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Reactivation of immobilized penicillin G acylase: Influence of cosolvents and catalytic modulators

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ABSTRACT

Reactivation of penicillin G acylase immobilized in glyoxyl-agarose after inactivation was studied with the purpose of increasing the lifespan of the biocatalyst by simple and reproducible strategies, considering unfolding–refolding and direct incubation in reactivation media. Reactivation yields were increased with respect to the control (fully aqueous medium) when cosolvents were added to the reactivation medium at concentrations below 50% (v/v). Best results were obtained with 30% (v/v) ethyleneglycol (EG) in both reactivation strategies. An increase in reactivation yield from 36.0 to 62.8% was obtained using the unfolding–refolding strategy, while an increase from 50.0 to 68.4% was obtained by direct incubation in aqueous media with respect to control. Catalytic modulators were also included in the reactivation medium: competitive inhibitors (phenylacetic acid and 2-thienylacetic acid) caused a reduction while non-competitive (7-ADCA and 6-APA) caused an increase in reactivation yield. Combining cosolvent and catalytic modulators, best results in both strategies were obtained with 30% (v/v) EG plus 100 mM 7-ADCA, where an increase in reactivation yield from 36.0 to 96.0% and from 50.0 to 98.0% was achieved with unfolding–refolding and direct incubation in reactivation media respectively. Apparent reactivation rate was higher in the case of direct incubation in reactivation media, best results being obtained when using 100 mM 7-ADCA and 30% (v/v) EG, with an increase with respect to the control (fully aqueous medium with no modulator) from 0.309 h^{-1} to 1.129 h^{-1}, while for unfolding–refolding strategy increase was only from 0.124 h^{-1} to 0.384 h^{-1}. Results indicate that direct incubation is a better strategy for penicillin G acylase reactivation and opens up the possibility of significantly increasing the operational lifespan of the biocatalyst by operating the reactor with repeated cycles of reaction and reactivation.
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1. Introduction

Enzymes are inherently labile catalysts so that increasing their lifespan under operation conditions is a major challenge and several strategies have been proposed to tackle the problem [1–3]. Among them, immobilization outstands since some of the features leading to inactivation, like aggregation and intermolecular interactions, can be suppressed or mitigated. When enzyme molecules are linked to the support via multipoint covalent attachment, the immobilized biocatalyst presents a higher resistance to any kind of distortion, like those produced by the action of organic solvents or heat [4]. However, even highly stabilized enzyme preparations may become inactivated after prolonged incubation in the presence of organic cosolvents or high temperatures, reducing the operational lifespan of the biocatalyst. The situation will be different depending on the cause of inactivation. When mild conditions are used (i.e. moderate temperatures and pH), primary structure of the enzyme should not be chemically modified. Thus, the only cause for inactivation of immobilized monomeric enzymes should be the conformational changes of their three-dimensional structure leading to an incorrect and less active conformation [5]. If this incorrect conformation can be reversed towards the original form through an appropriate strategy, partial or total recovery of the enzyme activity may be achieved. In this way, enzyme reactivation after partial inactivation appears as an additional and innovative tool that may help to increase the operational lifespan of the biocatalyst [6–8].

Unfolding–refolding and direct incubation in reactivation media are proposed as strategies for enzyme reactivation. Unfolding–refolding has been intensively used in the structural analysis field and applied to functionalize recombinant proteins [9]. Direct incubation in reactivation media is a simple washing process that may be useful as far as the reactivation process does not present kinetic barriers unable to be overcome [10,11]. If intense multipoint covalent attachment exists between the enzyme and

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the support, it is to be expected that the stability and reactivation yield will increase due to the number of the attachment points that serve as a guide towards a proper conformation and therefore to increased activity. It has been reported that the use of cosolvents and catalytic modulators like substrates and products of reaction have a strong effect during the enzyme inactivation process [12,13]; in fact, it has been postulated that any substance that interacts with the enzyme structure is a potential modulator of enzyme stability [14]. Additionally, the effect of different kinds of molecules in the correct refolding of the enzyme structure has been studied with respect to structure–activity relationships, being an important tool for the prediction of biocatalyst behavior [15–19].

Penicillin acylase (penicillin amidohydrolase, E.C. 3.5.1.11) has been chosen as a case study since it is a flexible enzyme able to catalyze several reactions of organic synthesis [20,21], many of them requiring to be conducted in non-aqueous media where stability of the biocatalyst is a major constraint [22–25]. In this work, we report the results of immobilized penicillin acylase reactivation and the effect of cosolvents and catalytic modulators (substrates and products of its hydrolytic activity) on the rate and level of enzyme recovery during the reactivation process.

2. Experimental

2.1. Chemicals and enzymes

Penicillin G acylase (PGA) from *Escherichia coli*, with 360 IU/mL and 25.7 mg/mL protein, was a product of Antibióticos S.A. (León, Spain) kindly donated by Dr. José Manuel Guisán (CSIC Madrid, Spain). The crude enzyme preparation was centrifuged and dialyzed prior to use and remained fully active at 5 °C during the whole working period. Penicillin G potassium salt (PenG-K) was donated by Natsus S.A. (Lima, Perú). 6-Aminopenicillanic acid (6-APA), phenylacetic acid (PAA) and 2-thyenylacetic acid (TA) were from Sigma (St. Louis, MO). Dioxane, EG, PEG and GL were from Merck (Darmstadt, Germany). 7-Amino-3-desacetoxycephalosporanic acid (7-ADCA) was kindly provided by Antibióticos S.A. (León, España). Agarose was from GE Healthcare (Uppsala, Sweden). Glyoxyl agarose was prepared as previously described [26]. All other reagents were of analytical grade.

2.2. Biocatalyst preparation

PGA was immobilized by multipoint covalent attachment to glyoxyl agarose (PGA–GA) as previously described [27].

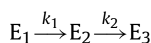
2.3. Determination of enzyme hydrolytic activity

Enzyme hydrolytic activity was determined using a pHstat (Mettler Toledo, DL50) to titrate the H^+ produced by the hydrolysis of 10 mM PenG-K in sodium phosphate buffer 0.1 M, pH 7.8 and 30 °C. 50 mM NaOH was employed as titrant solution. One international unit of hydrolytic activity (IU) of PGA was defined as the amount of enzyme that hydrolyzes 1 μmol of PenG-K per minute under the above conditions. Mass activity of hydrolysis is defined as the IU per unit mass of biocatalyst.

2.4. Inactivation of biocatalysts

Biocatalysts were incubated at 14 °C in 70% (v/v) dioxane in 5 mM phosphate buffer pH 7.4. Periodically, enzyme residual hydrolytic activity was determined until it reached 50% of its initial value. Temperature and apparent pH correspond to optimum values determined for the synthesis of β-lactam antibiotics with PGA [28].

Inactivation by cosolvent was modeled based on the deactivation theory proposed by Henley and Sadana [29]. Inactivation parameters were determined from the best-fit model of the experimental data, which was the one based on two-stage series inactivation mechanism with no residual activity. According to it, biocatalyst inactivation proceeds through two sequential steps leading to progressively less active enzyme species until a final completely inactive species is obtained, as represented in the following scheme:



$$E_1 \xrightarrow{k_1} E_2 \xrightarrow{k_2} E_3$$

where k_1 and k_2 are first-order transition rate constants, E_1, E_2 and E_3 are the corresponding enzyme species. The mathematical model representing this mechanism is:

$$\frac{e}{e_0} = \left[1 + \frac{\alpha k_1}{k_2 - k_1}\right] \exp^{(-k_1 \cdot t)} - \frac{\alpha k_1}{k_2 - k_1} \exp^{(-k_2 \cdot t)} \quad (1)$$

where e represents the activity at any time t, e_0 the initial activity and α the ratio of the specific activities of enzyme species E_2 and native enzyme species E_1.

2.5. Reactivation of biocatalysts

Two strategies were applied for the reactivation of biocatalysts partially inactivated by organic solvent, namely unfolding–refolding and direct incubation in reactivation media. Unfolding–refolding is based on protein refolding after complete unfolding to a random coil structure promoted by a chaotropic agent that removes the kinetic barriers that makes folding a very slow process. Once unfolded, refolding should occur rapidly and completely if the enzyme is incubated in a proper refolding medium. Direct incubation in reactivation media consists in a simple washing process of the enzyme in such reactivation media. If the conformational changes do not present significant kinetic barriers, it would be possible to reactivate the enzyme rather rapidly; otherwise the reactivation process could be too slow [10,11]. These strategies have already been tested for enzyme recovery [7,8,30], but the level of reactivation is well below 100% in the case of PGA so there is ample margin for improvement. The use of additives in the reactivation medium may improve both the level and rate of reactivation.

Biocatalysts were firstly inactivated down to 50% residual activity and then the inactivation medium was removed by filtration in a Gooch crucible fritted Pyrex disc. The recovered biocatalysts were then subjected to reactivation. In the unfolding–refolding strategy, biocatalysts were incubated in 8 M guanidine for 1 h at 25 °C and then the unfolding agent was removed and the biocatalysts incubated at 25 °C in 100 mM sodium phosphate buffer or in mixtures with cosolvent and/or modulators as described ahead. Periodically, its hydrolytic activity was determined as described above. In the strategy of direct incubation in the reactivation media, the recovered biocatalysts were re-incubated maintaining the same mass concentration of biocatalyst as in the inactivation stage; reactivation was conducted at 40 °C using fully aqueous medium (100 mM sodium phosphate buffer 7.4) or mixtures with cosolvent and/or modulators as described ahead. Periodically, the recovered hydrolytic activity was determined as described above.

In this study, the effect of adding different cosolvents (EG, PEG and GL) at varying concentrations (10, 20, 30, 40, 50, 80 and 100%, v/v) was evaluated on the level and rate of reactivation of the biocatalyst. Then, under the best conditions determined in terms of level and rate of reactivation, the effect of adding catalytic modulators to the reactivation media was undertaken. Two non-competitive inhibitors (the β-lactam nuclei 7-ADCA and 6-APA) and two competitive inhibitors (TA and PAA) of PGA were used as modulators

at concentrations of 100 mM, which is at least five times the corresponding values of the respective dissociation constants, ensuring that the modulators saturate their corresponding binding sites. The best cosolvent in terms of reactivation rate and level was selected and its effect, at the optimum concentration determined for each reactivation strategy, was determined in combination with modulators to evaluate the cosolvent–modulator interaction.

Reactivation of the biocatalyst was evaluated in terms of the following parameters: global yield of enzyme recovery (Y_g), reactivation yield (Y_r), and apparent reactivation rate (R_r).

Y_g is defined as the ratio of recovered activity after reactivation (e_f) to the initial activity (before inactivation) (e_0):

$$Y_g = \frac{e_f}{e_0} \quad (2)$$

Y_r is defined as the ratio of the recovered activity to the activity lost during inactivation. Recovered activity is defined as the difference between e_f and the residual activity obtained after the inactivation stage (e_i). Activity lost is defined as the difference between e_0 and final activity obtained after the reactivation stage (e_i):

$$Y_r = \frac{e_f - e_i}{e_0 - e_i} \quad (3)$$

R_r is defined as the ratio of reaction yield to the final reactivation time (t_f), this is, the time at which reactivation levels off:

$$R_r = \frac{(e_f - e_i)/(e_0 - e_i)}{t_f} = \frac{Y_r}{t_f} \quad (4)$$

Each experiment was conducted in triplicate and samples were assayed in duplicate. Differences obtained between experiments and samples, were statistically analyzed. Experimental error was in the range from 0.7% to 5.0%, with a 95% confidence interval.

3. Results and discussion

3.1. Inactivation of biocatalyst in the presence of dioxane

PGA–GA was inactivated in 70% (v/v) dioxane medium at 14 °C until reaching 50% of its initial activity. Time-course of inactivation is shown in Fig. 1. PGA–GA inactivation was well modeled (R^2 = 0.995) by a two-stage series mechanism [29], and the first order transition rate constants of the first and second stage of inactivation were 1.530 ± 0.053 h^{-1} and 0.026 ± 0.001 h^{-1} respectively. The value of α (ratio of specific activities of intermediate and initial enzyme species) was 0.697 ± 0.003. The first stage of inactivation is 58 times faster than the second one, indicating the high stability of the second species with respect to the native one.

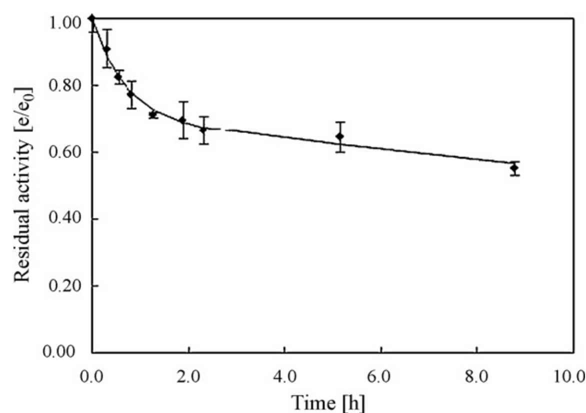


Fig. 1. Inactivation of PGA–GA at 14 °C in 70% (v/v) dioxane in phosphate buffer 5 mM pH 7.4 (■): experimental values; line: model.

3.2. Reactivation of partially inactivated biocatalysts

3.2.1. By unfolding–refolding

Firstly, the effect was studied of adding EG, PEG, and GL in different concentrations to the reactivation media. PGA–GA biocatalysts partially inactivated down to 50% of initial activity at 14 °C in the presence of 70% (v/v) dioxane were reactivated at 25 °C by the unfolding–refolding strategy. Results are summarized in Table 1 in terms of Y_g, Y_r and R_r. As seen, all three parameters increased with cosolvent concentration in the range from 0 to 30% (v/v), but were reduced at concentrations of 50% (v/v) or higher, being its magnitude dependent on the type of cosolvent. Inactivation of biocatalyst was observed at cosolvent concentrations of 80% or over as reflected by the negative values of the parameters Y_r and R_r. Best results were obtained with either PEG or EG at 30% (v/v), differences being not statistically significant between them, according to a Student's t-test performed where t-experimental was 0.967 and P value was 0.387; however, EG was selected for further studies since a sharp decrease in recovered activity was observed for PEG at concentrations higher than 30% (v/v). One possible explanation for the positive effect of polyols at moderate concentrations during the reactivation step is the preferential hydration of the enzyme that increases its chemical potential, mainly due to the strengthening of the interactions of polyols with the non-polar amino acid residues. This has been observed for water–polyol systems in dilute guanidine hydrochloride where the hydrophobic interactions are able to assist a better packing of the core structure, competing with the effect of guanidine hydrochloride under dilute conditions [31]. Nevertheless, at high cosolvent concentrations, the hydration level decreases and the positive effect disappears because these interactions are no longer selective for the non-polar-residues, but involve the whole protein molecule.

The next step was to evaluate the influence of adding different modulators to the reactivation medium in the absence of EG (con-

Table 1
Global yield of enzyme recovery (Y_g), reactivation yield (Y_r) and apparent reactivation rate (R_r) in reactivation of PGA–GA by unfolding–refolding at 25 °C in the presence of cosolvents at different concentrations (C).

C (%, v/v)	Y_g (%)			Y_r (%)			R_r (h^{-1})		
	EG	GL	PEG	EG	GL	PEG	EG	GL	PEG
0	68.0	68.0	68.0	36.0	36.0	36.0	0.124	0.124	0.124
10	74.7	69.0	70.0	49.4	38.0	40.0	0.170	0.131	0.138
20	75.3	70.8	76.8	50.7	41.6	53.6	0.175	0.144	0.185
30	81.4	76.5	80.0	62.8	53.1	60.0	0.217	0.183	0.207
40	71.7	65.0	60.0	43.4	30.0	20.00	0.150	0.103	0.069
50	72.5	63.0	55.0	44.9	26.0	10.0	0.155	0.090	0.034
80	20.0	18.0	15.0	−60.0	−64.0	−70.0	−0.207	−0.221	−0.241
100	15.0	16.0	5.0	−70.0	−68.0	−90.0	−0.241	−0.234	−0.310

Table 2
Effect of modulators on enzyme recovery (Y_g), reactivation yield (Y_r) and apparent reactivation rate (R_r) in reactivation of PGA–GA by unfolding–refolding at 25 °C.

	Ethyleneglycol concentration (%, v/v)					
	Y_g (%)		Y_r (%)		R_r (h^{-1})	
	0	30	0	30	0	30
No modulator	68.0	81.4	36.0	62.8	0.124	0.217
7-ADCA	92.0	98.0	84.0	96.0	0.336	0.384
6-APA	85.0	92.0	70.0	84.0	0.255	0.305
TA	65.0	75.0	30.0	50.0	0.155	0.192
PAA	63.0	73.0	26.0	46.0	0.100	0.177

Table 4
Effect of modulators on enzyme recovery (Y_g), reactivation yield (Y_r) and apparent reactivation rate (R_r) in reactivation of PGA–GA by direct incubation in reactivation medium at 40 °C.

	Ethyleneglycol concentration (%, v/v)					
	Y_g (%)		Y_r (%)		R_r (h^{-1})	
	0	30	0	30	0	30
No modulator	75.0	84.2	50.0	68.4	0.309	0.391
7-ADCA	93.0	99.0	86.0	98.0	0.965	1.129
6-APA	87.0	92.0	74.0	84.0	0.847	0.941
TA	69.0	78.0	38.0	56.0	0.217	0.635
PAA	69.3	75.0	38.5	50.0	0.224	0.833

trol) and in its presence at 30% (v/v). Results are summarized in Table 2. As seen, 6-APA and 7-ADCA had a positive effect on Y_r and R_r both in the presence and absence of EG, while the opposite occurred when TA and PAA were added. Cosolvent addition increased Y_g in all cases, irrespectively of the modulator added. In this case, the negative effect of PAA and TA became less significant with respect to the one of the control in the absence of cosolvent. These results can be explained based on the hypothesis raised by Teipel and Koshland [16,17] according to which when the biocatalyst is being refolded, there are intermediate conformers in a "correct" and "incorrect" form, that are in a dynamic equilibrium; the addition of a modulator to the reactivation medium will induce the formation of an enzyme–modulator complex only with the "correct" intermediate conformer, producing a shift in the equilibrium towards its formation at the expense of the free energy of binding [32]. Possibly this dynamic equilibrium only exists at the early stages of protein folding, when it would be expected that the enzyme is rather mobile. For an immobilized enzyme, this equilibrium also exists, despite of the higher rigidity of the enzyme molecule. Specifically, in the case of immobilization by multipoint covalent attachment it is to be expected that the stability and reactivation yield will increase due to the number of the attachment points acting as reference points that may help in obtaining the correct conformer and facilitate the interaction of the catalytic modulators with either the active or the modulation site. Best results for reactivation by unfolding–refolding were obtained in a reactivation medium with 30% (v/v) EG and 100 mM 7-ADCA where Y_r of 96% and a R_r value of 0.384 h^{-1} were obtained, the latter being 210% higher than the one obtained in the control (fully aqueous medium with no modulator). Time required for unfolding and refolding (about 3 h in total) is long enough to represent a serious drawback of this strategy in terms of reactor productivity.

3.2.2. By direct incubation in reactivation media

Taking into consideration that unfolding–refolding is a rather slow and complex process requiring the use of high concentrations of a chaotropic agent, a simpler strategy was envisaged consisting in direct incubation in reactivation medium after inactivation, where reactivation time may be decreased and the use of high concentrations of chemicals avoided. Results of reactivation of PGA–GA (partially inactivated in the presence of 70%, v/v dioxane at 14 °C) in the presence of EG, PEG, and GL, under this strategy are summarized in Table 3. All cosolvents had a positive effect when used at concentrations below 50% (v/v) as reflected by the increase in the values of Y_g, Y_r and R_r. However, these parameters decreased significantly when increasing the cosolvent concentration to 50% (v/v). R_r was more strongly dependent on cosolvent concentration than on the type of cosolvent, best values being obtained at 30% (v/v) with EG or PEG. At higher cosolvent concentrations, Y_r decreased sharply in the case of PEG and GL but slightly in the case of EG. Based on the above results, EG was selected for further studies, as in the case of the unfolding–refolding strategy. The effect of EG concentration was studied at levels over 50% (v/v). The biocatalyst could still be reactivated at 50% (v/v) EG but at concentrations of 80% (v/v) or higher inactivation ensued during the reactivation stage as reflected by the negative values of Y_r and R_r. Best EG concentration in terms of Y_g, Y_r and R_r was 30% (v/v) and was selected to evaluate the effect of adding catalytic modulators to the reactivation medium.

Finally, the addition of modulators to the reactivation media in the absence and presence of EG at 30% (v/v) was assessed. Results are summarized in Table 4. Non-competitive inhibitor β-lactam nuclei 7-ADCA and 6-APA increased all three reactivation parameters both in the absence and presence of EG. Competitive inhibitors TA and PAA presented the opposite behavior. Cosolvent improved the reactivation parameters for all modulators studied; however, its magnitude was different, being higher for the competitive inhibitors TA and PAA. Increase in R_r was quite significant, the highest value being obtained with 7-ADCA in the presence of EG 30% (v/v), representing an increase of 265% with respect to the control (fully aqueous medium with no modulator). It is remarkable that in the presence of the competitive inhibitors TA and PAA, EG produced an increase in R_r of 192 and 272% respectively, while in the case of the non-competitive inhibitors 7-ADCA and 6-APA the increase was only 17 and 11% respectively.

Table 3
Global yield of enzyme recovery (Y_g), reactivation yield (Y_r) and apparent reactivation rate (R_r) of PGA–GA by direct incubation in reactivation medium at 40 °C in the presence of cosolvents (C) at different concentrations.

C (%, v/v)	Y_g (%)			Y_r (%)			R_r (h^{-1})		
	EG	GL	PEG	EG	GL	PEG	EG	GL	PEG
0	75.0	75.0	75.0	50.0	50.0	50.0	0.309	0.309	0.309
10	77.9	76.6	76.9	55.8	53.2	53.8	0.344	0.304	0.307
20	80.7	nd	nd	61.4	nd	nd	0.351	nd	nd
30	84.2	81.2	83.9	68.4	62.4	67.8	0.391	0.357	0.387
40	72.0	nd	nd	44.0	nd	nd	0.280	nd	nd
50	68.0	67.5	63.4	36.0	35.0	26.8	0.215	0.213	0.167
80	23.6	nd	nd	−52.8	nd	nd	−0.298	nd	nd
100	17.6	nd	nd	−64.8	nd	nd	−0.411	nd	nd

nd: not determined.

3.3. Comparison between unfolding–refolding and direct incubation in reactivation media

Unfolding–refolding strategy presents several drawbacks, related to the use of a chaotropic agent and the values of R_r obtained, in spite of the fact that Y_g was similar for both strategies. R_r was smaller in unfolding–refolding strategy than in direct incubation in reactivation media, which can be explained because of the high protein loads of the derivatives used, which may promote protein–protein interactions during the unfolding step due to the great increase in molecular volume of the enzyme [10]. Besides, it should be noted that these interactions do not correspond to intermolecular aggregation, since immobilized derivatives are used.

Influence of cosolvents and catalytic modulators followed the same pattern in terms of Y_r for both strategies. Nevertheless, main differences were related to R_r: while in unfolding–refolding strategy one additional hour is required for the unfolding step (which has to be considered in R_r calculation), in direct incubation in reactivation media only the reactivation time has to be considered. As a consequence, R_r was nearly one third than for direct incubation in reactivation media at all conditions tested.

It should be noted that in the case of direct incubation in reactivation media the effect of cosolvent and catalytic modulators was more pronounced. At the best combination (30%, v/v EG plus 7-ADCA), an increase in R_r of 265% with respect to the control was obtained, while only 210% increase was obtained in the unfolding–refolding strategy.

For both strategies the β-lactam nuclei 6-APA and 7-ADCA (noncompetitive inhibitors) increased the values of the reactivation parameters with respect to the control (no cosolvent, no modulator), while the opposite was observed with TA and PAA (competitive inhibitors), in spite of the fact that all modulator molecules are small enough and, according to the theory raised by Teipel and Koshland [16,17], do not need a well conformed active site to interact with it. This may be explained by considering that 7-ADCA and 6-APA are bound to the enzyme at a non-catalytic site, while PAA and TA bind to the active site. This difference is significant because of the hydrophobic nature of the active site and its surroundings. It is well known that there are several non-polar amino acids that play an important role during penicillin G hydrolysis (α Phe-146; β Ser-1; β Phe 24; β Tyr-31; β Ala-69; β Phe-71; β Ala-241), but modulators interact via different mechanisms and non-polar interactions within the molecule differ significantly in the case of competitive inhibitors, noncompetitive inhibitors and the substrate penicillin G. It has been reported that the crystal structure of penicillin G acylase from *E. coli* presents significant differences when the enzyme is complexed with different molecules in the active site, suggesting that there are certain amino acid residues that may adopt distinct energetically favorable positions in order to accommodate a variety of compounds [33–35]. Structural analysis was done under nondynamic conditions; data were obtained from the crystal structures of PGA available in Protein Data Bank (1FXV; 1FXH; 1AJQ and 1GK9) and manual docking was done to obtain the structure of PGA complexed with the β-lactam nucleus 6-APA. When competitive inhibitors are present, hydrophobic interactions with amino acids located in the active site (β Phe-24; β Ser-67; β Tre-68; β Ile-177; β Ala-241) are enhanced in number and length (becoming shorter) as expected, while the opposite occurs with the amino acids associated to the non-catalytic binding site. On the other hand, when β-lactam nuclei are added interactions with the active site differ from those obtained in the presence of TA and PAA as expected, with the consequent modification of the behavior displayed by the enzyme after its reactivation. Furthermore, both 6-APA and penicillin G are able to interact with the amino acids at the noncatalytic binding site. In this case, the coordination of 6-APA and penicillin G with the enzyme reveals the displacement of α Phe-146 from the “closed” to “open” configuration, with a consequent displacement of the contiguous amino acid α Arg-145, which has been attributed a crucial role in specificity and stereoselectivity of PGA [35]. This is not the case with PAA and TA that cannot interact with the non-catalytic binding-site, which is a sound explanation for the differences observed in terms of enzyme activity expression.

4. Conclusions

Two reactivation strategies, namely refolding–unfolding and direct incubation in reactivation medium considering cosolvents and catalytic modulators have been developed that allowed improving the reactivation parameters of PGA–GA biocatalysts. Though results obtained in terms of Y_r did not differ significantly between both strategies, values were slightly higher in the latter. Besides, R_r values for direct incubation in reactivation medium were higher and the system is simpler not involving the additional step of unfolding nor the use of high concentrations of chaotropic agents.

Competitive inhibitors caused a reduction in reactivation parameters while the non-competitive inhibitor β-lactam nuclei increased them. The highest value of Y_r was obtained when 7-ADCA 100 mM and 30% (v/v) of EG were added to either the refolding or the reactivation media, where 98% and 99% respectively, of the enzyme activity lost could be recovered. On the other hand, R_r values were strongly dependent on the reactivation strategy used, being at least three times higher in the case of direct incubation in reactivation media. This strategy of enzyme reactivation by medium engineering is very promising, results suggesting that a large number of inactivation–reactivation cycles can be performed in the synthesis of β-lactam antibiotics with PGA before biocatalyst replacement. The use of this reactivation strategy will have a direct impact on the specific productivity of synthesis. This study is underway and the strategy is being applied to other enzyme biocatalysts.

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