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Characterization of the activity of penicillin G acylase immobilized onto nylon membranes grafted with different acrylic monomers by means of γ-radiation

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Abstract

Penicillin G acylase (PGA) has been immobilized onto nylon membranes grafted with methylmethacrylate (MMA) or diethyleneglycoldimethacrylate (DGDA) monomers by means of γ-radiation. Hexamethylenediamine (HMDA) has been used as spacer between the grafted membranes and the enzyme. Glutaraldehyde (GA) was used as crosslinking to couple the enzyme to the HMDA. The catalytic membranes so prepared were studied as a function of pH and temperature of the solution containing the substrate. The membranes showing the best characteristics were the ones grafted with DGDA. The dependence of the behavior of these membranes on several experimental conditions was studied, i.e., the temperature and duration of the aminoalkylation process, spacer concentration, the glutaraldehyde concentration and the enzyme concentration. The experimental conditions giving the best performance of the catalytic membranes have been deduced. The time requested to obtain 50% of substrate conversion, i.e., hydrolysis of cephalexin, has been studied as a function of its initial concentration.

Keywords: Immobilized enzymes; Biocatalytic membranes; Penicillin G acylase; γ-Radiation; Cephalexin

1. Introduction

The production of valuable compounds in the chemical, pharmaceutical and food industries benefits from the use of enzymes as biocatalysts. The advantages in using immobilized enzymes promoted the exploitation of catalytic membranes in industrial processes, notwithstanding that the immobilized biocatalyst [1] exhibited lower activity than that of an equivalent amount of the free form. This was essentially due to the interactions between the macromolecule and the supporting matrix. The knowledge of these interactions became relevant

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together with the study of the way in which they affect the microenvironment in which the immobilized enzyme is operating. For this reason, much effort has been put in obtaining new polymeric carriers and immobilization techniques able to increase the stability and yield of enzymatic processes [2–6].

Hydrophilic supports have been used, since enzymes normally operate in aqueous media, and all industrial processes involving the use of immobilized enzymes are done under isothermal conditions.

Recently it has been discovered that the enzyme activity of a catalytic membrane coupled to a hydrophobic membrane was increased when used in non-isothermal bioreactors. The activity increase was proportional to the temperature difference imposed across the membrane [7–13]. A prerequisite for obtaining these results was the presence of a hydrophobic membrane, able to induce the process of thermodialysis [14–18], i.e., transmembrane matter transport under non-isothermal conditions. The process of thermodialysis is considered to be one of the physical causes responsible for the increased activity of the catalytic membranes in the presence of temperature gradients.

With the aim of obtaining new catalytic membranes to be employed in the process of thermodialysis, we have recently modified commercial teflon or nylon membranes, making them at the same time catalytic and hydrophobic, by means of a double grafting technique and using γ-radiation [19–23]. One of these membranes, loaded with β-galactosidase [23], has given good reproducible results when employed in non-isothermal bioreactors. We used the β-galactosidase in view of its employment in the process of lactose hydrolysis in milk.

In this work we characterize under isothermal conditions a new catalytic and hydrophobic membrane, obtained from a nylon sheet preactivated by means of γ-radiation in the presence of different solutions of suitable monomers and loaded with penicillin G acylase (PGA; EC.3.5.1.11). We have chosen PGA since this enzyme plays a relevant role in pharmaceutical industries, catalyzing an important intermediate for the industrial production of semi-synthetic penicillins and cephalosphorins [24,25]. Besides this function, the enzyme can be used in additional biotechnological processes for resolution of racemic mixtures [26].

2. Materials and methods

2.1. Materials

As solid support to be grafted we used nylon Hydrolon membranes, a precious gift from the Italian Division of Pall (Pall Italia, Milano, Italy). These hydrophobic membranes have a nominal pore size of 0.2 μm. Pore size is related to the size of the minimum value of the diameter of the smallest particles that the membrane retains, since in the membrane there are no ''classical'' pores but irregular cavities crossing the membrane thickness.

All the chemicals were purchased from Sigma (Sigma Aldrich, Milano, Italy) and used without further purification. Diethylene glycol dimethacrylate (DGDA) or methylmethacrylate (MMA) were used as grafting monomers. Hexamethylendiamine (HMDA) (70% aqueous solution) was used as spacer between the grafted membrane and the enzyme. Glutaraldehyde (GA) aqueous solution (2.5%) was employed as bifunctional coupling agent for covalently binding the enzyme to the activated membrane.

The PGA and its substrate, i.e., cephalexin, were gifts from DSM, Geleen, The Netherlands. The enzyme specific activity, with cephalexin, was 250–300 μmole min^{-1} ml^{-1} for the original enzyme solution. The cephalexin had a purity of 92.5% w/w, 6% (w/w) being water and the remaining substances being impurities.

The PGA hydrolyses the cephalexin to phenylglycine (PG) and 7-aminodeacetoxy cephalosparinic acid (7-ADCA).

2.2. Methods

2.2.1. Catalytic membrane preparation

Membrane grafting was done by irradiation with γ-rays. The irradiation source was Caesium 137 in a γ-cell 1000 Elite by Nordion International, Canada. The average dose rate in the core of the radiation chamber (central dose rate) was 2.35×10^4 rad h^{-1}.

The nylon membranes to be grafted were immersed in a solution of ethanol in water (1:1 v/v) containing 10% (v/v) of DGDA or MMA and irradiated for 8 h. After treatment the membranes were washed with water to remove the homo-polymers adhered to the membrane surface and after that soaked in acetone for about 1 h. After a further washing with water the membrane was dried to estimate the amount of the grafting obtained. To evaluate the percentage of grafting we adopted the classical definition used for this parameter. The degree of grafting (X, %) was determined by the difference between membrane masses before, G_B, and after, G_A, the grafting according to the expression:

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

The result of these procedures is a nylon–polyDGDA or nylon–polyMMA grafted membrane ready to be activated for enzyme binding. The overall process of membrane activation and enzyme immobilization, illustrated in the following, is schematically represented in Fig. 1.

2.2.2. Membrane activation

To activate the grafted membranes, they were soaked in a HMDA 10% (v/v) aqueous solution for 1 h at room temperature. This temperature was used in all the aminoalkylation pro-

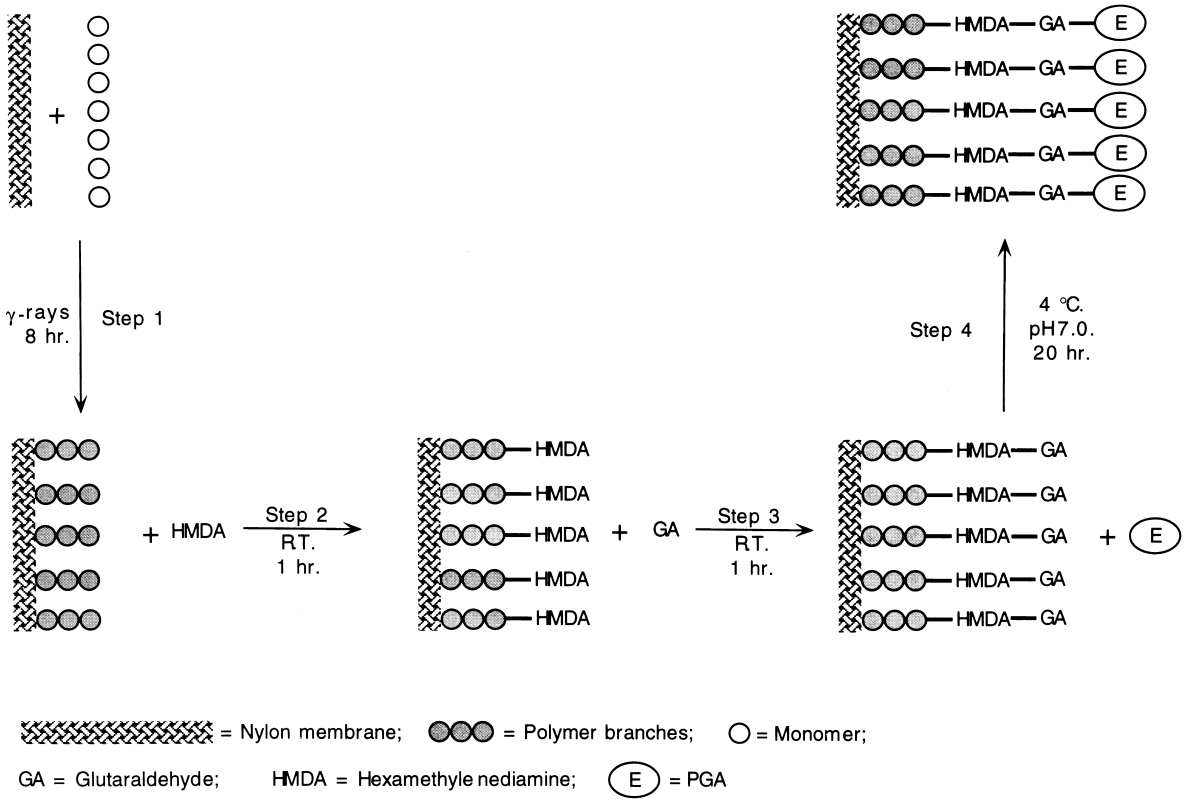

Fig. 1. Schematic illustration of the processes of activation of nylon grafted membranes and enzyme immobilization.

cesses, except when stated differently. After a further washing with water the aminoalkylated membranes were immersed, for 1 h at room temperature, in a GA 2.5% (v/v) aqueous solution. At this point the membranes were activated and ready to bind the enzyme.

2.2.3. PGA immobilization

To immobilize the enzyme, the activated membranes were immersed for 20 h at 4°C in a 0.1 M phosphate-buffer solution, pH 7.0, consisting of 10% (v/v) of the original enzyme solution. These experimental conditions were always applied, except when indicated otherwise.

After rinsing (with water) the membranes were ready for use. When not used directly, the catalytic membranes were stored at 4°C in 0.1 M phosphate-buffer solution, pH 7.0.

2.2.4. Determination of catalytic membrane activity

Catalytic membrane activity was determined by putting the membrane in a 25 ml of 0.1 M phosphate-buffer solution, pH 7.0, containing a 20 mM cephalexin concentration. Temperature, pH and cephalexin concentration were changed according to the experimental needs.

The enzyme activity is calculated using the amount of alkaline solution (0.5 N NaOH) needed to keep the cephalexin solution at the initial pH value. Membrane activity is expressed as μmole min^{-1}. Usually one experiment lasted 30 min.

Stability of the biocatalytic membranes was evaluated by analyzing their activity under the same experimental conditions every day. After 2 days, during which the membranes lost some activity, a stable condition was reached remaining unchanged for more than 2 months. Only these stabilized membranes have been used in the comparative experiments reported herein.

Under standard conditions, i.e., 25 ml of 0.1 M phosphate-buffer solution, pH 7.0 and $T =$ 30°C, containing a 20 mM cephalexin concentration, the absolute membrane activity was 1 μmole min^{-1}, corresponding to an activity of 285 μmole min^{-1} per m^2 of membrane surface.

2.2.5. Treatment of the experimental data

Every experimental point reported in the figures represents the average of three experiments done under the same conditions. The experimental errors never exceeded 5%.

3. Results and discussion

3.1. Biochemical characterization of the catalytic membranes

3.1.1. Effect of pH

Enzyme activity is markedly affected by environmental conditions, such as pH. The changes in optimum pH and pH-activity curve of immobilized enzymes with respect to free enzymes depend on the enzyme and/or the support charges. These changes are attributed to partition effects which, owing to electrostatic interactions with fixed charges on the support, cause different concentrations of charged species, such as substrate, products, hydrogen or hydroxyl ions etc., in the microenvironment of the immobilized enzyme and in the domain of the bulk solution. One of the main consequences of these partition effects is a shift in the optimum pH towards more alkaline or acid side for negatively or positively charged matrices, respectively [27]. Some authors also reported both a shift of optimum pH towards acid side and change of the pH activity profile when the enzyme immobilization occurs by a Schiff base formation [28].

In Fig. 2 the relative activity of PGA as a function of pH is reported for the free enzyme and immobilized form, either on the nylon–PDGDA or on nylon–PMMA membrane. From Fig. 2 it is evident how the free and immobilized enzyme have different pH values at which the optimum of their activity occurs. The optimum pH of the immobilized PGA is shifted

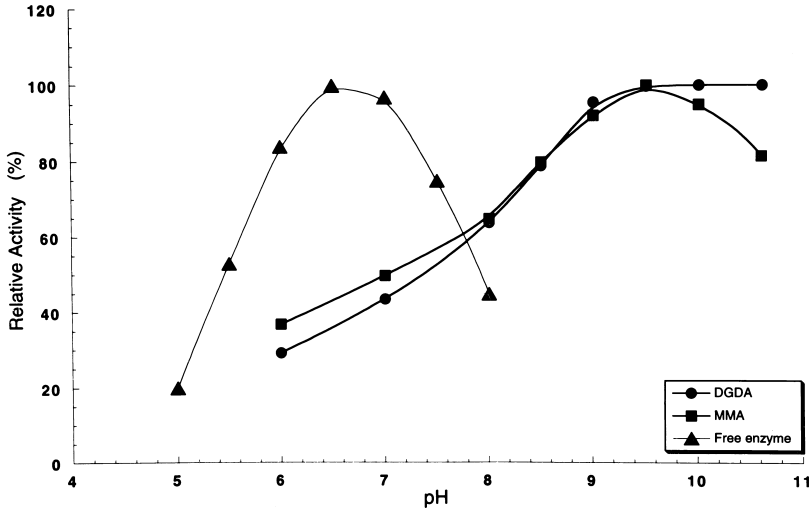

Fig. 2. Relative activity of the catalytic membrane as a function of pH at temperature = 30°C. Experimental condition for obtaining the catalytic membrane were: [DGDA] = [MMA] = 10% (v/v); [GA] = 2.5% (v/v); [HDMA] = 10% (v/v); $T_{\text{aminoalkylation process}}$ = 25°C; duration of aminoalkylation process = 1 h; [Enzyme] = 10% (v/v) of an enzyme solution of 250–300 μmole min^{-1} ml^{-1} at pH 7; [S] = 20 mM at pH = 7.

towards the alkaline side, in spite of the fact that immobilization occurs by a Schiff base formation. The optimum pH shifts about 3 pH units. The profiles of the activity–pH curves are different. While the free enzyme shows the well-known bell shape, the immobilized ones present a broader bell shape for the PGA immobilized on nylon–PMMA. A sigmoidal curve is found for PGA immobilized on nylon–PDGDA. Calling ''optimum pH range'' the range at which the relative activity is comprised between 95 and 100% this range occurs at pH = 6.1–6.9 for the free enzyme; at pH = 8.9–10.1 for the enzyme immobilized on nylon–PMMA; and at pH = 9.0–10.5 for the enzyme immobilized on nylon–PDGDA.

3.1.2. Effect of the temperature

In studying the dependence of enzyme activity on the temperature, a bell-shaped curve, with an optimum in the activity, is generally obtained. The curve for the immobilized enzyme can be broader, narrower or equal to the one of the free enzyme, while the optimum activity generally shows a shift towards higher temperatures upon immobilization. This can mean a higher resistance to enzyme thermal deactivation; this is the case when the structure of the enzyme is strengthened by the immobilization procedure. In Fig. 3 the temperature dependence of the PGA activity for the two types of catalytic membranes employed is reported. The temperature dependence of the activity of the free enzyme is also reported as reference curve. In all cases an approximately bell-shaped curve is found.

The free and immobilized enzymes exhibit a shared maximum activity at about 45°C. It is interesting to observe the marked difference between the behaviour of the enzyme immobilized on nylon–PDGDA membrane, which has a rather large temperature range in which the membrane has the optimum activity practically constant. Let us consider the ''optimum temperature range'' to be that temperature range at which the relative activity is between 95 and 100%. For free enzyme the optimum temperature range is 44–51°C, for the enzyme immobilized on nylon–PMMA 44–54°C, and for the enzyme immobilized on nylon–PDGDA 40–60°C. The nylon–PDGDA membrane is more suitable for industrial applications, especially in

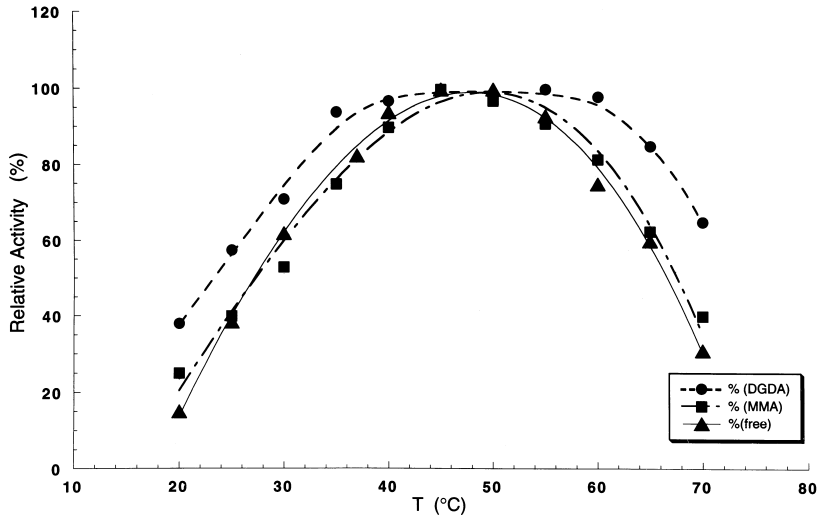

Fig. 3. Relative activity of the catalytic membrane as a function of temperature at pH = 7. Experimental conditions for obtaining the catalytic membrane were the same as in Fig. 2.

those cases in which high operational temperatures are required. With this consideration in mind, in Fig. 4 the percentage of enzyme inactivation as a function of temperature is reported. The reference temperature chosen is 45°C since at this temperature the free and immobilized enzymes have the common maximum of relative activity. From Fig. 4 it can be seen for instance, that at 70°C the active enzyme is reduced by about 70% for the free enzyme, 60% for the enzyme immobilized on nylon–PMMA membrane, and 35% for the PGA immobilized on nylon–PDGDA membrane. This interesting behaviour can be explained by considering the difference in branched structure constituted by the PMMA and PDGDA graft chains. While the

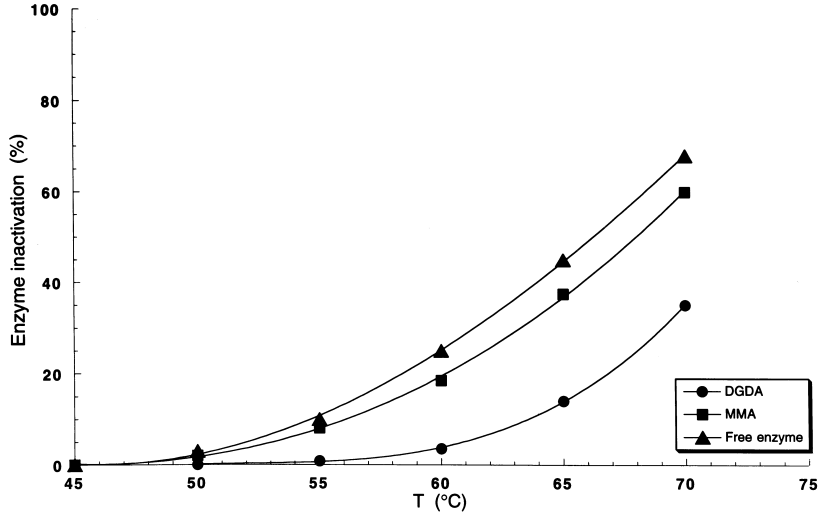

Fig. 4. Percent of enzyme inactivation as a function of temperature at pH = 7. Experimental conditions for obtaining the catalytic membrane were the same as in Fig. 2.

PMMA chains grow linearly, the DGDA monomers have two double bonds that allow the formation of branched and cross-linked polymeric structures. The latter results in a close-packed net structure, which probably limits somewhat the thermal agitation of the bound enzyme molecules, thus reducing the amount of macromolecules inactivated by the temperature increase.

3.2. Optimization of the aminoalkylation process

Direct immobilization of PGA on grafted membranes gave no appreciable enzymatic activity. This was probably due to strong interactions of the enzyme with the electrostatic charges on the nylon membrane, which alter somewhat the enzyme structure. That is why we have used HMDA as spacer to bind the enzyme far enough away from the electric field of the membrane. The aminoalkylation process in this case gives positive results since now the resulting catalytic membranes are active. For this reason we studied the yield of the catalytic membrane activity in dependence of the temperature and duration of the aminoalkylation process and of the HMDA and GA concentration.

All the experimental results reported below refer to nylon–PDGDA membranes, since these showed to be the most interesting for practical applications, as discussed above.

3.2.1. Dependence on the aminoalkylation temperature

In Fig. 5 the relative activity of the catalytic membrane is reported as a function of the temperature of the aminoalkylation process. The membranes prepared at 60°C exhibit optimum activity. The activity decrease at temperatures higher than 60°C can be explained by the fact that at these temperatures the aminoalkylation process can take place also on the nylon matrix, so that the density of the immobilized PGA molecules on the surface of the membrane increases. In spite of this increase the membrane activity decreases since, under these conditions, the occurrence of protein–protein interactions possibly reduces in fact the number of active macromolecules.

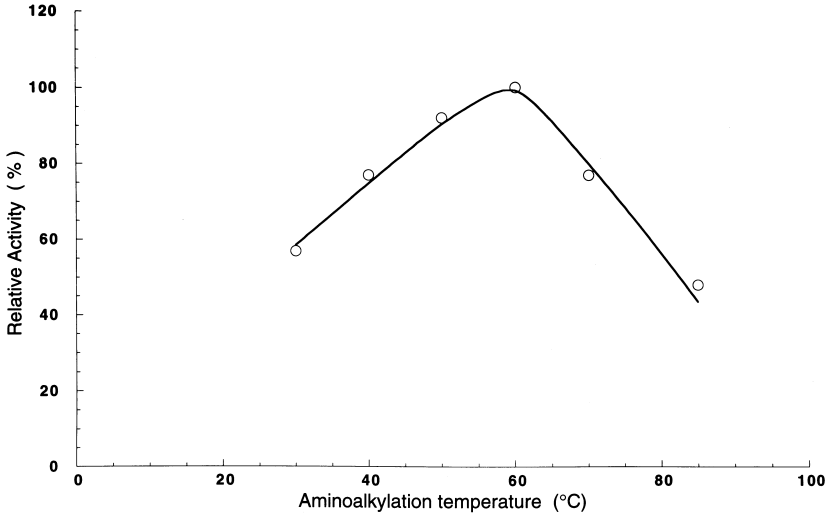

Fig. 5. Relative activity of the catalytic membrane as a function of aminoalkylation temperature. Experimental condition for obtaining the catalytic membrane were: [DGDA] = 10% (v/v); [GA] = 2.5% (v/v); [HDMA] = 10% (v/v); duration of aminoalkylation process = 1 h; [Enzyme] = 10% (v/v) of an enzyme solution of 250–300 μmole min^{-1} ml^{-1} at pH 7; [S] = 10 mM at pH. 9.5.

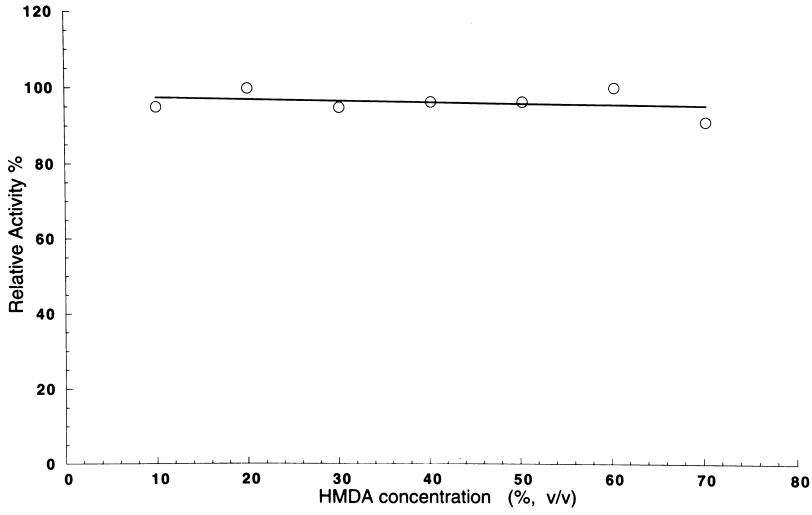

Fig. 6. Relative activity of the catalytic membrane on the HMDA concentration. Experimental conditions for obtaining the catalytic membrane were: [DGDA] = 10% (v/v); [GA] = 2.5% (v/v); $T_{\text{aminoalkylation process}} = 60°C$; duration of aminoalkylation process = 1 h; enzyme concentration is 10% (v/v) of an enzyme solution of 250–300 μmole min^{-1} ml^{-1} at pH 7; [S] = 10 mM at pH 9.5.

3.2.2. Dependence on the HMDA concentration

In Fig. 6 the relative activity of PGA immobilized on nylon–PDGDA membrane is reported as a function of the HMDA concentration used in preparing the catalytic membranes. No significant effect is observed: the activity of the catalytic membrane remains practically constant or only shows a small decrease. This may be explained considering that the 10% (v/v) HMDA concentration is sufficient to activate all the grafted polymeric branches on the membrane surface.

3.2.3. Dependence on the duration of aminoalkylation

Fig. 7 shows the effect of the duration of the aminoalkylation process on the activity of the catalytic membranes. The results indicate that

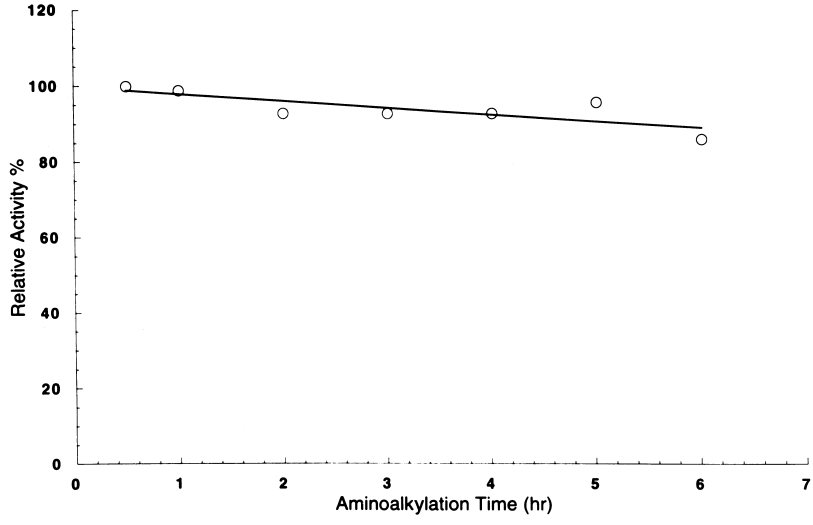

Fig. 7. Relative activity of the catalytic membrane on the duration of aminoalkylation process. Experimental conditions for obtaining the catalytic membrane were the same as in Fig. 5 with the exception of the duration of aminoalkylation.

increasing duration slightly decreases the activity of the catalytic membrane. This behaviour can be explained considering that when long reaction times are applied some aminoalkylation also occurs directly on the nylon surface, so that an increase in density of immobilized PGA can occur, with the result that possibly protein–protein interactions take place giving a slight decrease in activity.

3.2.4. Dependence on the GA concentration

The effect of GA concentration used for the activation of the grafted membranes on the activity of the catalytic membrane is shown in Fig. 8. GA concentration has no appreciable effects on the activity of the catalytic membranes. The same behaviour was observed also by other authors who immobilized PGA on acrylic copolymers [29].

3.3. Optimization of the immobilization process

Having found the best experimental conditions for the aminoalkylation process, what remains to be done for obtaining catalytic membranes giving high enzyme activities is the optimization of the immobilization process. Therefore, the most relevant factors affecting this process, namely the enzyme concentration and pH of the PGA solution used during the immobilization phase, have been studied.

3.3.1. Dependence on the enzyme concentration

The effect of the PGA concentration used during the immobilization phase on the activity of the catalytic membrane is shown in Fig. 9. Membrane activity increases with the increase of the enzyme concentration until a concentration of about 15% is reached; a further increase in enzyme concentration has no effect. These results are in agreement with those found in the literature [30].

3.3.2. Dependence on the pH of the PGA solution

In Fig. 10 the relative activity of different catalytic membranes is reported as a function of the pH of the PGA solution used in the immobilization phase. All the other experimental pa-

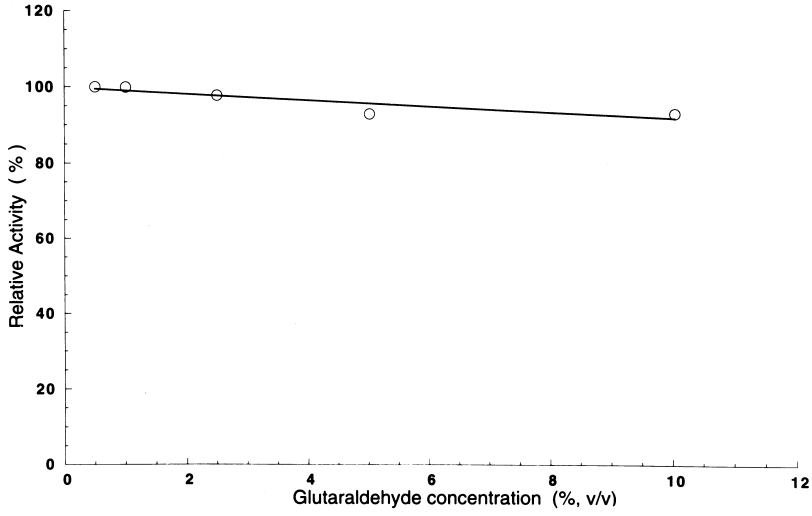

Fig. 8. Relative activity of the catalytic membrane as a function of GA concentration. Experimental conditions for obtaining the catalytic membrane were: [DGDA] = 10% (v/v); $T_{\text{aminoalkylation process}} = 60°C$; duration of aminoalkylation process = 0.5 h; [Enzyme] = 10% (v/v) of an enzyme solution of 250–300 μmole min^{-1} ml^{-1} at pH 7; [S] = 10 mM at pH 9.5.

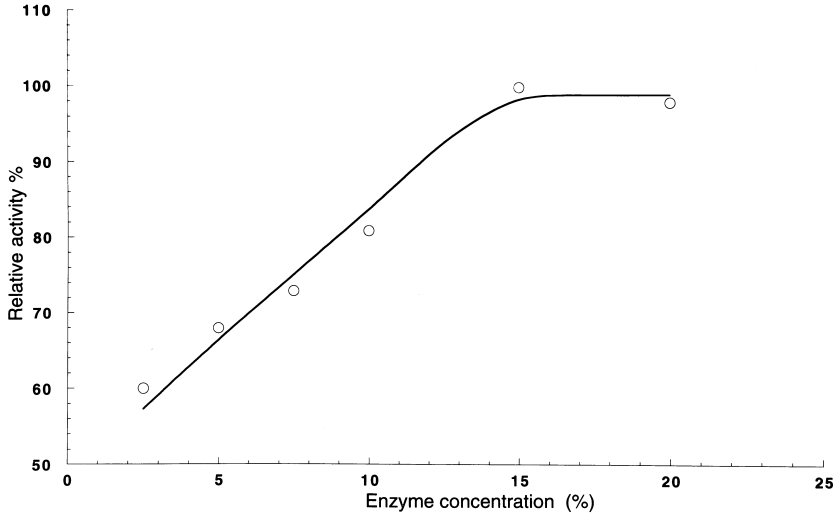

Fig. 9. Relative activity of the catalytic membrane as a function of enzyme concentration. Experimental conditions for obtaining the catalytic membrane were [DGDA] = 10% (v/v); [GA] = 0.5% (v/v); $T_{\text{aminoalkylation process}} = 60°C$; duration of aminoalkylation process = 0.5 h; [S] = 10 mM at pH 9.5.

rameters were kept constant. Before the immobilization phase the grafting percentage for each membrane was calculated. All the membranes had a grafting percentage value of $43 \pm 3\%$. Results in the figure show that the optimum membrane activity occurs using an enzyme solution at pH 7. The decrease of the membrane activity at pH values greater than this may be attributed to the instability of PGA at pH 8, as already reported by other authors [31].

3.4. Effect of the substrate concentration on the conversion

Since the enzyme should be used over longer times in industrial practice instead of measuring

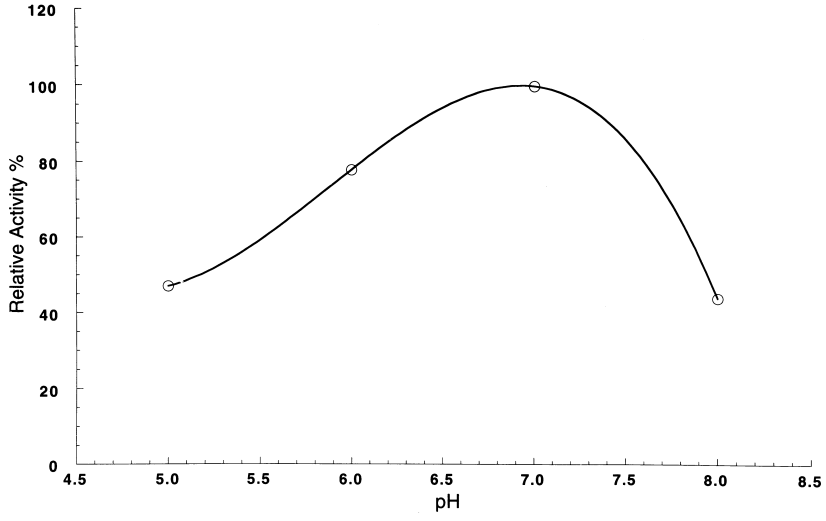

Fig. 10. Catalytic membrane activity as a function of pH of the PGA solution used for the immobilization process. Experimental conditions for obtaining the catalytic membrane were [DGDA] = 10% (v/v); [GA] = 0.5% (v/v); $T_{\text{aminoalkylation process}} = 60°C$; duration of aminoalkylation process = 0.5 h; [Enzyme] = 15% (v/v) of an enzyme solution of 250–300 μmole min^{-1} ml^{-1}; [S] = 10 mM at pH 9.5.

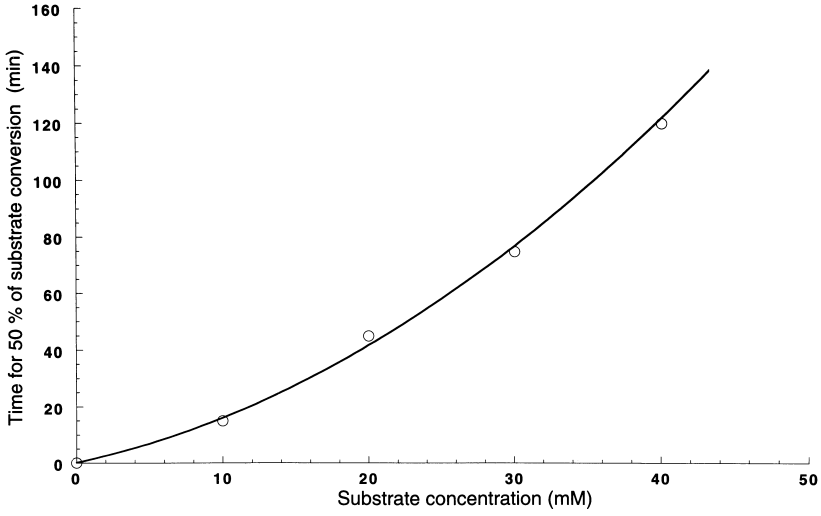

Fig. 11. Time for 50% of substrate conversion as a function of the initial substrate concentration. Experimental condition for obtaining the catalytic membrane were: [DGDA] = 10% (v/v); [GA] = 2.5% (v/v); [HDMA] = 10% (v/v); $T_{\text{aminoalkylation process}} = 25°C$; duration of aminoalkylation process = 1 h; [Enzyme] = 10% (v/v) of an enzyme solution of 250–300 μmole min^{-1} ml^{-1} at pH 7; [S] = 20 mM at pH 10.

initial activities only, we also monitored the activity over a longer period, i.e., to obtain a 50% hydrolysis of cephalexin at different concentrations (Fig. 11). These results indicate that by increasing the cephalexin concentration the time required for obtaining 50% of conversion exponentially increases.

4. Conclusions

Penicillin G acylase is immobilized onto a grafted nylon membrane. The highest yields are obtained if the membrane is treated first with a 10% (v/v) DGDA solution. This is followed by a 10% (v/v) HDMA solution treatment (at 60°C for the aminoalkylation process with duration of 0.5 h). After which a 2.5% (v/v) GA solution is applied. The last step is the application of a 15% (v/v) enzyme solution (the undiluted solution exhibiting an activity of 250–300 U/ml at pH 7).

By increasing the cephalexin concentration the times required for obtaining a 50% of substrate conversion exponentially increase. This circumstance should make our membranes not useful in industrial processes. A way for reducing the bioconversion times comes from the technology of non-isothermal bioreactors [6–13] where the reaction rate is increased linearly with the temperature difference applied across the catalytic membrane. The efficiency of the bioreactor increases from 20 to 40% when a temperature difference of 1°C is imposed across the membrane. The value of this efficiency increase depends on the enzyme and immobilization method. Experiments in this direction, using PGA immobilized on grafted nylon membranes, are reported in a different work [32].

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