Immobilization of penicillin G acylase on functionalized macroporous polymer beads

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Macroporous beaded polymers of varying pore sizes were synthesized coated with polyethyleneimine and derivatized with glutaraldehyde to generate aldehyde pendant groups. Penicillin G acylase (EC 3.5.1.11) was immobilized by covalent binding and the performance of immobilized enzyme was evaluated in batch mode. The expression was estimated as 43%. The shift in pH optima was marginal. The optimum temperature shifted from 40 to 57°C and K_m shifted from 31 to 12.8 μmol. Immobilization enhanced the thermal stability of the enzyme.

(Keywords: biocatalyst/enzyme immobilization; macroporous support; polyethyleneimine; penicillin acylase)

INTRODUCTION

Polymers with pendant amino groups have been widely used in derivatized/underivatized form as supports for immobilization of enzymes[1]. The generally accepted practice is to derivatize with glutaraldehyde to generate reactive aldehyde groups. Among the very many enzymes, penicillin acylases have received considerable attention owing to the commercial implications associated with production of 6-aminopenicillanic acid (6-APA), an important intermediate for semisynthetic penicillins[2]. A rich variety of macroporous polymeric supports have been reported for the immobilization of penicillin acylase[3,4]. In the present investigation, synthesis and modification of new hydrophilic macroporous spherical supports are described. The immobilization of penicillin G acylase on the support and the study with immobilized penicillin G acylase are presented.

EXPERIMENTAL

Materials

Hydroxyethyl methacrylate (HEMA) and divinylbenzene (DVB) were obtained from Fluka AG, Switzerland. Azobisisobutyronitrile (AIBN) was obtained from Scientific and Industrial Supplies Corporation, India. Cyclohexanol and lauryl alcohol were obtained from Loba Chemie, India. All other chemicals were of analytical grade from local suppliers. Distilled water was used as the reaction medium. *Escherichia coli* ATCC 11105 was obtained from the National Collection of Industrial Microorganisms, Pune, India.

Support preparation

Suspension polymerization was conducted in a 1 litre capacity double-walled cylindrical reactor under nitrogen blanket. The continuous phase comprised 2 wt% poly (vinylpyrrolidone) (PVP) in water. The discontinuous organic phase consisted of HEMA, DVB, polymerization initiator AIBN and a mixture of cyclohexanol and lauryl alcohol used as pore generating solvents. The ratio of aqueous to organic phase was set at 2.86:1.00. The discontinuous organic phase was introduced into the aqueous phase, stirring was started and the temperature was raised to 65 ± 0.1°C by circulating hot water. The polymerization was allowed to proceed for 3 h. The beaded polymer obtained was separated by decantation, washed with methanol and water, and dried at room temperature under vacuum. The beads passing through 40 mesh and retaining on 80 mesh (1 g) were coated under vacuum for 18 h with an aqueous solution (25 ml, 4% w/v) of branched polyethyleneimine (PEI) (*MW* 2000). The coated support was filtered, washed with glass distilled water and treated with excess of an aqueous glutaraldehyde (25% w/v) solution on a NBS shaker at a speed of 200 rpm for 2 h. The beads were filtered and washed repeatedly with distilled water till the filtrate was free from aldehyde. The beads were air dried and used for enzyme binding studies. Pore size distribution in resin was evaluated by mercury intrusion porosimetry using Quantachrome mercury porosimeter model 33.

Scanning electron microscopy

The surface morphology of crosslinked polymer beads showing the highest adsorption was examined on a Cambridge Stereoscan model 120. Standard procedures were used for preparation of samples.

Method of isolating penicillin G acylase

E. coli was grown in shake flasks at 26°C in 2% soyabean casein digest medium using 0.1 wt% phenylacetic acid as inducer. Cells were harvested after 24 h by centrifugation at 10 000*g* and stored at −20°C. Isolation and purification steps were conducted at 4°C. Cells were suspended in 0.05 M potassium phosphate buffer, pH 7.8, sonicated for 3 min in Biosonic III sonic oscillator and centrifuged at 10 000*g*. The cell debris was discarded. The supernatant was treated with streptomycin sulphate

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(1.4 g/100 ml) and the precipitate was removed by centrifugation (10 000*g*, 30 min). The clear supernatant was fractionated with ammonium sulphate. The penicillin acylase precipitating between 0.3 and 0.8 saturation was collected by centrifugation (10 000*g*, 20 min). The precipitate was dissolved in minimum volume of 0.05 M potassium phosphate buffer, pH 7.8, dialysed and clarified. The activity was estimated as described below.

Enzyme activity assay

The hydrolytic activity of soluble and immobilized penicillin G acylase preparations towards benzylpenicillin sodium salt was estimated by standard procedures. Protein was determined by the method of Lowry *et al.*[5]. One unit (U) of enzyme activity is defined as the quantity of enzyme required to liberate 1 μmol of 6-APA in 1 min under the assay conditions. Immobilized enzyme assay indicates the amount of enzyme expressed after immobilization. The percentage expressed of the bound enzyme is defined as the ratio of activity of the immobilized enzyme to that of the total enzyme bound to the support.

Immobilization of enzyme

First, 0.20 g of support (HD series, without treatment with PEI and glutaraldehyde) was weighed into a 50 ml Erlenmeyer flask and 3.0 ml of soluble enzyme in 0.1 M phosphate buffer, pH 7.5 (983 units) was added. Enzyme binding was conducted at 26°C by shaking at a speed of 200 rpm for 42 h. Similarly 0.20 g of support (HD 6, *Table 3*), derivatized with PEI and glutaraldehyde, was weighed into a 50 ml Erlenmeyer flask and 7.0 ml of soluble enzyme in 0.1 M phosphate buffer, pH 7.5 (1008 units) was added. The immobilized enzyme was filtered. The units of free enzyme as well as the protein content in the supernatant were estimated. The amount of penicillin acylase bound on the support is the difference between the units of enzyme added and that remaining in the supernatant.

Assay of immobilized enzyme

Penicillin acylase activity of immobilized enzyme (IME) was assayed at 40°C in a jacketed vessel under stirring. The assay medium was identical to that used for free enzyme. The volume of the reaction mixture was 20 ml. Aliquots of 0.1 ml were withdrawn after 15 min intervals to estimate the 6-APA formed.

RESULTS AND DISCUSSION

The HEMA–DVB macroporous beaded copolymers presented in *Table 1* were synthesized by suspension polymerization. The relative mole ratio of DVB was varied between 0.98 (HD 1) and 5.88 (HD 9) and the reactions were taken to virtual completion (>98%). The reactivity ratios of HEMA and DVB were not estimated. Since these would differ, the copolymers formed at different conversions would vary widely in compositions. The beads would differ in crosslink densities, and hence in pore size. However, global comparisons of the relative performances of the copolymers can be made. A mixed solvent system consisting of cyclohexanol (mole fraction 0.95) and lauryl alcohol (mole fraction 0.05) was used to generate the internal pores in all the copolymerizations. The average size of pores was estimated by mercury porosimetry and are presented in *Table 1*. The estimated average pore size increases with the DVB content in the copolymers. Similar observations are reported in the literature[6]. The size of the beaded particles was between 177 and 420 μm. A representative distribution is presented in *Table 2*. The surface morphology of a representative copolymer is presented in *Figure 1*. The beads are spherical (*Figure 1a*). The cross-section of

Table 1 Composition of hydroxyethyl methacrylate–divinylbenzene (HEMA–DVB) copolymers and their average pore size: poly(vinylpyrrolidone) (PVP) = 2 wt% in water; temperature = 65 ± 0.1°C; AIBN = 6.09×10^{-3} mol

Polymer code	HEMA (mole fraction)	DVB (mole fraction)	Water (ml)	Average pore size (Å)
HD 1	0.505	0.495	258	55
HD 2	0.454	0.546	265	53
HD 3	0.405	0.595	273	63
HD 4	0.358	0.642	279	59
HD 5	0.312	0.688	286	68
HD 6	0.239	0.761	292	69
HD 7	0.226	0.774	301	72
HD 8	0.185	0.815	307	77
HD 9	0.145	0.855	312	96

Table 2 Particle size distribution data of HD series

Particle size (μm)	Percentage
420	41.0
250	55.0
177	4.0

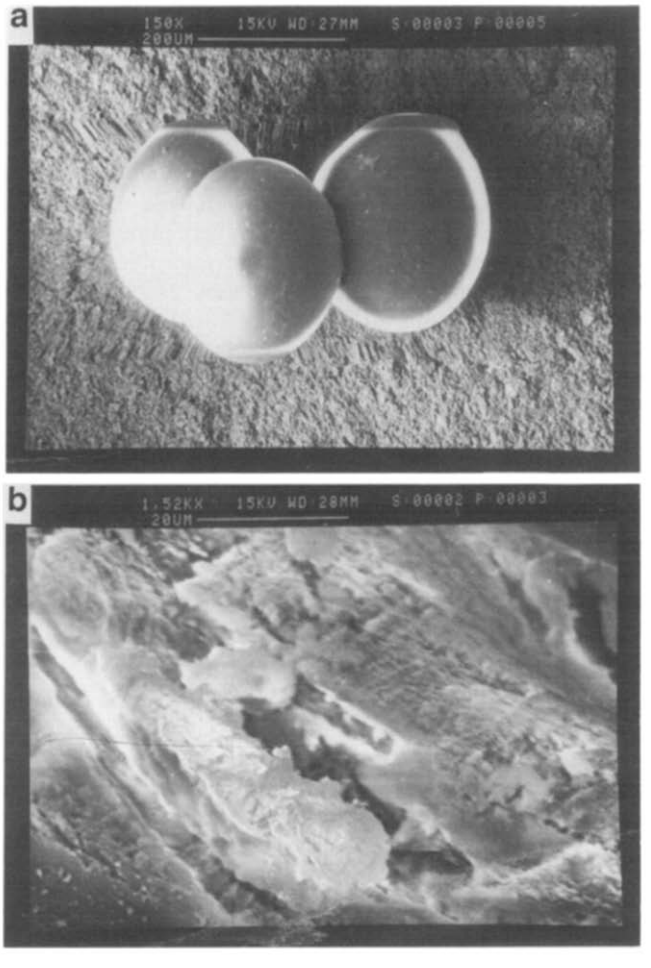

Figure 1 SEM micrograph of copolymer HD 6: (a) surface, (b) cross-section

Table 3 Percentage adsorption of penicillin G acylase on HEMA–DVB copolymers: amount of support taken = 0.20 g; total units loaded in each case = 983; total protein loaded = 76.80 mg

Polymer code	Units adsorbed	Adsorption (%)	Protein adsorbed (mg)	Protein adsorbed (%)
HD 1	6	0.61	37.8	49
HD 2	233	24	18.0	23
HD 3	420	43	33.0	43
HD 4	407	42	39.6	52
HD 5	392	40	34.8	45
HD 6	610	62	51.0	66
HD 7	212	22	43.8	57
HD 8	Nil	Nil	33.6	44
HD 9	Nil	Nil	33.0	43

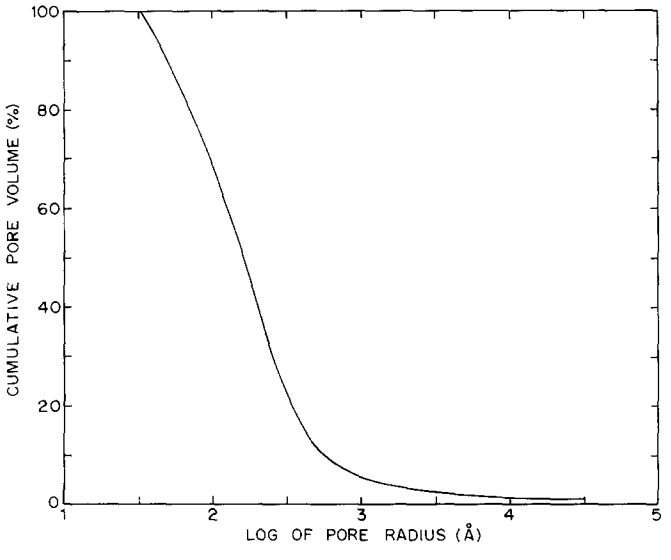

Figure 2 Pore size distribution in copolymer HD 6

the beads (*Figure 1b*) shows that the pores are present as irregular holes of varying dimensions. Penicillin G acylase was adsorbed on the copolymers under identical conditions. The results are presented in *Table 3*. Adsorption affinity increases from HD 1 to HD 6 and then falls. The factors contributing to enzyme adsorption are size of pores and hydrophobicity/hydrophilicity balance in the beads. In this series the pore size and hydrophobicity increases with DVB content, while hydrophilicity decreases. It is difficult to quantify the relative contributions of these factors to the adsorption and entrapment of enzyme within the beads. Optimal pore size is probably the most important of these factors[7]. The distribution in size of pores in copolymer HD 6 is presented in *Figure 2*. Copolymer HD 6 was used for covalent binding of penicillin H acylase by coating with polyethyleneimine and derivatizing with glutaraldehyde. Two approaches were investigated. In the first approach, the adsorbed enzyme was encased within a thin membrane on the surface of the beads by polyethyleneimine and glutaraldehyde treatments. The expression of enzyme immobilized by this technique was very poor and the approach was abandoned. The poor expression may be attributed to the inaccessibility of the substrate to the enzyme encased within the membrane. An alternative approach was adopted to overcome this difficulty. The copolymer was coated with polyethyleneimine and treated with an excess of glutaraldehyde solution to generate pendant aldehyde groups. The introduction of spacers between the support and enzyme is beneficial in minimizing the diffusional and steric effects, giving better expression for the immobilized enzyme. This is evident from the K_m of IME preparation. The K_m value towards benzylpenicillin at pH 7.8 in 0.1 M phosphate buffer is 12.8 μmol as compared to 30.1 μmol for free enzyme. An approximately 2.3-fold decrease in K_m was observed on covalent binding. Owing to the introduction of spacer in the form of glutaraldehyde, penicillin G acylase is immobilized on the surface and therefore it is freely available for catalysis. Carleysmith *et al.*[8], however, had observed enhancement in the K_m value of glutaraldehyde-modified enzyme compared to the native enzyme. The expression of this IME preparation was also good as compared to the previous approach.

Effect of pH

Immobilization marginally altered the pH optima, from 7.8 to 7.0–7.5, as presented in *Figure 3*. The broadening of the pH profile curve in the case of IME is due to the stability of IME over a wider pH range compared to the native enzyme.

Temperature effect

The effect of temperature after immobilization is shown in *Figure 4*. The optimal temperature of immobilized penicillin acylase is 57°C. The temperature–activity profiles of the native and immobilized enzyme are very distinct. Broadening of the temperature–activity profile in the case of bound enzyme near its optimum activity indicates that the bound enzyme is stable over a wider temperature range compared to the native enzyme.

Thermal stability

The temperature stability of the penicillin acylase was improved considerably after immobilization. No significant inactivation of IME was observed after incubation in 0.1 M phosphate buffer at 57°C for 2 h. The loss in initial activity was 10% vis-à-vis 95% for native enzyme.

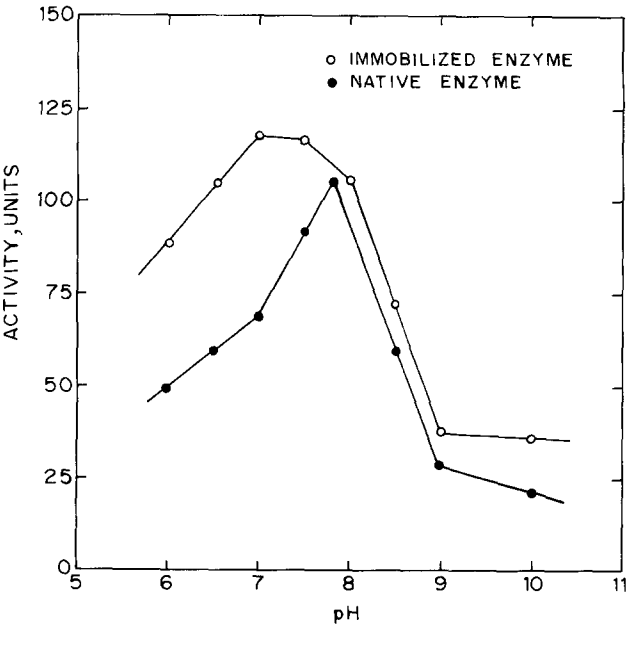

Figure 3 Effect of pH on enzyme activity of native (●) and immobilized (○) penicillin G acylase

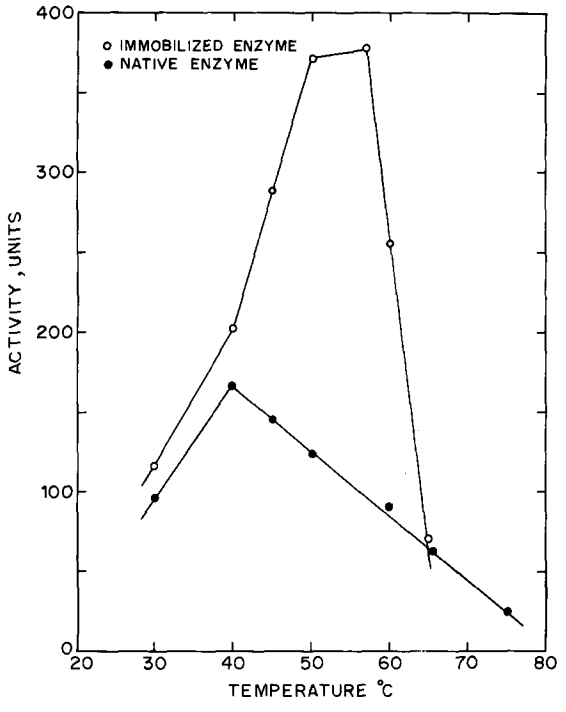

Figure 4 Temperature effect on the enzyme activity of native (●) and immobilized (○) penicillin G acylase

Operational stability

Operational stability was evaluated in a batch mode. Some 20 ml of the reaction mixture was used. The final substrate concentration was set at 4% w/v. The reaction mixture was stirred mechanically while incubating at 40°C, pH 7.8. The 6-APA formed was estimated as described earlier by withdrawing 0.1 ml aliquots of the reaction mixture at regular intervals, and adding immediately to 1.9 ml of 0.25 M citrate–phosphate buffer (CPB), pH 2.5, to arrest the reaction. After each cycle (about 95% conversion) the beads were washed thoroughly with buffer. No detectable loss in activity was observable after 32 work cycles (*Figure 5*).

CONCLUSION

The immobilization technique provides spherical beaded support having minimal diffusive limitations and amenable to easy scale-up. About 2645 units of enzyme, corresponding to about 17.5 mg of active enzyme, was bound per gram of bead. The activity expression in terms of units was 43%. The immobilized enzyme can be used repeatedly for 32 work cycles without any detectable change in the activity.

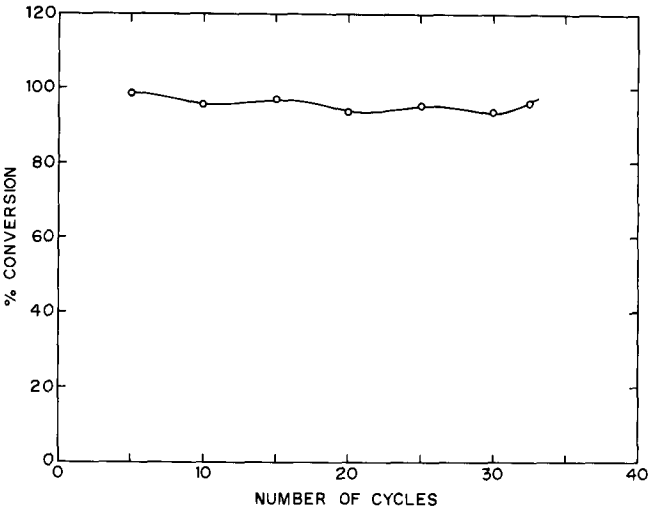

Figure 5 Operational stability of penicillin G acylase immobilized on macroporous HD 6 copolymer beads, treated with polyethyleneimine and derivatized with glutaraldehyde

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