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Reactivation of penicillin acylase biocatalysts: Effect of the intensity of enzyme–support attachment and enzyme load

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ABSTRACT

The influence of the intensity of enzyme–support attachment and enzyme load on the reactivation process has been studied considering three immobilized penicillin G acylase (PGA) biocatalysts: one immobilized by covalent attachment to cyanogen bromide Sepharose (CNBrSep-PGA) and two immobilized by multipoint covalent attachment to glyoxyl agarose with different intensity of attachment (Gx_1-PGA and Gx_2-PGA). The biocatalysts were inactivated in 70% (v/v) dioxane at 5 °C down to 25% residual activity. Biocatalysts were then recovered by filtration to remove the inactivation medium and re-incubated in aqueous medium to promote reactivation. The highest level of reactivation (73% recovery of activity with respect to the initial activity before inactivation) was obtained with Gx_1-PGA, corresponding to the biocatalyst with stronger multipoint interactions. For Gx_2-PGA and CNBrSep-PGA, only 33% and 31% of activity was recovered respectively. When the distortion caused by the organic solvent produces a inactive enzyme structure that cannot be significantly recovered by re-incubation in aqueous medium, complete unfolding of the enzyme molecule by chaotropic agents prior to its refolding by incubation in aqueous medium can be used to recover enzyme activity. When this strategy was used, 70% activity was recovered with Gx_1-PGA and Gx_2-PGA, but weakly linked CNBrSep-PGA was completely inactivated after unfolding and no activity was recovered by re-incubation in aqueous medium. With respect to enzyme load, full recovery of enzyme activity was obtained with Gx_1-PGA by reactivation in aqueous medium up to 74 IU/$g_{support}$. However, at higher enzyme loads recovery was significantly impaired because of intense protein–protein interaction during biocatalyst inactivation and reactivation.

1. Introduction

Enzyme performance in non-conventional media, particularly in organic solvents, has broadened its field of application considerably in recent years [1]. However, enzymes tend to be poorly stable in such media [2–5] and, among the many strategies to improve stability, enzyme immobilization outstands from a process perspective, favoring not only catalyst recovery but significantly increasing its lifespan [6,7]. This has profound technological impact because of the improvement of the efficiency of biocatalyst use leading to a dramatic decrease in the impact of the biocatalyst cost in process operation cost. Many studies of immobilization with different enzymes have been reported with such purpose [8–10]. However, despite increasing stability, enzyme inactivation is still present because of the stringent conditions usually required to perform reactions of synthesis. Therefore, enzyme biocatalysts have to be replaced frequently.

Multipoint covalent enzyme immobilization may strongly improve enzyme stability, activity and selectivity [11]. In fact, many industrial enzymes have been highly stabilized via multipoint covalent immobilization, including penicillin G acylase. A direct correlation between the number of enzyme–support links and enzyme stability has been sustained and the intensity of enzyme–support attachment can be easily modulated by changing experimental conditions like time and temperature of immobilization [12]. Also, previous reports suggest that when the enzyme is linked to a support by multi-point covalent attachment, inactivation by organic solvents at moderate temperatures can only occur by conformational changes promoted by the solvent that do not involve major chemical changes in the enzyme molecule; therefore, inactivation should be a completely or partially reversible phenomenon [13,14].

Enzyme reactivation after use, though being a powerful approach for increasing biocatalyst efficiency, has been seldom considered from a technological perspective [13,15–18]. Two main strategies have been proposed: washing and re-incubation in aqueous medium [14,16] and unfolding–refolding, in which the enzyme to be recovered is completely unfolded prior to

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incubation in aqueous medium [13,15,16]. Penicillin G acylase from *Escherichia coli*, α-chymotrypsin from bovine pancreas Type II, and lipase from *Thermomyces lanuginosus*, and glutamate dehydrogenase from *Thermus thermophillus* have been successfully reactivated after inactivation by organic solvents, recovering a substantial fraction of its original activity [16,18–20]. Reactivation is then worthwhile to be further explored to conceive new strategies aiming to the improvement of biocatalyst efficiency.

In the present work we studied the influence of the intensity of enzyme–support attachment and the enzyme load on the reactivation process. The underlying hypothesis is that increasing the number of enzyme–support linkages contributes to build-up a better scaffold for the correct refolding of the enzyme. It has been proposed that the enzyme, when unfolded, increases its molar volume favoring protein–protein interactions among neighbor enzyme molecules [16], which may affect the reactivation process. Reactivation then stems out a as a strategy to be considered in the toolbox for improving enzyme performance under process conditions.

Penicillin acylase (EC 3.5.1.11) is used as a model for a heterodimeric enzyme [21] that, beyond its use as a catalyst for the synthesis of β-lactam antibiotics, has been profusely used in important reactions of organic synthesis, like the resolution of racemic mixtures of alcohols, esters and amines by catalyzing enantioselective reactions of hydrolysis and synthesis [22–26], the non-specific acylation of nucleophiles [27,28] and the introduction of protecting groups in the synthesis of peptides [29,30]. Many such reactions need to be conducted in non-conventional media where the enzyme is exposed to harsh conditions, threatening its stability.

2. Materials and methods

2.1. Materials

Penicillin G acylase (PGA) from *E. coli*, with 220 ± 20 IU_H/mL and 21 ± 2 mg/mL protein, was kindly provided by Antibióticos S.A. (León, Spain). The enzyme was centrifuged at $12{,}000 \times g$ at 4 °C for 10 min and then dialyzed overnight at 4 °C against 5 mM phosphate buffer (5 L per 10 mL of sample) prior to use and remained fully active at 5 °C during the whole working period. Phenyl acetic acid (PAA) was from Sigma (St Louis, MO, USA) and dioxane was from Merck (Darmstadt, Germany). Penicillin G potassium salt (PenGK) was donated by Natsus S.A. (Lima, Perú). Cyanogen bromide Sepharose (CNBrSep) and cross-linked agarose beads were from GE Healthcare (Uppsala, Sweden). Glyoxyl agarose (Gx) was prepared as previously described [31]. All other reagents were of analytical grade.

2.2. Preparation of biocatalysts with different intensity of attachment

Three penicillin G acylase (PGA) biocatalysts were prepared: CNBrSep-PGA (cyanogen bromide Sepharose) and Gx_1-PGA and Gx_2-PGA (multipoint covalent attachment to glyoxyl agarose with two attachment intensities obtained by immobilization at two different times).

CNBrSep-PGA biocatalyst was produced according to the protocol from GE Healthcare. Immobilization of enzymes using CNBrSep was conducted at low temperature and for a few minutes to avoid any significant effect on the stability of the enzyme. CNBrSep (1 g) was added to 10 mL of 25 mM sodium phosphate containing PGA and gently stirred for 10 min, at 4 °C and pH 7.0. After this time, the support was filtered and washed with 100 mM sodium bicarbonate buffer at pH 8.3 and incubated in 100 mM Tris–HCl at pH 8 for 2 h to block the remaining CNBr groups. Finally, the immobilized preparations were washed with distilled water.

Gx_1-PGA and Gx_2-PGA biocatalysts were prepared as previously described [31,32], but varying the temperature and the enzyme–support contact time to achieve different intensity of enzyme–support attachment, as previously reported [12]. Immobilization was carried out by adding 1 g of fully activated support to 10 mL of 25 mM sodium bicarbonate buffer containing the enzyme at 25 °C and pH 10 and submitting the mixture to gentle stirring for three hours. Gx_1-PGA was produced under such conditions to promote a major level of enzyme–support attachment. To prepare Gx_2-PGA, the immobilization was carried out at 4 °C during only 50 min, obtaining a lower intensity of enzyme–support attachment. To stop the reaction of attachment, solid sodium borohydride was added to a final concentration of 1 mg/mL. After 30 min, the preparation was washed with an excess of distilled water. During immobilization, samples of supernatant, suspension and enzyme solution incubated in the presence of the inert support were taken at different times, and the activity and/or the protein concentration were assayed.

2.3. Preparation of biocatalysts with different protein loads

Multipoint attached biocatalyst Gx_1-PGA with different protein loads (2.4–35.4 mg protein/g support) was prepared adding 1 g of fully activated support to 10 mL of 25 mM sodium bicarbonate buffer containing the different concentrations at 25 °C and pH 10 and submitting the mixture to gentle stirring for three hours. The time and temperature of immobilization were the same for all biocatalysts in order to have equivalent levels of enzyme–support interaction, but different protein loads [9]. To stop the reaction of attachment, solid sodium borohydride was added to a final concentration of 1 mg/mL. After 30 min, the preparation was washed with an excess of distilled water. During immobilization, samples of supernatant, suspension and enzyme solution incubated in the presence of the inert support were taken at different times, and the activity and/or the protein concentration were assayed. With these values the amount of immobilized protein and enzyme activity was determined for each biocatalyst.

2.4. 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 at 30 °C. 50 mM NaOH was employed as titrant solution. One international unit of hydrolytic activity (IU_H) 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_H per unit mass of biocatalyst.

2.5. Inactivation of biocatalysts

All biocatalysts were incubated at 5 °C in 70% (v/v) dioxane in 10 mM phosphate buffer pH 7.4. Periodically, biocatalyst samples were taken and their residual PGA hydrolytic activity determined as described above.

Inactivation by cosolvent was modeled based on the deactivation theory proposed by Sadana and Henley [33]. 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 of 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 rates constants, and E_1, E_2 and E_3 are the corresponding enzyme species. The mathematical model representing this mechanism is:

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

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

Half-life (time at which the residual enzyme activity is half of its initial value; $t_{1/2}$) was used to compare the stability of the different biocatalysts, being determined by interpolation using the model represented in Eq. (1).

2.6. Reactivation of biocatalysts

Two strategies were applied for the reactivation of biocatalysts partially inactivated by organic solvent, namely, direct incubation in reactivation aqueous media and unfolding–refolding [14,16,18,20,34].

Biocatalysts were inactivated down to 25% residual activity and then the inactivation medium was removed, biocatalysts being recovered by filtration in a Gooch crucible fritted disc (porosity 10–15 μm). In the strategy of direct incubation in aqueous reactivation media, the recovered biocatalyst was re-incubated in 0.1 M sodium phosphate buffer pH 7.4 at 25 °C to promote reactivation. Volume of re-incubation was the same as the one in the inactivation stage. Periodically, the recovered hydrolytic activity was determined as described above. In the unfolding–refolding strategy, biocatalysts were incubated in 8 M guanidine for 1 h at 25 °C; then, the unfolding agent was removed by filtering it out and the biocatalysts were incubated in phosphate buffer as above. Periodically, its hydrolytic activity was determined as described before; in this case, reactivation rate is very high, reaching the final reactivated state in a few minutes.

Reactivation was modeled by making an extension of the deactivation theory [33]. The mechanism considered is a two step mechanism, where the partially inactivated specie E_i converts through two sequential steps to more active enzyme species, as represented in the following scheme:

$$E_i \xrightarrow{k_{r1}} E_{r1} \xrightarrow{k_{r2}} E_{r2}$$

where k_{r1} and k_{r2} are first-order transition rates constants of reactivation, E_i, E_{r1} and E_{r2} are the corresponding enzyme species before and during the period of reactivation in which the specific activity gradually increases up to the final stage of reactivation. The mathematical model representing this mechanism is:

$$\frac{e}{e_0} = \left((\phi - \eta) + (\gamma - \eta) \cdot \left(\frac{k_{r1}}{k_{r2} - k_{r1}}\right)\right) \exp(-k_{r1} \cdot t) - \left((\gamma - \eta) \cdot \left(\frac{k_{r1}}{k_{r2} - k_{r1}}\right) \exp(-k_{r2} \cdot t)\right) + \eta \quad (2)$$

where e is the activity recovered at time t, e_0 is the initial activity (before inactivation), ϕ, γ and η are the ratio of the specific activities of the enzyme species E_i, E_{r1} and E_{r2} with respect of that of the native enzyme species E_1 (before inactivation) respectively.

Two relevant parameters to evaluate the process are the reactivation capacity (ΔR) and recovered activity. ΔR is defined as the difference between the maximum activity achieved in the reactivation stage and the activity of the partially inactivated biocatalyst prior to reactivation:

$$\Delta R = (\eta - \phi) \quad (3)$$

Recovered activity is the percentage of the activity of the native enzyme (before inactivation) that is recovered after reactivation. Each experiment was conducted in triplicate and samples were assayed in duplicate. Differences obtained between experiments and samples, were statistically analyzed, experimental error being always below 5%.

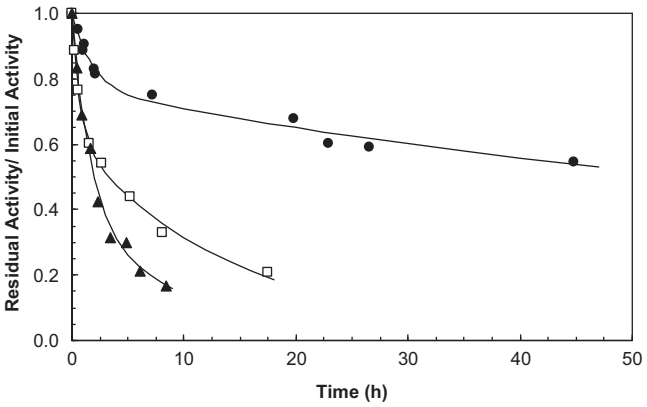

Fig. 1. Stability of biocatalyst in the presence of 70% (v/v) dioxane and 30% (v/v) phosphate buffer 5 mM pH 7.4 at 5 °C. CNBrSep-PGA (▲), Gx_1-PGA (●) and Gx_2-PGA (□).

Table 1
Inactivation parameters of different biocatalysts of PGA inactivated in the presence of 70% (v/v) dioxane in 30%(v/v) 5 mM phosphate buffer pH 7.4, at 5 °C.

Biocatalysts	α	k_1 (h^{-1})	k_2 (h^{-1})	R^2	$t_{1/2}$ (h)
Gx_1-PGA	0.76	0.57	0.014	0.994	56.2
Gx_2-PGA	0.59	1.43	0.067	0.997	3.3
CNBrSep-PGA	0.32	0.62	0.101	0.995	2.0

R: correlation coefficient.

2.7. SDS-PAGE analysis

SDS-PAGE was performed as previously described [35] in a SE 250-Mighty Small II electrophoretic unit (Hoefer Co.) using gels 12% polyacrylamide in a separation zone of 9 cm × 6 cm and a concentration zone of 5% polyacrylamide. 100 mg of biocatalyst was dissolved in 150 μl of SDS-loading buffer and boiled for 10 min. Then, supernatant was injected on the gel. The gels were stained following the Coomassie method [36]. Molecular weight markers were the LMW kit (14,400–94,000 Da) from GE Healthcare.

3. Results and discussion

3.1. Stability of biocatalysts in the presence of dioxane

CNBrSep-PGA, Gx_1-PGA and Gx_2-PGA were inactivated in 70% (v/v) dioxane in 10 mM phosphate buffer pH 7.4 at 5 °C, to evaluate their stability under such conditions. Time course of inactivation is presented in Fig. 1, revealing significant differences among them, being Gx_1-PGA the most stable and CNBrSep-PGA the most unstable, which is a reflection of the different intensities of enzyme–support attachment among those biocatalysts [12].

Inactivation was modeled according to Eq. (1) and the values of the corresponding parameters are in Table 1. In the case of CNBrSep-PGA, the enzyme is weakly linked and upon incubation

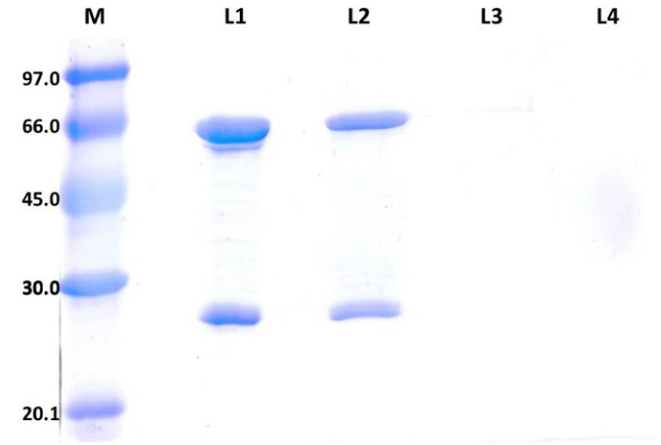

Fig. 2. SDS-PAGE of PGA biocatalysts. M: molecular weight marker. Lane 1: soluble PGA. Lane 2: eluate from CNBrSep-PGA. Lane 3: eluate from Gx_2-PGA. Lane 4: eluate from Gx_1-PGA.

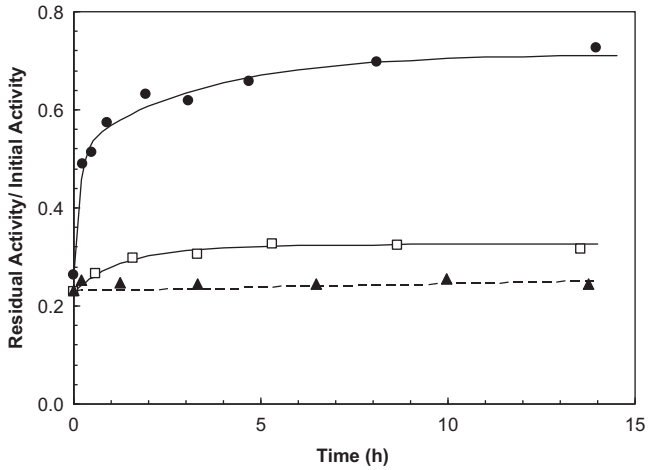

Fig. 3. Reactivation profiles of inactivated PGA by incubation in 100 mM phosphate buffer pH 7.4 at 40 °C: CNBrSep-PGA (▲); Gx_1-PGA (●); Gx_2-PGA (□).

in dioxane, subunit dissociation is likely to occur leading to rapid inactivation [37], which may also be caused by significant structural changes produced as a consequence of the low rigidity of the enzyme molecules in such support. In the case of Gx_1-PGA and Gx_2-PGA, multi-point covalent attachment, besides preventing subunit dissociation, produces a significant structural stabilization by increasing the rigidity of the enzyme molecules so reducing the conformational changes promoted by the cosolvent. In order to check that subunit dissociation occurs only in the case of CNBrSep-PGA, SDS-PAGE was performed to the three biocatalysts, as shown in Fig. 2. As seen, subunit dissociation is observed only in the case of CNBrSep-PGA as reflected by the appearance of two bands corresponding to both enzyme subunits. Such bands are not observed in the other two biocatalysts, which is in agreement with results previously reported showing that glyoxyl agarose support stabilizes the quaternary structure of the enzyme, precluding subunit dissociation [11].

Results on enzyme stability are in agreement with previous studies where a direct correlation was found between the number of enzyme–support linkages and the stability of the biocatalyst [12,38].

3.2. Reactivation of partially inactivated biocatalysts

Biocatalysts were inactivated in the presence of 70% (v/v) dioxane down to approximately 25% residual activity, to be then subjected to reactivation either by direct incubation in aqueous medium or by unfolding–refolding.

3.2.1. Reactivation of partially inactivated biocatalysts by incubation in fully aqueous medium

Results of reactivation of partially inactivated biocatalysts by direct incubation in aqueous medium are presented in Fig. 3, where significant differences can be observed among the three biocatalysts. In the case of CNBrSep-PGA, no reactivation occurred, which might well be the consequence of the irreversible dissociation of the subunits of PGA during inactivation in the presence of high dioxane concentration (Fig. 2). Recovery of activity of enzymes weakly linked to the support has only been reported for monomeric proteins like some lipases [14,20], trypsin and chymotrypsin [15], but not for multimeric enzymes like PGA.

Values of reactivation parameters for Gx_1-PGA and Gx_2-PGA, are presented in Table 2. In the case of Gx_2-PGA, as a consequence of the reduced number of enzyme–support linkages, the enzyme structure is not rigid enough to reverse the conformational changes produced during inactivation. In the case of Gx_1-PGA, bearing a higher number of enzyme–support linkages, the enzyme structure is sufficiently robust to withstand the harsh conditions during inactivation without significant conformational changes, being then prone to reactivation.

Table 3
Unfolding–refolding of partially inactivated biocatalysts. Unfolding agent was 8 M guanidine at 25 °C and refolding process was conducted in 100 mM phosphate buffer pH 7.4, at 40 °C.

Biocatalysts	Recovered activity (%)	ΔR
Gx_1-PGA	76 ± 1	47
Gx_2-PGA	67 ± 5	44
CNBrSepPGA	2.7 ± 0	−20

3.2.2. Reactivation of partially inactivated biocatalysts by unfolding–refolding

Results of reactivation of partially inactivated biocatalysts by unfolding–refolding are presented in Table 3 in terms of % of recovered activity. In this case, it was not possible to follow the course of reactivation since its rate was very high.

CNBrSep-PGA was not reactivated at all; even a negative value of ΔR was obtained, indicating that after inactivation by dioxane the enzyme kept on losing activity during "reactivation", which might be the consequence of subunit dissociation during unfolding in the presence of guanidine. These results suggest that a fully stabilized quaternary structure of the enzyme is needed to keep enzyme subunits linked to the matrix during unfolding. Values of ΔR are quite similar for Gx_1-PGA and Gx_2-PGA, indicating that the intensity of

Table 2
Reactivation parameters of different biocatalysts of PGA reincubated in 100 mM phosphate buffer pH 7.4, at 40 °C.

Biocatalyts	γ	η	k_{r1} (h^{-1})	k_{r2} (h^{-1})	R^2	Recovered activity (%)	ΔR
Gx_1-PGA	0.54	0.73	5.55	0.216	0.984	73 ± 1	45
Gx_2-PGA	0.33	0.36	0.75	0.010	0.981	32 ± 2	10
CNBrSep-PGA	–	–	–	–	–	25 ± 1	–

R: correlation coefficient.

Table 4
Effect of enzyme load on Gx_1-PGA reactivation by unfolding–refolding.

Enzyme loading (mg prot/g support)	Activity ($IU/g_{support}$)	Recovered activity (%)
35.4	372	75 ± 4
16.6	182	74 ± 2
10.7	103	72 ± 3
7.7	74	97 ± 4
5.6	51	98 ± 6
2.4	25	102 ± 7

multi-point covalent attachment of the enzyme to the support has no significant effect on refolding, since in these biocatalysts both subunits remained attached to the support, which is not the case with CNBrSep-PGA. Most probably, incubation in the presence of guanidine destroyed the non-native conformations promoted by the solvent during inactivation [39]. Enzyme conformation on both biocatalysts will be essentially the same after unfolding, no matter how many enzyme–support linkages they have, so virtually erasing its previous condition.

Weakly linked CNBrSep-PGA was not prone to reactivation in any of the strategies, reflecting that at least one linkage is required in each subunit for enzyme reactivation, which might not occur in an essentially single-point attachment. In the case of multi-point covalent attachment, the presence of multiple linkages stabilizes the quaternary structure by reducing the structural changes promoted by the organic solvent and, on the other hand, favors reactivation by offering a more robust scaffold for refolding.

3.3. Effect of enzyme load

The effect of enzyme load on Gx_1-PGA reactivation by unfolding–refolding was studied with biocatalysts prepared at different protein loads. Unfolding–refolding was selected as reactivation strategy in this case, because drastic conditions during unfolding allows one to better appreciate if interaction among neighbor protein molecules exists when the enzyme is inactivated. Results in Table 4 show that complete recovery of enzyme activity was only possible up to 7.7 mg protein/g support. At higher loads recovery dropped down to about 75%.

A tentative explanation is that no protein–protein interactions occur at low enzyme loads, since enzyme molecules are sufficiently far apart on the surface of the support, being then attainable a complete recovery of enzyme activity. However, the low specific activity of the biocatalyst precludes its use as a process biocatalyst. Interestingly, at enzyme loads over 10 mg protein/g support, the recovered activity remains almost constant up to protein loads in the order of 35 mg protein/g support. Even at such loads, enzyme recovery is quite significant, which highlights the potential of this strategy for increasing enzyme efficiency in a given production process.

4. Conclusions

The increase in the intensity of covalent interaction between the enzyme and the support, not only increased enzyme stability, but also the level of reactivation attainable by strategies in which enzyme refolding towards its native configuration is promoted. There is a clear correlation between the intensity of enzyme–support attachment in the biocatalyst and its propensity to reactivation.

Biocatalysts weakly immobilized to the support, as CNBrSep-PGA, could not be reactivated to any extent whatever the reactivation strategy used. In the case of biocatalysts immobilized by multi-point covalent attachment (Gx_1-PGA and Gx_2-PGA) there were significant differences between them in the level of reactivation according to the strategy employed. These differences were quite significant when reactivation was promoted by direct incubation in aqueous medium, but were reduced when using unfolding–refolding, since in that case the enzyme was exposed to a strong reagent which led to a completely unfolded configuration that erased the previous structural condition of the enzyme after inactivation.

Reactivation was affected by the protein load of the support and complete recovery of enzyme activity was only possible at low protein loadings. Protein–protein interaction, exacerbated at high loads, is likely to be the cause of only partial reactivation. Even so, 75% reactivation was obtained at 35 mg protein/g support, which is certainly significant in terms of increasing biocatalyst efficiency.

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