the immobilization and stabilization on glyoxyl agarose only involves the primary amino residues of the protein surface, which remained unaltered in the mutant PGA.[37]



Scheme 1.

Effect of Co-Immobilization of Native PGA with PEI on the Properties of the Enzyme

To attempt the covering of the native penicillin G acylase with PEI we used two strategies (Scheme 1). Firstly, the enzyme was immobilized on glyoxyl agarose; the aldehyde groups were reduced with sodium borohydride and then incubated in the presence of PEI of different sizes. In this case, PEI can only be incorporated into the immobilized derivative by the direct adsorption of the polymer on the enzyme surface. A similar strategy has allowed the stabilization of lipases against organic solvent denaturation.[39] For the second strategy (Scheme 1), PEI was added before the reduction of the immobilized enzyme preparation. Thus, PEI could react covalently with the aldehyde groups on the support, being in close contact with the portion of the surface of the immobilized protein involved in the immobilization. These conditions allowed us to coat the support surface with this polymer.[43] These treatments did not alter the enzyme activity, but did not promote an increase in its stability against heat or in the presence of organic solvents. In fact, using strategy 1, the titration of the enzyme derivatives with SPDP did not reveal a significant increase in the amount of primary amino groups

Table 1. Amino groups titration of mutant and native PGA glyoxyl derivatives coated with PEI polymer.

PGA preparation	μmol/g support[a]
Glyoxyl – native	0.045
Glyoxyl – native PEI – 600kD	0.043
Glyoxyl – mutant	0.044
Glyoxyl – mutant PEI 25kD	0.114
Glyoxyl – mutant PEI 60 kD	0.125
Glyoxyl – mutant PEI 600 kD	0.190

[a] Assay performed with *N*-succinimidyl-3-(2-pyridyldithio)-propionate (SPDP). See Experimental Section for further details.

(Table 1), suggesting that PEI was not adsorbed on the enzyme surface This result agrees with the observation that PGA is not adsorbed on supports coated with PEI.[32]

The failure of both treatments could be explained by the lack of polymer-enzyme interactions, since in this way, only those areas of the enzyme surface which are in close contact with the support can be protected by the polymer, while those areas of the protein oriented to the medium remain fully accessible to the organic solvent.

Effect of Co-Immobilization of Mutant PGA with PEI on the Properties of the Enzyme

Taking into account that the mutant PGA contains more Glu residues in its surface, we expected that the protein could interact better with PEI than the wild-type PGA. The strategy 1 (incubation of the immobilized mutant PGA with PEI) promoted an increase in the detected primary amino groups on the immobilized preparation (Table 1), suggesting that in this case the adsorption of the polymer on the enzyme surface was accomplished. This result agrees with the capacity of the mutant enzyme to become adsorbed on PEI-coated supports.[32] Interestingly, in this case the enzyme activity also remained unaltered by the PEI adsorption. However, this treatment promoted a slight decrease in thermal stability and only a small increase in the stability in the presence of an organic solvent (Figure 1 A and B). These results were similar to that observed when immobilized PGA was treated with aldehyde dextran[30] and the explanation may be similar, i.e., the large size of the polymer promotes steric hindrances to the interaction between the enzyme and the polymer in the neighboring of the support surface, leaving this protein area unprotected, and therefore, having a reduced stabilizing effect by this treatment.

Using the second strategy, the covalent co-immobilization (Scheme 1) of the PGA and the PEI, the enzyme activity remained unaltered. However, it presented a clear stabilizing effect of the enzyme in the presence of organic solvents, although the thermo-stability remained almost unaltered (Figure 1 A and B). This result suggested that now, using a polymer that is larger than the enzyme molecule, the co-immobilized polymer was able to interact and to cover the protein surface, generating a hydrophilic environment covering the protein molecules and decreasing the concentration of organic solvent molecules in the enzyme environment.

That way, this strategy permits us to take advantages of the stabilization of the enzyme by multipoint covalent attachment[33,37] and the generation of hydrophilic environments.[31]. The addition of both effects permits a much higher stability in the presence of organic solvents of these preparations than that achieved by just adsorbing the enzyme on PEI-coated

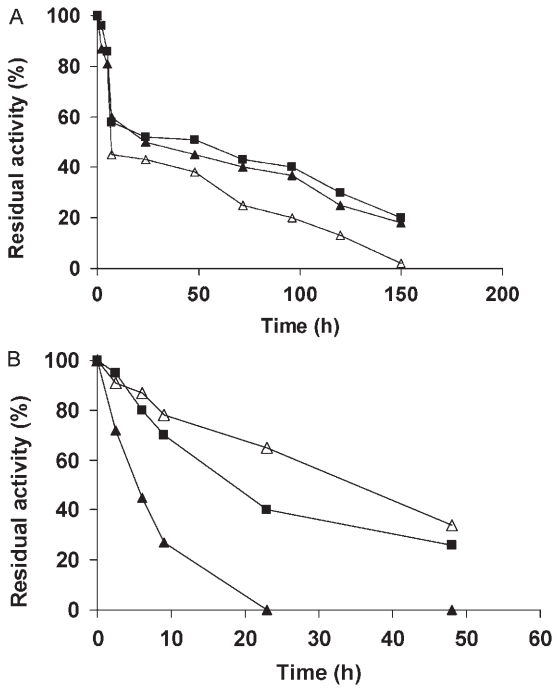


Figure 1. Different inactivation courses of mutant immobilized PGA after different treatments. Panel (**A**) Thermal inactivation. Experiments were carried out at 65 °C in 25 mM phosphate buffer pH 7.0. Panel (**B**) Inactivation by co-solvents. Experiments were carried out in presence of 75 % (v/v) dioxane in 25 mM sodium phospate buffer (pH 7.0) at 4 °C. Symbols: (▲) Mutant PGA glyoxyl derivative; (■) Mutant PGA glyoxyl derivative with adsorbed PEI 600 kD; (△) Mutant PGA glyoxyl derivative co-immobilized with PEI 600 kD.

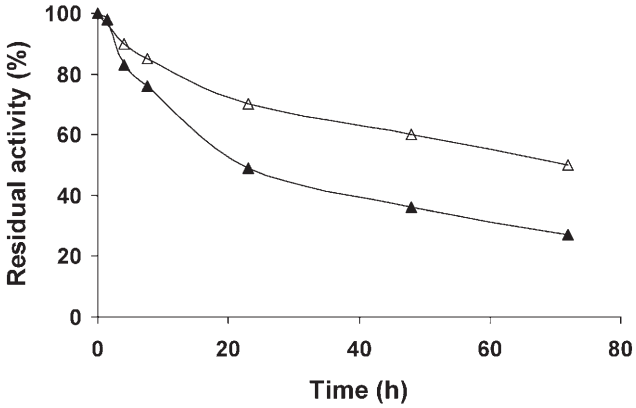


Figure 2. Co-solvent inactivation courses. (▲) Mutant PGA adsorbed on PEI 600kD agarose support as described in Experimental Section. (△) Mutant PGA co-immobilized with PEI 600 kD on glyoxyl agarose supports. Inactivation was carried out in the presence of 75 % (v/v) dioxane in 25 mM sodium phosphate buffer (pH 6.5) at 4 °C.

supports, where the enzyme was only protected by the hydrophilic environment[32] (Figure 2).

Conclusions

The use of site-directed mutagenesis to alter the properties of an enzyme may be a powerful tool to improve protein properties *via* physico-chemical tools.[36] Here we show that the increase of the negative charge in the protein surface has permitted the enzyme to interact with a cationic polymer which protected the enzyme surface from the interaction with an organic medium.

Depending on the final use of the biocatalyst, a light stabilization against organic solvents may be achieved by using a simple strategy, like adsorption on PEIcoated supports which allows the final reuse of the support after enzyme inactivation.[40,41,43] However, if a higher stabilization is required, it may be necessary to couple different stabilization strategies, as multipoint covalent attachment (that promotes a general rigidification of the protein structure[33,36] and generation of artificial environments such as those proposed here. The low stability of PGA against organic solvents requires full coverage of the enzyme by the polymer to get a significant protection, making it necessary to co-immobilize the enzyme with large polymers to get best results. These derivatives may be used in many reactions where stability in organic solvents is a limiting parameter.[14,22,23,25,26].

Experimental Section

Penicillin G was kindly provided by Antibióticos S.A. (León, Spain). Polyethyleneimine (PEI) with different molecular weights (25, 60, 600 kD), sodium borohydride, *N*-succinimidyl-3-(2-pyridyldithio)propionate and dithiotreitol were supplied by Sigma–Aldrich S.A. (St. Louis, MO, USA). Cross-linked 4% agarose beads (4BCL) were kindly donated by Hispanagar S.A. (Burgos, Spain). All other reagents were of analytical grade. PEI supports were prepared from glyoxyl agarose[33,42] as previously described.[43] Pure mutant enzyme was produced as described elsewhere.[32] The mutant enzyme keep almost unaltered its specific activity, its pH-dependent activity or its stability.[32]

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 plus 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 8.0 and 25 °C. All experiments were performed at least in triplicate and the results are presented as the mean value. Experimental error was never over 5%.

Immobilization of Native or Mutant PGA on Glyoxyl Agarose

Native or mutant PGA was immobilized as previously described.[35] Ten grams of glyoxyl agarose 4 BCL were added to 100 mL of a PGA solution (0.3 mg protein mL^{-1}) in sodium bicarbonate buffer 100 mM containing 100 mM phenylacetic acid and 20% glycerol. The suspension was then gently stirred for 3 h at pH 10.05 and 25 °C. After that 1 mg of solid $NaBH_4$ per mL of suspension was added. After 30 min the reduced derivatives were washed with 10 volumes of 25 mM potassium phosphate buffer at pH 7.5 and with an excess of distilled water.

Samples of the supernatants were periodically withdrawn and analyzed for protein concentration[44] and enzyme activity. 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 PGA activity in the supernatant of this reference suspension was fully preserved; therefore, the decrease in enzyme activity in the supernatant of the "immobilization suspension" can be directly correlated to the amount of immobilized enzyme after reaction with the glyoxyl groups.[33,42]

Adsorption of PEI on the PGA-Glyoxyl Derivatives

Enzyme glyoxyl derivatives (10 IU/g of support) (Scheme 1) were incubated in 20% of PEI (25, 60 and 600 kD) in 25 mM of phosphate buffer pH 7.0. The suspension was gently stirred for 2 h and then washed with 25 mM phosphate buffer pH 7.0. Derivatives were filtered under vacuum in order to remove the buffer except that filling the pores of the derivative.

Co-Immobilization of Enzyme and PEI on Glyoxyl Supports

PGA was immobilized on glyoxyl supports at 25 °C (10 IU/g support).[42] The enzyme was immobilized in less than 30 min, but the reaction was kept running for 3 h and then 20% of a PEI solution (with different molecular weight 25, 60 and 600 kD) was added. After 30 min under gentle stirring at 25 °C, the enzymes derivatives were reduced by adding 1 mg of solid $NaBH_4$ per mL of suspension. After 30 min, the derivatives were filtered and exhaustively washed with distilled water. Immobilized preparations of PGA were stored at 4 °C (see Scheme 1)

Immobilization of Mutant PGA on PEI-Agarose Supports

The immobilization of mutant PGA on PEI-agarose supports was carried out by incubating 1 g of PEI-agarose with 4 mL of enzyme (10 UI) in 5 mM sodium phosphate buffer pH 5.0 plus 150 mM of NaCl at 25 °C. During adsorption, samples of the supernatant and the suspension were withdrawn from the reaction 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 as described above using inert agarose. The PGA activity in the supernatant of this reference reaction was fully preserved in all cases and therefore, the decrease in PGA activity in the supernatant during the immobilization can be directly correlated to the amount of the enzyme adsorbed on the ionic exchangers.

Thermal Stability Assays

To determine the stability of the immobilized enzymes, the derivatives were incubated in 25 mM sodium phosphate buffer pH 7.0 at 65 °C. Samples of the suspension were periodically withdrawn and enzyme activity was analyzed as described above. PGA residual activity was expressed as a percentage of initial activity at the given incubation time.

Stability Assays in the Presence of Cosolvents

To determine the stability of the immobilized enzymes in the presence of a cosolvent, the derivatives were incubated with different percentages of dioxane (v/v) in 25 mM sodium phosphate buffer at the desired pH value 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.

Determination of Primary Amino Groups

The amount of primary amino residues was determined by the *N*-succinimidyl-3-(2-pyridyldithio)propionate assay as previously described.[46] Two hundred milligrams of enzyme-agarose derivative were mixed with 1 mL of 1 mM *N*-succinimidyl-3-(2-pyridyldithio)propionate (SPDP) in 10 mM phosphate buffer pH 8.0 and the mixture was incubated for 1 h at room temperature. Then, samples were reduced with a 50 mM ditiothreitol (DTT) at pH 8.0. Absorbance of the supernatant was measured at 343 nm.

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