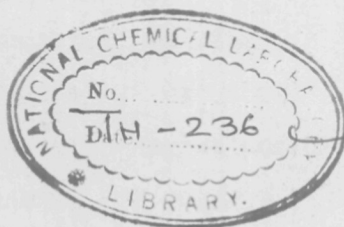
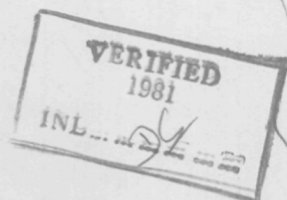


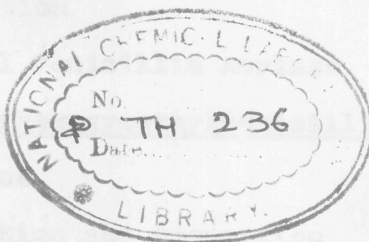
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IMMOBILIZED ENZYMES:
STUDIES ON PENICILLIN AMIDOHYDROLASE (EC 3.5.1.11)



A Thesis
Submitted to the
UNIVERSITY OF POONA
for the degree of
MASTER OF SCIENCE
(Partly by Papers and Partly by Research)

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ABBREVIATIONS

- 6-APA - 6-aminopenicillanic acid
- SDS - Sodium dodecyl sulphate
- CVB*- - Covalently bound- (eg. Sepharose -CVB-penicillin
acylase for penicillin acylase
covalently bound to Sepharose)
- PGME - DL-Phenylglycine methyl ester

- * Recommendations for standardisation of Nomenclature
in Enzyme Technology (P.V.Sundaram et al., 1972; Biotech.
Bioeng. Symp. 3, 15).

GENERAL INTRODUCTION

I PENICILLIN AMIDOHYDROLASE (EC 3.5.1.11)

Two distinct groups of enzymes hydrolyse the amide bonds in the penicillin molecule (Fig 1). The β -lactamases (penicillin β -lactam amidohydrolases, EC 3.5.2.6), which are enzymes of bacterial origin, cleave the amide bond in the β -lactam ring. The other group of enzymes, penicillin acylases (Penicillin amidohydrolase, EC 3.5.1.11), also known as penicillin amidases, catalyses reversibly the deacylation of penicillin with formation of 6-APA and the side chain. In addition to the penicillin acylases, a true penicillinamidase activity has also been reported, which cleaves penicillin amides to free ammonia. Such activity has been shown in the yeasts Rhodotorula gracilis and Torulopsis (Huang et al., 1963) towards amides of both benzyl- and phenoxyethylpenicillin as substrates.

I.1 MICROBIAL PENICILLIN ACYLASES

The occurrence of penicillin acylase was first reported by Sakaguchi and Murao (1950) in the mycelium of Penicillium chrysogenum and of Aspergillus oryzae without characterisation of the products. The products of the hydrolysis of benzylpenicillin were characterised later by Batchelor et al. (1961; 1961a) as 6-APA and the side chain, phenylacetic acid.

Several types of penicillin acylase are known which are characterised by their substrate preference (Hamilton-

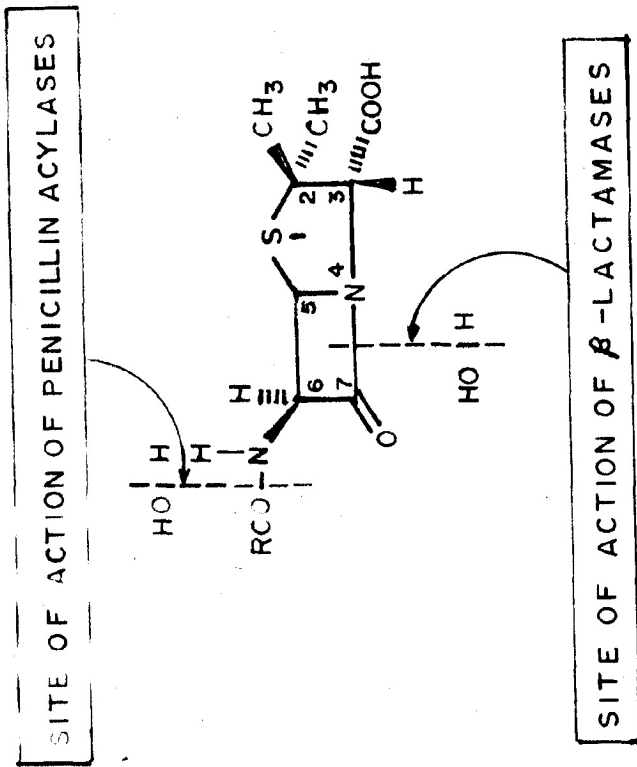


FIG. 1. SITES OF ACTION OF PENICILLIN HYDROLYSING ENZYMES

Miller, 1966; Vandamme and Voets, 1974). The features of the three main types of enzyme are described in Fig 2. The type I enzyme, which is generally fungal or actinomycete in origin, preferentially hydrolyses phenoxymethylpenicillin (Penicillin V). The type II enzyme, which is generally bacterial in origin, preferentially hydrolyses benzylpenicillin (Penicillin G). The non-preferred substrates in both types are hydrolysed at markedly slower rates than the preferred one. An interesting type of penicillin acylase reported more recently is of type III or ampicillin acylase, which hydrolyses ampicillin (D- α -aminobenzylpenicillin) specifically and is without action on benzylpenicillin, phenoxymethylpenicillin and semisynthetic penicillins other than ampicillin.

I.1.1 Type I (Phenoxymethylpenicillin) acylase

Occurrence

This type of penicillin acylases has been reported to occur in several strains of Penicillium, Cephalosporium, Aspergillus, Trichoderma, Fusarium and Gibberella (Vandamme and Voets 1974). The enzyme has also been isolated from several genera of yeasts (Torulopsis, Torula, Saccharomyces, Exosaccharomyces, Debaryomyces, Cryptococcus, Trichosporon, Rhodotorula), a few bacteria (Erwinia, Achromobacter, Micrococcus) and some actinomycetes (Micrococcidia, Streptomyces, Actinoplanes). (Vandamme and Voets, 1974).

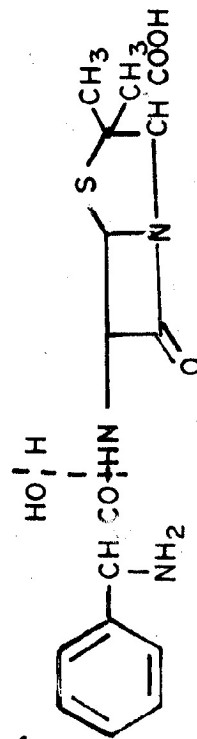
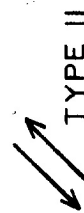
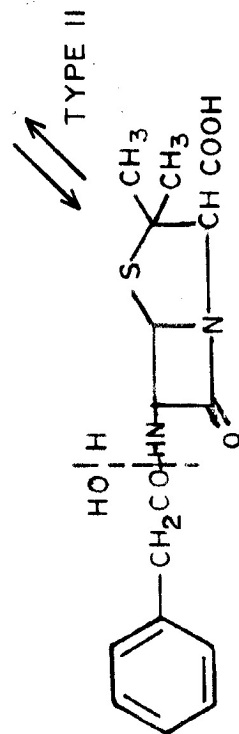
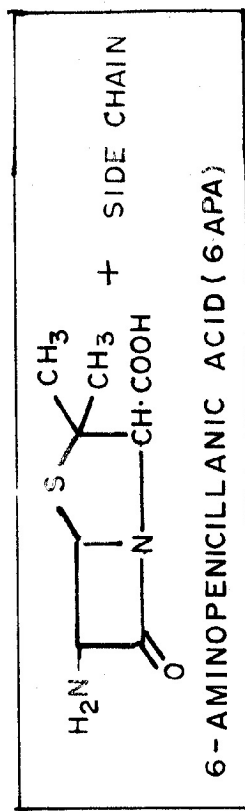
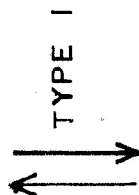
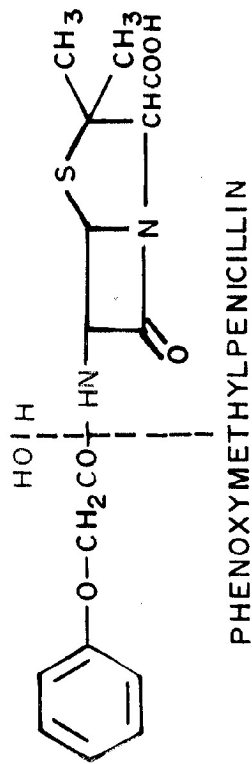


FIG.2. PENICILLIN AMIDOHYDROLYSES (EC 3.5.1.11)

Purification and Properties

The intracellular mycelial enzyme from Fusarium semitectum was partially purified by Waldschmidt-Leitz and Bretzel (1964), by precipitation with acetone and column chromatography on Amberlite IRC 50 and Sephadex G-25. The enzyme was shown to have a molecular weight ^{of} 65 000 and to contain two atoms of zinc/mol. The substrate specificity of the purified enzyme preparation was tested by Brandl (1972) with 52 different bio- and semisynthetic penicillins. The presence of bulky substitution as well as double bond in the side chain was shown to cause lowered susceptibility possibly due to steric hindrances. The intracellular enzyme in two strains of Penicillium chrysogenum was shown by Erickson and Bennett (1965) to hydrolyse phenoxymethylpenicillin at a rate six times faster than benzylpenicillin at the optimum pH of 8.5 and at a temperature of 30°C. The enzyme from Fusarium Sp was purified by Thadani et al. (1972). The mycelial enzyme activity was induced in the organism in the presence of phenoxyacetate or its derivatives and the enzyme specifically hydrolyses phenoxymethylpenicillin.

While the reports described above are of mycelium preparations, penicillin acylase activity has also been reported in fungal spores. Type I activity has been detected in spores of species of Fusarium (Singh et al., 1969; Vandamme et al., 1971). The spore acylase has been reported to be highly specific towards phenoxymethyl-

penicillin. An extracellular acylase has been reported by Batchelor et al. (1961) in the actinomycete, Streptomyces lavendulae.

A type I penicillin acylase has been reported to be present as a constitutive enzyme in Erwinia aroidae, while penicillin acylases as a class are generally induced enzymes whose biosynthesis is dependent on the presence of inducers like the side chain acid (Vandamme et al., 1971a). The Erwinia enzyme was purified partially by these authors and shown to have a molecular weight^{of} 62 000.

The reversibility of the hydrolytic action by the type I acylases was first demonstrated by Erickson and Dean (1966), using mycelial extracts of Penicillium chrysogenum strains at pH 6.8 to obtain enzymic synthesis of phenoxy-methyl- and benzylpenicillin from 6-APA and the side chain acid. The formation of benzylpenicillin from phenoxyacetyl CoA and 6-APA has been demonstrated in extracts of Penicillium chrysogenum (Spencer, 1968; Spencer and Maung, 1970; Spencer and Burnham, 1973).

I.1.2 Type II (benzylpenicillin) acylase

Occurrence

The type II enzyme occurs as an induced activity in several gram-positive and gram-negative bacteria. These include Rhodospseudomonas, Pseudomonas, Xanthomonas, Alcaligenes, Erwinia, Anatochaeter, Escherichia, Aerobacter, Kluyvera, Bacillus and Mycobacterium (Vandamme and Voets, 1974).

Among the actinomycetes, its occurrence has been shown in Nocardia and Streptomyces. The production of the type II enzyme by Nocardia has been reported by Huang et al. (1960). Streptomyces antibioticus has been reported to produce an intracellular type II penicillin acylase and an extra cellular type I enzyme (Nara et al., 1971). Among fungi, several strains of Neurospora crassa have been shown to produce type II penicillin acylase (Rossi et al., 1973).

Penicillin acylase biosynthesis in E. coli has been shown to be stimulated by phenylacetic acid and its derivatives (Levitov et al., 1967).

While the enzyme activity generally occurs intracellularly, Chiang and Bennett (1967), have shown that in the gram-positive organism, Bacillus megaterium, the activity is extracellular.

Purification and Properties

Chiang and Bennett (1967) obtained the enzyme from Bacillus megaterium ultracentrifugally homogeneous by repeated adsorption and elution from Celite followed by fractionation on CM-cellulose and showed it to have a molecular weight of 120 000. The enzyme is inhibited competitively by phenylacetic acid and non-competitively by 6-APA. The K_m for benzylpenicillin is 4.5 mM (Ryu et al., 1972).

The intracellular enzyme from E. coli NCIB 8743A was partially purified by Balasingham et al. (1972) and

Warburton et al. (1972) and was found to have a K_m value of 0.67 mM for benzylpenicillin at 37°C at pH 8.0. The enzyme was shown to be inhibited by benzylpenicillin as well as the products of hydrolysis. Inhibition by phenyl-acetic acid was found to be competitive, while that of 6-APA was non-competitive. The enzyme from E.coli ATCC 9637 was obtained in electrophoretically homogeneous form by Bondareva et al. (1969,1969a) and shown to cleave phenoxymethylpenicillin and ampicillin, in addition to benzylpenicillin. Self et al. (1969) partially purified the enzyme from this source and reported a K_m value of 7.6 mM for benzylpenicillin.

The enzyme from E.coli ATCC 11105 was obtained crystalline by Kutsbach and Hauenbusch (1974) after 100-fold purification involving ammonium sulphate fractionation followed by column chromatography on SE-sephadex and DEAE-sephadex. Crystals were obtained at 0.7 saturation with ammonium sulphate. Disc electrophoresis of the crystallised enzyme showed a single band in some samples and two in others. Isoelectric focussing however indicated a main band and two to four weaker bands. The native enzyme had a molecular weight of 70 000 with subunits of molecular size of 20 500 in SDS-polyacrylamide gel electrophoresis system.

Holt and Stewart (1964) detected an extracellular type II acylase activity in the culture supernatants of

E. coli NCIB 9465 with a pH optimum of 5.5 for the hydrolysis, which is markedly lower than that of other penicillin acylases.

The production, partial purification and properties of the enzyme from Kluyvera citrophila has been reported by Nara et al. (1971,1971a), Okaeni et al. (1973a,b), Shimizu et al. (1975a). Enzyme production is induced by phenyl-acetic acid and repressed by glucose, fructose and maltose. The enzyme was purified 120-fold by DEAE-cellulose and hydroxylapatite chromatography. The relative rate of hydrolysis of ampicillin was 60% of that of benzylpenicillin. In addition some semisynthetic cephalosporins were shown to be hydrolysed to 7-aminosephalosporanic acid. The enzyme had a molecular weight of 63 000.

Several procedures are described in the patent literature for the purification of the enzyme on an industrial scale because of its application for manufacture of 6-APA, a key intermediate for the production of semi-synthetic penicillin. The isolation of crystalline enzyme in high yield has been claimed by Kutzbach (1975). In this process the enzyme is adsorbed on aluminosilicate and then eluted and crystallised.

I.1.2(a) Enzymic synthesis of penicillins by type II penicillin acylase

The reversibility of the hydrolytic cleavage by the type II enzyme has been shown at acidic pH value of 4.5 to 6.5 compared to the general alkaline pH optimum of 7.5 to

9.0 for the forward hydrolysis reaction. The reverse reaction has been studied with the E.coli enzyme by Bauer et al. (1970), Bondareva et al. (1969, 1969a), Kaufmann and Bauer (1960), Self et al. (1969) and Cole (1969a,b). The catalysis of penicillin formation has also been studied with enzymes from Alcaligenes faecalis (Claridge et al., 1960) and Kluyvera eutrophila (Nara et al., 1971b). Since the substrate specificity for the synthetic and hydrolytic reactions was similar, the involvement of a single enzyme in both processes was assumed. This assumption was substantiated by Bondareva et al. (1969a), by showing the synthetic activity with the electrophoretically homogeneous enzyme from E.coli. The synthetic reaction rate was shown to be enhanced in the presence of esters, amides and other energy-rich forms of the side chain instead of the free acid (Okachi et al., 1972, 1972a, 1973a; Takasawa et al., 1972, Nara et al., 1971, 1971a, 1972).

1.1.3 Type III (Ampicillin) acylase

The occurrence of a penicillin acylase which specifically cleaves ampicillin to 6-APA and D-phenylglycine has been reported in several strains of Pseudomonas (Nara et al., 1972, Okachi et al., 1973b). The hydrolysis of ampicillin by these enzymes is optimal at pH 7.5. The enzyme cleaves ampicillin specifically and is without action on benzylpenicillin, phenoxymethylpenicillin and semisynthetic penicillins other than ampicillin.

The isolation of mutants of this organism, which are penicillinase-deficient has been reported by Okachi et al. (1972,1972a,1973).

Synthesis of ampicillin from 6-APA and DL-phenyl glycine and esters of both D-and L-and DL-phenylglycine at pH 5.5-6.0 and 34°C has been described by Okachi et al. (1972a).

I.2 NON-MICROBIAL PENICILLIN ACYLASE

Penicillin acylase activity^{has} also been reported in the higher forms of life. Whether these non-microbial enzymes are true penicillin acylase or broad spectrum amidases is not clear (Cole,1964). Type I acylase activity has been reported in bovine and kidney^{and} liver, spleen and lung homogenates (Cole,1964; Weitnauer,1964). Alburn, Grant and Clark (1964) have reported that ficin preparation from the trees of the genus Ficus also cleaved phenoxymethylpenicillin to 6-APA.

1.3 IMMOBILIZED PENICILLIN ACYLASE

Since penicillin acylase is an industrially important enzyme used in the manufacture of 6-APA, a key intermediate for the production of semisynthetic penicillins, several immobilized systems have been described specially in patent literature. Immobilized systems of the enzyme can be repeatedly reused which has obvious economic advantages. The use of immobilized enzyme in place of soluble preparations also excludes possible allergenic macromolecular impurities from the product (Delin et al., 1972). The latter is an overriding consideration particularly in the drug industry.

Immobilized penicillin acylase preparations described in the literature have been reviewed in two recent articles by Abbott (1976) and Subramanian et al. (1978).

Table I summarizes the data from these reviews together with more recent examples.

TABLE 1: IMMOBILIZED SYSTEMS OF PENICILLIN ACYLASES

Carrier/Activation Procedure	Activity Immobi- lized	Reference
<u>ENZYME ADSORPTION</u>		
Bentonite	--	Henser <u>et al.</u> (1969).
DEAE-cellulose/Succinylated enzyme	90	Miyasaki <u>et al.</u> (1972).
DEAE-sephadex/Succinylated penicillin amidase	--	Kanogashira <u>et al.</u> (1972)
<u>ENZYME COVALENTLY LINKED</u>		
Cellulose/trichloro-g-triazine	65	Self <u>et al.</u> (1969).
DEAE-cellulose/Carboxymethyl aminodichloro-g-triazine	100	Self <u>et al.</u> (1969).
DEAE-cellulose/aminodichloro- g-triazine	45-81	Warburton <u>et al.</u> (1972).
CM-cellulose/amide method	35	Self <u>et al.</u> (1969).
Acrylamide-maleic acid-N,N'- methylene bisacrylamide copolymer	98	Hueper (1973).
Sephadex(Sepharose)/CNBr	30	Dehn <u>et al.</u> (1972).
CNBr-activated polysaccharide hexamethylenediamine/Gluta- raldehyde	--	Smith (1976).
Cellulose/CNBr	85	Subramanian <u>et al.</u> (1978).
Maleic anhydride-tetraethylene glycol-dimethacrylate copolymer	65	Beemer ^{<u>et al.</u>} (1973), Hueper (1973a).

TABLE 1 (continued)

Maleic anhydride-methacrylic acid-tetraethyleneglycol dimethacrylate copolymer	92	^{et al} Boemer (1973a), Hueper (1973a).
Maleic anhydride-Vinylmethyl-ether copolymer/enzyme + octadecylamine	70	Smith (1976a).
Polymerization of CNBr-activated Hydroxymethacrylate-enzyme product	50	Hansher (1974).
Polymerization of N(p-amino-phenyl)methacrylamide diazonium salt-enzyme product	--	Hansher (1974).
Polymerization of methacryloyl chloride-enzyme product	--	Hansher (1974).
Cellulose 2-(3'-amino-4'-methoxyphenyl)sulfonyl ethyl ether/Glutaraldehyde	--	Nys <u>et al.</u> (1977).
<u>INTERMOLECULAR CROSSLINKING</u>		
Inert filler/Glutaraldehyde	--	Anotz (1974).
Methacrylate resin Amberlite XAD-7/Glutaraldehyde and $H_2N(CH_2)_3.NH_2$	--	Savidge <u>et al.</u> (1974).
DEAE-cellulose, CM-cellulose /Glutaraldehyde	--	Savidge and Powell (1975).
Porous Silica/adipic acid diazide	--	Pedoseev <u>et al.</u> (1977).
<u>ENTRAPPED/ENCAPSULATED ENZYME</u>		
Cellulose triacetate fiber	--	Dinelli (1972).
Collagen membrane	--	Wang and Vieth (1973).
Polymer encapsulation of soluble complex obtained by treating enzyme with CNBr-activated dextran or starch	--	Hueper (1974).
Polyacrylamide	87	Handel <u>et al.</u> (1975).

TABLE 1 (continued)

Glycidylmethacrylate-N,N'
methylene bisacrylamide
copolymer

--

Hansher and
Lozanov (1978).

IMMOBILIZED WHOLE CELLS

E. coli cells in polyacryl-
amide gel

--

Sato et al.
(1976).

1.3.1 Enzyme Immobilized by Adsorption

The kinetics of the enzyme from Bacillus megaterium immobilized by adsorption on Bentonite have been reported by Ryu et al. (1972). The enzyme from Bacillus circulans has been succinylated and adsorbed on DEAE-sephadex and this system has been used both for 6-APA preparation from benzylpenicillin (Niyazaki et al., 1972), as well as the preparation of ampicillin from 6-APA and D(-) phenylglycine methyl ester (Kamogashira et al., 1972).

1.3.2 Enzyme Immobilized by Covalent linkage

Several examples of the enzyme covalently attached to carrier are documented in the literature. Cellulose activated with trichloro-g-triazine and DEAE-cellulose activated with 2-carboxymethylamino-4,6-dichloro-g-triazine and 2 amino-4,6-dichloro-g-triazine have been used for coupling the enzyme from E. coli (Self et al., 1969, Warburton et al., 1972). The kinetic properties of the latter DEAE-cellulose derivative and its performance in various reactor types have been documented (Warburton et al., 1972, 1973). CN-cellulose hydrazide has been used for coupling of the enzyme from E. coli (Self et al., 1969). CNBr-activated Sepharose and Sephadex have been used for binding of enzyme from E. coli (Delin et al., 1972). Alkali-treated cellulose has also been used for CNBr-activation and the efficient immobilization of the enzyme from E. coli from dilute solution (Subramanian et al., 1978). Several synthetic,

hydrophilic reactive copolymers similar to and including the ethylene-maleic anhydride (EMA) system described first by Levin et al., (1964) have been applied for the immobilization of the E. coli enzyme. These include the oxide derivative of EMA copolymer (Savidge et al., 1969), polyacrylamide-maleic acid copolymer obtained by heating at 80°C, maleic anhydride-polyvinylmethacrylate copolymer derivatives and maleic anhydride-vinylmethyl ether copolymer (Hueper, 1973; Boemer et al., 1973; Hueper et al., 1973a; Boemer et al., 1973a; Smith 1976a). The polymerization of enzyme monomer derivatives has also been used for immobilizing penicillin acylase from Proteus rettgeri (Hausher 1974). These include enzyme coupled to CNBr-activated hydroxymethacrylate, N(p-aminophenyl)methacrylamide diazonium salt or methacryloyl chloride. Cellulose 2-(3'-amino-4'-methoxyphenyl)sulfonyl ether has been treated with glutaraldehyde and then coupled with enzyme (Nys et al., 1977).

1.3.3 Enzyme Immobilized by Intermolecular Crosslinking

Examples of the enzyme immobilized by intermolecular crosslinking are also available. Crosslinking with bifunctional reagents like glutaraldehyde has been carried out and inert fillers like cellulose included for ease of use (Anots, 1974). Nonionic porous methacrylate resin fillers have been used with glutaraldehyde in the presence of water soluble diamine for crosslinking the enzyme from E. coli

(Savidge et al., 1974). The enzyme has been initially adsorbed on ion exchange resins like DEAE- and CM-cellulose and then crosslinked with glutaraldehyde (Savidge and Powell, 1975). Adipic acid diacid has been used for crosslinking the enzyme adsorbed on porous silica (Fedoseev et al., 1977).

I.3.4 Enzyme Immobilized by Entrapment/Encapsulation

Encapsulation has also been used for immobilizing the enzyme. The enzyme from E. coli encapsulated in wet-spun cellulose triacetate fibers has been used by Dinelli for the production of 6-APA (Dinelli, 1972; Marconi et al., 1973). The use of the triacetate fiber entrapped enzyme for the preparation of semisynthetic penicillins from 6-APA and side chain ester has also been reported (Marconi et al., 1975). The enzyme from Bacillus megaterium has been immobilized in collagen membrane by impregnation of the preswollen support (Wang and Vieth, 1973). Polymer encapsulation of a soluble complex obtained by treating the enzyme from E. coli with CNBr-activated dextran or starch has also been used (Hueper, 1974). Polyacrylamide entrapment of the E. coli enzyme has been described (Mandel et al., 1975). The entrapment of enzyme from Proteus rettgeri in glycidyl methacrylate-N,N'-methylene bisacrylamide copolymer has also been reported (Hamsher and Lomanov, 1978).

I.3.5 Immobilized Microbial Whole cells

E. coli cells entrapped in polyacrylamide gel have also been used for the hydrolysis of benzylpenicillin to 6-APA (Sato et al., 1976).

PRESENT INVESTIGATION

STUDIES ON PENICILLIN ACYLASE (EC 3.5.1.11)

from Kluyvera citrophila

6-APA, the key intermediate for the production of semisynthetic penicillin can be obtained by three different procedures, namely; 1) Fermentation, 2) Chemical cleavage of biosynthetic penicillin and 3) Enzymic cleavage of biosynthetic penicillin. Fermentation procedures for manufacture of 6-APA are only of historical significance as yields are uneconomically low (Carrington et al., 1971; Abbott, 1976). Chemical and enzymic hydrolysis of biosynthetic penicillin, such as benzylpenicillin and phoxymethylpenicillin convert the substrates almost quantitatively to 6-APA and the side chain. In the early procedures were either whole cells of microorganisms or crude soluble extracts of activity were used, chemical process was the preferred route. The more recent developments in enzyme immobilization has lead in recent years to the preferred use of such systems in place of the low temperature chemical conversion (Dunnill and Lilly, 1978). Besides the reuse potential, the immobilized enzyme preparation has the added advantage of excluding macromolecular contaminants and yielding finally nonallergenic products (Delin et al., 1972).

Work had been initiated in the National Chemical Laboratory in collaboration with Hindustan Antibiotics Ltd for the development of the immobilized penicillin acylase based on the enzyme from high enzyme-yielding strains of E. coli (NCIM 2400) and Fusarium Sp HA 7.5-5. The partially purified

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enzymes have been immobilized on a cellulose support (Subramanian et al., 1978) and the usefulness of the preparations established in pilot plant trials (Borkar et al., 1978).

In a programme to study the behaviour of immobilized preparations of the enzyme from other sources, the enzyme from ^drecently reported source, Kluyvera citrophila ATCC 21255, has been studied in the present investigation. The enzyme from this source has not been immobilized hitherto. The enzyme was purified to a high degree by a modification of the procedure originally described by Shimizu et al., (1975a). The immobilization of the purified enzyme by covalent attachment to a variety of supports, namely Sepharose & controlled-pore ceramics of TiO_2 , Al_2O_3 and SiO_2 have been carried out and some of the properties of the native and immobilized enzyme have been studied. The immobilization of penicillin acylase on inorganic supports has not been reported earlier in the literature.

The results of the investigation are presented in the following two parts:-

Part I : Purification and properties of penicillin acylase from Kluyvera citrophila.

Part II: Studies on immobilized penicillin acylase from Kluyvera citrophila.

The literature references for the General Introduction as well as component Parts of the Present Investigation^{are} collectively listed in the final section.

The significance of the findings are summarised in the following section.

GENERAL SUMMARY

GENERAL SUMMARY

A procedure has been described for the partial purification of penicillin acylase from Kluyvera citrophila ATCC 21285. The purified enzyme has no detectable β -lactamase activity. Polyacrylamide gel electrophoresis shows the presence of three components, all of which have penicillin acylase activity. SDS-polyacrylamide gel electrophoresis yields almost exclusively a single component. The enzyme has a pH optimum of 7.5 for the hydrolysis of benzylpenicillin with a K_m value of 2.7 mM towards the substrate and an activation energy of 10.9 Kcal/mol. The enzyme has a molecular weight of 60 000 to 67 000 and probably consists of a single polypeptide chain. The enzyme is characteristic of type II acylase and preferentially hydrolyses benzylpenicillin, the relative rates of hydrolysis of phenoxymethylpenicillin and ampicillin being 10 and 40%, respectively, of that of the preferred substrate.

The preparation and properties of immobilized penicillin acylase from Kluyvera citrophila ATCC 21285 are described. The partially purified enzyme has been immobilized on CNBr-activated sepharose as well as on the aldehyde derivative of silanized controlled-pore ceramics.

The enzyme is quantitatively immobilized by covalent binding to CNBr-activated Sepharose 4B. The immobilized enzyme shows an efficiency of about 67% on the basis of the bound protein. The Sepharose-CVB-penicillin acylase

has a pH optimum of 7.5, for benzylpenicillin cleavage activity, similar to that of the native enzyme. The calculated activation energy and temperature coefficient values are 12.5 Kcal/mol and 2.12, respectively. The immobilized preparation has good operational stability and no detectable loss in activity was observed in 22 cycles of use.

Results are reported for the first time on the immobilization of penicillin acylase by covalent attachment to inorganic supports. The partially purified enzyme from K. citrophila was immobilized by covalent binding via glutaraldehyde to γ -aminopropyltriethoxysilane derivatives of the controlled-pore ceramics: TiO_2 , Al_2O_3 and SiO_2 . High binding efficiency could be obtained with adequate amounts of the activated support. Activated TiO_2 binds 90% activity from solution under these conditions; and the immobilized enzyme has an efficiency of 90% of the soluble preparation. The TiO_2 -CVB-penicillin acylase has a pH optimum of 7.5 for hydrolytic activity, similar to that of the native enzyme. The calculated K_m value of the immobilized enzyme is 2.5 mM, comparable to the K_m value of 2.7 mM of the soluble enzyme. The activation energy and temperature coefficient values are 11.2 Kcal/mol and 1.9, respectively, similar to those of the soluble enzyme.

The TiO_2 -CVB-penicillin acylase shows good operational stability with no detectable loss in activity

over 10 cycles of use which were tried out. Al_2O_3 -CVB-penicillin acylase also shows no loss of activity over the 4 cycles of use tried out. SiO_2 -CVB-penicillin acylase, however, loses approximately 20% activity in every cycle of use, probably through leaching out by hydrolytic cleavage of the $-\text{Si}-\text{O}-$ silane bonds.

The TiO_2 -CVB-penicillin acylase catalyzes formation of ampicillin from 6-APA and DL-PCMB at pH 6.5 and 40°C ; the yield being about 50% on the basis of 6-APA initially taken.

PART I

PURIFICATION AND PROPERTIES OF PENICILLIN ACYLASE
from Kluyvera citrophila

SUMMARY

A procedure has been described for the partial purification of penicillin acylase from Kluyvera citreophila ATCC 21285. The purified enzyme has no detectable B-lactamase activity. Polyacrylamide gel electrophoresis shows the presence of three components, all of which have penicillin acylase activity. SDS-polyacrylamide gel electrophoresis yields almost exclusively a single component. The enzyme has a pH optimum of 7.5 for the hydrolysis of benzylpenicillin with a K_m value of 2.7 mM towards the substrate and an activation energy of 10.9 Kcal/mol. The enzyme has a molecular weight of 60 000 to 67 000 and probably consists of a single polypeptide chain. The enzyme is characteristic of type II acylase and preferentially hydrolyses benzylpenicillin, the relative rates of hydrolysis of phenoxymethylpenicillin and ampicillin being 10 and 40%, respectively, of that of the preferred substrate.

INTRODUCTION

Wide variations have been observed in the properties of penicillin acylases from different microbial sources and even from strains of the same microorganism (Vandamme and Voets, 1974). A source in which a type II or benzylpenicillin acylase activity has been reported recently is Kluyvera citreophila KY 3641 (ATCC 21285) (Nara et al., 1971; 1971a; Okaichi et al., 1973a,b). The enzyme from this source has

not been immobilized hitherto. A study of the purification, properties and immobilization of the enzyme from this source was undertaken under a programme of work on penicillin acylase systems suitable for industrial production of 6-APA.

Preliminary studies on the purification and properties of penicillin acylase from Kluyvera citrophila are described in this Part of the thesis.

MATERIALS

All chemicals were of analytical grade. Streptomycin sulphate, primary standard grade 6-aminopenicillanic acid (6-APA), benzylpenicillin, phenoxymethylpenicillin and ampicillin were obtained from Hindustan Antibiotics Ltd, Poona. Crystalline bovine serum albumin was obtained from Sigma Chemical Co., USA. Sephadex G-200 was from Pharmacia fine chemicals, Sweden. DEAE-cellulose was chromatography grade DE-52 from Whatman Ltd, UK. Acrylamide monomer, N,N'-methylenebisacrylamide, N,N,N',N'-tetra~~methylethyl~~ methylethyl enediamine, riboflavin were from Eastman Organic Chemicals, USA. Sodium dodecyl sulphate was from HICO products Ltd, Bombay.

EXPERIMENTAL PROCEDURE

Penicillin acylase activity

Enzyme activity measurements were made at 40°C by determining the 6-APA formed with p-dimethylaminobenzaldehyde. The coloured Schiff's base which was obtained with the

reagent was assayed according to the procedure described by Bernstein and Evans (1965). The details are indicated below.

Colorimetric determination of 6-APA: Citrate-phosphate buffer, pH 2.5, was prepared by dissolving 56 g Citric acid, H_2O and 17.5 g $Na_2HPO_4 \cdot 12 H_2O$ in 1L H_2O . The colour reagent was prepared by dissolving 1 g of p-dimethylamino-benzaldehyde in 50 ml ethanol and adding 10 ml of 0.01% w/v hydroquinone and 110 ml of the citrate-phosphate buffer, pH 2.5.

Samples containing 6-APA were mixed with an equal volume of the colour reagent and read after 3 min at 415 nm. The standard curve of 6-APA is shown in Fig I.1 .

Assay of Penicillin acylase activity: The assay was done essentially as described by Balasingham et al. (1972). The test system contained 2% w/v substrate in 0.1M potassium phosphate buffer, pH 7.5. One unit of enzyme activity is defined as the amount of enzyme catalysing the hydrolysis 1 μ mol/min of substrate under assay condition.

β -lactamase activity

β -lactamase activity was checked by the microiodometric procedure as described by Hess and O'Callaghan (1975). The assay was standardized with a **β -lactamase** preparation obtained from Hindustan Antibiotics Ltd, Poona.

Protein assay

Protein was determined by the method of Lowry et al. (1951) at 500 nm, standardized against crystalline bovine

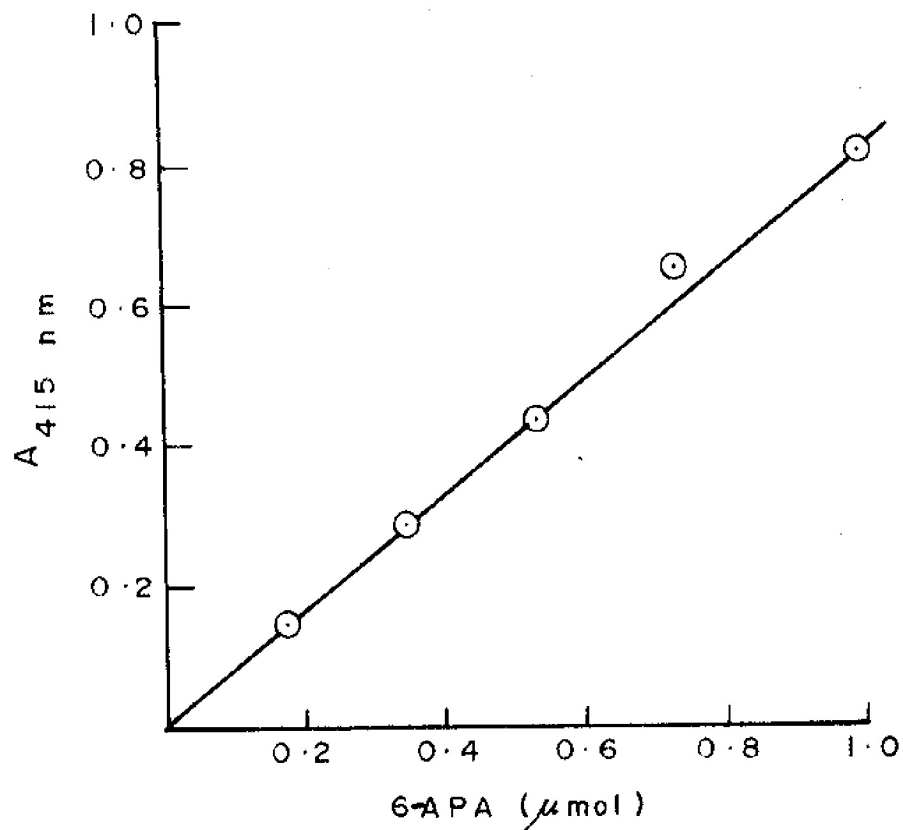


FIG. 1. STANDARD CURVE OF 6-APA. ESTIMATIONS WERE CARRIED OUT WITH p-DIMETHYLAMINO BENZALDEHYDE AS DESCRIBED IN THE TEXT.

serum albumin.

Optical density

All optical density measurements were made on a Beckman Model 26 spectrophotometer using cuvettes of 10 mm light path.

pH measurements

A Philips precision pH meter PR 9405 with the combination of glass electrode was used for all pH measurements.

Polyacrylamide gel electrophoresis

Disc electrophoresis was carried out according to Davis (1964), using 7% polyacrylamide gel. Riboflavin was used for polymerisation and Tris-HCl buffer, pH 8.3 was used in the separating gel. Neither sample nor stacking gels were used. 50-100 µg of sample was made dense with 20% sucrose and loaded on top of the gel in a volume of 0.2 ml. Tris-Glycine electrode buffer, pH 8.3 was layered on top. Bromophenol blue was incorporated as tracking dye. Electrophoresis runs were carried out at 4°C with a current of 4 mA/tube applied till the tracking dye had migrated to the bottom of the gel. Protein bands were visualised by staining with 0.5% amidoblack 10B in 7% acetic acid. Destaining was done by diffusion in a 7% acetic acid solution.

SDS-Polyacrylamide gel electrophoresis

SDS-polyacrylamide gel electrophoresis was carried out according to the procedure of Weber and Osborn (1969). 7.5% polyacrylamide gels were prepared using 0.07% ammonium

per sulphate for polymerisation. Approximately $50 \frac{-100}{\mu\text{g}}$ of protein in about 0.2 ml was loaded and the running buffer, 0.1M sodium phosphate pH 7.3 containing 0.1% SDS, was layered carefully on the top. Electrophoresis was carried out at room temperature at 8 mA/tube. Bromophenol blue was used as tracking dye and relative mobilities were calculated with respect to dye. Gels were stained and destained as described earlier.

Growth and Extraction of Enzymes From K. citrophila cells

Organism : Kluyvera citrophila ATCC 21285 was obtained from the American Type Culture Collection. The organism was routinely maintained on nutrient agar medium and subcultured every month. The culture was grown at 30°C for 24 h.

Growth of organism : Kluyvera citrophila ATCC 21285 was grown separately on 1) Medium described by Shimizu et al., (1975a) and 2) Modified nutrient medium to determine whether the culture could be grown effectively on a cheaper medium.

The former medium was of the following composition : Yeast extract, 5 g; Peptone, 10 g; NaCl, 2 g; phenylacetic acid 15 mM; Na-Glutamate, 10 g made upto 1 L after adjusting pH to 7.2 with 1 N NaOH. The supplemented nutrient medium had ^{the} following composition : Beef extract, 10 g; Peptone, 10 g; NaCl, 5 g; phenylacetic acid, 15 mM and water to make 1 L after adjusting pH to 7.2 by adding 1 N NaOH. The culture was grown in

shake flasks for 48 h at 30°C and cells harvested on the centrifuge. Sonic extracts obtained after 2 min exposure to 20 Kc (300 W) at 0-5°C, indicated that cells of the organisms grown in both media had similar penicillin acylase activity. The organism was therefore grown on the modified nutrient media for enzyme isolation.

4.5 L lot of the nutrient medium supplemented with phenylacetic acid were taken in 10 L flat-bottomed flasks at 30°C, inoculated with 250 ml seed of the Kluyvera citrophila grown in the same medium for 24 h at 30°C in shake flasks. The inoculated flask was aerated at 0.55 L/min for 24 h at 30°C. Cells from two lots were pooled and harvested by centrifugation at 4°C in a Sorvall RC 5B centrifuge at 11 000 x g for 20 min. Yield of wet cells was approximately 10 g from 10 L media.

Extraction of cells

Wet cells were suspended in 0.1 M potassium phosphate buffer, pH 7.5 (10 ml/g). The suspension was sonicated at 20 Kc (300 W) for 2 min in the Biosonic III (Bronwill Scientific Co., USA) sonic oscillator with cooling in ice. The cell debris was removed by centrifugation at 15 000 x g for 20 min at 4°C. The cell-free extract (100 ml) contained 120 U of activity and 750 mg protein.

Purification of Penicillin acylase from Kluyvera citrophila

The enzyme was purified by a modification of the procedure described by Shimizu et al., (1975a), in which the

final hydroxylapatite chromatography was replaced by filtration through Sephadex G-200. All operations were carried out at 0-4°C.

Streptomycin sulphate treatment

The extract of sonicated cells was treated with streptomycin sulphate (1.4 g/100 ml) under constant stirring to a final concentration of 1% of the base. The precipitated nucleic acids were removed by centrifugation at 50 000 x g for 30 min.

Ammonium sulphate precipitation

The supernatant from the streptomycin step (100 ml) was stirred constantly and treated with powdered ammonium sulphate to 0.45 saturation (25.6 g/100 ml). After 30 min stirring, the faint precipitate was removed by centrifugation at 10 000 x g for 30 min and was discarded. To the clear supernatant more powdered ammonium sulphate was added to 0.9 saturation and stirring continued for 30 min. The precipitate was then separated by centrifugation at 10 000 x g for 30 min and the supernatant was then discarded. The precipitate was 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.

DEAE-cellulose column chromatography

The dialyzed solution after ammonium sulphate precipitation was centrifuged at 10 000 x g for 20 min and the clear supernatant was loaded on a DEAE-cellulose column

(28 x 1 cm) equilibrated with 0.01 M potassium phosphate buffer, pH 7.5. The column was washed with 250 ml of 0.01 M potassium phosphate buffer, pH 7.5 at a flow rate of about 50 ml/h. Fractions of about 15 ml were collected on an automatic fraction collector. The enzyme was eluted between 30 to 90 ml. The fractions with penicillin acylase activity were pooled and concentrated by ultrafiltration through a PM-10 membrane to a final volume of about 3 to 4 ml.

Sephadex G-200 filtration

The enzyme solution from the ultrafiltration step was loaded on Sephadex G-200 (100 x 2 cm) equilibrated with 0.05 M potassium phosphate buffer, pH 7.5. The column was eluted with buffer of the same composition at a flow rate of 4 ml/h and fractions of 1 ml were collected on an automatic fraction collector. A drop from each sample was assayed for penicillin acylase activity. The elution behaviour is described under RESULTS on molecular weight estimation. Fractions containing enzyme activity were pooled. The pooled fraction contained about 3.8 mg protein with 30 U activity.

**TABLE I.1 : PURIFICATION OF PENICILLIN ACYLASE FROM
*Kluyvera citreophila***

Wet weight of cells: 8 g

Fraction	Volume ml	Total Activity U/g ¹ h	Total Protein mg	Specific Activity U/mg	Yield %
Cell-free extract	100	120	750	0.16	100
Ammonium sulphate fraction (0.5-0.9 sat.) dissolved in 0.01 M potassium phosphate buffer and dialyzed	42	83	453	0.18	69
DEAE-cellulose fraction concen- trated by ultrafiltration	4	52	22	2.4	43
Sephadex G-200 fraction	5	30	3.8	8.0	25

RESULTS

Purification

The results obtained in a typical experiment are summarized in Table I.1.

The ammonium sulphate precipitation was used mainly to reduce the volume of the crude extract of sonicated cells. Removal of nucleic acids with streptomycin sulphate and the ammonium sulphate precipitation step did not increase the specific activity of the enzyme. DEAE-cellulose chromatography resulted in approximately 15-fold purification and the final gel filtration step a further 3 to 4-fold purification. The overall purification was about 50-fold with a recovery of about 25% of initial activity. The specific activity of the final preparation was 8 U/mg compared to a value of approximately 13 reported by Shimizu *et al.*, (1975a).

Properties of partially purified Penicillin acylase

Absence of β -lactamase activity

The purified enzyme was checked for β -lactamase activity as described under experimental procedures. The assay indicated complete absence of β -lactamase activity in the purified enzyme preparation.

Disc electrophoresis

Electrophoresis in polyacrylamide gel showed the presence of three components. The electrophoresis profile is represented in Fig I.2. The protein components from regions in unstained gels when cut out and qualitatively

FIG. I.2

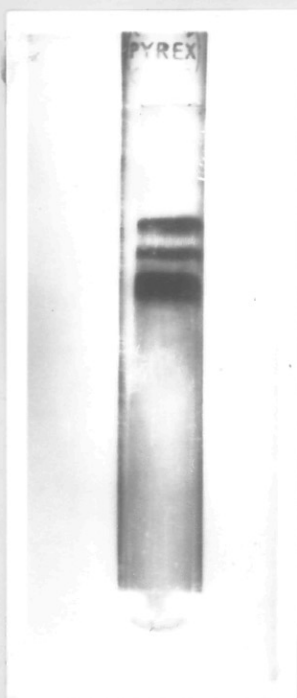


FIG. I.3



Fig 1.2 : POLYACRYLAMIDE GEL ELECTROPHORESIS OF
PURIFIED PENICILLIN ACYLASE FROM
Kluyvera citrophila

7% gel; Tris-glycine buffer, pH 8.3;

4 mA/tube; 1.5 h; ^{14}C ; Protein loaded 100 μg .

Segments from unstained gel corresponding to all 3 components showed presence of Penicillin acylase activity.

Fig 1.3 : SDS-POLYACRYLAMIDE GEL ELECTROPHORESIS OF
PURIFIED PENICILLIN ACYLASE FROM
Kluyvera citrophila

0.1 M sodium phosphate buffer, pH 7.3;

SDS, 0.1%; 8 mA/tube; 4 h.

Total protein loaded, 100 μg .

checked for penicillin acylase activity showed that all three components were active. This might probably indicate the presence of either three different types of penicillin acylases in the organism or the presence of isozymes of the same type. No further work has been done in the present studies to characterise the nature of these components.

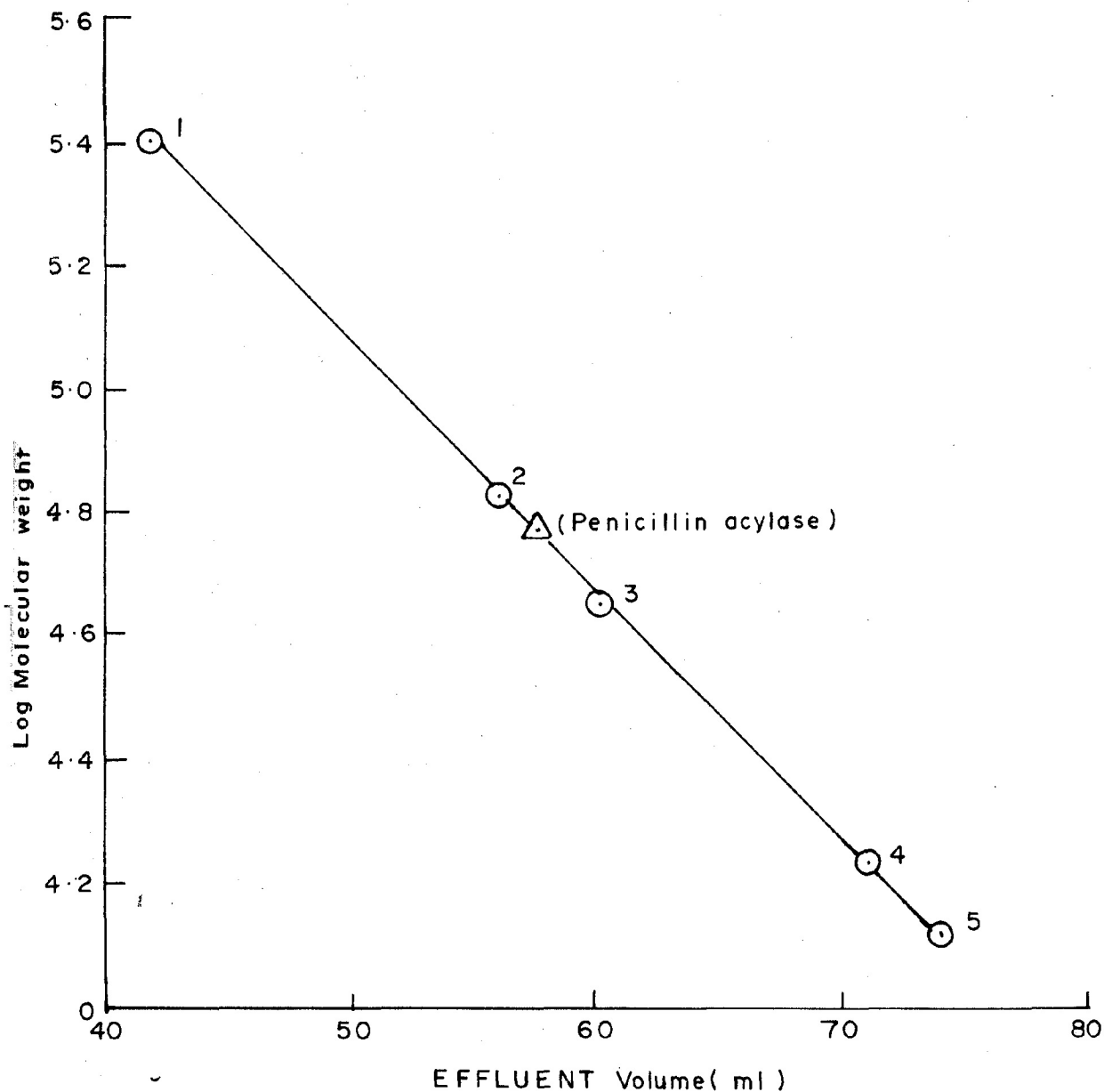
SDS-gel electrophoresis

SDS-polyacrylamide gel electrophoresis indicated the presence of one major component only. The electrophoresis pattern is shown in Fig I.3. The presence of atleast four very faintly stained bands can be detected in Fig I.3. The presence of a single major component might suggest that the heterogeneity observed in gel electrophoresis under native conditions could either be due to artefacts such as those in which asparagine and glutamine amide bonds are hydrolysed or of differing enzyme types of similar molecular size but varying net charge. The presence of aggregates that still retain activity is unlikely, since no evidence for this was obtained in gel filtration through Sephadex G-200.

Molecular Weight Determination

1) Native Enzyme

The molecular weight of the enzyme was determined by gel filtration through a Sephadex G-200 column (100 x 2 cm) calibrated with standard proteins. The results are shown in Fig I.4. The activity emerges in a single symmetrical



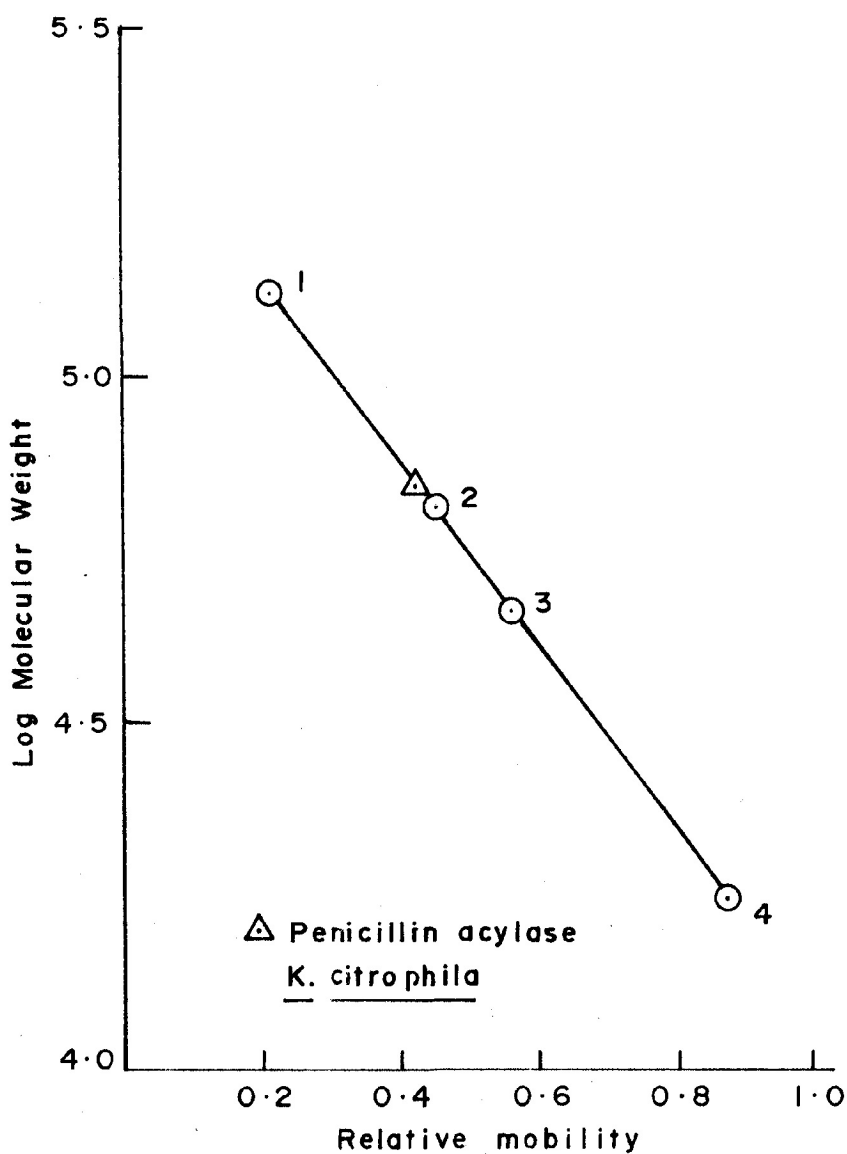
I.4 . MOLECULAR WEIGHT DETERMINATION BY GEL FILTRATION THROUGH SEPHADEX G-200 (100 x 2 cm). MOLECULAR WEIGHT OF MARKER PROTEINS:
 (1) CATALASE (250 000); (2) BOVINE SERUM ALBUMIN (68 000);
 (3) OVALBUMIN (45 000); (4) MYOGLOBIN (17 500); (5) CYTOCHROME C (13 000); (Δ) PENICILLIN ACYLASE FROM *K. citrophila*.

peak and had a calculated molecular weight of 60 000.

As stated earlier, no evidence was obtained for aggregates with penicillin acylase activity.

2) Subunit molecular weight

For determination of subunit molecular weight, the partially purified enzyme and marker proteins were treated separately in 1% 2-mercaptoethanol and incubated for 3 h, at 37°C. The solutions were dialysed against 0.1% SDS, 0.1% 2-mercaptoethanol-0.1 M sodium phosphate buffer, pH 7.2. Electrophoresis were carried out simultaneously but in separate gels. The enzyme sample migrated almost exclusively as a single component (Fig I.3). The molecular weight of this component was determined from a calibration curve obtained with marker proteins. A typical calibration curve of the logarithm of molecular weights of marker proteins versus relative mobility is shown in Fig I.5. The molecular weight of the main component was 67 000. This would indicate that the enzyme probably consists of a single polypeptide chain, since the native enzyme also has approximately the same molecular weight.



5. MOLECULAR WEIGHT DETERMINATION BY SDS-POLYACRYLAMIDE GEL ELECTROPHORESIS. CALIBRATION CURVE FOR 7.5 % GEL, MOLECULAR WEIGHT OF MARKER PROTEINS: (1), BOVINE SERUM ALBUMIN DIMER (136000), (2), BOVINE SERUM ALBUMIN MONOMER (68000), (3) OVALBUMIN (45000), (4), MYOGLOBIN (17500)

ENZYME KINETICS

Enzyme kinetics of Penicillin hydrolysis

1) pH Optimum

The activity dependence of the enzyme on the pH of the test medium for the cleavage of benzylpenicillin is shown in Fig I.6. It will be seen from Fig I.6 that the optimum pH for the enzyme is 7.5. This is in agreement with the value reported by Nara *et al.* (1971).

2) Effect of substrate concentration

Effect of substrate concentration on the enzyme activity of the partially purified preparation towards benzylpenicillin is shown in Fig I.7. The K_m of the enzyme was calculated from the Lineweaver-Burk plot. The K_m value for benzylpenicillin was 2.7 mM.

3) Effect of temperature

The effect of temperature on the enzyme activity of the partially purified fraction towards benzylpenicillin is shown in Fig I.8. The calculated activation energy from Fig I.8 was 10.9 Kcal/mol, which corresponds to a temperature coefficient, Q_{10} of 1.85 for hydrolysis of benzylpenicillin.

4) Substrate specificity

The relative rates of hydrolysis of benzylpenicillin, phenoxymethylpenicillin and ampicillin are shown in Table I.2. The enzymic hydrolysis of benzylpenicillin is approximately 10 times faster than that of phenoxymethylpenicillin and 2.5 times faster than that of

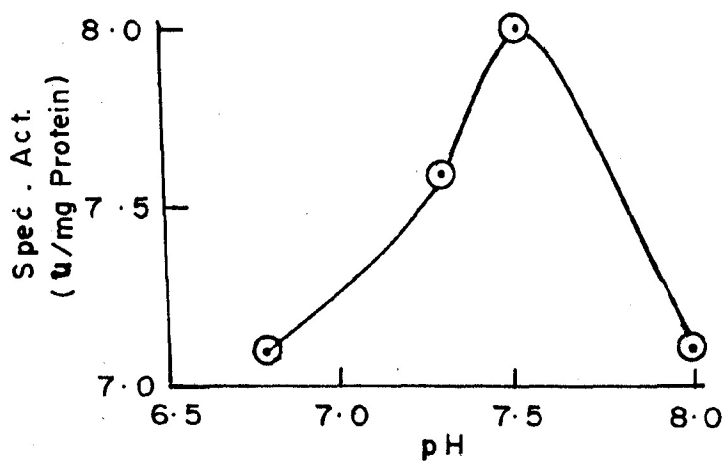


FIG.I.6 EFFECT OF pH ON INITIAL RATES OF HYDROLYTIC ACTIVITY OF PURIFIED PENICILLIN ACYLASE FROM *K.citreophila* TOWARDS BENZYL PENICILLIN. 0.1 M POTASSIUM PHOSPHATE BUFFERS; 40° C.

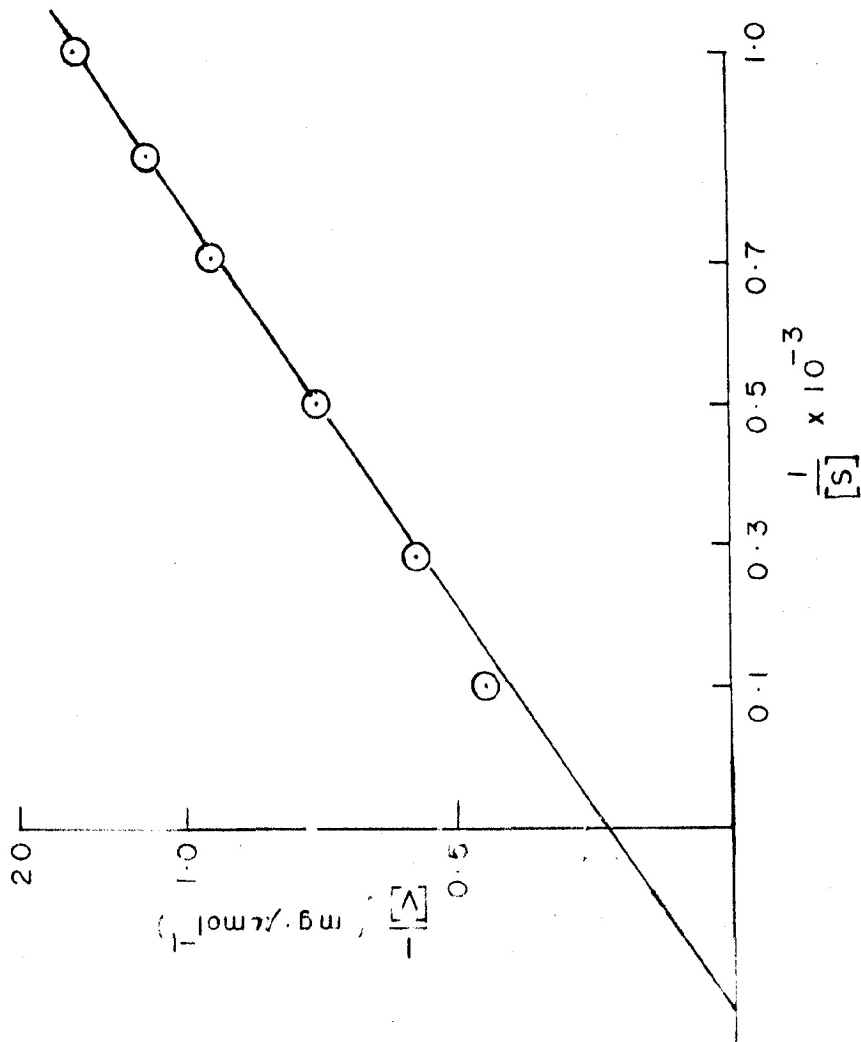


FIG 1. 7. LINEWEAVER-BURK PLOT OF INITIAL RATES OF HYDROLYSIS OF BENZYL PENICILLIN BY PURIFIED PENICILLIN ACYLASE FROM *E. coli*, pH 7.5, 40°C

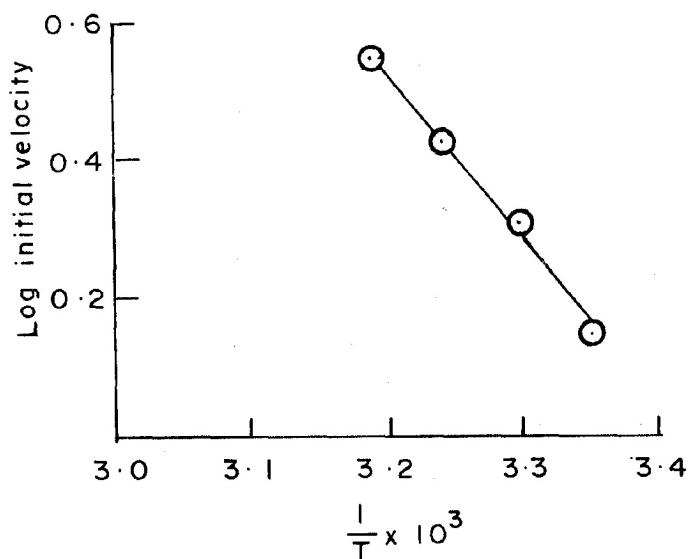


FIG. I. 8. EFFECT OF TEMPERATURE ON HYDROLYTIC ACTIVITY OF PENICILLIN ACYLASE FROM K. citrophila TOWARDS BENZYL PENICILLIN. SUBSTRATE, 0.1 M; POTASSIUM PHOSPHATE BUFFER, pH 7.5.

ampicillin. The substrate specificity is characteristic of type II, which preferentially hydrolyses benzylpenicillin, like most other enzymes from bacterial sources.

TABLE 1.2 : RELATIVE RATES OF HYDROLYSIS OF BIO/SEMISYNTHETIC PENICILLINS BY PARTIALLY PURIFIED PENICILLIN ACYLASE FROM *Kluyvera citrophila*

Substrates, 0.1 M; 0.1 M potassium phosphate buffer, pH 7.5; temperature 40°C.

Substrate	Relative rates of hydrolysis * %
Benzylpenicillin	100
Phenoxyethylpenicillin	10
Ampicillin	40

* Relative rates calculated from initial rates of hydrolysis.

DISCUSSION

The purified preparation of penicillin acylase from Kluyvera citrophila obtained by the present procedure shows three components on polyacrylamide gel electrophoresis, all three having enzyme activity. In SDS-polyacrylamide gel electrophoresis, however, only a single major component is observed together with faint traces of at least four other components. The molecular weight of the native enzyme determined by gel filtration is 60 000 and that obtained for the major component in SDS-gel electrophoresis is 67 000. This might indicate the presence of isozymes of penicillin acylase in the organism.

As described above the molecular weight of the enzyme from K. citrophila has been found to be 60 000 to 67 000 both from gel filtration and from SDS-polyacrylamide gel electrophoresis. This value is in close agreement with the value of 63 000 reported by Shimizu et al, (1975a), by thin layer chromatography on Sephadex G-100. The present data obtained from SDS-polyacrylamide gel electrophoresis would indicate that the enzyme molecule consists of a single polypeptide chain. The crystalline enzyme from E. coli ATCC 11105 has been reported to have a molecular weight 70 000 by thin layer gel chromatography, while SDS-polyacrylamide gel electrophoresis indicated the presence of a 20 500 molecular weight species in addition to one of 71 000. The enzyme from B. megaterium has a molecular weight of 120 000 (Acevedo et al., 1973).

The partially purified enzyme from K.sitrophila has a pH optimum of 7.5 for the hydrolysis of benzylpenicillin. Similar pH optimum has been reported in most of the Type II enzymes; the exception being the E.coli enzyme (NCIB 9465) which has an unusually low pH optimum of 5.5 (Holt and Stewart, 1964).

The K_m value of the partially purified enzyme from K.sitrophila is 2.7 mM for benzylpenicillin. Shimizu et al. (1975a) reported a K_m of 1.4 mM for cephalixin hydrolysis.

The crystalline enzyme from ^{E.Coli} ATCC 11105 has K_m of 0.02 mM towards benzylpenicillin. The enzyme from E.coli NCIB 8743 A has been reported to have a K_m 0.67 mM for benzylpenicillin (Balasingham et al., 1972). The enzyme from B.negativerium has a K_m of 4.5 mM for benzylpenicillin (Acevedo et al., 1973). The enzyme from E.coli NCIB 8743 A, when present in the intact cell, has been reported to have an unusually high K_m value of about 30 mM (Cole 1969). The enzyme from E.coli NCIM 2400 has been shown to have a K_m of 0.04 mM for benzylpenicillin (Borker et al., 1978).

The preferred substrate of the K.sitrophila enzyme is benzylpenicillin, which characterizes it as a Type II Penicillin acylase.

PART II

STUDIES ON IMMOBILIZED PENICILLIN ACYLASE

from Kluyvera citrophila

SUMMARY

The preparation and properties of immobilized penicillin acylase from Kluyvera citrophila ATCC 21285 are described. The partially purified enzyme has been immobilized on CNBr-activated sepharose as well as on the aldehyde derivative of silanized controlled-pore ceramics.

The enzyme is quantitatively immobilized by covalent binding to CNBr-activated Sepharose 4B. The immobilized enzyme shows an efficiency of about 67% on the basis of the bound protein. The Sepharose-CVE-penicillin acylase has a pH optimum of 7.5, for benzylpenicillin cleavage activity, similar to that of the native enzyme. The calculated activation energy and temperature coefficient values are 12.5 Kcal/mol and 2.12, respectively. The immobilized preparation has good operational stability and no detectable loss in activity was observed in 22 cycles of use.

Results are reported for the first time on the immobilization of penicillin acylase by covalent attachment to inorganic supports. The partially purified enzyme from K.citrophila was immobilized by covalent binding via glutaraldehyde to γ -aminopropyltriethoxysilane derivatives of the controlled-pore ceramics: TiO_2 , Al_2O_3 and SiO_2 . High binding efficiency could be obtained with adequate amounts of the activated support. Activated TiO_2 binds 90% activity from solution under these condition and the

immobilized enzyme has an efficiency of 90% of the soluble preparation. The TiO_2 -CVB-penicillin acylase has a pH optimum of 7.5 for hydrolytic activity, similar to that of the native enzyme. The calculated K_m value of the immobilized enzyme is 2.5 mM, comparable to the K_m value of 2.7 mM of the soluble enzyme. The activation energy and temperature coefficient values are 11.2 Kcal/mol and 1.9, respectively, similar to those of the soluble enzyme.

The TiO_2 -CVB-penicillin acylase shows good operational stability with no detectable loss in activity over 10 cycles of use which were tried out. Al_2O_3 -CVB-penicillin acylase also shows no loss of activity over the 4 cycles of use tried out. SiO_2 -CVB-penicillin acylase, however, loses approximately 20% activity in every cycle of use, probably through leaching out by hydrolytic cleavage of the $-\text{Si}-\text{O}-$ silane bonds.

The TiO_2 -CVB-penicillin acylase catalyzes formation of ampicillin from 6-APA and DL-PGME at pH 6.5 and 40°C ; the yield being about 50% on the basis of 6-APA initially taken.

INTRODUCTION

Amongst the few documented applications of immobilized enzymes in industrial processes is that of immobilized penicillin acylase for the production of 6-APA, a key intermediate for the manufacture of semisynthetic penicillins (Abbott, 1976). The properties of enzymes

Abbreviations : PGME:- DL-Phenylglycine methyl ester

from various sources immobilized by diverse procedures on a variety of supports has already been described briefly in the General Introduction.

The immobilization of the type II penicillin acylase from K. citrophila ATCC 21285 is described here. The enzyme from this source has not been immobilized hitherto. The immobilization has been carried out with the purified enzyme by covalently binding to CNBr-activated sepharose and to silanized derivatives of the controlled-pore ceramics TiO_2 , Al_2O_3 and SiO_2 . The immobilization of penicillin acylases by covalent binding on such inorganic supports has not been reported earlier. Some of the properties of the immobilized systems are described.

MATERIALS

Penicillin acylase from *Kluyvera citrophila* ATCC 21285

The enzyme was purified as described in Part I of this thesis. Enzyme frozen at -20°C was stable over prolonged periods and no detectable loss in activity was observed even after 3 months of storage at this temperature. Enzyme samples used for immobilization had a specific activity of about 5 to 8 U/mg protein.

Supports: Sepharose 4B was obtained from Pharmacia Fine Chemicals. The support was washed free of preservative before use.

Controlled-pore TiO_2 , Al_2O_3 and SiO_2 were gifts from Corning Glass Work USA. The specifications of these

supports are :

TiO_2 (Lot 2511-T), Pore diameter 560 Å; Surface area 10 m^2/g ; 30/45 mesh.

Al_2O_3 (Lot 2011-A), Pore diameter 225 Å; Surface area 90 m^2/g ; 30/45 mesh.

SiO_2 Pore diameter 425 Å; Surface area 40 m^2/g ; 45/80 mesh.

The ceramics were cleaned before use by boiling with 5% nitric acid for 45 min and were then cooled and washed with distilled water and dried at 115°C for 24 h.

Chemicals All organic solvents were redistilled before use. Buffers were prepared from analytical grade reagents. Cyanogen bromide and glutaraldehyde (50% in H_2O) were obtained from Fluka, Switzerland. γ -Aminopropyltriethoxysilane was obtained from Pierce Chemical Company, USA. Silicone DC 100 was obtained from Dow Corning, USA. DL-Phenylglycine and Ethanolamine (2-aminoethanol) were obtained from the Organic Synthesis and Chemical Engineering Divisions of this laboratory.

EXPERIMENTAL PROCEDURES

Preparation of Phenylglycine methyl ester-HCl

The ester was prepared according to the procedure described by Greenstein and Winitz (1961). A stream of dry HCl gas was passed rapidly through a suspension of 10 g DL-phenylglycine in 150 ml of absolute methanol without external cooling. After all the amino acid had gone into

solution, the hot reaction mixture was cooled in an ice bath and passing of HCl gas continued until saturation. The reaction mixture was protected from atmospheric moisture with a CaCl_2 guard tube and left 3-4 h at room temperature. The reaction mixture was taken to dryness in vacuo. The dry solid was dissolved in the minimum amount of hot methanol, decolorised with activated charcoal, filtered and the filtrate treated till turbidity with petroleum ether (B.P. $50-60^\circ\text{C}$) and left overnight at -20°C . White crystal of the ester hydrochloride appeared (Yield, 3.1 g).

CNBr-Activation of Sepharose

The activation of Sepharose 4B with CNBr was carried out by a modification of the method described by Porath et al. (1967); in which 1M Na_2CO_3 was used for controlling 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 2M Na_2CO_3 (1.5 vol). An aqueous solution of CNBr (100 mg/ml; 0.5 vol) was added to the beads and the mixture gently agitated for 10 min at $18-22^\circ\text{C}$. The mixture was then cooled with addition of crushed ice. The activated beads were washed rapidly on a sintered glass filter. ~~Washings~~ with ice cold 0.1M NaHCO_3 containing 0.17 M NaCl (25 vol). Filtration and washings were completed within 10 min by applying gentle suction. The washed beads were immediately added

to ice cold enzyme solution in a 0.05M 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 filtrate pH 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 1M ethanolamine at pH 8 at 0-5°C for 1 h. The beads were finally filtered off, washed with 0.1M potassium phosphate buffer, pH 7.5 (10 vol) and stored at 0-4°C in the same buffer.

Immobilization of penicillin acylase on controlled-pore ceramic

Enzyme was covalently bound to the surface of the carrier ceramic through silanization with γ -aminopropyl-triethoxysilane followed by coupling of the protein with the use of the bifunctional reagent, glutaraldehyde, according to the procedure described by Westall (1976).

Preparation of alkylamine carrier: The cleaned carrier after drying (1 g) was added to 10% w/v γ -aminopropyl-triethoxysilane dissolved in toluene (75 ml). The mixture was refluxed on a heating mantle for 16 h, then cooled and the carrier filtered off and washed thoroughly first with toluene and then with acetone. The alkylamine carrier so obtained was air dried.

Preparation of glutaraldehyde derivative of alkylamine

carrier: The alkylamine carrier (1 g) was treated with 2.5% w/v glutaraldehyde solution (10 ml), so as to cover the support. The mixture was 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) in 0.1M potassium phosphate buffer, pH 7.5. The mixture was stirred for 1 h at room temperature. The carrier was filtered off and washed with 0.1M potassium phosphate buffer, pH 7.5. The washings and filtrate were assayed for enzyme activity.

HYDROLYTIC ACTIVITY

Assay of immobilized penicillin acylase

Immobilized enzyme preparations were assayed in stirred water-jacketed reaction vessels maintained at 40°C. The assay medium was similar to that described in Part I of this thesis for the assay of 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 as described earlier with p-dimethylaminobenzaldehyde according to the method of

Benstein and Evans (1965).

Determination of operational stability

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 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 0.5-1M aqueous ammonia solution dispensed from a microburette. The progress of reaction was monitored by assay of 6-APA in samples withdrawn periodically. The assay was^{as} described in Part I of this thesis. The completion of hydrolysis was apparent from the cessation of proton release. After the completion of reaction, the immobilized enzyme were filtered off 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

Enzymic synthesis of ampicillin from 6-APA and Phenylglycine methyl ester

The formation of ampicillin catalysed by immobilized penicillin acylase from *E. clostriphila* was followed essentially as described by Okachi et al. (1972a). The reaction mixture contained in a total volume of 6.5 ml, 6-APA, 50 mg; DL-phenylglycine methyl ester HCl, 125 mg; potassium phosphate buffer, 0.03M, final pH adjusted to 6.5 with 1N NaOH. The reaction mixture was treated with 250 mg TiO_2 -CVB-penicillin acylase (8 U). The mixture was stirred with a tefloncoated magnetic stirring bar and 0.2 ml aliquots were withdrawn every hour by means of a tuberculin syringe fitted with a sintered disc at the nozzle. 10 μl of the clear filtrate from the syringe were diluted to 1 ml and 0.1-0.2 ml of the diluted solution analysed for 6-APA with p-dimethyaninobenzaldehyde as described earlier. Both phenylglycine methyl ester and ampicillin gave no colour with the reagent and did not interfere in the assay of 6-APA. 1-2 μl samples were taken for characterization of the product by reversed-phase thin-layer chromatography on silica gel.

Detection of ampicillin by reversed-phase thin-layer chromatography

Ampicillin was detected chromatographically by the procedure of Biagi et al. (1969). 20 x 20 cm glass plates were

coated with Silica gel G in the usual manner to a thickness of 2 mm. A stationary nonpolar phase was obtained by impregnating the silica gel layer with Silicone DC 100. This was done by developing the plates preliminary in a 5% solution of the silicone oil in diethyl ether inside a covered chamber for 16 h. The plates were then removed and dried at room temperature and separately spotted with the reaction mixture as well as authentic samples of ampicillin, 6-APA and DL-phenylglycine methyl ester. The plates were then developed with 0.05M sodium acetate-veronal buffer, pH 7.4, mixed with acetone (50 vol buffer to 1 vol acetone). The developed plates were sprayed with dilute alkaline solution of KMnO_4 and the compounds visualized by heating for a few minutes in an oven at 120°C . Yellow or white spots appeared on an intense pink background.

RESULTS

The data on immobilization of partially purified enzyme from K. citrophila on CNBr-activated Sepharose-4B are summarised in TABLE II.1

TABLE II.1 : PREPARATION OF SEPHAROSE-CVB-PENICILLIN ACYLASE

Sepharose-4B beads (3 g wet wt) were activated with CNBr in the presence of 1M Na_2CO_3 as described under Experimental Procedures. Activated beads were treated with the indicated amount of penicillin acylase (3 ml) for 16 h at 4°C. Activity of the immobilized enzyme was determined by benzylpenicillin cleavage and is compared with the corresponding activity of native enzyme on the basis of total protein bound.

Soluble Penicillin Acylase		Total Protein in filtrate + washings	Protein bound	Immobilized enzyme Activity	
Total Activity U	Total Protein mg			bound	Effi- ciency (a)
		mg	%	U/g wet beads	%
10	1.2	0	100	2.24	67

(a) Calculated from activity taken up from solution and that obtained on assay

It can be seen from TABLE II.1 that quantitative binding of protein to CNBr-activated Sepharose is obtained under the conditions used. The immobilized enzyme showed

only about 67% of the activity calculated from the protein bound. 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 nonporous (Subramanian et al. 1978).

Properties of Sepharose-CVB-penicillin acylase

pH optimum

The dependence of benzylpenicillin cleavage activity of the Sepharose 4B-CVB-penicillin acylase on the pH of the test medium is shown in Fig II.1. It will be seen from the Fig II.1 that the optimum pH of the immobilized enzyme is 7.5, which corresponds to that of the soluble enzyme. Apparently the positive charge conferred on the support by the isourea groups formed during the coupling of the CNBr-activated support to protein (Perath, 1974) has negligible effect on the pH optimum of the hydrolysis reaction.

Effect of temperature

The effect of temperature on the activity of the sepharose bound enzyme is shown in Fig II.2. The calculated activation energy was 12.5 Kcal/mol and temperature coefficient Q_{10} value, 2.12. The soluble enzyme has a slightly lower value of 10.9 Kcal/mol.

Progress curve of hydrolysis of benzylpenicillin

Progress curve of hydrolysis of benzylpenicillin

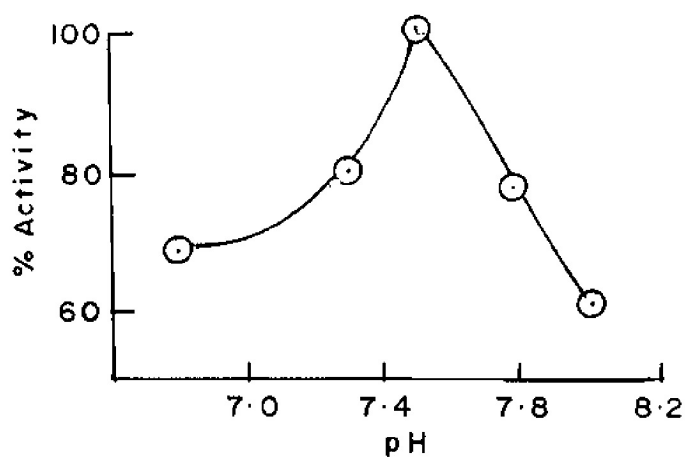


FIG.II.1 EFFECT OF pH ON INITIAL RATES OF HYDROLYTIC ACTIVITY OF SEPHAROSE-CYB-PENICILLIN ACYLASE (*K.citrophila*) TOWARDS BENZYL-PENICILLIN. 0.1M SUBSTRATE, 0.1M POTASSIUM PHOSPHATE BUFFERS; 40°C. INITIAL RATES EXPRESSED AS % MAXIMUM ACTIVITY OBTAINED.

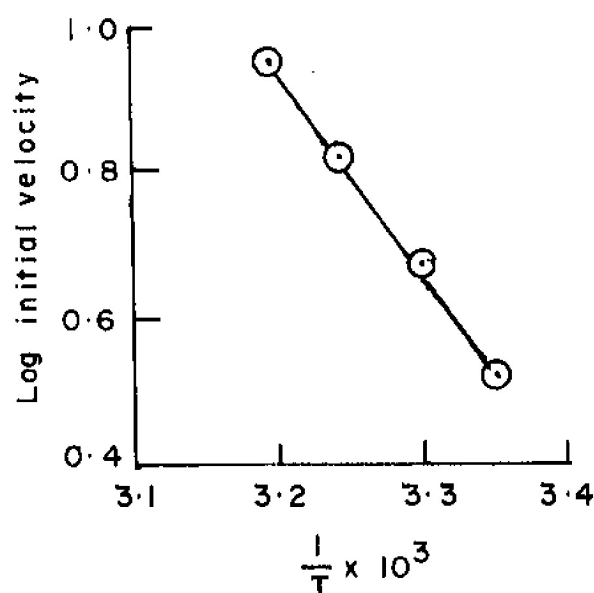


FIG II.2 EFFECT OF TEMPERATURE ON INITIAL RATES OF HYDROLYSIS OF BENZYL PENICILLIN BY SEPHAROSE-CVB-PENICILLIN ACYLASE (*K.citrophila*). 0.1M SUBSTRATE; 0.1M POTASSIUM PHOSPHATE BUFFER, pH 7.5.

at 2% substrate level is shown in Fig II.3. It will be seen that hydrolysis of the substrate is quantitative in a period corresponding to about twice that calculated from the initial hydrolysis rate.

Operational stability

Operational stability was determined as described under experimental procedures after every 5 cycles of use, each of a duration of about 2-3 h required for complete hydrolysis (95-100%) of benzylpenicillin. The data is shown in Fig II.4. It will be apparent that no detectable loss in activity is observed in 22 cycles of use, which was carried out over a period of about two weeks.

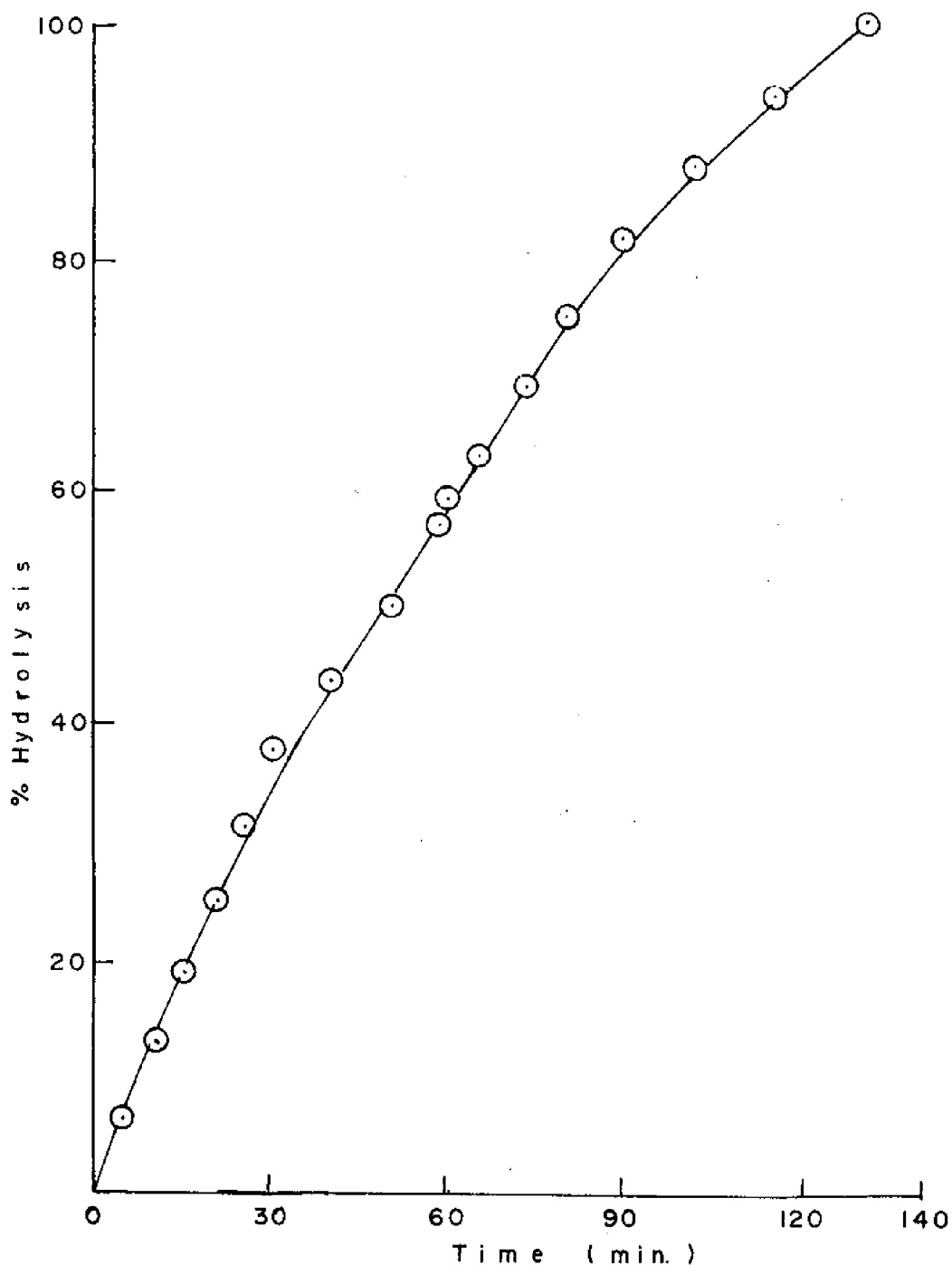


FIG.II.3 PROGRESS CURVE OF HYDROLYSIS OF BENZYL-PENICILLIN BY SEPHAROSE-CVB-PENICILLIN ACYLASE (*K.citrephila*). 10 ml, 2% (w/v) BENZYL-PENICILLIN K-SALT; pH MAINTAINED AT 7.5 WITH ADDITION OF Aq. NH_4OH ; 3g WET SEPHAROSE BEADS (6.7U) 40°C

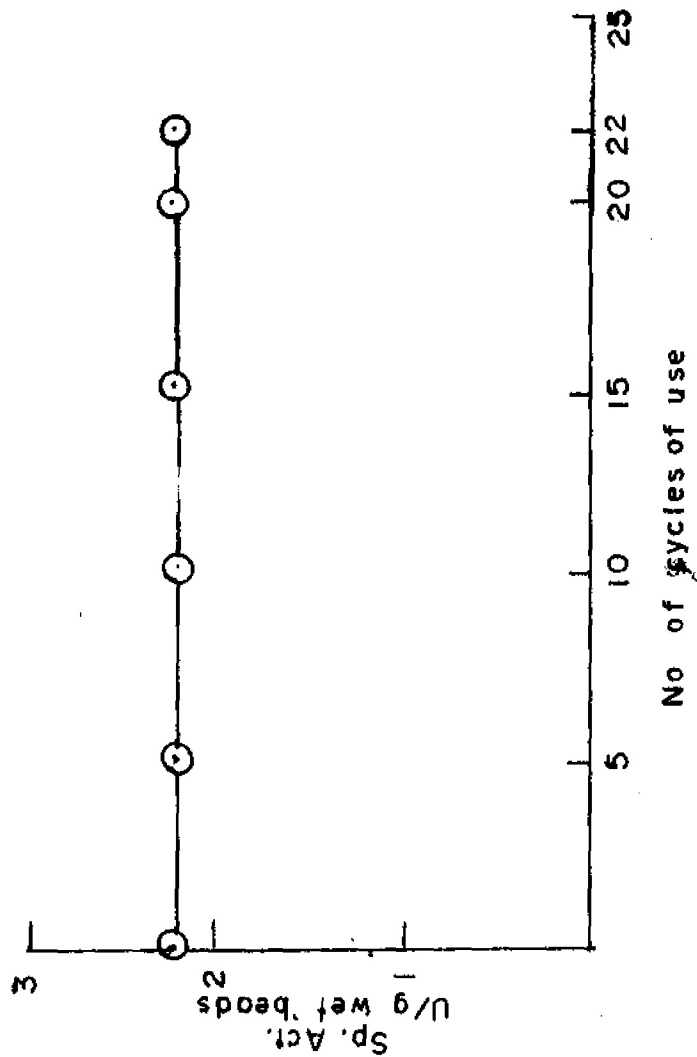


FIG II.4 OPERATIONAL STABILITY OF SEPHAROSE-CVB-PENICILLIN ACYLASE (K. citrophila). THE IMMOBILIZED ENZYME WAS RECOVERED BY FILTRATION AND USED IN SUCCESSIVE CYCLES OF BATCH OPERATION. THE SPECIFIC ACTIVITY OF THE PREPARATION WAS CHECKED AFTER EVERY 5 CYCLES OF USE AND AT THE END OF THE LAST CYCLE. NO DETECTABLE DECREASE IN SPECIFIC ACTIVITY OR ENHANCED TIME FOR 95-100% HYDROLYSIS OF SUBSTRATE WERE OBSERVED.

IMMOBILIZATION OF PENICILLIN ACYLASE ON CONTROLLED-PORE CERAMICS

The data on immobilization of the partially purified enzyme from *K. citrophila* on the silanized controlled-pore ceramics, TiO_2 , Al_2O_3 and SiO_2 are presented in TABLE II.2.

TABLE II.2 : IMMOBILIZATION OF PENICILLIN ACYLASE ON CONTROLLED-PORE CERAMICS

Cleaned ceramics were silanized with γ -aminopropyl-triethoxysilane and coupled to the partially purified enzyme through glyoxaldehyde.

Ceramics 100 mg
Partially purified Penicillin acylase 6 U/mg

Support	Pore diameter	Soluble enzyme		Immobilized enzyme	
		Total Activity		Activity Bound	Specific activity
		Amt. taken	Amt. recovered in filtrate + washings		
(mg)	(Å)	(U)	(U)	(%)	(U/g)
		(a)	(b)	(a)	(b)
TiO_2	560	6.8	3.3	52	36
Al_2O_3	425	6.8	3.6	47	25
SiO_2	225	5.0	0.2	96	30

(a) Calculated from the difference between activity taken and activity recovered in filtrate and washings.

(b) Value obtained on assay

It will be seen from TABLE II.2 that the binding to support varies from 47% to 96%. The highest efficiency of binding is observed with SiO_2 . The specific activity of

the TiO_2 -CVR-penicillin acylase preparation determined experimentally corresponded approximately to the value calculated from the units of activity taken and activity recovered in filtrate and washing. This would indicate that diffusional restrictions are negligible in this support. The enzyme bound on Al_2O_3 shows an experimentally^{al} determined activity corresponding to 78% of that picked^{up} from solution, while the enzyme immobilized on SiO_2 shows an efficiency of only 60% of the activity taken up^{on the support} from solution. The pore diameters of the TiO_2 , Al_2O_3 and SiO_2 were 560 Å, 225 Å and 425 Å respectively.

An experiment was carried out with TiO_2 to determine whether the binding efficiency could be enhanced by increasing the ratio of support to enzyme protein. In this experiment the units of activity taken corresponded to the calculated amount from the data obtained with the 100 mg lot of TiO_2 (TABLE II.2). The data are summarized in TABLE II.3.

TABLE II.3 EFFECT OF SUPPORT/PROTEIN RATIO ON THE EFFICIENCY OF BINDING OF PENICILLIN ACYLASE ACTIVITY TO SILANIZED TiO_2 VIA GLUTARALDEHYDE

Support	Soluble enzyme		Immobilized enzyme	
	Total Activity		Activity bound	Specific activity
	Amount taken	Amount recovered in filtrate + washing		
	(U)	(U)	(%) (a)	(U/g) (b)
TiO_2 (100 mg)	6.8	3.3	52	36
TiO_2 (500 mg)	16.6	1.5	90	28

(a) Calculated from activity taken and activity recovered in filtrate and washings.

(b) Experimentally determined.

It will be apparent from TABLE II.3 that an efficiency as high as 90% binding is obtained when the proportion of support to enzyme protein is increased. It is also seen from TABLE II.3 that the bound enzyme has an experimentally determined activity of 28 U/g, which is 90% of the calculated activity of 30.2 U/g picked up by activated support from the solution. This again confirms that diffusional restrictions in the enzyme bound to controlled-pore TiO_2 of 560 Å pore diameter are negligible.

**PROPERTIES OF PENICILLIN ACYLASE IMMOBILIZED ON
CONTROLLED-PORE CERAMICS**

HYDROLYTIC ACTIVITY

TiO₂-GVB-Penicillin acylase (K. citreophila)

pH Optimum : The dependence of activity of penicillin acylase immobilized on TiO₂ through the aldehyde-silane derivative is shown in Fig II.5. The pH optimum was 7.5 which is similar to that of the soluble enzyme as well as of enzyme immobilized on CNBr-activated sepharose.

Effect of substrate concentration : Effect of benzylpenicillin concentration on enzyme activity of penicillin acylase immobilized on TiO₂ is shown in Fig II.6. The K_m value calculated from the Lineweaver-Burk plot was 2.5 mM, which is similar to that of the soluble enzyme.

Effect of temperature : Activity dependence of penicillin acylase immobilized on TiO₂ on temperature is shown in Fig II.7. The calculated activation energy was 11.2 Kcal/mol, which is similar to that of the soluble enzyme. The calculated temperature coefficient, Q₁₀, was 1.9.

Progress curve of hydrolysis of benzylpenicillin : Progress curve of hydrolysis of benzylpenicillin at 2% substrate level is shown in Fig II.8. As in the case of enzyme immobilized on Sepharose, the hydrolysis of substrate is quantitative in a period corresponding to less than twice that calculated from the initial hydrolysis rate.

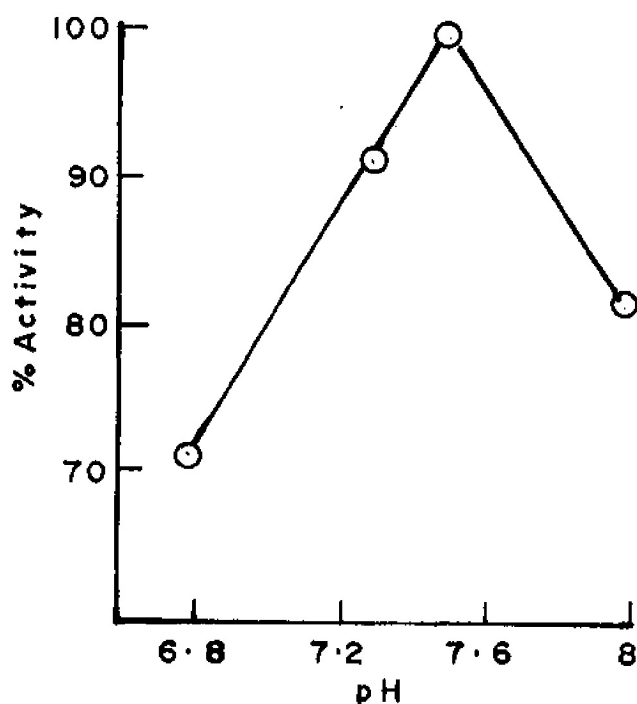


FIG. II.5 EFFECT OF pH ON HYDROLYTIC ACTIVITY OF TiO_2 -CVB-PENICILLIN ACYLASE (*K. citrophila*) TOWARDS BENZYL PENICILLIN. 0.1 M SUBSTRATE IN 0.1M POTASSIUM PHOSPHATE BUFFER, pH 6.8-8.0; 40°C . INITIAL RATES EXPRESSED AS % MAXIMUM ACTIVITY OBTAINED.

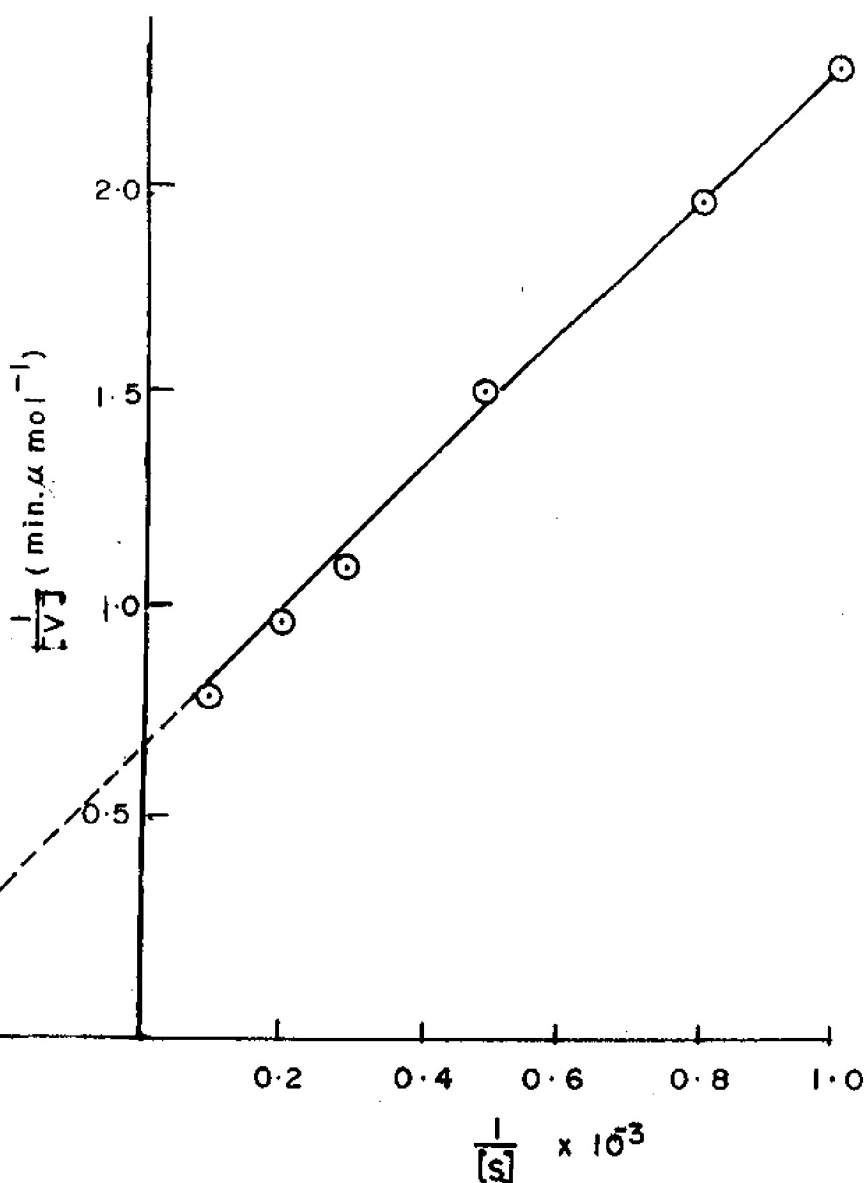


FIG.II.6. LINEWEAVER-BURK PLOT OF INITIAL RATES OF HYDROLYSIS OF BENZYL-PENICILLIN BY TiO_2 -CUE-PENICILLIN ACYLASE (*K.citrophila*). 0.1.M POTASSIUM PHOSPHATE BUFFER, pH 7.5; 40°C

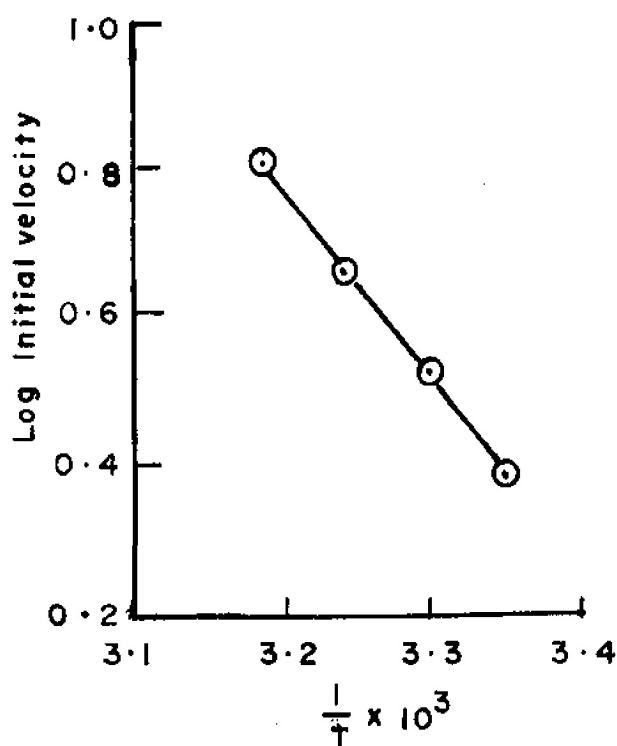


FIG.II.7 EFFECT OF TEMPERATURE ON HYDROLYTIC ACTIVITY OF T10₂-CVB- PENICILLIN ACYLASE (K.citrophila) TOWARDS BENZYL PENICILLIN . SUBSTRATES, 0.1 M; POTASSIUM PHOSPHATE BUFFER, pH 7.5, 0.1 M.

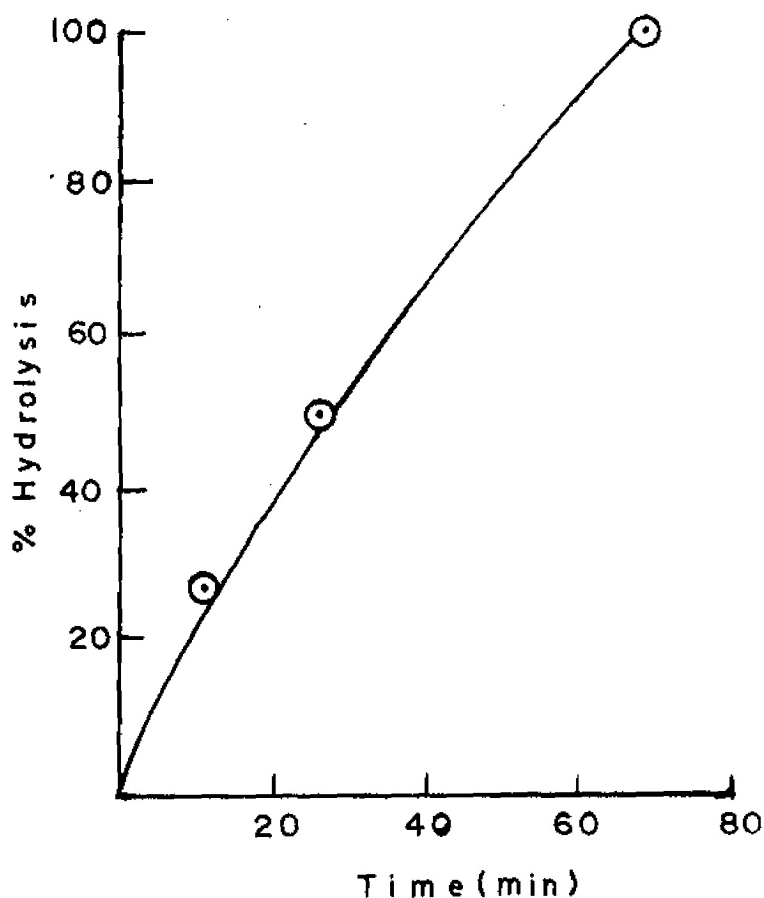


FIG.II.8. PROGRESS CURVE OF HYDROLYSIS OF BENZYL PENICILLIN
 BY TiO_2 -CVB-PENICILLIN ACYLASE (*K.citrophila*)
 10 ml. 2% W/V BENZYL PENICILLIN K-SALT: pH MAINTAINED
 AT 7.5 WITH ADDITION OF NH_4OH A_d 40°C. 500 mg TiO_2
 - ENZYME (14 U)

Operational stability of Penicillin acylase immobilized on Controlled-pore Ceramics

TiO₂-CVR-penicillin acylase : Operational stability was determined as described under Experimental Procedure by determining the specific activity of the immobilized enzyme after every 5 cycles of use. The data are presented graphically in Fig II.9. It can be seen from Fig II.9 that no detectable loss in activity occurs even after 10 successive cycles of use. Further prolonged use has not been carried out.

Al₂O₃-CVR-penicillin acylase : The experiments on Al₂O₃ were carried out only over a limited number of cycles of use. 4 cycles of use ^{were} ~~was~~ found to have no effect on the activity of the immobilized enzyme. The results are shown graphically in Fig II.10.

SiO₂-CVR-penicillin acylase : The studies with SiO₂-immobilized enzyme were also only over a limited number of cycles of use. The data are summarized in Fig II.11. It can be seen from Fig II.11 that approximately 20% loss in activity occurs in every cycle of use of enzyme immobilized on SiO₂. The leakage of bound enzyme from SiO₂, particularly at alkaline pHs has been reported in the literature. Glucoamylase bound to SiO₂ has been shown to leak activity (Weetall and Havewala, 1972) even at acid pH of 4.5. This has been explained on the susceptibility of the -Si-O-Si- alkylamine bonds to hydrolytic cleavage. The silanization

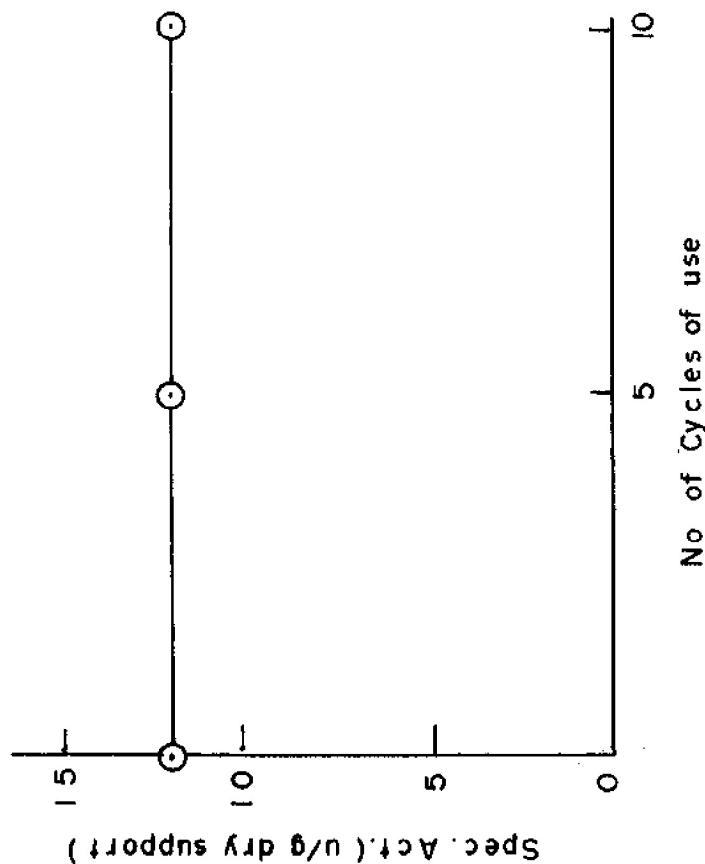


FIG. II-9. OPERATIONAL STABILITY OF TiO_2 -CVB-PENICILLIN ACYLASE (K.citrophila)
 THE IMMOBILIZED ENZYME WAS RECOVERED BY FILTRATION AND USED IN
 SUCCESSIVE CYCLES OF BATCH OPERATION. THE SPECIFIC ACTIVITY OF
 THE PREPARATION WAS CHECKED AFTER EVERY 5 CYCLES OF USE.
 NC: DETECTABLE DECREASE IN SPECIFIC ACTIVITY OR ENHANCED TIME
 FOR 95-100% HYDROLYSIS OF SUBSTRATE WERE OBSERVED.

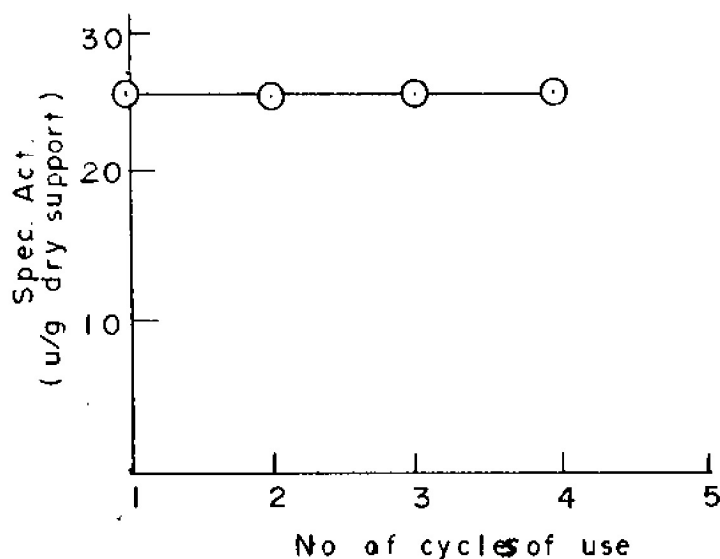


FIG. II.10, OPERATIONAL STABILITY OF Al_2O_3 -CVB-PENICILLIN ACYLASE (*K. citrophila*). THE IMMOBILIZED ENZYME WAS RECOVERED BY FILTRATION AND USED IN SUCCESSIVE CYCLES OF BATCH OPERATION. THE SPECIFIC ACTIVITY OF THE PREPARATION WAS CHECKED AFTER EVERY CYCLE OF USE.

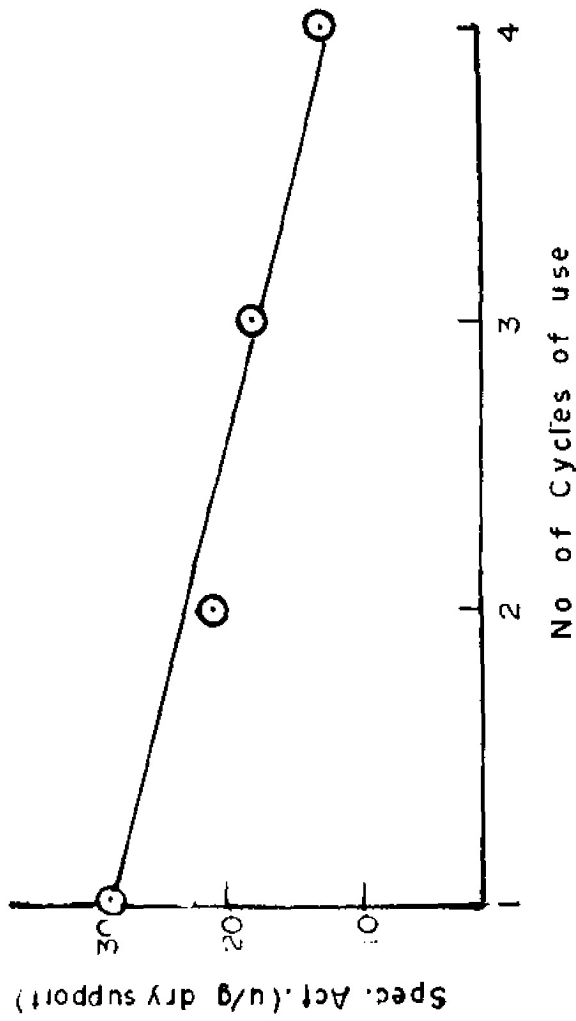


FIG. II.11. OPERATIONAL STABILITY OF SiO_2 -CMB-PENICILLIN ACYLASE (*K. citrophila*)
THE IMMOBILIZED ENZYME WAS RECOVERED BY FILTRATION AND USED IN
SUCCESSIVE CYCLES OF BATCH OPERATION. THE SPECIFIC ACTIVITY OF THE
PREPARATION WAS CHECKED AFTER EVERY CYCLE OF USE.

occurs superficially at the exposed surfaces of the support and the cleavage of these bonds results in leakage of the bound enzyme. Such leakage has been overcome through ZrO_2 -coating of the support which results in the more resistant $-Zr-O-Si-$ alkylamine bonds on silanization.

SYNTHETIC ACTIVITY

Synthesis of ampicillin : Synthesis of ampicillin from 6-APA and DL-phenylglycine methyl ester catalyzed by partially purified penicillin acylase immobilized on silanized- TiO_2 was determined at pH 6.5 and $40^\circ C$. The pH used^{was} that shown to be optimum for the soluble enzyme for the synthetic reaction by Okashi et al. (1972a). The progress of the reaction was monitored by estimating disappearance of 6-APA by the p-dimethylaminobenzaldehyde method described in the Experimental Procedure. The progress curve for the synthesis is shown in Fig II.12.

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. Conversion calculated from data presented by Okashi et al. (1972a) for the soluble enzyme from K. citreophilis, 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. Mareoni et al. (1973) have reported 45% conversion of 6-APA to ampicillin in case of the fibre entrapped E. coli enzyme while Kamogashira et al. (1972) estimated 70% conversion with DEAE-sephadex adsorbed succinylated enzyme from E. coli.

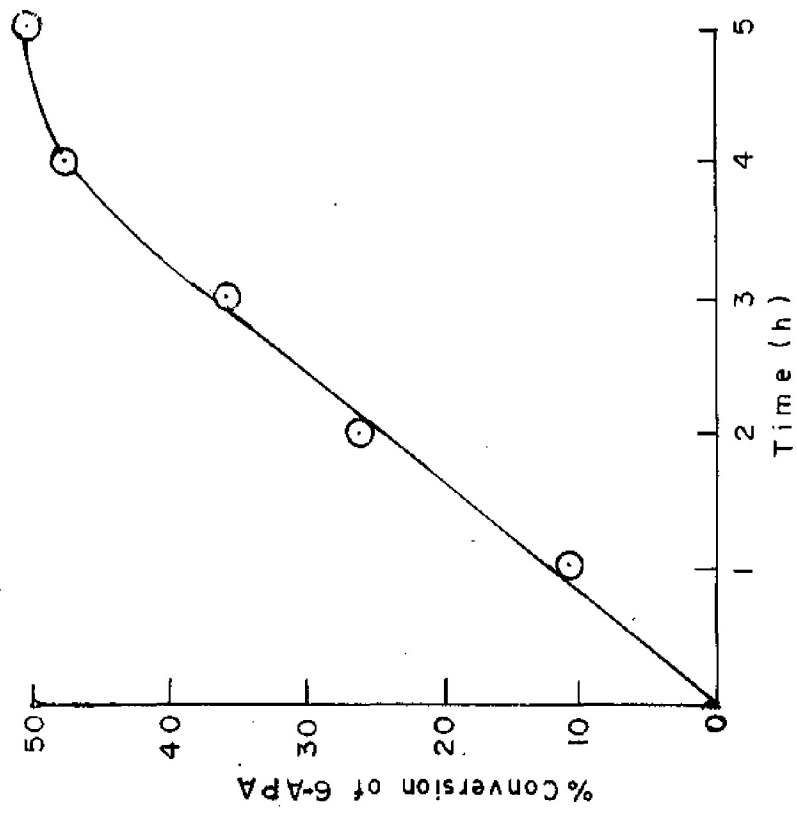


FIG. II. 12. PROGRESS CURVE OF FORMATION OF AMPICILLIN FROM 6-APA AND PHENYLGLYCINE METHYL ESTER. 1102-CVF-PENICILLIN ACYLASE
K. citrophila (250 mg SUPPORT, 7 U); 10 ml, REACTION
 MIXTURE CONTAINING: 6-APA, 50 mg; DL-PGME, 125 mg;
 0.03 M POTASSIUM PHOSPHATE BUFFER, pH 6.5; 40° C.
 ALIQUOTS WERE WITHDRAWN EVERY h FOR 6-APA ASSAY.

Characterization of the product

The product obtained in the experiment described above was characterized by reversed-phase thin-layer chromatography as described in the Experimental Procedure. The diagrammatic representation of the chromatogram is shown in Fig II.13. The reaction mixture at the end of 5 h incubation qualitatively shows presence of a spot corresponding to that obtained with an authentic sample of ampicillin, in addition to those due to reactants.

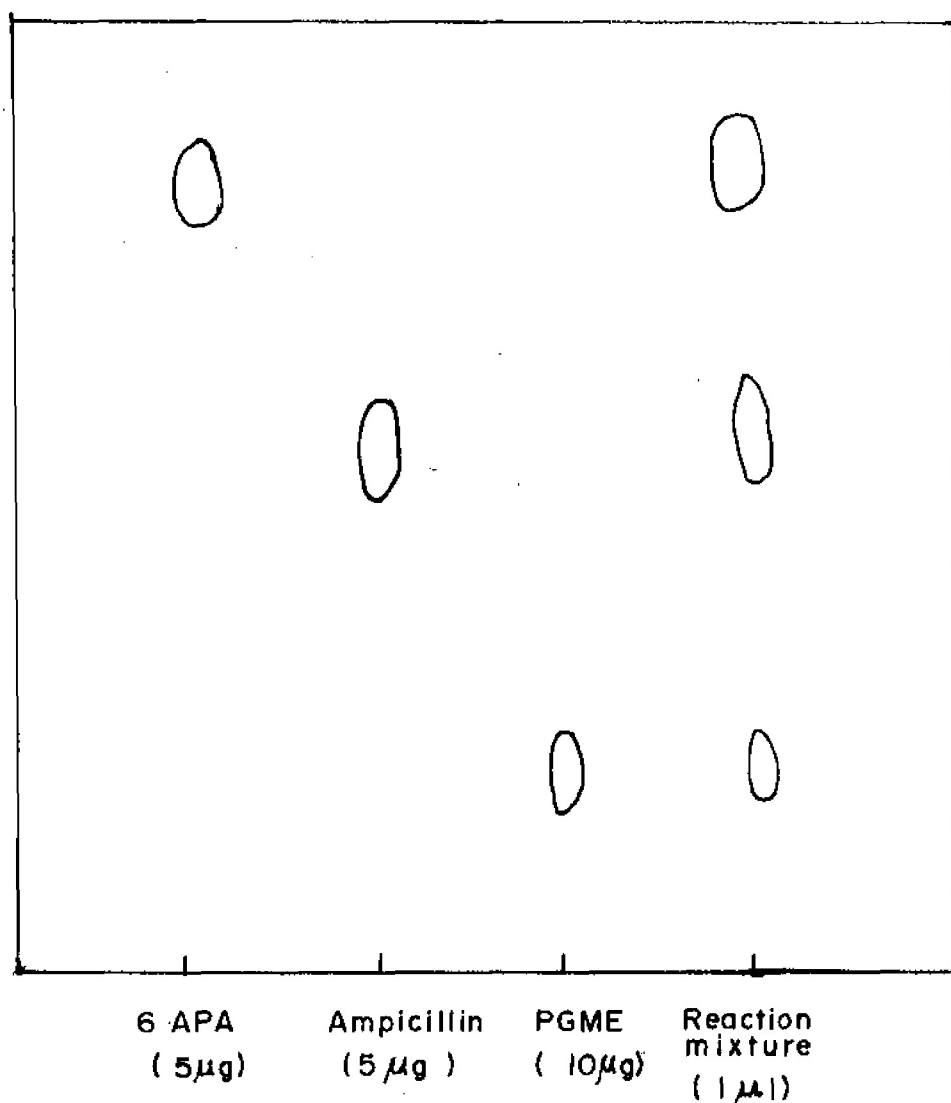


FIG.II.13. DIAGRAMMATIC REPRESENTATION OF TLC.

CHARACTERIZATION OF PRODUCTS OF REACTION
BETWEEN 6APA AND PHENYLGLYCINE METHYL ESTER
IN THE PRESENCE OF TiO_2 — CMB-PENICILLIN ACYLASE
FROM *K. citrophila*.

REACTION MIXTURE;—50 mg 6-APA, 125 mgDLPGME, TiO_2 —
ENZYME 250 mg (7 U), TOTAL VOL. 6.5 ml; pH 6.5,
TEMPERATURE 40° C.

REACTION MIXTURE(1µl) SPOTTED ON SILICONE-COATED
SILICA GEL G AND DEVELOPED WITH 0.05 M SODIUM ACETATE-
VERONAL BUFFER,pH 7.4/ACETONE (50 : 1 v/v).
SPOTS VISUALIZED WITH ALKALINE KMnO_4 .

DISCUSSION

The partially purified penicillin acylase from *E. citreophila* is immobilized quantitatively in presence of an excess of CNBr-activated Sepharose 4B and in approximately 90% efficiency by the aldehyde derivative of silanized TiO_2 , when adequate support is taken. The enzyme immobilized on Sepharose shows an efficiency of 67% of bound activity, the enzyme immobilized on TiO_2 shows 90-100% efficiency. This could be explained probably on ^{account of} diffusional restrictions in the case of the gel and the absence of this in the case of the Controlled-pore ceramic support. The pH optimum of the enzyme has been shown to be the same as that of the soluble enzyme in the case of both supports.

The enzyme immobilized on TiO_2 has a K_m value similar to that of the soluble enzyme; the values for the immobilized and the soluble enzyme being 2.5 mM and 2.7 mM, respectively.

The calculated activation energy of the enzyme immobilized on TiO_2 is 11.2 Kcal/mol, which is in close agreement to the value obtained with the free enzyme. Enzyme immobilized on Sepharose has an activation energy of 12.5 Kcal/mol compared to a value of 10.9 Kcal/mol of soluble enzyme. Theoretically diffusional restrictions should result in lowering of the activation energy, while any enhanced temperature stability due to immobilization should

result in an increase in this value. Enhancement of the activation energy has been reported in the case of glucose isomerase immobilized on fine glass particles, the values being 17.8 Kcal/mol (Lee et al. 1976) for the immobilized enzyme compared to 14.6 Kcal/mol (Danno, 1970) reported for the soluble enzyme.

The enzyme immobilized on CNBr-activated Sepharose has high operational stability and no loss in activity could be detected even after 22 cycles of use. Such stability has been reported in the enzyme from E. coli immobilized on CNBr-activated Sephadex G-200 (Ekstrom et al., 1974).

The enzyme immobilized on Controlled-pore TiO_2 also showed high operational stability and in the 10 cycles of use, to which the immobilized enzyme preparation was put, no detectable loss in activity was observed. Controlled-pore Al_2O_3 tested over 4 cycles of use also showed no measurable losses. Controlled-pore SiO_2 on the other hand was found to lose approximately 20% bound enzyme activity in every cycle of use. Such behaviour of SiO_2 -immobilized enzymes has been reported earlier and attributed to the ease with which the surface $-\text{Si}-\text{O}-\text{silane}$ bonds are hydrolysed, particularly at alkaline pH values (Westall and Havewalla, 1972).

The application of penicillin acylase from K. citrophila immobilized on TiO_2 for the conversion of 6-APA to ampicillin in the presence of phenylglycine methyl ester was examined

in preliminary experiments using an excess of ester. Approximately 50% of the 6-APA could be converted to a product resembling ampicillin in thin-layer chromatography.

The present investigation establishes the usefulness of immobilized penicillin acylase from K. citrophila both for hydrolysis of benzylpenicillin to 6-APA as well as for the synthesis of ampicillin from 6-APA and phenylglycine methyl ester. These are the first reports on the behaviour of immobilized enzymes obtained from this source and also of penicillin acylase covalently bound to inorganic supports.

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