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Immobilization of Penicillin Acylase in Porous Beads of Polyacrylamide Gel

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ABSTRACT

A procedure is described for the immobilization of benzylpenicillin acylase from *Escherichia coli* within uniformly spherical, porous polyacrylamide gel beads. Aqueous solutions of the enzyme and sodium alginate and of acrylamide monomer, N,N'-methylene-bis-acrylamide, N,N,N',N'-tetramethylethylenediamine (TEMED) and sodium alginate are cooled separately, mixed, and dropped immediately into ice-cold, buffered calcium formate solution, pH 8.5, to give calcium alginate-coated beads. The beads are left for 30–60 min in the cold calcium formate solution for polyacrylamide gel formation. The beads are then treated with a solution of glutaraldehyde and the calcium alginate subsequently leached out with a solution of potassium phosphate.

Modification of the native enzyme with glutaraldehyde results in a slight enhancement in the rate of hydrolysis of benzylpenicillin at pH 7.8 and 0.05*M* substrate concentration. The enzyme entrapped in porous polyacrylamide gel beads shows no measurable diffusional limitation in stirred reactors, catalyzing the hydrolysis of the substrate at a rate comparable to that of the glutaraldehyde-modified native enzyme.

The immobilized enzyme preparation has been used in batch mode over 90 cycles without any apparent loss in hydrolytic activity.

Index Entries: *E. coli* penicillin acylase; immobilized penicillin acylase; glutaraldehyde crosslinking within polyacrylamide beads.

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INTRODUCTION

We reported earlier a process for the entrapment of yeast cells in porous polyacrylamide gel beads and the application of the immobilized cells for ethanolic fermentation of sugars at high volumetric productivities (*1*). The procedure depends on the *in situ* polymerization of cooled acrylamide—N,N'-methylene-bis-acrylamide monomers within calcium alginate-coated beads containing pregrown yeast cells and the subsequent differential leaching out of the calcium alginate.

In the present paper we describe a modification of this procedure for the entrapment of benzylpenicillin acylase (EC 3.5.1.11) from *E. coli*. Immobilized penicillin acylase preparations find wide application in the manufacture of 6-aminopenicillanic acid (6-APA), the key intermediate in the manufacture of semisynthetic penicillins (*2*). The use of synthetic polymers of porous and spherical form for the entrapment of the enzyme could be expected to contribute both to an enhancement of the operational stability of the matrix and to the abatement of diffusional limitation.

MATERIALS AND METHODS

Chemicals

Acrylamide monomer, N,N'-methylene-bis-acrylamide and N,N,N,N'-tetramethylethylenediamine (TEMED) were obtained from Eastman Kodak Co. (Rochester, NY, USA). Sodium alginate was Protanal type LF 120 from Protan AS (Drammen, Norway). DEAE-Sepharose CL-6B was obtained from Pharmacia Fine Chemicals (Uppsala, Sweden).

Microorganism

Escherichia coli ATCC 11105 was obtained from the National Collection of Industrial Microorganisms, Pune, India.

E. coli was grown in shake flasks at 26°C in 2% soyabean—casein digest medium with 0.1% phenylacetic acid added as inducer. Cells were harvested after 24 h at 10,000 × g and stored at −20°C till required.

Enzyme Preparation

All steps were carried out at 4°C. Cells of *E. coli* were suspended in 0.05*M* potassium phosphate buffer, pH 7.8, and sonicated for a total period of 3 min using a Biosonic III sonic oscillator. The sonicate was centrifuged at 10,000 × g and the cell debris was discarded. The supernatant was stirred and treated with streptomycin sulfate (1.4 g/100 mL) and the precipitate removed by centrifugation (10,000 × g, 30 min). The clear supernatant was fractionated with ammonium sulfate and the penicillin acylase activity that precipitated between 0.3 and 0.8 saturation was col-

lected by centrifugation (10,000 × g, 20 min). The precipitate was dissolved in the minimum vol of 0.05*M* potassium phosphate buffer, pH 7.8, and dialyzed overnight against 100 vol of buffer of the same composition. The dialysate was clarified by centrifugation and the clear supernatant loaded on a DEAE-Sepharose CL-6B column (2.5×30 cm) equilibrated with 0.01*M* potassium phosphate buffer, pH 7.5. The enzyme was eluted out of the column with the same buffer. Fractions containing penicillin acylase activity were pooled, concentrated, and stored at −20°C.

Solutions of the enzyme were assayed for hydrolytic activity toward benzylpenicillin sodium salt at 37°C essentially as described by Balasingham et al. (*3*) using a test system containing 0.05*M* benzylpenicillin sodium salt in 0.1*M* potassium phosphate buffer, pH 7.8, and *p*-dimethylaminobenzaldehyde for assay of 6-APA formed (*4*). One unit (U) of enzyme activity is defined as that required for catalyzing the hydrolysis of 1 μmol substrate in 1 min. Protein was determined by the method of Lowry et al. (*5*) using crystalline bovine serum albumin as standard.

The enzyme preparation had a specific activity of 15 U/mg protein after the final purification step. This preparation was used for immobilization and characterization.

Enzyme Immobilization

An aqueous solution containing acrylamide 15% (w/v), N,N'-methylene-bis-acrylamide, 0.8% (w/v), sodium alginate 0.5% (w/v), and TEMED 1% (v/v) and a solution of the enzyme (50 U/mL) and sodium alginate 0.5% (w/v) were separately cooled in an ice bath and pumped at equal flow rates into a cooled mixing chamber through a double-walled tubular device similar to that described earlier (*1*). The enzyme and the acrylamide monomer solutions were mixed rapidly and remained in contact only for a very short period of time before passing into a gently stirred solution containing calcium formate (3% w/v), ammonium persulfate (0.5% w/v) in 0.05*M* sodium tetraborate buffer, pH 8.5, maintained at 4°C. The calcium alginate-coated beads that formed were left in the buffered calcium formate solution for 0.5–1 h for polymerization of the acrylamide. The beads were then removed by pouring through a nylon net and suspended for 1 h in 1 vol of 1% (w/v) glutaraldehyde prepared in 0.85% NaCl solution for intermolecular crosslinking of the protein. Calcium alginate was then leached out by washing with 0.05*M* potassium phosphate buffer, pH 7.8, till the washings were clear. The beads thus obtained were uniformly spherical and about 1.5–2.0 mm in diameter.

Immobilized enzyme preparations were assayed for penicillin acylase activity in stirred, water-jacketed vessels maintained at 37°C. The assay medium was similar to that used for the soluble enzyme. The final vol of the assay mixture was 10 mL. Aliquots (10 μL) were withdrawn at 15-min intervals and the 6-APA formed was determined with *p*-dimethylaminobenzaldehyde as described earlier.

Table 1
Comparative Kinetics of *E. coli* Penicillin Acylase Preparations

Preparation	V_{max} (U/mg protein)	K_m (μM)
Native enzyme	15.0	30.1
Glutaraldehyde-modified soluble enzyme	16.5	18.9
Glutaraldehyde-modified polyacrylamide gel entrapped enzyme	16.5	20.0

Operational Stability

The operational stability of the immobilized penicillin acylase preparation was determined in repeated cycles of use. The beads with immobilized penicillin acylase (50 U) were suspended in a final vol of 50 mL of 0.1*M* potassium phosphate buffer, pH 7.8, containing 40 mg/mL of sodium salt of benzylpenicillin in a stirred, water-jacketed reaction vessel maintained at 37°C. Aqueous ammonia (1.2*N*) was added from a microburette to maintain pH at 7.8. After every complete conversion (95–100%), the beads were washed thoroughly free of product on a nylon net and 6-APA was determined in filtrate and washings. The beads were reused repeatedly and the initial rates of hydrolysis were used as a measure of the residual activity.

RESULTS

Modification of Native Enzyme with Glutaraldehyde

Preliminary experiments were carried out to determine the effect of glutaraldehyde on the native enzyme. An aqueous solution of the enzyme (10 U/mL) was treated with glutaraldehyde (1% w/v in 1 mL final vol) for 1 h at 25°C and the excess reagent removed by exhaustive dialysis. The resulting pale yellow opalescent solution of modified enzyme showed a slightly enhanced hydrolytic activity toward 0.05*M* benzylpenicillin at pH 7.8, compared to the native enzyme (Table 1). This finding is in keeping with an earlier report. Carleysmith et al. (*6*) had observed that treatment of penicillin acylase from *E. coli* with glutaraldehyde yields a modified enzyme that shows a shift in the substrate-hydrolytic activity profile toward higher concentrations of benzylpenicillin such that at pH 8.0 and benzylpenicillin concentrations above 0.01*M*, the modified enzyme showed slight enhancement in activity compared to the native enzyme.

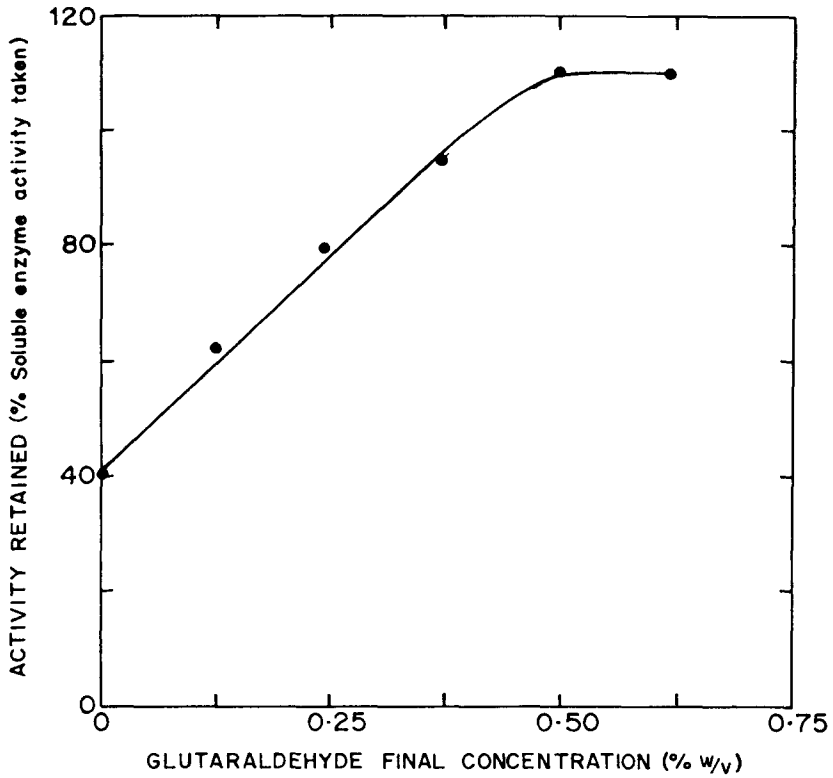

Fig. 1. Effect of glutaraldehyde concentration on crosslinking of penicillin acylase within polyacrylamide beads. The calcium alginate coated polyacrylamide beads were treated for 1 h at 4°C with an equal vol 0.85% NaCl solution containing glutaraldehyde at concentrations varying from 0–1.25% w/v. Activity retained within beads after exhaustive leaching with 0.05*M* potassium phosphate buffer, pH 7.8, is expressed as % of activity of the native enzyme taken for entrapment.

Crosslinking of Entrapped Enzyme

Conditions were optimized for the crosslinking of enzyme entrapped in polyacrylamide gel such that no activity was lost on leaching of the calcium alginate-coated polyacrylamide beads with potassium phosphate buffer, pH 7.8. The effects of treatment with glutaraldehyde solutions of concentrations varying from 0–0.625% w/v is shown in Fig. 1.

It can be seen from Fig. 1 that at 0.5% final concentration of glutaraldehyde, the enzyme activity in the beads after differential leaching with phosphate buffer is comparable to that of an equivalent amount of the soluble enzyme modified with glutaraldehyde.

Kinetic Properties of Immobilized Enzyme

Table 1 compares some of the kinetic properties of penicillin acylase entrapped and fixed by glutaraldehyde treatment in porous polyacrylamide

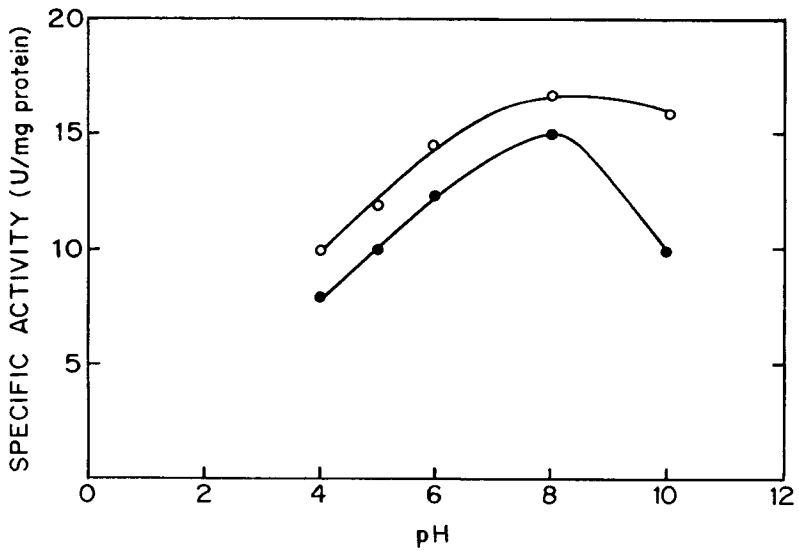

Fig. 2. Effect of pH on initial rates of hydrolytic activity of *E. coli* penicillin acylase toward 0.05*M* benzylpenicillin at 37°C. 0.1*M* citrate-phosphate buffer (pH 4–6), 0.1*M* potassium phosphate buffer (pH 7–8), 0.1*M* Tris glycine buffer (pH 10). (—●—) Native enzyme; (—○—) enzyme entrapped in porous acrylamide gel and modified with glutaraldehyde.

gel beads with those of the native enzyme and the soluble enzyme modified with glutaraldehyde. As stated earlier, the V_{max} values of both glutaraldehyde-modified soluble enzyme and of the modified enzyme entrapped in polyacrylamide gel are about 10% higher than that of the native enzyme preparation. The K_m value toward benzylpenicillin at pH 7.8 in presence of 0.1*M* potassium phosphate is 30.1 μM in the case of the native enzyme, compared to corresponding values of 18.9 μM and 20 μM for the glutaraldehyde-modified soluble enzyme and the modified enzyme entrapped in porous polyacrylamide gel. Carleysmith et al. (*6*), however, had reported enhancement in the K_m value of glutaraldehyde-modified enzyme compared to the native enzyme.

Although both native and entrapped enzyme have an optimum pH of 7.8 for hydrolytic activity, the pH activity profiles are distinct, the entrapped enzyme showing a broadening near its optimum activity (Fig. 2).

The temperature stability of the immobilized enzyme is markedly better than that of the native enzyme; incubation at 50°C for 1 h in 0.1*M* potassium phosphate buffer, pH 7.8, at a protein concentration of 1.67 mg/mL gel resulting in only 10% loss of initial activity in the case of the entrapped enzyme compared to a loss of 90% of initial activity of the native enzyme at the same concentration in the buffer of pH 7.8.

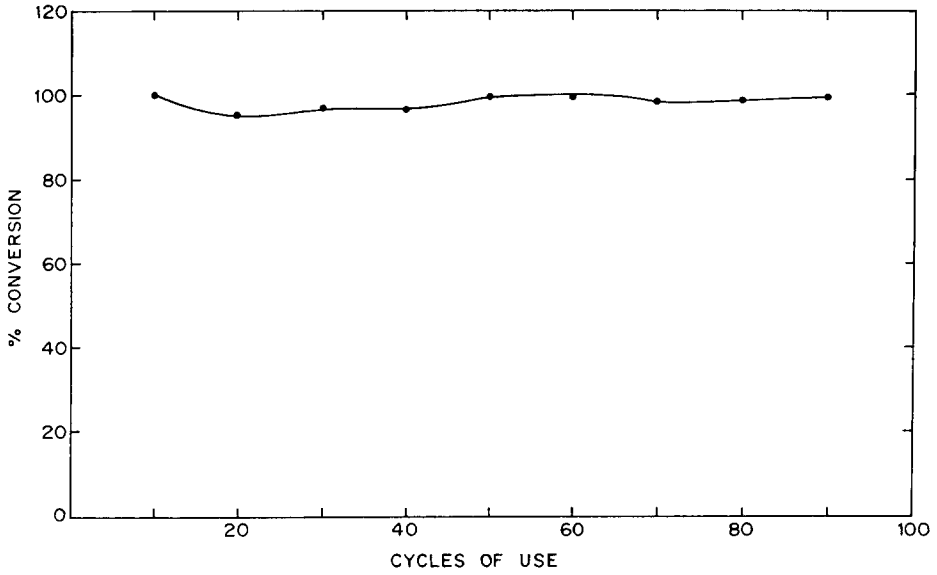

Fig. 3. Operational stability of *E. coli* penicillin acylase entrapped in porous polyacrylamide beads modified with glutaraldehyde. Beads were reused in successive cycles of batch operations after completion of 95–100% hydrolysis of benzylpenicillin Na salt 4% (w/v) at 37°C and pH 7.8. The activity was determined from initial rates of hydrolysis.

Operational Stability

The performance of the entrapped enzyme in repeated reuse is shown graphically in Fig. 3. The immobilized enzyme preparation showed no detectable loss in activity even after 90 successive cycles of use.

DISCUSSION

The entrapment of enzymes within gel particles of relatively large size has the attendant disadvantage of diffusional limitation despite the ease in handling and recovering such systems. The effect of particle size of Amberlite XAD7, a macroporous resin carrier, on the efficiency of benzylpenicillin acylase covalently bound to the carrier with glutaraldehyde has been described by Carleysmith et al. (*6*). The rate of deacylation of benzylpenicillin catalyzed by the immobilized enzyme was shown to increase when the support was ground from its original 1 mm diameter spherical form to particles of 15 μm mean diameter, the ground preparation showing the same efficiency as the native enzyme. In the present

studies benzylpenicillin acylase entrapped and crosslinked with glutaraldehyde within porous spherical polyacrylamide beads of 1.5–2 mm diameter have been found to have slightly enhanced hydrolytic activity compared to the native enzyme but comparable to that of the glutaraldehyde-modified soluble enzyme. The porous structure of the gel beads could be expected to alleviate diffusional limitation.

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