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Immobilization of penicillin G acylase onto chemically grafted nylon particles

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Abstract

Nylon particles, grafted with diethylene glycol dimethacrylate (DGDA) using potassium persulphate as initiator, were treated with hexamethylene diamine (HMDA). The aminoalkylated particles were activated with glutaraldehyde and finally, penicillin G acylase (PA) was immobilized to these activated particles.

Both the conditions of the aminoalkylation- and the immobilization process were optimized. The hydrolysis of cephalexin was used as model conversion. The retention of activity of the immobilized enzyme was 12%. This value improved to 30% by adding phenyl acetic acid (PAA), as active-site protecting agent, to the enzyme solution. The results suggest formation of multi-point attachment between the enzyme and the matrix.

Keywords: Immobilized enzymes; Penicillin G acylase; Nylon; Chemical grafting; Cephalexin

1. Introduction

Immobilized penicillin G acylase (PA) is one of the few immobilized enzymes applied on industrial scale for the production of semi-synthetic antibiotics [1–4]. Many supports have been investigated for immobilization of the enzyme [5–8], including nylon [9]. Since nylon has a few free end groups for covalent attachment of enzyme molecules, it must be pre-treated to generate potentially reactive centers [10]. In spite of the great potential of the grafting technique to create reactive centers on copolymer matrices, very few papers were published about grafted nylon used for enzyme immobilization [11–15]. The value of graft copolymers is that a variety of matrices, possessing different physical, chemical and morphological characteristics, can be made. By careful selection of the matrix and the grafted monomer, it is possible to vary the hydrophilic/hydrophobic nature of the immobilized support that could improve enzyme activity and stability and also give easier handling and storage. The structure of the immobilized enzyme

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system, using graft copolymers, suggests that the enzyme is more available for reaction than is the case with enzyme entrapped in a gel where problems of diffusion of reactants and products can arise especially if the products are competitive inhibitors. Additionally, the number of reactive groups used for coupling the enzyme can be more closely controlled. Recently, the authors used nylon membranes grafted with different vinyl monomers to immobilize different enzymes including PA [16,17].

In the present work, nylon particles, which are cheaper than membranes, grafted with diethylene glycol dimethacrylate (DGDA) monomer was used as matrix for the immobilization of PA to be used in stirred tank bioreactor which is more economical than the membrane one. The activation and the immobilization conditions were optimized and the efficiency of the immobilization process is reported on.

2. Materials and methods

2.1. Chemicals

Nylon 6,6 pellets of 5 mm diameter, were used as solid support to be grafted with DGDA. Potassium persulphate (KPS) served as initiator for the grafting process. 1,6-Hexamethylene diamine (HMDA), 70% aqueous solution, was used as spacer between the grafted membrane and the enzyme. Glutaraldehyde (GA), 25% aqueous solution, was used as coupling agent for covalent binding of the enzyme to the activated HMDA-nylon beads. All the chemicals were purchased from Aldrich and used without any further purification. The enzyme, PA, and the substrate used here, cephalexin, were gifts from DSM-Anti-infectives (Delft and Geleen, The Netherlands). The specific activity, with cephalexin as a substrate, was 2500 IU ml^{-1} for the original enzyme solution. The cephalexin had a purity of 92.5% w/w and 6% (w/w) of water. Phenyl acetic acid (PAA), 98.5%, from Acros Organics, NJ, USA, was used as active-site protector.

2.2. Nylon-particles grafting (matrix functionalization)

The nylon pellets were first ground to 1 mm in diameter and then immersed in 5% DGDA solution in ethanol:water (1:1) in the presence of 0.5% (w/v) potassium persulphate. The temperature was raised to 60°C and the mixture stirred for 1 h. The obtained matrix was washed with ethanol to remove the unreacted monomer and then dried at 80°C. The percentage of grafting, $X\%$, is defined as the difference between particles masses before, G_B, and after the grafting, G_A, according to the following equation:

$$X\% = \frac{G_A - G_B}{G_B} \times 100(\%).$$

Under these conditions, a grafting percentage of about 8% was usually obtained.

2.3. Matrix activation

To activate the grafted particles, they were soaked in a 1,6-HMDA aqueous solution of a defined concentration, reaction time and temperature as specified later in the text. After washing with water, the aminoalkylated nylon particles were immersed for 1 h at room temperature in a glutaraldehyde 2.5% (v/v) aqueous solution of specified pH. At this point, the particles were activated and ready to bind the enzyme.

2.4. Enzyme immobilization

The activated particles (2 g) were immersed for 16 h at 25°C in 20 ml of the enzyme solution, prepared by diluting 10 times the orig-

inal enzyme solution with 0.1 M phosphate-buffer solution pH 7.0; the suspension was gently stirred during immobilization. These experimental conditions were always applied, except where indicated otherwise. After washing with water and buffer, the immobilized enzyme was ready for use.

In case of studying the effect of adding PAA as active-site protector, it is added to the enzyme buffer solution with final concentration 15 mM.

2.5. Determination of catalytic activity

Catalytic activity was determined as follows: 2 g of immobilized PA was put in 50 ml cephalexin solution (20 mM) at pH 7.0 and 30°C. The enzyme activity was measured through the amount of alkaline solution (0.05 N NaOH) required to keep the treated cephalexin solution at pH 7.0 (using an automatic pH control unit). The activity is expressed in μmol min^{-1} per 2 g of immobilized enzyme. The amount of enzyme immobilized on the matrix, is expressed as percentage immobilization yield (IMY%), obtained from the difference between the enzyme activity in solution before, *B*, and after the immobilization, *A*, divided by activity in solution before immobilization, *B*, times 100%. The percentage of activity retention (RTA%) was calculated by dividing the measured immobilized enzyme activity by the expected activity (i.e. $B - A$), times 100%. When the catalytic matrices were not in use, they were stored at 4°C in 0.1 M phosphate-buffer solution, pH 7.0.

2.6. Reliability of the experimental data

The experimental errors fall in the range of 3–6% for the grafting and in the range of 6–10% for the activity based on double experiments.

3. Results and discussion

3.1. Optimization of the aminoalkylation process

Since the activity of the immobilized enzyme depends on the amount of functional groups created on the matrix, optimization of the conditions of the activation process, including aminoalkylation, glutaraldehyde activation and enzyme binding are required. The dependency of the catalytic activity of the immobilized enzyme on the concentration of HMDA, temperature and duration of the aminoalkylation process were studied first.

3.1.1. Effect of the HMDA concentration

In Fig. 1, the activity of immobilized PA is presented as a function of the HMDA concentration. The figure shows that the activity increases with the concentration of HMDA, reaching a maximum value at 4–6%. If the concentration is increased to 10% than the activity decreases by 50%. Other authors [18] have found that carriers for acylase immobilization should not have a high amino-group concentration in order to prevent multi-point enzyme bonding, resulting in deactivation of the retained activity. From the above results we can accuse the formation of multi-bonds between

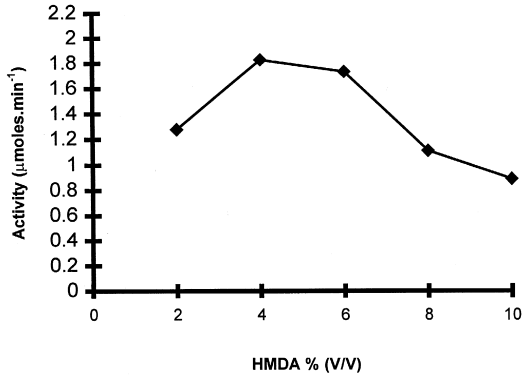

Fig. 1. Activity of the catalytic matrix as a function of the HMDA concentration. Experimental conditions for obtaining the catalytic matrix were: [DGDA] = 5% (v/v); [GA] = 2.5 % (v/v); $T_{\text{aminoalkylation process}}$ = 25°C; duration of aminoalkylation process = 1 h; [Enzyme] = 2500 U at pH 7; [S] = 20 mM at pH 7.0.

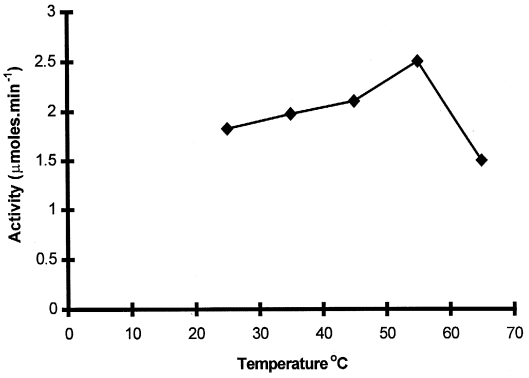

Fig. 2. Activity of the catalytic beads as a function of aminoalkylation temperature. Experimental condition for obtaining the catalytic matrix were: [DGDA] = 5% (v/v); [GA] = 2.5 % (v/v); [HDMA] = 2% (v/v); duration of aminoalkylation process = 1 h; [Enzyme] = 2500 U at pH 7; [S] = 20 mM at pH. 7.0.

the enzyme molecule and the support for the decrease in activity of the immobilized enzyme with increase of HMDA used concentration.

3.1.2. Effect of the aminoalkylation temperature

The dependency of the catalytic activity on the temperature of the aminoalkylation process is shown in Fig. 2. Optimum activity is observed for the matrix prepared at 55°C. Increasing the temperature to 65°C resulted in 40% less activity. The decrease in activity at temperatures higher than 55°C may be explained by considering that at these temperatures the amination process may takes place on the nylon matrix in addition to the polymer graft branches. In this case, the density of the amine groups available to attach PA molecules directly on the surface of the matrix may be increased. Consequently, the enzyme molecules attach with more than one bond, which in turn leads to a decrease in expressed activity.

3.1.3. Effect of the aminoalkylation reaction time

The obtained results indicate that increasing the reaction time from 7.5 to 75 min decreases the activity by 20%. The minimum handling time is 7.5 min and this clearly is enough to obtain the highest maximum activity. This indicates that the process mainly occurred on the surface of the particles. This is in agreement with the non-porous nature of the nylon particles.

3.2. Optimization of the glutaraldehyde-activation process

The effect of the activation conditions, i.e. glutaraldehyde concentration, reaction time, and reaction-pH, were optimized.

3.2.1. Effect of glutaraldehyde concentration

The effect of the glutaraldehyde concentration used for the activation of the aminoalkylated matrix on the activity is shown in Fig. 3. Increasing the glutaraldehyde concentration increased the activity reaching its maximum value at 2.5%. Beyond this concentration the activity decreased with the glutaraldehyde concentration; at 5% glutaraldehyde, the relative activity was 55%.

3.2.2. Effect of the activation time

No significant effect of the activation time was observed in the range of 1–3 h. At 4 h, the activity started to decrease and dropped to 59% at 5 h. Prolonging the reaction time to 24 h decreased the activity to 43%. One hour of

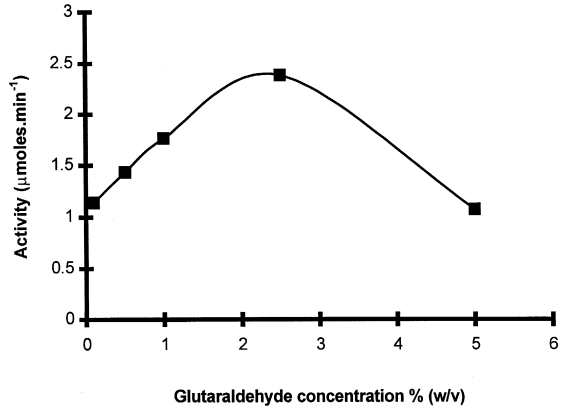

Fig. 3. Activity of the catalytic matrix as a function of glutaraldehyde concentration. Experimental conditions for obtaining the catalytic beads were: [DGDA] = 5% (v/v); $T_{\text{aminoalkylation process}}$ = 55°C; duration of aminoalkylation process = 7.5 min; [Enzyme] = 2500 U at pH 7; [S] = 20 mM at pH 7.0.

reaction time has been chosen for doing the rest of the experiments.

3.2.3. Effect of pH of the glutaraldehyde solution

A clear effect of the pH of the glutaraldehyde solution was found in the studied range of 6–10 (Fig. 4). The maximum activity was found at pH 9.0. Dramatic loss of activity occurred at both lower and higher pH, with only 50% of relative activity at pH 10 and 20% at pH 6. These results are in full agreement with those obtained by other authors, who studied the glutaraldehyde activation step in detail with the immobilization of invertase on Nylon 6 tube. They used *O*-alkylation technique to create active functional groups on the surface of the nylon tube [19].

3.3. Optimization of the enzyme immobilization process

Finally, the immobilization conditions were optimized, i.e. enzyme amount, pH of the enzyme solution and the immobilization time. In addition, the effect of PAA as active-site protector was studied.

3.3.1. Effect of the enzyme concentration

The effect of the PA concentration used during the immobilization step on the catalytic activity is shown in Fig. 5. The activity initially sharply increases with enzyme concentration until 25 U ml^{-1}; after that further increase of enzyme concentration has no effect. These results agree with those found in the literature [20] in which PA was immobilized onto a copolymer of butyl acrylate and ethylene glycol dimethacrylate.

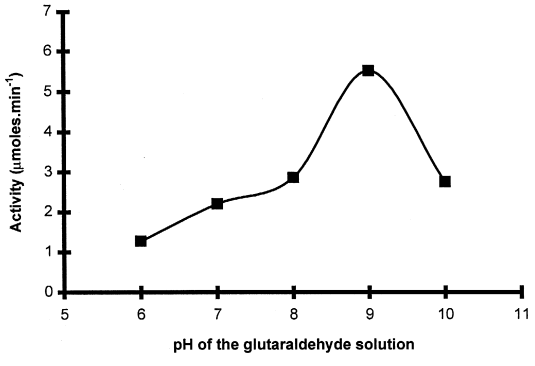

Fig. 4. Activity of the catalytic matrix as a function of pH of the glutaraldehyde solution. Experimental conditions for obtaining the catalytic beads were: [DGDA] = 5% (v/v); [GA] = 2.5 % (v/v); $T_{\text{aminoalkylation process}}$ = 55°C; duration of aminoalkylation process = 7.5 min; [Enzyme] = 2500 U at pH 7; [S] = 20 mM at pH 7.0.

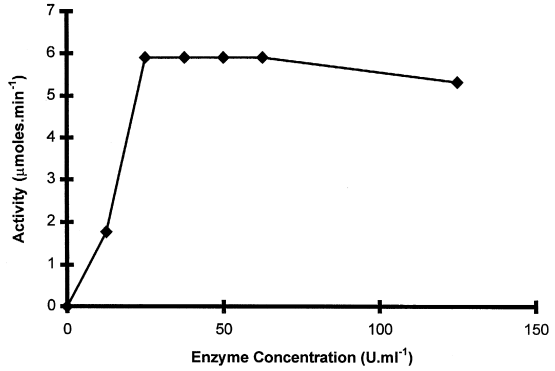

Fig. 5. Activity of the catalytic matrix as a function of enzyme concentration. Experimental conditions for obtaining the catalytic beads were: [DGDA] = 5% (v/v); [GA] = 2.5 % (v/v) at pH 9.0; $T_{\text{aminoalkylation process}}$ = 55°C; duration of aminoalkylation process = 7.5 min; [S] = 20 mM at pH 7.0.

3.3.2. Effect of the pH of the enzyme solution

In Table 1, the activity of the immobilized enzyme prepared using enzyme solutions with different pH is presented; the results show maximum activity in the range of 7.0–8.0. Especially at the highest pH tested, i.e. 10, the activity drops to less than 25% of the maximum activity. This may be explained by the follow-

Table 1
Effect of the pH of the immobilization solution on the activity, IMY% and RTA%
Experimental conditions for obtaining the catalytic beads were: [DGDA] = 5% (v/v); [GA] = 2.5 % (v/v) at pH 9.0; $T_{\text{aminoalkylation process}}$ = 55°C; duration of aminoalkylation process = 7.5 min; [Enzyme] = 250 U; [S] = 20 mM at pH 7.0.

pH	Activity/2 g (μmol min^{-1})	IMY%	RTA%
6.0	4.87	10.6	8.2
7.0	5.60	20.0	11.3
8.0	5.60	24.0	9.0
9.0	4.55	81.3	2.3
10.0	1.38	42.5	1.3

Table 2
Effect of the immobilization time on the activity, IMY% and RTA%
Experimental conditions for obtaining the catalytic beads were: [DGDA] = 5% (v/v); [GA] = 2.5 % (v/v) at pH 9.0; $T_{\text{aminoalkylation process}}$ = 55°C; duration of aminoalkylation process = 7.5 min; [Enzyme] = 250 U; [S] = 20 mM at pH 7.0; [PAA] = 15 mM.

Immobilization time (h)	IMY%	Activity (−PAA) (μmol min^{-1})	Activity (+PAA) (μmol min^{-1})	RTA% (−PAA)	RTA (+PAA)
1	ND	2.0	ND	ND	ND
2	16	4.85	12	12	30.77
4	18	5.78	11	15	27.5
6	21	6.3	11.5	12	21.7
8	20	6.1	ND	12	ND
16	21	6.0	ND	11.3	ND
24	ND	ND	11.73	ND	22.3

ing reasons: decrease of amount of enzyme immobilized at this pH (Table 1), wrong orientation of the immobilized enzyme at the alkaline pH, the formation of multi-point attachment with the matrix, and instability of PA at this high pH. The latter explanation has already been reported before [21] and is the most probable explanation. The results in the table also show that the retained activity (RTA%) has the same trend as the activity, reaching a maximum at neutral pH. This may be explained by the effect of the pH on the immobilization yield percent, which increased with higher rate than that of the activity and has its maximum at alkaline pH. The retained activity is the result of dividing the measured activity by the expected activity of the immobilized enzyme (see Materials and methods).

3.3.3. Effect of immobilization time

The effect of the immobilization time on the activity is shown in Table 2. One hour of immobilization gives an activity of 2 μmol min^{-1} (30% of maximum); doubling of this time increases this value to 4.85 μmol min^{-1}, reaching 5.78 and 6.3 μmol min^{-1} after 4 and 6 h, respectively. Further increase of the immobilization time to 16 h has no effect. This result is easy to explain by the fact that the amount of immobilized enzyme (IMY%) shows the same trend (Table 2). Since the retained activity depends on both the activity and the amount of immobilized enzyme (IMY%), as mentioned in Materials and methods, and they shown the same behavior, so the retained activity of the system was affected slightly by changing the immobilization time.

3.3.4. Effect of the PAA

From the results in Tables 1 and 2, it is clear that most of the enzyme activity is lost in the immobilization process. Therefore, PAA, an inhibitor of the enzyme, was added to protect the active site during the immobilization process. The effect of PAA on the activity and RTA%, for different immobilization times, is also shown in Table 2. The results show that both the activity and RTA% increased by adding PAA to the immobilization solution as protecting agent for the enzyme active site. The increase by a factor of 2 indeed indicates that bonds are formed, with loss of activity, between amino groups in the active site and the aldehyde groups on the matrix in the absence of PAA. This result is in agreement with the result obtained by another group [21] in which they immobilized PA into an agarose matrix. They found that the retention of activity of the prepared derivative in the absence of PAA was 51% and improved to 98% by the addition of PAA to the immobilization matrix.

4. Conclusions

PA was immobilized on poly-diethyleneglycoldimethacrylate (pDGDA) grafted nylon of

8% grafting. After optimization of the factors affecting the activity of the immobilized enzyme, 12% of the activity is retained. Binding in the wrong orientation of the immobilized enzyme, multi-point attachment to the matrix and binding with groups in the active site, are the probable reasons, which cause the loss of activity. It was found that the increase of the amine-groups concentration on the matrix surface had a negative influence on the activity. By protecting the active site of the PA through the immobilization process using PAA, both the activity and the retention of activity were improved by a factor of 2. The latter was reaching 30%.

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