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Immobilization of *Kluyvera citrophila* penicillins acylase on controlled-pore ceramics

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Abstract. Penicillin acylase was purified from *Kluyvera citrophila* and immobilized on glutaraldehyde derivatives of silanized controlled-pore ceramics. The behaviour of the enzyme attached to TiO_2, Al_2O_3 and SiO_2 in the hydrolytic reaction are compared with that of the native enzyme as well as of the enzyme bound to CNBr-activated Sepharose 4B. The enzyme immobilized on TiO2 shows an efficiency of about 95% on the basis of protein bound. The penicillin acylase attached to SiO_2, unlike the enzyme immobilized on TiO_2, Al_2O_3 and Sepharose looses activity markedly in every cycle of use.

Keywords. *Kluyvera citrophila;* penicillin acylase; controlled-pore ceramics; immobili zation; 6-aminopenicillanic acid;

Introduction

Penicillin acylase (penicillin amidohydrolase, EC 3·5·1·11 also known as penicillin amidase), catalyses the deacylation of penicillins with the formation of 6-aminopenicillanic acid (6-APA) and the side chain. The immobilized enzyme finds industrial application in the manufacture of 6-APA required for the production of semisyn thetic penicillins and several methods have been described in the literature for the attachment of the enzyme to solid supports (Abbott, 1976; Subramanian *et al.,* 1978; Park *et al.,* 1982; Savidge, 1984; Lowe, 1985). These procedures include adsorption on carriers, inter-molecular crosslinking, entrapment in gels and covalent attachment to polysaccharide carriers and to synthetic polymers.

The present paper reports the immobilization of penicillin acylase from *Kluyvera citrophila* by covalent attachment to silanized derivatives of controlled-pore ceramics of titania, alumina and silica. The properties of the enzyme attached to the inorganic carriers are compared with those of the native enzyme and of the enzyme attached covalently to Sepharose. The *K. citrophila* enzyme, like most of the other penicillin acylases of bacterial origin, preferentially hydrolyses benzylpenicillin (Shimizu *et al.,* 1975).

Materials and methods

Benzylpenicillin, Phenoxymethylpenicillin, ampicillin, 6-APA and streptomycin sulphate were obtained from Hindustan Antibiotics Ltd., Poona.

DL-Phenylglycine methyl ester was prepared by esterification of the DL-acid as described by Greenstein and Winitz (1961). Crystalline bovine serum albumin (BSA) and other marker proteins were obtained from Sigma Chemical Co., St. Louis,

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Abbreviations used: 6-APA, 6-Aminopenicillanic acid; BSA, bovine serum albumin; M_r , molecular weight; SDS, sodium dodecyl sulphate; PAGE, Polyacrylamide gel electrophoresis.

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Missouri, USA. Sephadex G-200 and Sepharose 4B were from Pharmacia Fine Chemicals, Sweden. DEAE-cellulose was chromatography grade DE-52 from Whatman Ltd., UK.

The following controlled-pore glass and ceramics were gifts from Corning Glass Works, USA; SiO_2 (pore diameter 425 Å; surface area 40 m^2/g, 45-80 mesh), TiO_2 (Lot 2511-T, pore diameter 560 Å; surface area $10m^2/g$, 30-45 mesh), Al_2O_3 (Lot 2011-A, pore diameter 225 Å; surface area $90m^2/g$, 30-45 mesh). The inorganic supports were cleaned before use by boiling with 5% nitric acid for 45 min followed by cooling, washing with distilled water and drying at 115°C for 24 h.

Cyanogen bromide and glutaraldehyde were obtained from Fluka, Switzerland and γ-aminopropyltriethoxysilane was obtained from Pierce Chemical Co., USA.

Growth of organism

K. citrophila ATCC 21285 was obtained from the American Type Culture Collection. The culture was maintained on nutrient agar medium and subcultured every month. For obtaining cells required for the isolation of penicillin acylase the following growth medium was used: beef extract, 10 g; Difco peptone, 10 g; NaCl, 5 g; phenyl-acetic acid, 2·04g; and water to make 1 L after adjustment of pH to 7·2 with 1 N NaOH. The organism was grown in shake flasks for 48 h at 30°C and cells were harvested by centrifugation.

Extraction of cells

Wet cells suspended in 0·1 M potassium phosphate buffer, pH 7·5 (10 ml/g) were cooled in an ice bath and sonicated for 2 min in the Biosonic III Sonic oscillator (Bronwill Scientific Co., USA). The cell debris was removed by centrifugation at 15000 *g* for 20 min at 4°C.

Purification of penicillin acylase

The initial steps in the purification procedure were essentially similar to those used by Shimizu *et al.* (1975). All operations were carried out at 0-4°C. Batches of 100 ml each of the extract of sonicated cells were processed at a time. The extract was treated with streptomycin sulphate (1·4 g) under constant stirring and precipitated nucleic acids were removed by centrifugation at 50,000 *g* for 30 min.

The supernatant from the streptomycin step was stirred and fractionated with powdered ammonium sulphate. Penicillin acylase activity which precipitated mainly between 0·45 and 0·9 saturation was collected by centrifugation and dissolved in 0·01 M potassium phosphate buffer, pH 7·5 (17 ml) and dialyzed against 1 L of the buffer of the same concentration for 16 h with 3 changes. The dialyzed ammonium sulphate fraction was centrifuged at 10,000 *g* for 20 min and the clear supernatant was loaded on a DEAE-cellulose column (1× 28cm) equilibrated with 0·01 M potassium phosphate buffer; pH 7·5. The column was eluted with 250 ml of 0·01 M potassium phosphate buffer, pH 7·5 at a flow rate of about 50 ml/h and the fractions with penicillin acylase activity (30-90 ml eluate fractions) were pooled and concen-

trated by ultrafiltration through a PM-10 membrane to a final volume of about 3-4 ml.

The concentrated enzyme solution was loaded on Sephadex G-200 (2 × 100 cm) equilibrated with 0·05 M potassium phosphate buffer, pH 7·5 and eluted with buffer of the same composition at a flow rate of 4 ml/h and fractions of 1 ml volume were collected, and fractions containing enzyme activity were pooled and stored at - 20°C.

Estimation of molecular weight

The molecular weight (M_r) value of the enzyme was estimated by gel filtration through Sephadex G-200 (2 × 100 cm) column standardized with catalase (250,000), BSA (68000), ovalbumin (45000), myoglobin (17500) and cytochrome *c* (13000). Subunit M_r was determined by sodium dodecyl sulphate (SDS)-polyacrylamide gel electrophoresis (PAGE) in 7·5% gels (Weber and Osborn, 1969) using myoglobin, ovalbumin and BSA monomer and dimer as markers.

Assay of enzyme activities

Soluble enzyme: The assay for penicillin acylase activity was done essentially as described by Balasingham *et al.* (1972). The test system contained 2% w/v substrate in 0·1 M potassium phosphate buffer, pH 7·5. Enzyme activity measurements were made at 40°C by determining 6-APA formed with *p*-dimethylaminobenzaldehyde according to the procedure described by Bomstein and Evans (1965). One unit of enzyme activity is defined as the amount of enzyme catalysing the hydrolysis of 1 μmol of substrate in 1 min under assay condition.

ß-Lactamase activity was checked by the micro-iodometric procedure described by Ross and O'Callaghan (1975).

Immobilized enzyme: Preparations were assayed in stirred, water-jacketed vessels maintained at 40°C. The assay medium was similar to that used for the soluble enzyme. The final volume of the reaction mixture was 50 ml. Aliquots were withdrawn at 15 min intervals and rapidly filtered off through a sintered glass filter under suction and the 6-APA in the filtrate was assayed with *p*-dimethylamino-benzaldehyde as described earlier.

Enzyme kinetics: The K_m for benzylpenicillin was calculated from the double reciprocal plots by least squares analysis. Studies on the temperature dependence of activity where in the temperature range of 25°-40°C. The initial reaction rates (v) were measured as described under assay of enzyme activity. The activation energy was calculated from $\log_{10} v$ *versus* $1/T$ plots.

Protein assay

Protein was determined by the method of Lowry *et al.* (1951) at 500 nm using crystalline BSA as standard.

Immobilization of penicillin acylase on CNBr-activated Sepharose

Sepharose 4B was activated with CNBr using 1 M N a_2 CO_3 for controlling the pH during activation as recommended by March *et al.* (1974).

One volume of an aqueous slurry of washed Sepharose 4B beads (1:1 w/v) was treated with 2 M Na_2CO_3 (1·5 volume). An aqueous solution of CNBr (100 mg/ml; 0·5 volume) was added to the beads and the mixture agitated gently for 10 min at 18°-22°C. The mixture was then cooled with addition of crushed ice. The activated beads were washed rapidly on a sintered glass filter with ice cold 0·1 M $NaHCO_3$ containing 0·17 M NaCl (25 volumes). Filtration and washing were completed within 10 min by applying gentle suction. The washed beads were immediately added to an ice cold solution of the purified enzyme in 0·05 M potassium phosphate buffer, pH 7·5 (0·4 mg protein/ml; 3-4 U/g wet beads). The beads were gently agitated for 16 h at 0°-4°C. The beads were then filtered off and washed with cold coupling buffer. The pH of the filtrate was approximately 8 at the end of the coupling reaction. The filtrate and washings were separately tested for activity and protein. Any unreacted, activated groups on the support were masked by treatment with 1 M ethanolamine at pH 8 at 0°-5°C for 1 h. The beads were finally filtered off, washed with 0·1 M potassium phosphate buffer, pH 7·5 (10 volumes) and stored at 0°-4°C in the same buffer.

Controlled pore glass and ceramic supports

Enzyme was covalently bound to the surface of the inorganic carriers through silanization with γ -aminopropyltriethoxysilane followed by coupling of the protein with the use of glutaraldehyde according to the procedure described by Weetall (1976).

Preparation of alkylamine carrier: The cleaned dry carrier (1 g) was treated with 10% (w/v) γ-aminopropyltriethoxysilane dissolved in toluene (75 ml) and refluxed for 16 h. The mixture was then cooled and the carrier filtered off, washed thoroughly first with toluene and then with acetone and air dried.

Preparation of glutaraldehyde derivative of alkylamine carrier: The alkylamine carrier (1 g) was covered with 2·5% aqueous glutaraldehyde solution (10 ml) and agitated with a teflon-coated magnetic stirring bar for 1 h at room temperature. The carrier was then filtered off on a Buchner funnel and washed with distilled water to remove excess of glutaraldehyde.

Coupling of enzyme to the aldehyde carrier: The aldehyde derivative of the carrier (1 g) was treated with the enzyme solution (5 ml containing 5 mg protein and about 30 U activity in 0·1 M potassium phosphate buffer, pH 7·5). The mixture was stirred for 1 h at room temperature. The carrier was filtered off and washed with 0·1 M potassium phosphate bluffer, pH 7·5. The filtrate and washings were pooled and assayed for enzyme activity.

Hydrolytic activity of immobilized enzyme

The operational stability of immobilized penicillin acylase preparations was determined in repeated cycles of use at 40°C and a substrate concentration of approximately 2% benzylpenicillin K-salt taken in a 10 ml capacity water jacketed glass reaction vessel provided with a combination glass electrode probe for monitoring pH and a teflon-coated magnetic stirring bar for continuous agitation. 9 ml reaction mixture containing 200 mg benzylpenicillin K-salt was treated with an adequate amount of immobilized enzyme for complete hydrolysis of the substrate in 2-3 h. The pH was maintained at 7·5 with addition of 0·5-1·0 M aqueous ammonia solution dispensed from a microburette. The progress of reaction was monitored by assay of 6-APA in samples withdrawn periodically. The completion of hydrolysis was apparent from the cessation of proton release. After the completion of reaction, the immobilized enzyme was filtered of and washed with water till washings were free from 6-APA. Determination of 6-APA in filtrate and washings established 95-100% hydrolysis of the substrate.

Aliquots of the immobilized enzyme were assayed for enzyme activity after set cycles of use.

Synthetic activity in ampicillin production from 6-APA and phenylglycine methyl ester

Synthetic activity was followed essentially as described by Takasawa *et al.* (1972). The reaction mixture (6·5 ml) contained 6-APA, 50 mg; DL-phenylglycine methyl ester HCl, 125 mg; potassium phosphate buffer, 0·03 M, final pH adjusted to 6·5 with 1 N NaOH. This system was maintained at 40°C. Reaction was initiated with addition of the purified enzyme immobilized on controlled-pore TiO_2 (8 U, 250 mg) and the mixture stirred continuously. Aliquots were withdrawn hourly for assay of 6-APA using *p*-dimethylaminobenzaldehyde. Neither phenylglycine methyl ester nor ampicillin gave any colour with the reagent and their presence did not interfere in the assay. Aliquots were also taken for characterization of the product by reversed phase thin layer chromatography on silica gel (Biagi *et al.,* 1969).

Results

Purification of K. citrophila penicillin acylase

The results obtained in a typical batch are summarized in table 1.

The overall purification was about 50-fold with a recovery of about 25 % of initial activity. The specific activity of the final preparation towards benzylpenicillin was 8 U/mg. Shimizu *et al.* (1975) have reported a value of about 14·5 U/mg for the enzyme purified from *K. citrophila* (KY 7844).

Properties of K. citrophila penicillin acylase

Disc electrophoresis of the Sephadex fraction carried out in 7·5% polyacrylamide at pH 8·3 according to Davis (1964) showed the presence of 3 bands (figure 1A).

Table 1. Purification of penicillin acylase from *K. citrophila.*

Purification step	Volume (ml)	Total activity (U)	Total protein (mg)	Specific activity (U/mg)	Yield (%)
Cell free extract	100	120	750	0·16	100
Ammonium sulphate fraction (0·45–0·90 sat.) dissolved in 0·01 M potassium phosphate buffer, pH 7·5 and dialysed	42	83	453	0·18	69
DEAE-Cellulose fraction concentrated by ultrafiltration	4	52	22	2·4	43
Sephadex G-200 fraction	5	30	3·8	8·0	25

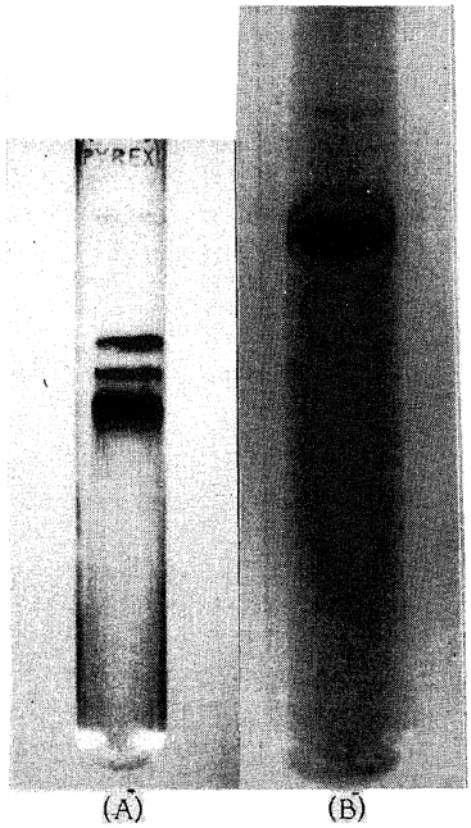

Figure 1. PAGE of *K. citrophila* penicillin acylase. (A), 7% Polyacrylamide gel; Tris glycine buffer, pH 8·3; 4 mA/tube; 1·5 h; 4°C; Protein loaded 100 μg; (B), SDS polyacrylamide gel; 0·1 M sodium phosphate buffer, pH 7·3; SDS, 0·1%; 8 mA/tube; 4h. Total protein loaded, 100 μg.

Segments cut out from the unstained gel when assayed for penicillin acylase activity showed that all the 3 components were active. SDS-PAGE showed almost exclusively a single band of M_r 67000 (Figure 1B). Shimizu *et al.* (1975) have purified the enzyme from another strain of *K. citrophila* (KY 7844) and reported the presence

of only a single component on isoelectric focusing. The reasons for the multiple components obtained in the present work are not clear. Such heterogeneity in peni cillin acylases has been reported for the enzymes obtained from other bacterial sources. Vandamme and Voets (1975) have reported the presence of 2 active components in the enzyme purified from *Erwinia aroideae.* Kashe *et al.* (1984) have separated atleast 5 active components from the enzyme purified from *Escherichia coli.*

The M_r estimated by gel filtration was approximately 60000 which is in agreement with the reported value of 63000±3000 for the enzyme from this source (Shimizu *et al.,* 1975). The optimum pH for the cleavage of benzylpenicillin was 7·5 in agreement with the value reported by Okachi *et al.* (1972). The K_m value for benzylpenicillin was found to be 2·7 mM. The calculated activation energy was 10·9 Kcal/mol corresponding to a temperature coefficient Q_{10} of 1·85 for the hydrolysis of benzylpenicillin.

The rates of hydrolysis of Phenoxymethylpenicillin and ampicillin were 10% and 40%, respectively of the rate with benzylpenicillin (table 2).

Table 2. Relative rates of hydrolysis of penicillins by

Substrate	Relative rates of hydrolysis (%)
Benzylpenicillin	100
Phenoxymethylpenicillin	10
Ampicillin	40

Substrates of 0·1 M; 0·1 M potassium phosphate buffer, pH 7·5; temperature 40°C. Relative rates calculated from initial rates of hydrolysis.

The enzyme was stable when stored at - 20° C and no detectable loss in activity was observed even after a period of 3 months.

Immobilization of penicillin acylase

Data on immobilization of the purified enzyme from *K. citrophila* on CNBr-activated Sepharose 4B and silanized controlled-pore inorganic supports are summarized in table 3.

It is seen from table 3 that protein is quantitatively bound to CNBr-activated Sepharose under the conditions used, the immobilized enzyme showing, however, an efficiency of only 67% on the basis of the activity calculated from the bound protein. This could be due to diffusional restrictions within the gel matrix. Inactivation on chemical coupling is unlikely since penicillin acylase from *E. coli* coupled to CNBr-activated cellulose shows 100% efficiency, the support in this case being non-porous (Subramanian *et al.,* 1978). Under appropriate conditions, a high proportion of the activity (90%) is also picked up from solution by the glutaraldehyde derivative of the silanized controlled-pore TiO_2. In the case of enzyme immobilized on TiO_2, the efficiencies of 93% and 97% would indicate that diffusional restriction effects are

Table 3. Immobilization of *K. citrophila* penicillin acylase.

Supports (initial amount taken)	Soluble enzyme total activity		Immobilized enzyme		
	Amount taken (U)	Amount recovered in filtrate + washings (U)	Activity[a] bound (%)	Specific[b] activity (U/g)	Efficiency (%)
Sepharose 4B (3 g wet wt.)	10	0	100	55[c]	67
TiO_2 (100 mg)	6·8	3·3	52	32	97
TiO_2 (500 mg)	16·6	1·5	90	28	93
Al_2O_3 (100 mg)	6·8	3·6	47	25	78
SiO_2 (100 mg)	5·0	0·2	96	30	62·5

Sepharose was activated with CNBr and the inorganic supports were glutaraldehyde derivatives of the alkylamine carriers.
[a]Calculated from the difference between activity taken and activity recovered in filtrate and washings.
[b]Value obtained on assay.
[c]Calculated value on dry weight basis.

negligible. With Al_2O_3 and SiO_2 markedly low efficiently values of 78% and 62·5%, respectively, were observed.

Properties of immobilized penicillin acylase

The pH optimum for the hydrolysis of benzylpenicillin by the enzyme immobilized on CNBr-activated Sepharose and on silanized TiO_2 was 7·5, similar to that of the soluble enzyme. The K_m and activation energy values for the enzyme immobilized on TiO_2 were 2·5 mM and 11·2 Kcal/mol, respectively, which are essentially similar to the values obtained with the soluble enzyme.

Operational stability in the hydrolytic reaction

The enzyme immobilized on Sepharose was stable over the 22 cycles of use which were carried out, showing no detectable loss in activity in the repeated reuses. Such stability has been reported with *E. coli* penicillin acylase immobilized on CNBr-activated Sephadex G-200 (Ekstrom *et al.,* 1974). Similar results were obtained with the enzyme immobilized on the silanized controlled-pore TiO_2 and Al_2O_3 over the 10 and 4 cycles, respectively, through which the preparations were used. In marked contrast, the SiO_2 immobilized enzyme lost approximately 20% of its activity in every reuse during the 4 cycles of operation. Figure 2 summarizes the data obtained with the inorganic supports.

Enzymatic synthesis of ampicillin

Synthesis of ampicillin from 6-APA and DL-phenylglycine methyl ester was monitored as described under 'materials and methods' using the penicillin acylase

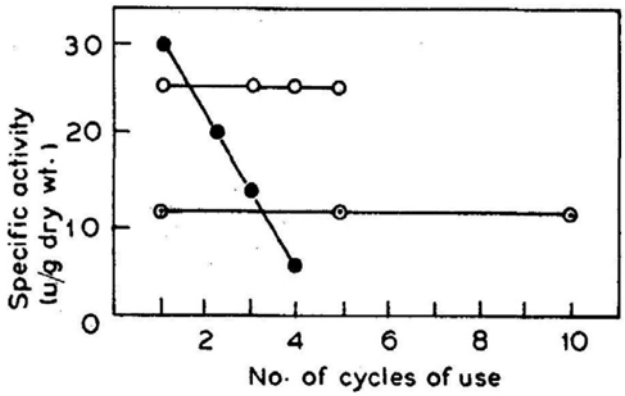

Figure 2. Operational stability of *K. citrophila* penicillin acylase immobilized by covalent attachment to silanized controlled-pore ceramics.
(⊙), Silanized Ti O_2; (O), Silanized $A1_2O_3$; (●), Silanized SiO_2.

immobilized on silanized TiO_2. The pH used was 6·5 which has been reported to be the optimum for the synthetic reaction catalysed by *K. citrophila* cells (Okachi *et al.,* 1972). DL-Phenylglycine was taken in 2·5 fold molar excess. The progress curve of ampicillin formation is shown in figure 3.

It can be seen from the curve that approximately 50% conversion of 6-APA is obtained in a period of 5 h under the conditions of the experiment. The reaction mixture at this stage showed the presence of ampicillin and the reactants on reversed

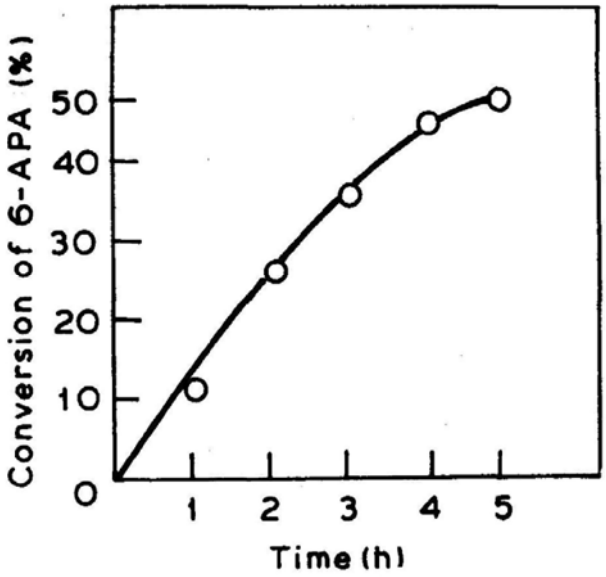

Figure 3. Progress curve of formation of ampicillin from 6-APA and DL-phenylglycine methyl ester. Reaction mixture (10 ml) containing 6-APA (50 mg); DL-phenylglycine methyl ester (125 mg); 0·03 M potassium phosphate buffer, pH 6·5, *K. citrophila* penicillin acylase immobilized on silanized Ti O_2 (250 mg support, 7 U). Temperature 40°C.

phase thin layer chromatography. The data presented by Takasawa *et al.* (1972) for whole cells of *K. citrophila* indicate approximately 38% conversion when DL-phenylglycine methyl ester is used in 2·5 fold excess and 66% when D-phenylglycine methyl ester is used.

Discussion

Controlled-pore ceramics have several desirable features which make them attractive supports for the immobilization of enzymes. Thus they have extreme rigidity, very high surface area, porosity of any desired and narrow range which could exclude microbes and protect the enzyme molecules held within the pores from microbial attack (Messing, 1974). The supports also lend themselves to regeneration after pyrolysis or chemical treatment. Despite the advantages, the use of such supports for the immobilization of penicillin acylase has not been reported hitherto in the literature. The choice of pore size for a particular application depends on the size and shape of the enzyme to be attached, the recommended pore size being approximately twice the large diameter of the enzyme molecule (Messing, 1974). The present studies indicate that controlled pore $A1_2O_3$ of 225 Å pore size has adequate porosity for the binding of *K. citrophila* penicillin acylase of $M_r \sim 65000$.

The enzyme immobilized on controlled-pore TiO_2 has shown 93-97% efficiently compared to the value of 67% obtained with the enzyme bound on Sepharose 4B. The lower value obtained with Sepharose could reflect diffusional restrictions in the case of the gel matrix.

The enzyme immobilized on controlled-pore TiO_2 and $A1_2O_3$ shows no detectable loss in activity in the limited cycles of use through which the immobilized enzymes were operated. In marked contrast the enzyme bound on controlled pore SiO_2 looses activity to the extent of about 20% in every cycle of use.

Leakage of glucoamylase covalently attached to silanized SiO_2 has been reported by Weetall and Havewala (1972), loss of activity being observed even at the acid pH value of 4·5. This was attributed to the dissolution of the glass and the consequent loss of the enzyme bound superficially at the silylated exposed surfaces of the support. The leakage was overcome by them through ZrO_2-coating of the support which results in a more resistant surface and stabler -Zr -O - Silane bonds attaching the enzyme to this surface. The marked loss in activity at pH 7·5 (range pH 7·0-pH 8·0) in every cycle of use of the controlled-pore SiO_2 immobilized penicillin acylase is probably due to the hydrolytic cleavage of the silyl groups attaching the enzyme to the surface of the support. In contrast the higher stability of the linkage to TiO_2 and $A1_2O_3$ precludes such a leakage of the attached enzyme.

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