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Immobilization and stabilization of glutaryl acylase on aminated sepabeads supports by the glutaraldehyde crosslinking method

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Abstract

We have investigated the use of the glutaraldehyde chemistry to stabilize glutaryl acylase (GAC). GAC has been immobilized on different aminated supports (ethylendiamine (EA) or polyethylenimine (PEI) coated supports) and the effect of the treatment with glutaraldehyde on both stability and activity has been analyzed. It was determined that immobilization on aminated supports increased the enzyme stability, and that this stabilization increased with the size of the polyethylenimine. The treatment with glutaraldehyde presented a low impact on the enzyme activity (activity recoveries were over 80%) and greatly improved the enzyme stability. A similar treatment using the enzyme immobilized on supports that cannot react with glutaraldehyde did not give rise to stabilization, suggesting that this stabilization was due to a reaction between the enzyme and the support through the glutaraldehyde chemistry. Curiously, the larger the PEI, the lower the stabilization observed. Therefore, final differences between different immobilized GAC preparations treated with glutaraldehyde were not very significant. Other variables, such as glutaraldehyde concentration, were found to have a great impact on the final results (optimal concentration was in the range 0.5–0.65%). After optimization, the stability of the best immobilized GAC was over 250-fold higher than that of the soluble enzyme, retaining 90% of the immobilized activity and much more stable than commercially available GAC preparations or the enzyme immobilized on pre-activated supports. The simple preparation of this immobilized GAC, its good activity and stability, make this strategy very suitable for its industrial implementation.

Keywords: Enzyme stabilization; Glutaraldehyde; Aminated supports; Sepabeads; Glutaryl acylase

1. Introduction

Glutaryl-7-aminocephalosporanic acid acylase (GAC) is an industrially relevant enzyme used in the industrial production of 7-amino cephalosporanic acid (7-ACA) from cephalosporin C (CPC) [1]. Because of the lack of a suitable enzyme able to carry out the direct hydrolysis of CPC [2] industrially, the current process for the production of 7-ACA involves a three-step reaction with two different enzymes [3].

The application of enzymes at industrial level usually requires their immobilization on solid supports. Thus, it seems very convenient to couple immobilization to stabilization. Several examples have been previously reported, in which GAC has been immobilized onto different supports and through different methodologies [4–8].

The covalent immobilization of proteins using the glutaraldehyde chemistry is one simple alternative to immobilize proteins. There are several possibilities of using glutaraldehyde for this purpose, such as the immobilization of enzymes on supports previously activated with glutaraldehyde [9–16] or the treatment with glutaraldehyde of proteins adsorbed onto supports having primary amino groups [17,18]. Following this last strategy, all the primary amino groups of the enzyme and the support will be activated with one molecule of glutaraldehyde and it has been shown that these may permit to have an intense cross-linking under a broad range of reaction conditions [19].

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2. Materials and methods

2.1. Materials

Epoxy-Sepabeads (ECEP3) and Sepabeads EA (a support activated with ethylendiamine) supports were kindly donated by Resindion S.R.L (Mitsubishi Chemical Corporation). Glutaryl 7-ACA was kindly donated by Bioferma Murcia S. A (Murcia, Spain). Glutaryl 7-ACA acylase from *Pseudomonas* sp. (GAC) was supplied by Roche (purity approximately 80%). Immobilized commercial glutaryl acylase (GAC) was supplied by Roche. Polyethylenimine (PEI) of 700 and 25,000 Da were supplied by Sigma–Aldrich (St. Louise, USA), polyethylenimine of 70,000 Da was supplied by Nipon Shokuba (Tokio, Japan), polyethylenimine (PEI) of 600,000 Da and Glutaraldehyde solution (25% v/v) (glut) were supplied by Fluka (Buch, Switzerland). All other reagents were of analytical grade.

3. Methods

All results represent averages of at least three experiments. The experimental error was never higher than 5%.

3.1. Preparation of GAC

One millilitre of commercial GAC was diluted with 4 mL of 25 mM potassium phosphate solution pH 7.0 and then dialyzed against 5 L of 5 mM potassium phosphate buffer pH 7.0 for 24 h at 4 °C. The dialyzed enzyme was then centrifuged (12,000 rpm for 30 min at 4 °C), and the supernatant (containing 16 IU/mL and 11 mg protein/mL) was used as the enzymatic preparation for further experiments. The recovered activity accounted for more than 90% of the initial activity.

3.2. Assay of GAC activity

GAC activity was measured with a pH-stat using an automatic titrator (Crison micro TT 2050). The assays were carried out by adding 1–2 IU of GAC to 10 mL of a 10 mM solution of glutaryl 7-ACA in 0.1 M potassium phosphate buffer, at 25 °C and pH 7.5, titrating the reaction mixture with 25 mM NaOH. In the case of the commercial immobilized GAC from Roche, or the experiments to determine the loading capacity, 100 mM glutaryl 7-ACA was used.

One unit of glutaryl 7-ACA acylase activity was defined as the amount of enzyme necessary to produce 1 μmol of glutaric acid per minute under the previously described conditions. Moreover, GAC activity was double-checked by HPLC. Samples were withdrawn at different times. The initial rate of different GAC preparations was calculated as the conversion of glutaryl 7-ACA to 7-ACA at different times. No-side-reaction was detected. The column used was a C8 Kromasil (5 μm, 250 mm × 16 mm) and the mobile phase was 20 mM ammonium acetate/acetonitrile 95/5 (v/v) at pH 5.2.

3.3. Preparation of Sepabeads-PEI supports

Ten grams of Epoxy-Sepabeads (ECEP3) were incubated with 90 mL HCl 2 M, in order to open all epoxy groups to diol groups. Then, the support was washed with a large excess of distilled water. The washed support was incubated with 90 mL of 11 mM sodium periodate during 2 h to oxidize all the diol groups to glyoxyl groups. Then, the Sepabeads activated with glyoxyl groups was washed with an excess of distilled water. Next, 7 g of glyoxyl Sepabeads was added to 10 g of polyethylenimine of different sizes (600, 70, 25 and 0.7 kDa) in 80 mL distilled water (pH was previously adjusted at pH 10. The suspension was kept under mild stirring at 25 °C for 3 h [20]. Then, the suspensions were reduced by adding solid sodium borohydride to a final concentration of 10 mg/mL and were left under mild stirring for 2 h at 25 °C. Finally, the reduced PEI coated-supports were filtered and sequentially washed with 100 mM sodium acetate buffer at pH 4, 100 mM sodium borate buffer at pH 9, and with an excess of distilled water. The PEI-supports were stored at 4 °C until further use.

3.4. Adsorption of GAC onto Sepabeads-PEI

Five grams of Sepabeads-PEI were added to 5 mL of GAC (16 IU/mL) in 5 mM potassium phosphate buffer at pH 7. This suspension was kept under mild stirring for 3 h at 25 °C. Afterwards, the suspension was filtered and the biocatalyst (Sepabeads-PEI-GAC) was stored at 4 °C until further use.

3.5. Treatment of the adsorbed glutaryl acylase with glutaraldehyde

One gram of Sepabeads-PEI-GAC (prepared as described above) was added to 4 mL of a glutaraldehyde solution at different concentrations This suspension was kept under mild stirring for 1 h at 25 °C. Then, the suspension was filtered and washed with 20 mM potassium phosphate at pH 7. The immobilized GAC preparations were incubated for 16 h at 25 °C and then stored at 4 °C until further use. This immobilized GAC preparation was named Sepabeads-PEI-GAC-glut *n*%, *n* being the % v/v of glutaraldehyde used in the treatment.

3.6. Optimization of the cross-linking conditions for the Sepabeads-PEI-GAC

Two samples of Sepabeads-PEI-GAC-glut 0.5% were incubated at 4 °C and 37 °C to permit a more intense support-enzyme reaction during different times (1, 5, 13, 30 days) in order to improve the reaction of glutaraldehyde. After this time immobilized GAC preparations were reduced. For this, 1 gram of immobilized preparation was diluted in 10 mL of 100 mM sodium bicarbonate at pH 10, then 10 mg sodium

borohydride was added and the suspension was gently stirred for 1 h at 4 °C.

3.7. Loading capacity

Different amounts of GAC (20, 40, 60, 80, 100 and120 IU/mL) were offered to 1 g of Sepabeads-PEI600 at pH 7, 25 °C during 2 h. Then all immobilized preparations with different enzyme loads were treated with 0.5% glutaraldehyde solution under the optimal conditions. The expressed activity (IU/g support) of immobilized preparations with different loads was compared with the offered activity for each load. The activities of these immobilized preparations were analyzed by pHSTAT and HPLC with a mixture reaction containing 100 mM of glutaryl 7-ACA in the same conditions that were describe above.

4. Results

4.1. Immobilization of GAC on different supports

The immobilization of GAC via ionic adsorption on Sepabeads EA was very fast and the full activity was recovered after the immobilization process. When the GAC was immobilized onto Sepabeads-EA pre-activated with glutaraldehyde, the immobilization yield was also 100% and the expressed activity was 90%. Similarly, when GAC was adsorbed onto Sepabeads EA and then treated with glutaraldehyde, the immobilized GAC retained more than 90% of its initial activity (data not shown).

The treatment with 1 M of NaCl of the different immobilized GAC preparations, fully released the enzyme adsorbed on Sepabeads EA, as expected from the ionic nature of the adsorption. However, the enzyme was not released at all when it was immobilized onto Sepabeads EA pre-activated with glutaraldehyde (Sepabeads EA-glut) or when the ionically adsorbed GAC preparation was treated with glutaraldehyde. This showed that, there is, at least, a covalent link between the enzyme and the support through the glutaraldehyde chemistry.

All immobilized preparations of GAC showed a higher stability than the soluble preparations of GAC (Fig. 1). The GAC covalently immobilized on Sepabeads EA pre-activated with glutaraldehyde was more stable than GAC only ionically adsorbed on the support. However, the treatment of the latter with glutaraldehyde rendered the product with the highest thermal stability.

The high stability of the GAC adsorbed on Sepabeads-EA further cross-linked with glutaraldehyde was not due to the chemical modification of the enzyme because when the enzyme was immobilized on Sepabeads EA-glut and then treated with a 0.5% glutaraldehyde solution, it did not alter its stability. Similarly, the enzyme immobilized on glyoxyl-agarose did not improve its stability by treatment with glutaraldehyde. That suggests that only if the support can react with the glutaraldehyde, it was possible to achieve stabilization.

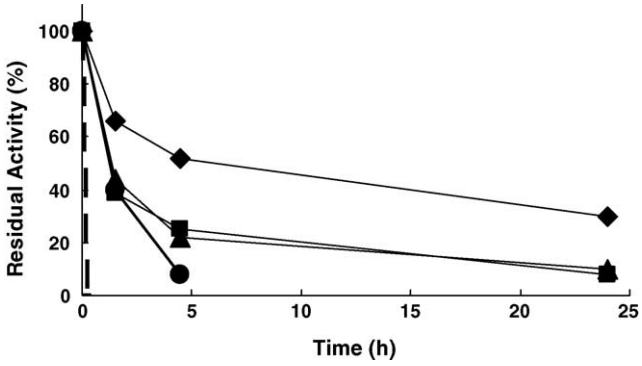

Fig. 1. Thermal inactivation course of GAC immobilized on different supports. All GAC preparations were thermally inactivated at 47 °C in 5 mM potassium phosphate buffer pH 7. Soluble GAC (- - -); GAC ionically adsorbed onto Sepabeads EA (●); GAC covalently immobilized on Sepabeads EA-glut (▲); GAC covalently immobilized on Sepabeads EA-glut and further treatment with 0.5% glutaraldehyde solution (■); GAC ionically adsorbed on Sepabeads EA and further cross-linking with 0.5% glutaraldehyde solution (♦).

Thus, we can conclude that the high stability of GAC adsorbed on Sepabeads-EA and then treated with glutaraldehyde is due to the crosslinking reaction between amino groups placed on the support and on the enzyme, not to intermolecular crosslinking of the GAC molecules.

4.2. Effect of the support

The enzyme stability was compared when adsorbed on Sepabeads EA and supports coated with PEI of different size. The observed stabilization was related to the size of the PEI (Fig. 2A). Thus, the coating of the support with PEI of 70–600 kDa increased the stability of the immobilized preparation four to five-fold in comparison with the preparation on a support coated with PEI of 0.7 kDa.

The stability of all preparations was significantly improved by treatment with glutaraldehyde. Curiously, all immobilized GAC preparations treated with glutaraldehyde showed approximately the same stability (Fig. 2B).

4.3. Effect of glutaraldehyde concentration

Fig. 3 shows that the enzyme activity was not very affected by the concentration of glutaraldehyde during the treatment. Curiously, modifications with low glutaraldehyde concentrations presented a higher inactivating effect of the enzyme adsorbed in PEI-Sepabeads, while approx 90% of the activity was recovered using concentrations in the range 0.5–2% This effect was not found when treating GAC immobilized on glyoxyl agarose, where the activity recovery was always approx 90%.

On the other hand, in the case of the stability, a maximum stabilization factor (over 250-fold higher than the soluble enzyme) was found when using 0.5–0.65% (v/v) glutaralde-

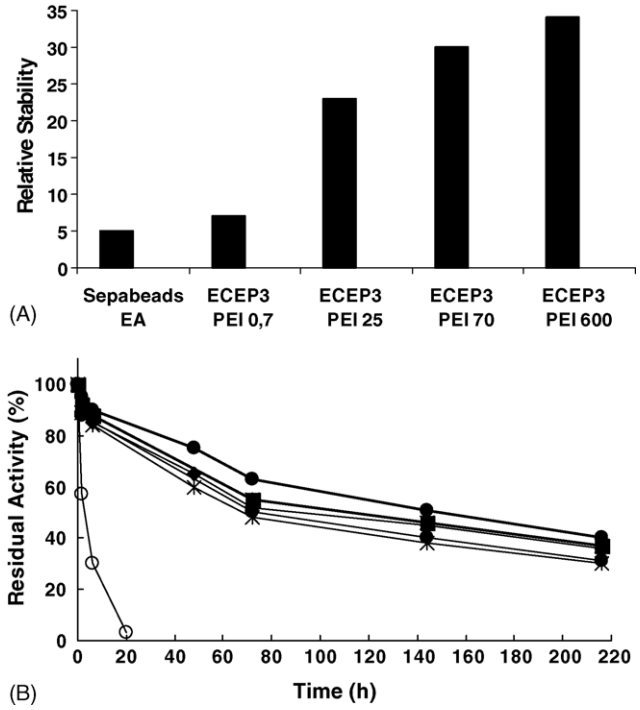

Fig. 2. (A) Effect of PEI size on the stability of immobilized glutaryl acylase. All immobilized GAC were thermally inactivated at 45 °C in 5 mM potassium phosphate buffer pH 7. The relative stability was defined as the ratio between the half-lives of the immobilized preparation and soluble enzyme, respectively. (B) Effect of glutaraldehyde treatment on the stability of GAC immobilized on different supports inactivation was performed incubating 3 IU/mL in 5 mM potassium phosphate buffer pH 7 at 45 °C. GAC ionically adsorbed onto Sepabeads EA and further cross-linking 0.5% glutaraldehyde solution (⚹); Sepabeads PEI 0.7-glut 0.5% (●); ECEP3-PEI25-GAC-glut 0.5% (♦); ECEP3-PEI70-GAC-glut 0.5% (■); ECEP3-PEI600-GAC-glut 0.5% (▲); ECEP3-PEI600-GAC, without cross-linking with glutaraldehyde (○).

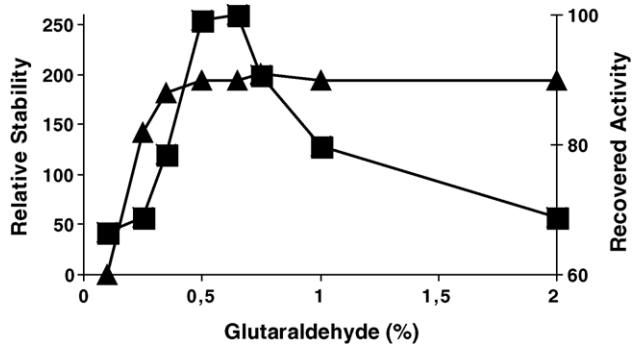

Fig. 3. Effect of glutaraldehyde concentration on the stability and activity of glutaryl acylase during the cross-linking of GAC immobilized on Sepabeads-PEI600. All immobilized GAC were thermally inactivated at 47 °C in 5 mM potassium phosphate buffer pH 7. The relative stability was defined as the ratio between the half-lives of the immobilized preparations and soluble enzyme respectively. Recovered activity (▲) and relative stability (■).

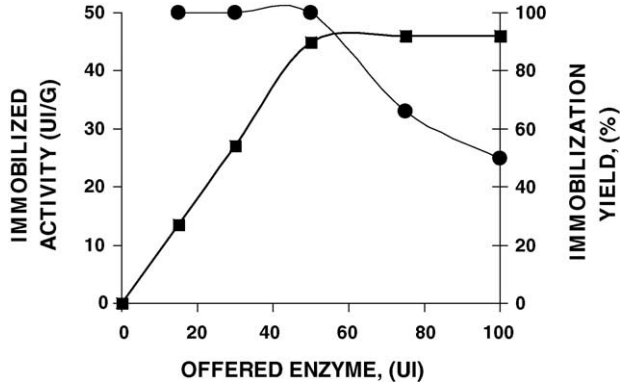

Fig. 4. Loading capacity of Sepabeads ECEP3-PEI 600. The experiment was carried out offering different enzyme concentrations to 1 g of support at pH 7 and 25 °C and further cross-linking with a 0.5% glutaraldehyde solution; immobilized activity (●) and immobilization yield (■).

hyde (Fig. 3). Results using 0.5 or 0.65% glutaraldehyde were very similar and thus, we decided to use 0.5% glutaraldehyde.

4.4. Loading capacity of the Sepabedas-PEI-GAC-glut 0.5%

Different amounts of GAC were offered to the Sepabeads-PEI600 support. It was observed that 100% of the GAC was immobilized up to 50 IU per gram of wet support. Higher loadings did not promote an increment of the activity of the immobilized preparation, and promoted a decrease in the immobilization yield. These immobilized GAC preparations were cross-linked with 0.5% glutaraldehyde under the optimal conditions (Fig. 4). All preparations lost approximately 10% of activity after cross-linking with glutaraldehyde. Thus, the optimal preparations showed a final activity of 45 IU/g wet support under the assayed conditions.

Fig. 5 shows that the optimal immobilized GAC preparation presented a higher stability than the commercially available immobilized GAC, which was more stable than the preparation immobilized on Sepabeads-EA pre-activated with glutaraldehyde.

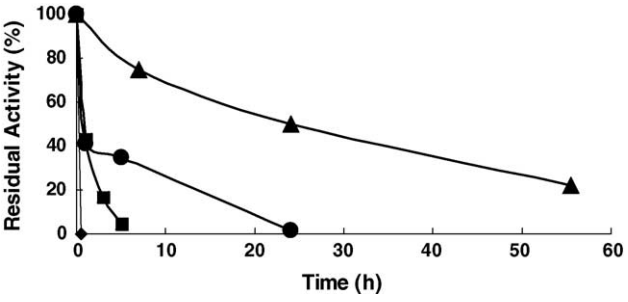

Fig. 5. Thermal inactivation course of different glutaryl acylase preparations. Inactivation was performed incubating 3 IU/mL of each preparation in 5 mM potassium phosphate buffer pH 7 at 47 °C: (●) Immobilized GAC from, Roche; (▲) ECEP3-PEI600-GAC glut 0.5%; (♦) Soluble-GAC; (■) GAC adsorbed on Sepabeads EA.

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