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Preparation of artificial hyper-hydrophilic micro-environments (polymeric salts) surrounding enzyme molecules New enzyme derivatives to be used in any reaction medium

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Abstract

Although enzymes usually undergo rapid inactivations in the presence of organic media, the mechanism of these inactivations is often quite simple. An immobilized enzyme, fully dispersed inside porous supports, incubated in the presence of medium–high concentrations of water-miscible organic cosolvents under mild conditions, is mainly inactivated by the interaction of the enzyme with cosolvent molecules. Thus, the only inactivating effect is the promotion of conformational changes on enzyme structure.

In this paper, we propose an optimized strategy to stabilize immobilized enzymes against the presence of organic solvent: the generation of a hyper-hydrophilic shell surrounding each individual protein molecule by using several layers of different polymers. We have optimized different variables, such as the size of the polymers, the number of polymer layers, the correct assembly of the hydrophilization protocol, etc.

After building a shell formed by different layers of polyethylenimine and dextran aldehyde, the addition of dextran sulfate promoted a qualitative increase in the enzyme stability.

As an example, penicillin G acylase (PGA) has been immobilized-stabilized on Sepabeads (a rigid support that does not swell when changed from aqueous to anhydrous media), and the protocol to hydrophilize the protein nano-environment has been applied. This protocol originates derivatives able to stand even 90% of dioxane without significant losses of activity after several days, while conventional derivatives were readily inactivated under these conditions.

Keywords: Enzymes in organic solvents; Stabilization of enzymes; Hydrophilization of enzyme environments; Polymeric salts

1. Introduction

The use of organic solvents and cosolvents may greatly improve the performance of many interesting enzyme biotransformations. In this way, the use of water–cosolvent mixtures as well as of anhydrous media may permit to dissolve hydrophobic compounds, to shift thermodynamical equilibria towards synthesis [1,2], etc. However, enzyme molecules are usually very unstable under such experimental conditions. This lack of stability strongly limits the industrial implementation of such

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interesting biotransformations in non-conventional media [3,4].

In fact, an immobilized enzyme (therefore fully dispersed and immobilized inside a porous support) usually undergoes very rapid inactivation when it is exposed to the presence of medium–high concentrations of water-soluble organic solvents or organic solvents in anhydrous systems, even at neutral pH values and moderate temperatures. However, the exact mechanism of these dramatic inactivations should be quite simple. Under these mild conditions, immobilized enzymes hardly undergo chemical modifications (the cosolvents are chemically inert and pH and temperature are very mild) and aggregation is prevented by the dispersion on the support surface. For all these reasons, we can assume that, under these conditions, the unique cause of these dramatic inactivations of enzyme molecules is their interaction with organic cosolvents and the main inactivating effect is the promotion of conformational changes on the enzyme structure.

On the basis of this simple mechanism of dramatic inactivation, two clear strategies for enzyme stabilization against organic cosolvents can be proposed:

(a) *The prevention of the inactivation cause*: promotion of a drastic diminution of the cosolvent concentration in the immediate surroundings of the enzyme molecules.
(b) *The prevention of inactivating effects*: a drastic "rigidification" of enzyme molecules in order to reduce the intensity of conformational changes induced by cosolvents.

In this paper, we have focused in the first phenomenon: when an enzyme has to work in the presence of medium–high concentrations of miscible organic cosolvents (e.g. 50–90%) in the bulk solution, its effective stability may be greatly increased if we are able to greatly reduce the actual concentration of cosolvent existing in the micro-environment of the enzyme molecule (e.g. 15–30%) [5]. This goal could be achieved via generation of an hydrophilic shell surrounding every enzyme molecule. Obviously, this hydrophilic shell can not be constituted by a very close structure not allowing the transfer of substrates and products from/to the bulk solution to/from the enzyme active center. On the contrary, the hydrophilic shell must have a very open structure and the only limitation to the access of cosolvent molecules has to be achieved through "partition effects".

In a previous paper, by using poliethylenimine plus aldehyde dextran, we have been able to significantly improve the stability against the effects of organic cosolvents of the enzyme penicillin G acylase (PGA) immobilized-stabilized in glyoxyl agarose.

Now, we have considered the optimization of the system.

First, we have used a new support, Sepabeads (an epoxy-acrylic resin from Resindion srl), that due to its production process have some advantages, one of the most significant being that it is so rigid that it did not swell or change its morphology when incubated in water or organic media. This support may be used in a stirred tank or in a column with very good performance.

Secondly, we have optimized the preparation of the hydrophilic micro-environment. In order to get such very strong partition effects between cosolvents and the enzyme environment, the open hydrophilic shell surrounding every enzyme molecule has to be an extremely hydrophilic one. To achieve this objective, the use of some "random coil" polyfunctional polymers is here proposed. The following polymers were utilized:

(a) *Polyethylenimine* is commercially available and there are preparations with very different MWs. This polymer contains a very high concentration of primary, secondary and tertiary amino groups. Most of them are ionized at neutral pH value and hence this polymer is able to become adsorbed on proteins and other polyanionic polymers. In addition to that, the presence of non-ionized primary amino groups also allows the covalent attachment of this polymer on aldehyde supports and aldehyde polymers.
(b) *Aldehyde dextran* can be obtained via periodate oxidation of commercial dextrans with different MW [6]. These polyfunctional polymers may covalently react with primary amino groups placed in enzymes, support or other polymers (e.g. PEI).
(c) *Dextran sulfate* is also commercially available in different MW preparations. This is a very hydrophilic polymer able to become adsorbed on polyethylenimine with formation of polymeric salts (very similar to a polymeric ammonium sulfate salt).

By using different combinations of these polymers, we have optimized the preparation of artificial hyper-hydrophilic micro-environments formed by polymeric salts and completely surrounding multipoint covalent immobilized ones.

As a model enzyme, we have chosen penicillin G acylase from *Escherichia coli*. The use of PGA in organic media has the following very interesting industrial prospects.

(a) The hydrolysis of non-crystallized penicillin G in the presence of water saturated with immiscible organic solvents.
(b) The synthesis of semi-synthetic antibiotics by direct condensation of non-activated side chains with antibiotic nuclei in water–cosolvents mixtures [5,7–10].
(c) The enantioselective synthesis of chiral amides by condensation of phenyl acetic acid and analogues with chiral amines in water–cosolvent mixtures as well as in anhydrous media [11–13].

2. Materials and methods

2.1. Materials

Penicillin G acylase (10 IU/mg determined as described below) was kindly donated by Antibioticos S.A. (Leon, Spain). All other reagents were of analytical grade. Aldehyde dextrans (MW 6000 Da) were obtained by total oxidation of dextrans (34 mg/ml) with sodium periodate as previously reported [6]. Polyethyleneimine was from Sigma. Penicillin G acylase (300 IU/ml of packed support) was immobilized in Sepabeads following a protocol described elsewhere [14], keeping a final 70% activity. Poorly activated glutaraldehyde support (5 μmol/ml) was prepared as previously described [15] but using Sepabeads modified with 5 μmol/ml with ethylendiamine [16]. A total of 7 g of wet (vacuum dried) Sepabeads corresponds to a packed volume of 10 ml.

2.2. Hydrophilization of multipoint covalently immobilized derivatives

The protocol to fully hydrophilize the micro-environment of the immobilized enzyme has been optimized considering the size of the co-immobilized PEI, the number of layers, the beneficial effects of different dextran sulfate polymers, etc.

2.2.1. Co-immobilization of polyethylenimine and the enzyme

The immobilization of the enzyme and the polyethylenimine molecules was accomplished in two steps:

(a) the preliminary immobilization via multipoint covalent attachment of the enzyme on Sepabeads epoxyde as previously described [14];
(b) the subsequent immobilization of the polyethylenimine using some of the remaining epoxy groups on the support.

The standard protocol to prepare the co-immobilized derivative was as follows: 20 g of polyethylenimine (MW from 700 to 1,000,000 Da) were added to 50 ml of 200 mM phenylacetic acid, the pH value was adjusted to 9 and the volume was adjusted to 100 ml. Then, 7 g of PGA-Sepeabeds derivative was added.

After 16 h of reaction, the enzyme/polyamine co-immobilized derivative was washed to eliminate the excess of PEI and re-suspended in 100 ml of 3 M glycine–100 mM phenyl acetic acid at pH 8.5. After 24 h, no free epoxy groups could be detected. The derivative was washed with an excess of distilled water.

2.2.2. Modification with aldehyde dextran

Then, 7 g of wet immobilized penicillin G acylase-PEI were suspended in 145 ml of aldehyde dextran (6000 Da) prepared as previously described [7] at pH 7. The reaction mixture was maintained at 25 °C under very gently stirring for 12 h.

2.2.3. Multilayer PEI-aldehyde dextran

The derivative described above was washed with an excess of distilled water to eliminate the excess of aldehyde dextran. Then, 7 g of the derivative was re-suspended in 100 ml of distilled water containing 2 g of polyethylenimine (25 kDa) adjusted to pH 8 by adding HCl. After 12 h, soluble PEI was washed and a new layer of aldehyde dextran was added following the protocol described above. Successive additions/washings of PEI and aldehyde dextran were performed to build a three layers of PEI/aldehyde dextran on the support surface. Then, the reaction volume was increased to 500 ml by adding 100 mM sodium bicarbonate/100 mM phenyl acetic acid/25% (v/v) glycerol

pH 10.05. Then, 500 mg of solid sodium borohydride were added. After 30 min, the modified enzyme derivative was abundantly washed with distilled water.

2.2.4. Generation of "polymeric salts"

A total of 10 ml of derivative was suspended in 150 ml a solution of 32 mg/ml dextran sulfate (6000 Da) (the pH was previously adjusted at pH 7 by adding the necessary NaOH). After 1 h under very gentle stirring, the derivatives were washed with distilled water.

The whole hydrophilization treatment promoted a slight decrease in the enzyme activity (mainly by the first aldehyde dextran treatment) by around a 20% (activity determined by the assay described below).

2.3. Assays of enzyme activity

Enzyme activity was evaluated using a pHstat to titrate the release of H^+ produced by the hydrolysis of 10 mM penicillin G in 0.1 M sodium phosphate/0.5 M NaCl at pH 8 and 37 °C. A total of 100 mM NaOH was employed as titrating agent.

2.4. Inactivation of PGA-derivatives in the presence of water-soluble organic cosolvents

Enzyme derivatives were washed and equilibrated with cooled solutions (4 °C) of the desired water/organic cosolvent mixture at the pH and concentration indicated. Then, the enzyme derivatives were re-suspended in that solution and incubated at the temperature indicated. Samples were withdrawn periodically and the activity was checked following the above assay. Stability is expressed as the half-life of the each enzyme derivative under each inactivation condition. Stabilization factor was calculated as the ratio between the half-lives of the reference and that of the studied derivative (actual or estimated from the inactivation courses if stability was too high). Experiments were carried out in triplicate, standard error was never over 2%.

2.5. Inactivation of PGA-derivatives in the presence of organic solvents

A total of 300 mg of wet PGA derivatives were incubated in 5 ml of 2-pentanone and 5 g of molecular sieve (UOP type 4A from Fluka) was added to dry the derivative. Periodically, samples of derivative were taken of this "drying suspension" and the activity of the derivatives was assayed in a synthetic reaction. The reaction was the amidation reaction catalyzed by the enzyme using phenylacetyl methyl ester and octyl amine, that was carried out in anhydrous pentanone (dried with molecular sieves) (Abian et al., in preparation).

3. Results and discussion

3.1. Direct formation of polymeric salts around the enzyme molecules

Fig. 1 shows that the co-immobilization of PEIs and PGA is not enough to promote a significant stabilization of the enzyme, even using very large polymers that are much larger that the PGA molecule (stabilization factor was around a two-fold factor). However, it is possible to find in the literature some examples where this treatment alone has got a positive and remarkable effect on the enzyme stability of immobilized preparation in the presence of organic solvents [17,18]. This low effect of PEI in the stability of immobilized PGA may be related to the fact that PGA hardly becomes adsorbed to PEI supports, therefore, it may be expected that PEI is not adsorbed to the enzyme either. In this way, only the area of the enzyme next to the support may have a certain degree of protection.

Afterwards, we tried to generate a polymeric salt covering the enzyme molecules by adding dextran sulfate to the PGA-PEI derivative. However, again no improvement in the enzyme stability was detected even when using the largest PEI (Fig. 2). This result contrasts with the high stabilizing effect of the protocol found when using aldehyde dextran [19] and suggest that the addition of anionic and cationic polymers is not enough to generate a shell *covering* the enzyme molecules. Apparently, both polymers interact with each other but did not involve the enzyme molecules in that process, perhaps because the final result is a very compact layer of polymeric salts on the surface of the support.

The failure to improve the stability of the enzyme by using PEI and sulfate dextran suggested that a direct stabilizing effect of the polymers on the enzyme stability should not be expected.

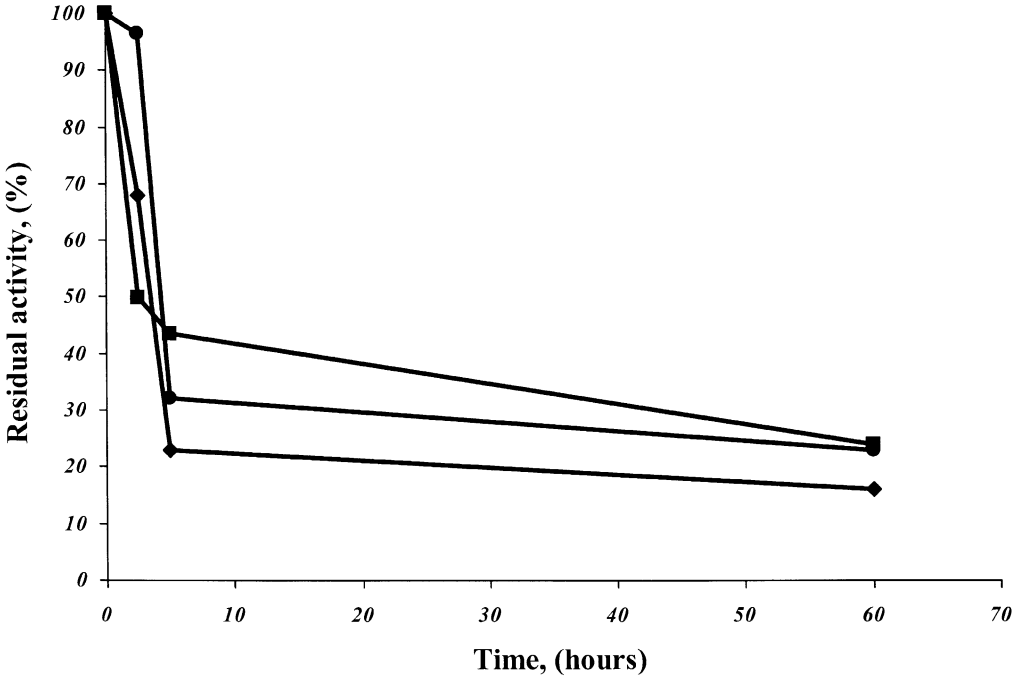

Fig. 1. Effect of the co-immobilization of PEIs and PGA in Sepabeads derivatives on their stability in organic cosolvent. Inactivation was performed at pH 7 and 4 °C in 75% dioxane. PEIs with different MWs were tested. Unmodified Sepabeads PGA (rhombus), Sepabeads PGA co-immobilized with low MW PEI (circles), Sepabeads PGA co-immobilized with high MW PEI (squares).

3.2. New strategy: use of aldehyde dextran to prevent the collapsing of the hydrophilic net

In order to prevent the possible collapse of the PEI-sulfate dextran composite, we have performed a previous modification of the PEI-PGA derivative with aldehyde dextran. This modification has a positive and significant effect on the enzyme stability in the presence of dioxane (Fig. 3). In fact, the hydrophilized derivative presented its activity almost unaltered after incubation in dioxane under conditions in which the conventional one lost more than 60%. After 10 days, the activity of these new derivatives still was over 60%.

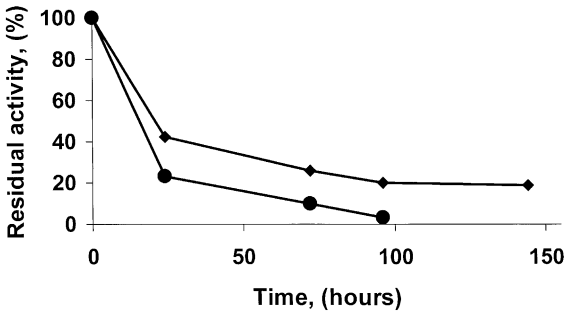

Fig. 2. Effect of the addition of dextran sulfate on the stability of PEI-PGA Sepabeads derivative in the presence of organic cosolvents. Inactivation was performed at pH 7 and 20 °C in 90% dioxane. Unmodified Sepabeads PGA (rhombus), Sepabeads PGA-PEI 1,000,000 Da co-immobilized with dextran sulfate (circles).

The effect of the size of the PEI co-immobilized with the PGA on the stability of the derivative is also clear (Fig. 3). Apparently, the larger the PEI, the larger the stabilization effect of the aldehyde dextran modification, perhaps because covering in a large extension the enzyme molecules.

Using this PEI-PGA-aldehyde dextran derivatives, the addition of sulfate dextran presented a significant stabilizing effect in the presence of organic media (Fig. 4).

Thus, the three-step strategy to create the hydrophilic environment around the enzyme molecules seems to fulfill the expectations: a high increment of the enzyme stability in the presence of organic cosolvent.

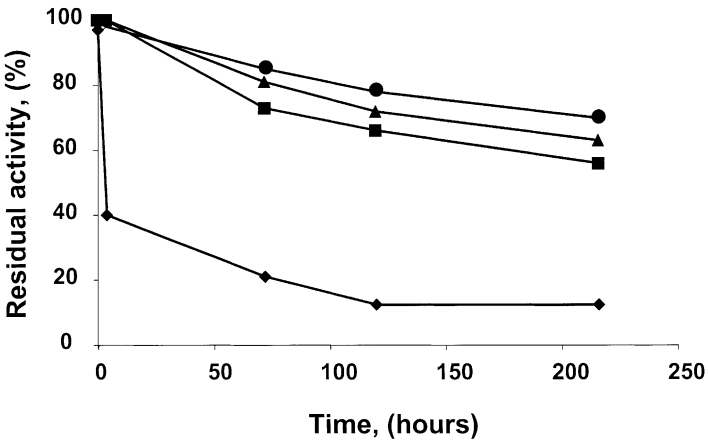

Fig. 3. Effect of the addition of aldehyde dextran on the stability in the presence of organic cosolvents of PGA Sepabeads derivatives co-immobilized with PEI of differents MW. Inactivation was performed at pH 7 and 20 °C in 90% dioxane. Unmodified Sepabeads PGA (rhombus), PEI 25,000-PGA Sepabeads derivative (squares), PEI 60,000-PGA Sepabeads derivative (triangles), PEI 1 million-PGA Sepabeads derivative (circles).

However, the thermal stability of this composite was not improved, in fact, resulted slightly lower that the unmodified derivative (results not shown). This result suggested that the stabilization was not derived from a direct rigidification of the enzyme structure through chemical or physical cross-linking.

3.3. Effect of the polymeric-saline layer thickness on the enzyme stability

Bearing in mind the hypothesis that the main effect of this hyper-saline environment is to promote a partition of the organic solvent molecules, it may be expected that the thicker the shell, the fewer the organic solvent molecules around the enzyme molecules and, therefore, the higher stabilizing effects. To increase this thickness, we have performed several cycles of reaction of aldehyde dextran and PEI, following the protocol described in methods, to a total of three layers of this composite.

In fact, Fig. 5 shows that the stability of the PGA derivative increased when we built several aldehyde dextran/PEI layers. To prepare these new layers, we have used medium size polymers to permit the

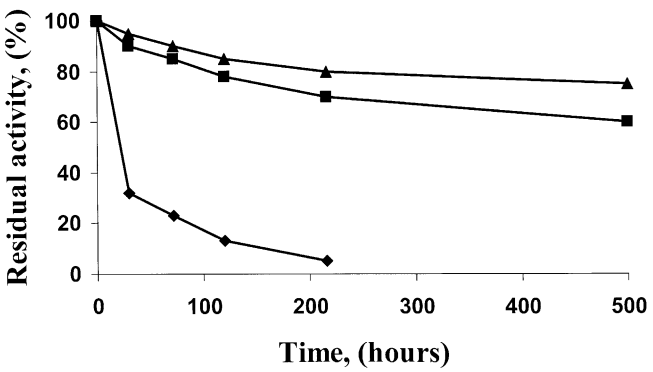

Fig. 4. Effect of the addition of dextran sulfate on the stability in the presence of organic cosolvent of PEI-PGA-aldehyde dextran derivatives. Inactivation was performed at pH 7 and 20 °C in 90% dioxane. Unmodified Sepabeads PGA (rhombus), PEI 1 million-PGA-aldehyde dextran Sepabeads derivative (squares), PEI 1 million-PGA-aldehyde dextran–dextran sulfate Sepabeads derivative (triangles).

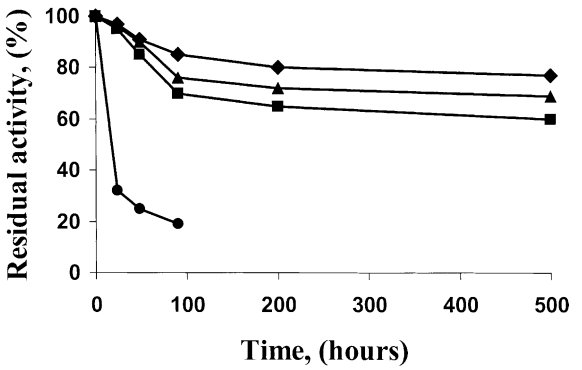

Fig. 5. Stability in organic media of Sepabeads-PGA derivatives after the addition of several layers of PEI-aldehyde dextran. Inactivation was performed at pH 7 and 20 °C in 90% dioxane. Unmodified Sepabeads-PGA (circles), one layer PGA-Sepabeads derivative (squares), two layer PGA-Sepabeads derivative (triangles), three layer PGA-Sepabeads derivative (rhombus). The MWs of the PEIs used were 10^6 Da in the first layer and 25×10^3 Da in the second and third layers.

preparation of several layers of polymers (larger polymers could "close the pores"), reaching a maximum stabilization effect when using three layers of polymers.

Fig. 6 shows how the effect of the sulfate dextran addition in these polymer multilayer derivatives permits a further increase in the stability of the enzyme derivatives in the presence of organic cosolvents. Now the enzyme may stand up to 90% of dioxane without a significant loss of activity after several days of incubation.

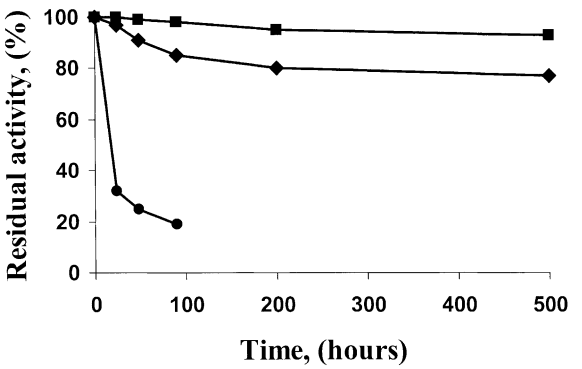

Fig. 6. Increase of stability in organic cosolvent of the multilayer Sepabeads-PGA derivative when dextran sulfate is added. Inactivation was performed at pH 7 and 20 °C in 90% dioxane. Unmodified Sepabeads-PGA (circles), three layer PGA-Sepabeads derivative (rhombus), three layer PGA-dextran sulfate Sepabeads derivative (squares).

4. Characterization of the new PGA derivative

4.1. Stability in the presence of different organic cosolvents

Fig. 7 shows that the optimal derivative prepared in this paper may stand 90% of different organic solvents without significant loss of activity. Thus, enzyme derivatives are very stable in the presence of hydrophobic solvents dioxane, tetraglyme, diglyme, etc. Using the solvents with the lowest deleterious effects (e.g. tetraglyme) [10], it may be possible to use even 95% of some of these organic cosolvents (Fig. 7).

However, the stabilizing effect is not always so high, depending on the nature of the organic cosolvent. Using polar organic solvents the stabilizing effect is lower, although also very significant (Fig. 8A). For instance, while using standard derivatives, stability is much higher in the presence of dimethylacetamide than in the presence of dioxane (Fig. 8B), the new derivatives presented an inversion in the stability, the stability in dioxane (that may be used even at 90%) being much higher than in DMA (Fig. 8B).

This might be associated to the hypothesized mechanism of stabilization: DMA is much more hydrophilic

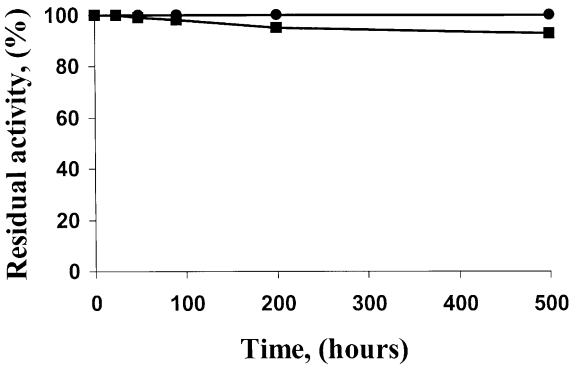

Fig. 7. Stability of the optimal derivative of Sepabeabs-PGA multilayer dextran sulfate derivative in the presence of different organic cosolvents. Inactivation was performed at pH 7 and 20 °C in 90% of dioxane and 95% of diglyme and tetraglyme. Three layer PGA-dextran sulfate Sepabeads derivative incubated in 90% of dioxane (squares), in 95% of diglyme and in 95% of tetraglyme (circles).

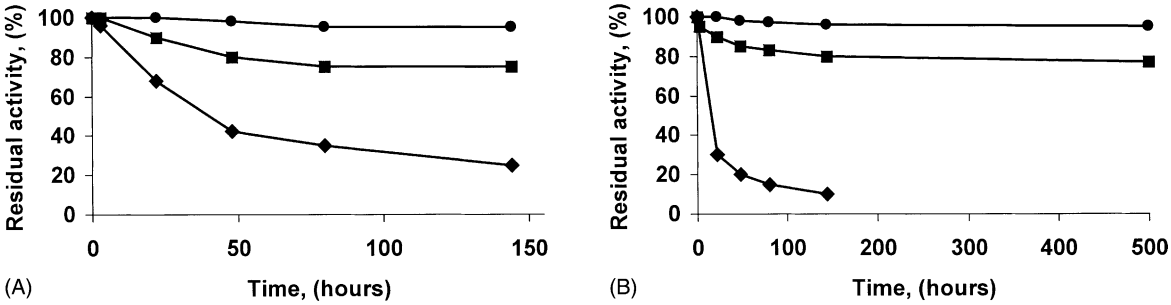

Fig. 8. Stabilization of the optimal multilayer Sepabeads-PGA dextran sulfate derivative in the presence of organic cosolvents with different polarity. Inactivation was performed at pH 7 and 20 °C in (A) 50% dimetilacetamide and (B) 90% dioxane. (A and B) Unmodified Sepabeads-PGA (rhombus), three layer PGA-Sepabeads derivative (squares), three layer PGA-dextran sulfate Sepabeads derivative (circles).

than dioxane and thus the partition effect of the polymeric salt micro-environment may be expected to be lower.

4.2. Stability in anhydrous systems

This physicochemical strategy to modify enzymes may also present some stabilizing effects when the derivative is exposed to anhydrous media. To check this, different PGA derivatives have been incubated in the presence of different anhydrous organic solvents. Fig. 9 shows the results.

Two facts can be extracted from this Figure: glutaraldehyde derivative was completely inactive under these conditions (activity determination was not possible), the standard derivative (that is 10,000-fold more stable than the soluble enzyme in experiments of thermal inactivation [14]) presented very low initial activity and became fully inactivated after only some few hours, while the new multilayer hydrophilized derivative was much more active and kept high activity values even after 24 h of drying.

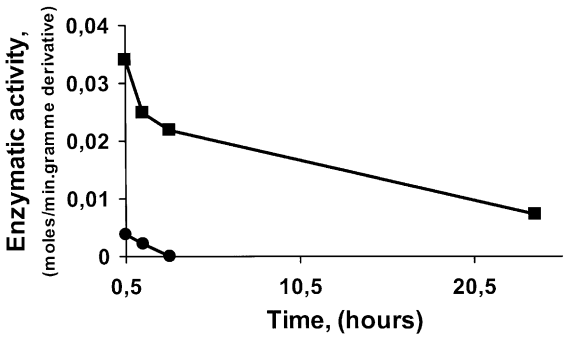

Fig. 9. Inactivation of Sepabeads derivative in anhydrous medium in presence of an organic solvent. Residual enzymatic activity was measured in the anhydrous medium, using a amidation reaction in pentanone. Unmodified Sepabeads-PGA (circles), three layer PGA-dextran sulfate Sepabeads derivative (squares).

We will like to remark that these new derivatives, because of the support properties, may be used in anhydrous media without suffering significant changes in the biocatalyst volume. Therefore, they have good prospects of being utilized in anhydrous media.

5. Conclusions

The generation of “polymeric salts micro-environments” has promoted an impressive stabilization of the PGA stability against the deleterious effects of organic media. The positive effect of the PEI-aldehyde dextran strategy has been highly improved by the use of sulfate dextran that generates this new polymeric salts.

These hyper-hydrophilic micro-environments (“polymeric ammonium sulfate”) seems to be very useful, not only to reduce the concentration of cosolvent in the enzyme environment but also to prevent the direct interaction of immobilized enzyme molecules with solvent interfaces when these immobilized enzymes are used in anhydrous media.

At first glance, this strategy could be performed to stabilize any other enzyme to be used in organic media.

In the case of the PGA, it opens new and exciting possibilities for the massive use of this interesting biocatalyst in many reactions that are hardly possible

now because the current lack of stability of the available PGA preparations on organic solvents and even more in water/organic cosolvent mixtures [Guisan and coworkers, in preparation].

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