MICROBIAL ENZYMES: STUDIES ON PENICILLIN ACYLASE (EC 3.5.1.11) FROM *Escherichia coli*

A THESIS
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Dedicated

to

My Parents

C O N T E N T S

DECLARATION

Certified that the work incorporated in the thesis **"Microbial Enzymes: Studies on Penicillin Acylase (EC 3.5.1.11) from Escherichia coli"** submitted by Mrs. Asmita A. Prabhune was carried out by the candidate under my supervision. Such material as has been obtained from other sources has been duly acknolwedged in this thesis.

H SivaRaman

Dr. (Mrs.) H. SivaRaman
Research Guide

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Aprabhune

(Asmita Prabhune)

ABBREVIATIONS

6-APA	6-aminopenicillanic acid
NBS	N-bromosuccinimide
HNBBr	2-hydroxy-5-nitrobenzyl bromide
PMSF	Phenylmethylsulphonyl fluoride
CTAB	N-cetyl-N,-N,N-trimethylammonium bromide
SDS	Sodium dodecylsulphate
DEAE-Sepharose	Diethylaminoethyl-Sepharose
Tris	Tris(hydroxymethyl) amino-methane

ABSTRACT

INTRODUCTION :

Penicillin acylases (penicillin amidohydrolases, EC 3.5.1.11) are enzymes of microbial origin that catalyse the hydrolysis of penicillins to the corresponding side chain and the β-lactam nucleus , 6- aminopenicillanic acid (6- APA) . The cleavage reaction catalysed by the enzymes, which proceeds to virtual completion under appropriate conditions of pH, temperature and substrate concentration, is currently the preferred route for the manufacture of 6-APA, the key intermediate in the production of semisynthetic penicillins.

The enzymes are classed under the following three types depending on their substrate preference or specificity; the penicillin G acylases which are usually of bacterial origin and catalyse the hydrolysis of benzylpenicillin (penicillin G) preferentially, the phenoxymethyl penicillin acylases (penicillin V) which are usually of fungal origin and catalyse the hydrolysis of (penicillin V) phenoxymethylpenicillin preferentially, and the bacterial ampicillin acylases which catalyse the hydrolysis of ampicillin (D-aminobenzylpenicillin) specifically.

Extensive studies have been reported in the literature on the production of these enzymes, the immobilization of the enzyme

activity and the cloning of the relevant genes. Relatively little is known however about the structure - function relationships and the active -site residues involved in the enzyme action.

The **THESIS** embodies work on *Escherichia coli* penicillin acylase, one of the most studied among enzymes of this group. The investigations carried out on the enzyme from *E. coli* ATCC 11105 and *E. coli* NCIM 2350 include studies on enzyme production, characterization of active site residues, whole cell and enzyme immobilization.

The **THESIS** is presented in 5 sections, **Part I** of which is a **GENERAL INTRODUCTION** which comprises a review of the relevant literature. The present work is set out in the remaining **Parts** and the following are the significant finding from these studies. The salient findings from these investigations are summarized briefly below:

PART I: GENERAL INTRODUCTION

PART II : PRODUCTION AND PURIFICATION OF BENZYLPENICILLIN ACYLASE FROM *ESCHERICHIA COLI* AND ISOLATION OF CONSTITUTIVE MUTANT

Benzylpenicillin acylase production by *Escherichia coli* NCIM 2350 was standardized using complex sources of carbon and

nitrogen to yield high cell-bound activity under shake flask conditions.

A modified purification procedure was developed for obtaining pure benzylpenicillin acylase from *E.coli* cells. The procedure involved sonication of cells for extraction of enzyme activity, removal of nucleic acids by streptomycin sulphate treatment, fractional precipitation with ammonium sulphate, DEAE-Sepharose column chromatography and gel filtration through Sephadex G-200. The homogeneity of the preparation was established by disc electrophoresis in polyacrylamide gel which showed the presence of a single protein band and by SDS-polyacrylamide gel electrophoresis which showed the presence of the two component polypeptide bands of $M_r \backsim 20,000$ (α-subunit) and $M_r \sim 70,000$ (β-subunit). The pure enzyme had a specific activity of 27 - 36 μmole.$min^{-1}mg^{-1}$ in hydrolysis of benzylpenicillin at pH 7.8 and 40°C.

A mutant designated as *E. coli* UV-2 was isolated by UV-irradiation of *E. coli* NCIM 2350. Unlike the parent strain, the mutant produced benzylpenicillin acylase constitutively. The enzyme produced by the mutant remained cell-bound as in the parent strain. The mutant produced higher levels of enzyme activity constitutively than the parent strain in presence of phenylacetic acid. Glucose (1 g l^{-1}) repressed completely the induced enzyme production by the parent strain. The mutant,

however, was more resistant to repression by glucose, producing about 60% of the activity obtained in absence of glucose.

PART III: ESSENTIAL ARGININE AND TRYPTOPHAN RESIDUES IN THE PUTATIVE SIDE CHAIN BINDING SUBUNIT OF PENICILLIN ACYLASE FROM *ESCHERICHIA COLI*.

Incubation of penicillin acylase from *Escherichia coli* with phenylglyoxal or 2,3-butanedione results in enzyme inactivation. Benzylpenicillin, phenylacetamide and phenylacetate protected the enzyme against the inactivation, indicating the presence of arginine at or near the catalytic site. The reactions follow pseudo first-order kinetics and the inactivation kinetics indicate the presence of a single essential arginine moiety.

The enzyme is also inactivated by incubation with the tryptophan-modifying reagents, N-bromosuccinimide (NBS) and 2-hydroxy-5-nitrobenzyl bromide (HNBBr). Benzylpenicillin, phenylacetate and 6-aminopenicillanic acid (6-APA) significantly lower the extent of inactivation by the tryptophan-modifying reagents. The inactivation reaction kinetics are indicative of the involvement of a single essential tryptophan residue.

Subunit-complementation experiments in which various combinations of the physically isolated α and β-subunits derived from untreated enzyme and the phenylglyoxal-inactivated or

the NBS-inactivated enzyme were assayed for recovery of enzymatic activity, indicated that both the essential arginine and the essential tryptophan residues are present in the putative substrate-binding α-subunit of the enzyme molecule. These are the first reports on the essential amino acid residues involved in the binding domain of the α-subunit of penicillin acylase.

PART IV: IMMOBILIZATION OF PERMEABILIZED *ESCHERICHIA COLI* WITH BENZYLPENICILLIN ACYLASE ACTIVITY.

Escherichia coli cells with penicillin acylase activity were sequentially treated at pH 7.8 with aqueous solutions of N-cetyl-N,N,N-trimethylammonium bromide and glutaraldehyde and then immobilized within porous polyacrylamide beads. The immobilized whole cells showed enhanced hydrolysis rates in the conversion of benzylpenicillin to 6-APA compared to untreated cells immobilized and used under identical conditions.

The immobilized system showed no apparent loss in enzyme activity when used repeatedly over 90 cycles for 6-APA production from 4% benzylpenicillin.

PART V: IMMOBILIZATION OF PENICILLIN ACYLASE IN POROUS BEADS OF POLYACRYLAMIDE GEL.

A procedure is described for the immobilization of benzylpenicillin acylase from *Escherichia coli* within uniformly

spherical,porous polyacrylamide gel beads. Aqueous solutions of the enzyme and sodium alginate and acrylamide monomer, N,N´-methylene-bis-acrylamide,N,N,N´N´-tetramethylethylenediamine (TEMED) and sodium alginate are cooled separately, mixed and dropped immediately into ice-cold, buffered calcium formate solution, pH 8.5, to give calcium alginate-coated beads. The beads are left for 60 min in the cold calcium formate solution for polyacrylamide gel formation.The beads are then treated with a solution of glutaraldehyde and the calcium alginate subsequently leached out with a solution of potassium phosphate buffer.

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

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

PART I

GENERAL INTRODUCTION

I.1. ENZYMATIC CLEAVAGE OF PENICILLINS

The hydrolytic cleavage of the penicillin molecule is brought about by two distinct classes of enzymes; namely β-lactamases (penicillin β-lactam hydrolases EC 3.5.2.6) which catalyse the opening up of the β - lactam ring and penicillin acylases (penicillin amidohydrolase EC 3.5.1.11; also known less commonly as penicillin amidases) which catalyse reversibly the deacylation to 6-aminopenicillanic acid (6-APA) and the carboxylic acid side chains **(Fig.I.1)**. These two types of activities are exhibited by several genera of microorganisms , the former being more widespread in it´s distribution than the latter (Vandamme and Voets ,1974; Vandamme, 1980).

I. 2. PENICILLIN ACYLASE (EC 3.5.1.11)

The earliest reports on the enzymatic hydrolysis of penicillins to 6-APA were of Sakaguchi and Murao (1950; 1955) with enzymes from *Penicillium chrysogenum* and from *Aspergillus oryzae.*

Many bacteria, actinomycetes, fungi and yeasts have subsequently been shown to produce the enzyme, intracellularly or cell-bound in some cases and extracellularly in others (Vandamme and Voets, 1974; Vandammme, 1980; Sudakaran and Borkar, 1985).

The enzymes catalyse the hydrolysis of penicillins to 6-APA and the side chain carboxylate in almost quantitative yield under appropriate conditions of pH, usually in the alkaline range (Cole, 1967). The reverse reaction in the synthetic direction proceeds at a slightly acid pH, particularly with an activated form of the side chain such as the amide or the ester derivative.

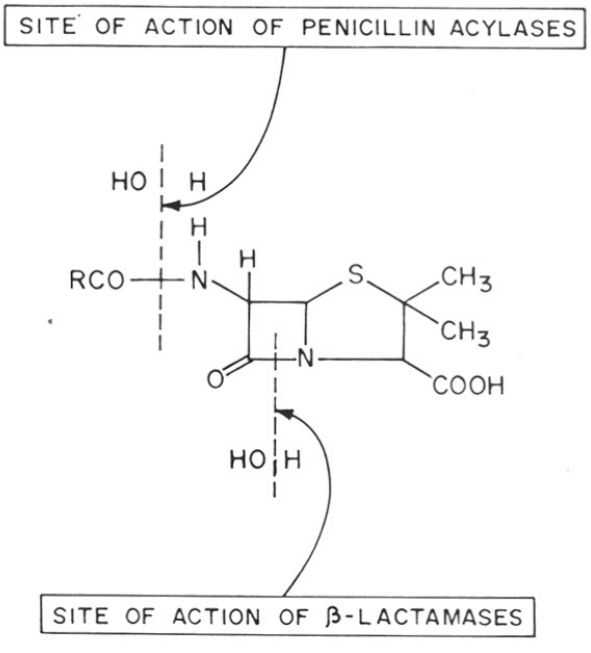


FIG. I-1: SITE OF ACTION OF PENICILLIN HYDROLYSING ENZYMES

The conversion of 6-APA to penicillin catalysed by penicillin acylase is only partial even in presence of an excess of the side chain derivative, reported values ranging between 45% - 70% at equilibrium in the case of ampicillin synthesis (Okachi *et al.*, 1972; Kamogashira *et al.*, 1972; Marconi *et al.*, 1973).

Besides penicillins, penicillin acylases catalyse the hydrolysis of amides, acylamino acids and ester derivatives of the carboxylic acid side chains of penicillins, substrate susceptibility being determined by the acyl moiety and some of the non-penicillin compounds being better substrates than the corresponding penicillins (Cole, 1964; Kaufmann and Bauer, 1964; Kutzbach and Rauenbusch, 1974).

I.2.i Types of Penicillin Acylases

Penicillin acylases have been grouped under three types depending on their substrate preference or specificity towards phenoxymethylpenicillin (penicillin V); benzylpenicillin (penicillin G) or ampicillin (D-phenylglycylpenicillin) **Fig.I.2.** The types are:

(a) **Phenoxymethylpenicillin acylases** (described earlier as type I) generally of fungal origin which catalyse the hydrolysis of phenoxymethylpenicillin preferentially,

(b) **Benzylpenicillin acylases** (type II) mostly of bacterial origin, which catalyse the hydrolysis of benzylpenicillin preferentially, and

(c) **Ampicillin acylase** (type III) reported hitherto only in *Pseudomonads* which catalyses the hydrolysis of ampicillin specifically.

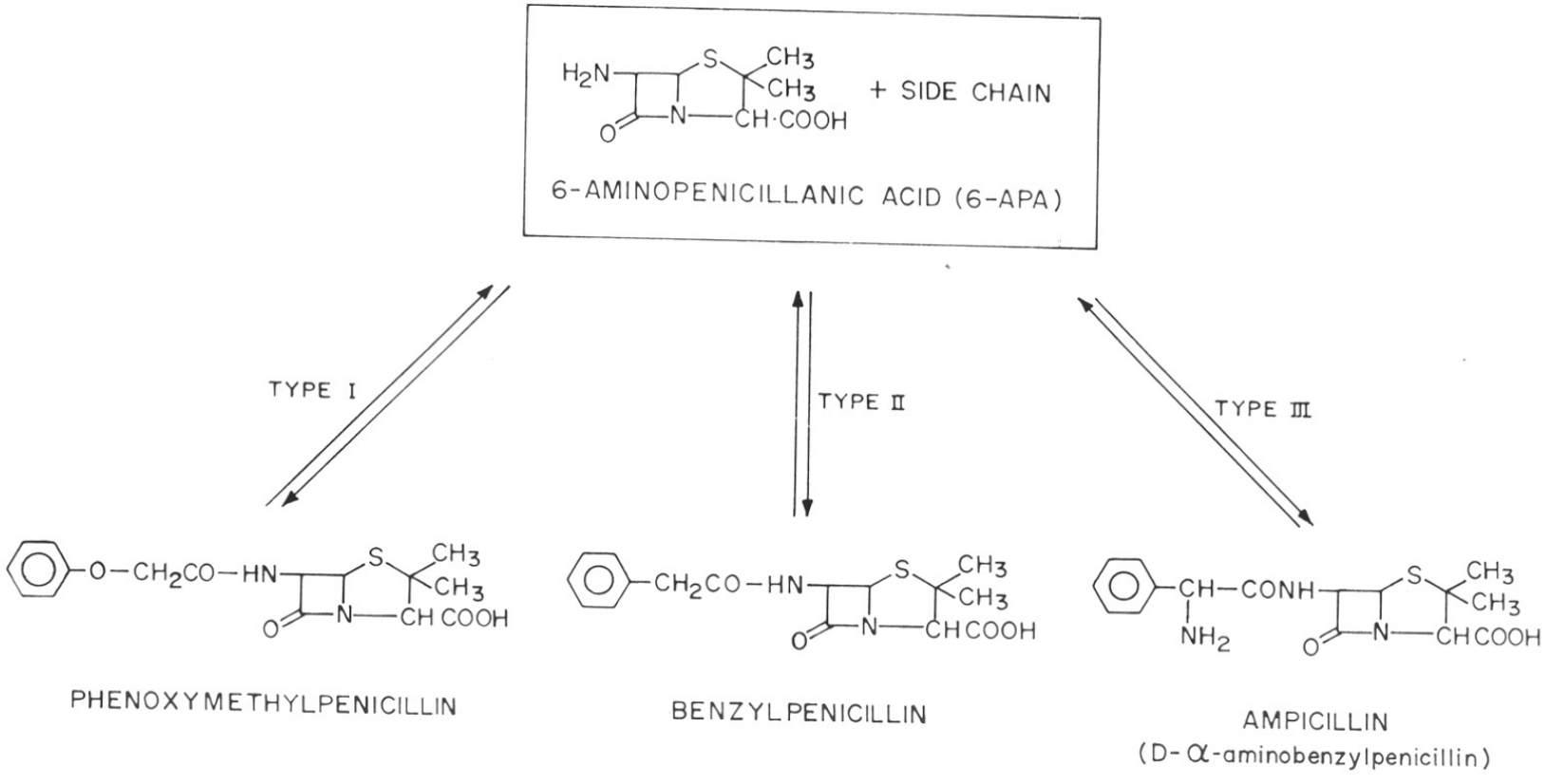


FIG. I·2 : PENICILLIN AMIDOHYDROLASES (EC 3·5·1·11)

I.2.ii. Physiological Role

The role of penicillin acylases in the physiology of the microorganisms that produce these enzymes is not understood clearly. The early hypothesis that the role is to confer resistance towards β-lactam antibiotics that may be present in the natural habitats (Holt and Stewart, 1964) has been disputed mainly on account of the low affinity that the acylases have for β-lactam antibiotics and the unphysiological, alkaline pH for the hydrolytic activity (Sutherland, 1964; Cole and Sutherland, 1966). The currently favoured hypothesis is that the enzymes on account of their preference for the acyl moiety are probably involved in the utilization of naturally occurring aromatic compounds such as derivatives of phenylacetic acid which are abundant in plant detritus (Valle *et al.*, 1991).

I.2 .iii. Industrial Applications

Penicillin acylases have found extensive industrial application in the manufacture of 6-APA, the key intermediate in the production of semi-synthetic penicillins. The global annual production of 6-APA in 1985 had been 4200 tons and the estimated 1990 production was 5250 tons (Shewale and SivaRaman, 1989). The bulk of the intermediate is produced through enzymatic hydrolysis of biosynthetic penicillins, mainly benzylpenicillin, using immobilized penicillin acylase in preference to the chemical deacylation route (Vandamme, 1988).

On account of its industrial importance, extensive literature exists particularly on the production, immobilization and application of penicillin acylases. Several reviews have also

been published including those by Hamilton-Miller (1966); Cole (1967); Vandamme and Voets (1974); Cole *et al.*(1975); Savidge (1975); Vanderhaeghe (1975); Abbott (1976); Vandamme (1980); Mahajan (1984); Francetic (1988) and Shewale and SivaRaman (1989).

The present GENERAL REVIEW focusses mainly on aspects relevant to the investigations reported in the thesis. These include an overview of the more important sources of the enzymes, approaches adopted for strain improvement, purification and molecular aspects of penicillin acylases, molecular biology of penicillin acylase genes and some immobilized microbial whole cell and enzyme systems.

I.2.iv. Screening Methods

The approach of screening for penicillin - resistance (Holt and Stewart,1964) was ineffective as β -lactamase activity is more widespread in distribution and the procedure does not distinguish between the two degradative activities.The alternative approach of selective growth on mineral medium containing penicillin or phenylacetic acid as sole source of carbon and testing for hydrolysis of acylamino acids was adopted by Kameda *et al.*,(1961). Selective growth on corn steep liquor medium and other penicillin fermentation media have also been employed for screening for penicillin acylase- producers (Batchelor *et al*,1961;Haung *et al*,1963). Mineral medium with N-acetylglycine,glycylglycylglycine or phenylacetamide as sole carbon and nitrogen source has been used by Voets (1973) and Vandamme(1973) for selective growth of penicillin acylase-

producers.Amide substrates were used by Walton (1964)in a soil enrichment procedure and the isolates screened with the chromogenic substrate, N-phenylacetyl-4-nitroanilide, a colourless compound that liberates yellow 4-nitroaniline on hydrolysis.Other chromogenic substrates that have been applied are 6-nitro-3-phenylacetamido benzoate(Kutzbach and Rauenbusch,1974) and 2-nitro-4-phenylacetamido benzoate (Nys *et al.*, 1977).In yet another procedure,paper discs soaked with phenyacetyl-4-aminobenzoate show up as red spots after diazotization and coupling with H-acid(1-amino-8-hydroxynaphthalene-3,6-disulphonic acid) when enzyme activity is present(Szewczuk,*et al.*,1980). The paper discs are left 30 s in contact with bacterial cultures on agar plates prior to assay.

A microbiological screening method based on *Serratia marscecens* ATCC 27117 which is sensitive to 6-APA has been described by Meevootisom *et al.*,(1983).

I.2.v. Occurrence

Examples of microorganisms that produce the three types of penicillin acylases are set out in **Tables** I.1,I.2 and I.3.The enzymes in the wild type are generally induced in presence of the side chain or it´s derivatives. Strains show variation in enzyme production even within species.

(a) **Phenoxymethylpenicillin Acylases**

Penicillin acylases of most fungi are associated with the mycelia. Exceptions are the presence of the activity in spores of *Fusarium moniliforme* AYF 255,*F. conglutinans* AYF 254 (Singh *et al.*,1969) and *F. moniliforme* (Vandamme *et al.*,1971). While most

of this type of acylases are also active towards benzylpenicillin and ampicillin. the enzymes from a *Cephalosporium* CM 149137(Claridge *et al* ,1963), *Fusarium semitectum* BC805 (Baumann *et al*., 1971)and a *Fusarium* sp(Thadani *et al* .,1972) have been reported to be specific towards phenoxymethylpenicillin and the enzyme from the yeast, *Rhodotorula glutinis* (Vandamme, 1973; Vandamme and Voets, 1973) has also been reported to hydrolyse phenoxymethylpenicillin specifically.

The bacterial enzyme from *Erwinia aroidae*(Vandamme, 1972; Voets and Vandamme, 1973) has been reported to be constitutive, specific for phenoxymethylpenicillin and to have no synthetic activity.

(b) **Benzylpenicillin Acylases**

The enzymes are mainly of bacterial origin, exceptions being the enzyme from *Neurospora crassa* (Rossi *et al* .,1973). The enzyme from *Bacillus megaterium* (Chiang and Bennett, 1967) and from *Arthrobacter viscosus* (Ohashi *et al* , 1988) are extracellular, while in other cases the enzyme remains cell-bound. The enzymes from *Escherichia coli*, which has been studied the most, remains in the periplasmic space (Schumacher *et al* ., 1986).

(c) **Ampicillin Acylases**

The enzymes , obtained hitherto only from *Psuedomonas*, have been shown to be specific towards ampicillin and to have no activity against phenoxymethylpenicillin and benzylpenicillin.

I.2.vi.Regulation of Enzyme Production

Enzyme production has been studied the most in the bacterial

systems, particularly in *E. coli*. Enzyme production in wild strains of *E.coli* and several other microorganisms has generally been reported to be induced by the presence of the side chain carboxylates of the penicillin molecule.

Benzylpenicillin acylase production by *E.coli* is stimulated in presence of phenylacetic acid (Kaufmann and Bauer, 1968) or ammonium phenylacetate (Sikyta and Slezak, 1964), 1 mg of the acid/ml stimulating 8-fold the enzyme production in cells (Levitov *et al.*, 1967). The highest productivity by cells has been reported when phenylacetate was the sole source of carbon and energy (Vojtisek and Slezak, 1975).

Enzyme synthesis in *E. coli* is repressed at high concentrations of dissolved oxygen (Kleiner and Lopatev,1972;Vojtisek and Slezak,1975).Complete catabolite repression by glucose and partial repression by acetate have been reported (Vojtisek and Slezak, 1975). cAMP stimulates enzyme production and overcomes completely repression by glucose in *E. coli* (Gang and Shaikh, 1976)and diminishes catabolite repression in *K.citrophila* (Takesava *et al.*,1972;Shimizu *et al.*,1975 a,b).

Enzyme production by *E. coli* is optimal at temperatures between 24°C - 28°C, no enzyme activity being produced when grown at 37°C (Vojtisek and Slezak, 1975). Unlike in *E.coli*,benzylpenicillin acylase is produced constitutively by *P.rettgeri* ATCC 31052 and enzyme production is not subject to catabolite repression by glucose but is repressed by the C_4 dicarboxylic acids of Krebs cycle *viz.* succinate,fumarate and malate (Daumy *et al.*,1982)

TABLE I.1: Microorganisms that produce phenoxymethylpenicillin acylase

Organism	References
Fungi	
Penicillium chrysogenum Q 176	Sakaguchi and Murao(1950)
P. chrysogenum A 9342	Claridge *et al.* (1963)
P. chrysogenum wis 49408	Erickson and Bennett (1965)
Bovista plumbea NRRL 3501, 3824	Schneider and Roher(1976)
Cephalosporium sp.	Cole (1966)
C. acremonium ATCC 11550	Dennen *et al.* (1971)
Aspergillus niger sp.	Vandamme *et al.* (1971)
Emericellopsis minim (Stolk) IMI 69015	Cole and Rolinson (1961)
Cephalosporium CMI 49137	Claridge *et al.* (1963)
Pleurotus ostreatus	Brandl (1965)
Botrytis cinerea	Batchelor *et al* (1961)
Fusarium sp. 755	Thadani *et al.* (1972)
F. semitectum BC 805	Baumann *et al.* (1971)
Gibberella fugikuroi	Vasilescu *et al.* (1969)
Yeasts	
Rhodotorula glutinis	Vandamme and Voets (1973)
Cryptococcus, Saccharomyces, Trichosporon Torulopsis	Batchelor *et al.* (1961)
Zygosaccharomyces	Cole (1966,1967)
Debaromyces, Torula	

Bacteria	
Erwinia aroidae	Voets and Vandamme (1972)
Achromobacter (NCIB 9424)	Cole(1964)
Micrococcus ureae KY 3769	Nara *et al.* (1971)
Streptomyces lavendulae B R L 198	Batchelor *et al.* (1961)
Bacillus sphaericus	Carlson and Emborg (1981)
Beijerinckia indica var. *penicillanicum*	Ambedkar *et al.* (1991)
Actinomycetales	
Nocardia globerula KY 3901	Nara *et al.* (1971)
Sreotomyces lavendulae BRL 198	Batchelor *et al.*(1961)
*S. netropsis*2814	Haupt and Thrum.(1967)

TABLE I.2. Microorganisms that produce benzylpenicillin acylase

Organisms	References
Bacteria	
Rhodopsuedomonas sphaeroides	Nara *et al.* (1971)
Psuedomonas aeruginosa KY 3591, KY 8501	Okachi *et al.*(1973)
Psuedomonas sp.	Huang *et al.* (1960)
Xanthomonas sp.	Huang *et al.* (1963)
Alcaligenes faecalis BRL 1237, 1238	Cole and Sutherland (1966)
Flavobacterium	Huang *et al.* (1963)
Azotobacter chroococcum Beij C 12 Pr	Lepidi *et al.* (1970)

Escherichia sp.	Kolinson *et al.* (1960)
E. coli ATCC 9637	Kaufmann and Baurer (1960)
E. coli NCIB 9465	Holt and Stewart (1964)
E. coli BMN, KY 8219, KY 8268, KY 8275, KY 8289	Okachi (1973)
E. coli ATCC 11105	Bauer *et al.* (1971)
E. coli NCIB 8134, 8879, 8949	Cole (1967)
Aerobacter cloacae	Claridge *et al.* (1960)
Proteus morganii KY 4035, KY 405	Okachi *et al.* (1973)
Proteus rettgeri F D 13424	Huang *et al.* (1963)
Proteus rettgeri ATCC 9919, 9250	Cole (1967)
Bacillus subtilis var *niger*	Claridge *et al.* (1960)
Bacillus megaterium ATCC 14945(extracellular)	Chiang and Bennett (1967)
Actinomycetales	
Mycobacterium phlei	Claridge *et al.* (1960)
Streptomyces ambofaciens SPSL 15	Nara *et al.* (1971)
Nocardia F D 46973, ATCC 13635	Huang *et al.* (1960)
Kluyvera citrophila KY3641	Nara *et al.* (1971)
Arthrobacter viscous ATCC 15294	Ohashi *et al* (1988).
Fungi	
Neurospora crassa	Rossi *et al.* (1973)

TABLE I. 3.Microbial sources of Ampicillin acylase

Organisms	References
Psuedomonas melanogenum	
KY 3987, KY 4030, KY 4031	Okachi *et al.* (1973)
Psuedomonas ovalis KY 3962	Okachi *et al.* (1973)

I.2.vii. Strain Improvement

Several hyperproducing strains with desirable characteristics have been claimed in patents and reports, although details of the performances of industrial strains are not available. An overview of the strategies adopted for obtaining benzylpenicillin acylase hyperproducers and mutants with altered specificities are presented briefly here with only illustrative examples being cited. More examples are available in reviews by Savidge (1974) and Shewale and SivaRaman (1989).

The main focus and objectives of strain improvement programmes have been directed towards obtaining

(a) β-lactamases - less mutants,

(b) hyperproducers of penicillin acylase,

(c) constitutive mutants not under catabolite repression, and

(d) strains with altered substrate specificities.

Mutants have been isolated or obtained by various approaches including (a) clonal selection after spontaneous mutation,(b) clonal selection after induced mutagenesis and (c) cloning of the penicillin acylase (*pac*) gene.

(a) Spontaneous Mutation

A constitutive hyperproducer, *E. coli* strain 5, has been derived from *E. coli* ATCC 9637 by spontaneous mutation under environmental stress (Sikyta and Kyslik, 1981). The mutant has been claimed to give 20-fold enhancement in enzyme production compared to the parent strain. Spontaneous mutants of *E. coli* ATCC 9637 and *P. rettgeri* ATCC 31052 with altered substrate specificities have been obtained by subjecting the organisms to growth on different amides as sole source of nitrogen (Daumy *et al.*, 1985 a).

(b) Induced Mutagenesis

Constitutive mutants of *E. coli* (Mayer *et al.*, 1980) and *B. megaterium* (Son *et al.*, 1982) with 1.8 and 4-fold enhancement of enzyme productivity, respectively, have been obtained through UV irradiation. *E. coli* mutants with 2- to 6-fold enhancement of activity have been claimed after mutagenesis with N-methyl-N-nitro-N-nitrosoguanidine (Marancenbaum and Park, 1979; Morita and Iwata, 1979).

(c) Constructs by Recombinant DNA Techniques

E. coli strain 5K (pHM12) was constructed by initially cosmid packaging the total genome of *E. coli* ATCC 11105, subcloning into the multicopy plasmid pBR 322 from which the β-lactamase gene was deleted for obtaining the pHM 6 hybrid (Mayer *et al*, 1981), and mutating the hybrid by UV radiation to give *E. coli* 5K (pHM 12). The construct was constitutive and the cell extract gave enzyme with 8-fold higher specific activity than in cell extracts of *E.coli* ATCC 11105 (Mayer *et al.*, 1981).

The *pac* gene from *P. rettgeri* has been cloned into *E. coli* K12 which normally does not produce the acylase (Daumy *et al.*, 1986). Unlike in *P. rettgeri*, the acylase activity expressed by the construct was cell-bound, regulated by glucose and not repressed by succinate. The *pac* gene from hyperproducing mutant strains *E. coli* 194-3 and *B. megaterium* UNI have been cloned into the nonproducer *E. coli* DH1 on pACYC184 plasmid vector (Meevootisom and Sauders, 1987). The Bacillus penicillin acylase was produced intracellularly in the *E.coli* recombinant unlike the normal extracellular production of the enzyme by *B.megaterium*. Enzyme production by the *E.coli* recombinants was higher than in the original strains. Enzyme production in *E. coli* recombinants carrying *pac* genes from both *E. coli* 194 and the Bacillus were constitutive.

Standard recombinant DNA technology by *pac* gene cloning in *E. coli* does not produce the expected high levels of activity. The reason for this failure is the post-translational processing of the gene product, a large enzymatically inactive precursor polypeptide being secreted into the periplasmic space where the active heterodimeric (α,β) enzyme forms and accumulates. The limitation is attributed to the saturation of the secretion mechanism across the cytoplasmic membrane, the number of secreting channels in *E. coli* being estimated to be about 20,000 (Valle *et al.*, 1991). The alternate approach of expressing separately genes corresponding to the α- and β- subunits of the α,β heterodimeric enzyme molecule and reassociating the separate subunits isolated from the intracellular inclusion bodies into

the active enzyme poses problems of low yields (Lindsay and Pain, 1991). These aspects are discussed in greater detail later in the GENERAL INTRODUCTION (Section I.3).

Among other examples of *pac* gene cloning are the heterologous expression of the gene encoding phenoxymethylpenicillin acylase from *B. sphaericus* in *E. coli* (Olsson and Uhlen, 1986) and the expression of the benzylpenicillin acylase gene from *A. viscosus* in *E. coli* (pHYM-1) (Ohashi *et al* .,1988).

I.2.viii. Enzyme Assay Procedures

Assay procedures are based in general either on determination of penicillin degradation products, *viz* 6-APA or side chain carboxylates or on the use of chromogenic substrates.

The earliest procedures involved the use of penicillin substrates and paper chromatography to separate 6-APA from the substrate, treatment of the 6-APA with phenylacetylchloride followed by bioassay of the benzylpenicillin formed (Batchelor *et al.*,1961 a). Subsequently, solvent extraction had been used for the separation of the penicillin substrates, followed by the colorimetric assay of 6-APA with hydroxylamine (Cole *et al.*,1975).

^{35}S Labelled benzylpenicillin had also been used as substrate, the 6-APA separated by chromatography being assayed by autoradiography (Preuss and Johnson,1965).

Direct and more rapid assay procedures were developed later. These include colorimetric assay of the 6-APA produced without separation of the substrate ,using p-dimethylaminobenzaldehyde

(Bomstein and Evans,1965) and titrimetric determination of side chain carboxylic acid released using a pH stat (Sjoberg *et al.*, 1967) or indicator (Findlater and Orsi, 1973).Assay of 6-APA with fluorescamine (Baker,1985) and with D-glucosamine (Shaikh *et al.*,1973) have also been reported as well as the assay of phenylacetic acid by gas chromatography(Chiang and Bennett,1967) and the substrate and products by HPLC (Daumy *et al.*,1982; Tiwari and Goldberg,1988)

Colorimetric assay of penicillin acylase activity has been carried out using phenylacetyl-4- aminobenzoate as substrate, the amino benzoate released being assyed by diazotization and coupling with H-acid (Szewczuk *et al*,1980). 6-APA produced in the reaction has been assyed microbiologically with *Serratia marscescens* ATCC 27117 which is insensitive to penicillins but sensitive to 6-APA (Oostendorp,1972).

The use of chromogenic substrates permits continuous monitoring of the enzyme catalysed hydrolysis. Among these, the most commonly used is 6-nitro-3-phenylacetamido benzoate (Kutzbach and Rauenbusch,1974) which has better solubility properties than phenyacetyl-4- nitroanilide (Walton,1964).

I.2.ix. Enzyme Purification and Kinetic Properties

(a) Purification of benzylpenicillin acylases

The enzyme from *E. coli* had been obtained crystalline for the first time by Kutzbach and Rauenbusch (1974) from cell extracts of *E. coli* ATCC 11105 by adjusting pH to 5.0 and fractionating the supernatant by column chromatography on SE-Sephadex/CM-Sephadex and DEAE-Sephadex followed by

crystallization from 0.45 saturation ammonium sulphate, pH 6.0. The crystalline enzyme was homogeneous in polyacrylamide gel electrophoresis but isoelectric focussing revealed a main band and several minor bands, all showing enzyme activities. Kasche *et al.* (1984) have also reported multiple active components in the enzyme purified from *E. coli*.

Hydrophobic-chromatography has been applied for purification of the *E. coli* enzyme (Mahajan and Borkar, 1984; Sudhakaran and Shewale, 1987; Karyekar and Hegde, 1991).

The enzyme from *P. rettgeri* has been purified by Daumy *et al.* (1985 b). Cell sonicates were treated with protamine sulphate, the supernatant fractionated with ammonium sulphate, ion-exchange chromatography and gel filtration.

K. citrophila penicillin acylase purification has been reported by Barbero *et al.*. (1986). To carry out the purification, *E.coli* (pYKH5) recombinant carrying the *pac* gene from *K.citrophila* was used. Cells were submitted to osmotic shock by treatment with 33mM EDTA + 20% sucrose followed by lysis with distilled water.The aqueous extract was then fractionated with ammonium sulphate, gel filtration and DEAE-cellulose column chromatography.

Purification of the enzyme from *A. viscosus* has been reported by Ohashi *et al.* (1988). The enzyme which is extracellular was adsorbed from the culture broth with calcium phosphate gel containing Celite,the adsorbed enzyme extracted, precipitated with ammonium sulphate and fractionated by column chromatography on hydroxyapatite, CM-Sephadex, DEAE-

cellulose and gel filtration.

Penicillin acylase from *B.megaterium* which is also extracellular has been purified by Chiang and Bennett (1967) by Celite adsorption,extraction with 24% (w/v) ammonium sulphate, concentration in vaccuo to precipitate the enzyme, CM- cellulose column chromatography and a second cycle of Celite adsorption, ammonium sulphate extraction and precipitation.

I.2.x. Kinetic Properties of Benzylpenicillin Acylases

Some of the kinetic properties of benzylpenicillin acylases are summarized in **Table I.4.**

Benzylpenicillin acylases as stated earlier, exhibit low specificity hydrolysing amide and acylamino acid derivatives of phenylacetic acid. The *E. coli* enzyme has been reported to hydrolyse phenylacetylglycine at twice the initial rate of hydrolysis of benzylpenicillin (Cole, 1964), while phenylacetamide is hydrolysed about 60% faster than benzylpenicillin (Kutzbach and Rauenbusch,1974). Phenylacetyl derivatives of L-amino acids have been reported to be markedly better as substrates than derivatives of the D-isomers (Kutzbach and Rauenbusch, 1974; Plaskie *et al.*, 1978).

I.2.xi. Mechanism of Action and Active Site Residues

The formation of an acyl-enzyme intermediate has been postulated from the kinetics of the enzyme catalyzed reactions (Konecny, 1981 a, b; Konecny *et al.*, 1983).The presence of an essential hydroxyamino acid residue had been established by titration with phenylmethane sulphonylfluoride(PMSF), the serine reagent inactivating completely an equimolar amount of the enzyme

Table I.4 General kinetic properties of benzylpenicillin acylases in the hydrolysis of benzylpenicillin

Enzyme sourse (purity)	Specific activity ($\mu Mol\,min^{-1}mg^{-1}$)	pH optimum	K_m (mM)	Product inhibition	Reference
E. coli ATCC 11105 (crystalline)	23¶ at pH 7.5, 37°C	8.1	0.02	PA* (competitive) K_i 0.2 mM, 6-APA# non-competitive K_i 15 mM.	Kutzbach and Rauenbusch (1974)
E. coli NCIB 8743A (partially purified)	7 at pH 8.0, 37°C	8-9	0.67	PA (competitive) K_i 4.8 mM, 6-APA non-competitive K_i 7.1 mM	Balasingham *et al.* (1972)
K. citrophila / *E. coli* (pYKHS) clone	30 at pH 8.0, 37°C	8.0	-	-	Barbero *et al.* (1986)
K. citrophila ATCC 21285 (partially purified)	8.4 at pH 7.5, 40°C	7.5	2.70		Bodhe and SivaRaman (1987)
A. viscosus ATCC 15294	67 at pH 7.0, 37°C	6-7	0.42		Ohas hi *et al.* (1988)
B. megaterium ATCC 14945	32 at pH 8.7, 37°C	8-9	4.5	PA (competitive) K_i 0.45 M, 6-APA non-competitive K_i 2.6×10^{-2} M	Chiang and Bennnett (1967)

¶Calculated from reported activity towards 6-nitro-3-phenylacetamido benzoate. *Phenyl acetic acid; #6-aminopenicillanic acid.

from *E. coli* (Kutzbach and Rauenbusch, 1974), *P. rettgri* (Daumy *et al.*, 1985) or *K. citrophila*(Martin *et al* .,1991). The presence of the essential PMSF-sensitive residue in the large β-subunit of the enzyme from *P. rettgeri* was demonstrated by Daumy *et al.* (1985 b) by subunit -complementation. The enzyme inactivated with PMSF as well as untreated enzyme were separately dissociated with urea and the subunits separated by polyacrylamide gel electrophoresis. The separated subunits were mixed and reassociated in different combinations. The mixture then showed no enzyme activity when the large β-subunit was the one isolated from PMSF-treated enzyme.

The presence of the PMSF - sensitive residue in the β-subunits of the *E. coli* and *K. citrophila* enzymes have been established by the use of ^{35}S-labelled PMSF and SDS-polyacrylamide gel electrophoresis of the modified enzyme and assaying gel slices for radioactivity (Slade *et al.*,1991,Martin *et al.*,1991).Efforts to identify and locate the essential PMSF - sensitive residue have also been made recently. Site- directed mutagenesis of serine 838 (number is of precursor polypeptide chain residue, see **Fig.I 4.**) to cysteine ruled out the involvement of this residue (Sizmann *et al.*, 1990). The strategy of site-directed chemical mutagenesis (Neet and Koshland, 1966; Polgar and Bender,1977) was used by Slade *et al.* (1991) to identify the essential hydroxyamino residue in the enzyme from *E. coli* and by Martin *et al.*,(1991) in the *K.citrophila* enzyme. The PMSF -inactivated penicillin acylases in both cases were treated with potassium thioacetate to yield acetylthiol enzymes which were

subsequently converted to thiol enzymes. Since native *E. coli* and *K. citrophila* benzylpenicillin acylases do not contain any cysteine residues the newly formed cysteine residue derived from PMSF-inactivated enzyme was tagged with iodo (2-^{3}H)acetic acid which was shown by SDS-polyacrylamide gel electrophoresis and sequence analysis to be associated with the β-chain amino-terminal residue indicating the conversien of serine - 290 to S-carboxymethyl-cysteine. The catalytic activity of the mutant enzyme was less than 0.02% of the normal enzyme. The unusual location of the catalytically active serine at the N-terminus of the β-subunit had led Slade *et al.* (1991) to propose that serine - 290 probably lies close to the active site of benzylpenicillin acylase in an environment favouring selective modifications. The possibility was also suggested that the acetylthiol group might have migrated during the preparation of the thiol enzyme due to the possibility of the serine -290 being located spatially in the proximity of an initially modified group.

Several lines of evidences have indicated that the active site of *E. coli* benzylpenicillin acylase may comprise a hydrophobic environment. These include the observations of Klyosov *et al.*, (1977a, b) that the *E. coli* enzyme is inhibited by aliphatic alcohols, the inhibitory effect varying with the hydrophobicity of the alcohols. In more recent studies, Marquez *et al.*, (1988) have shown the binding of benzylpenicillin sulfoxide, a substrate analogue and a competitive inhibitor, results in changes in the circular dichroism of aromatic residues. The hydrophobic environment and the requirement for

specific hydrophobic interaction with the phenylacetyl side chain of benzylpenicillin before any acylenzyme intermediate can form had been indicated in inactivation studies with serine - modifying reagents (Kutzbach and Rauenbusch, 1974). While phenylmethanesulphonyl fluoride(PMSF), which structurally resembles the side chain of benzylpenicillin, inactivates the enzyme 100%,diisopropylfluorophosphate does so only to the extent of 16%. Further site-directed mutagenesis of Met- 168 to Ala in α- subunits of the enzymes from *E.coli* (Williams and Zuzel, 1985) and from *K. citrophila* (Martin *et al.*, 1990., Preito *et al.*,1990) has been shown to alter the substrate specificities of these enzymes. In the latter case, the initial rate of hydrolysis of benzylpenicillin catalysed by the mutant enzyme was markedly lowered, while the rate of hydrolysis of 6-bromohexyl-6-APA was enhanced four - fold. PMSF - sensitivity of the mutant was also significantly decreased and more than 1 mole of the inhibitor was required for the complete inactivation of a mole of mutant enzyme. These could be explained on the basis of conformational changes in the mutant resulting in changes in enzyme-substrate binding geometry for acylation of the active site serine.

Spontaneous mutants of *E. coli* and *P. rettgeri* benzylpenicillin acylases with altered specificities for the side chain of the penicillin molecule have been obtained by positive selection procedure of growth on the amide derivatives of these side chains (Daumy *et al.*, 1985 a). Subunit -complementation studies, identical to the approach used for locating the catalytically active PMSF -sensitive residue, showed that the

substrate specificity resides in the small α-subunit of the *P. rettgeri* enzyme indicating that the α-subunit contains the substrate binding site (Daumy *et al.*, 1985 b).

I.3. MOLECULAR ASPECTS

Molecular aspects of penicillin acylases are discussed here briefly and compared with the closely related cephalosporin acylases. The latter have close structural relationships with the penicillin acylases and are of two distinct types, namely, 7 - β-(4-carboxybutanamido)cephalosporanic acid acylase (more commonly known as glutaryl- 7- amino cephalosporanic acid acylases (GL 7-ACA acylase), which hydrolyses 7-β-(4-carboxybutanamido) cephalosporanic acid and cephalosporin acylase which hydrolyses cephalosporin C (Vandamme, 1988; Matsuda and Komatsu, 1985; Matsuda *et al.*, 1987).

I.3.i. Molecular Weight and Subunit Structure

The molecular weight and subunit structure data of the well characterized benzylpenicillin acylases and related enzymes are summarized in **Table I.5.**

The earliest evidence for the heterodimeric (α,β) structure of benzylpenicillin acylase from *E. coli* was the report of Kutzbach and Rauenbusch (1974) of the presence of two protein bands in SDS-polyacrylamide gel electrophoretograms of the crystalline enzyme. This was confirmed by Bock and co-workers (Bock *et al.*, 1983 a,b). All benzylpenicillin acylases reported hitherto, with the exception of the *B. megaterium* enzyme, have been shown to resemble the *E. coli* enzyme both in M_r value and subunit composition (**Table I.5**). Thus the M_r values of the

Table I.5 M_r of holoenzyme and subunits of penicillin and cephalosporin acylases

Enzyme	Holoenzyme (M_r)	Subunits (M_r)		Reference
		α	β	
BENZYLPENICILLIN ACYLASES				
E. coli	85,500	20,500	65,000	Schumacher et al. (1986)
K. citrophila	85,200	23,600	61,600	Barbero et al. (1986)
P. rettgeri	90,000	24,500	66,000	Daumy et al. (1985a; b)
A. viscosus	81,000	24,000	60,000	Ohashi et al. (1988)
B. megaterium	120,000	Not reported		Chiang and Bennett (1967)
PHENOXYMETHYL PENICILLIN ACYLASES				
B. sphaericus	140,000	35,000 homotetramer		Olsson and Uhlen (1986) Olsson et al. (1985)
B. plumbea	88,000	not determined		Schneider and Roher (1976)
E. aroidae	62,000	not determined		Vandamme and Voets (1975)
F. semitectum	65,000	not determined		Walschmidt and Bretzel (1964)
B. megaterium	58,000	not determined		Meevootisom and Saunders (1987)
AMPICILLIN ACYLASE				
P. melanogenum	146,000	72,000 homodimer		Kim and Byun (1990)

Table I.5 contd.

Enzyme	Holoenzyme (M_r)	Subunits (M_r)		Reference
		α	β	
CEPHALOSPORIN ACYLASES				
GL-7 ACA acylase				
P. melanogenum sp. GK 16	70,000	16,000	54,000	Matsuda and Komatsu (1985)
GL-7 ACA acylase I				
Pseudomonas sp. SE 83	58,100	38,000	19,900	Matsuda et al. (1987a)
GL-7 ACA acylase II				
Pseudomonas sp. SE 83	83,600	25,400	58,200	Matsuda et al. (1987a; b)

holoenzymes range from 80,000 - 90,000, of the α-subunits between 20,000 - 25,000 and of the β-subunits between 60,000 - 66,000. The exception is the less studied enzyme from *B. megaterium* which has a reported M_r of 120,000. The structural features of the cephalosporin acylases closely resemble that of *E. coli*.

In marked contrast the ampicillin acylase from *P. melanogenum* has an M_r value of 146,000 with a homodimeric structure and the only phenoxymethylpenicillin acylase that has been studied in detail; namely, the enzyme from *B. sphaericus* has an M_r of 140,000 with a homotetrameric structure.

I.3.ii.Post- Translational Pathway of Maturation:

The heterodimeric structure of the bacterial benzylpenicillin acylases have been shown to result from post-translational processing pathway of maturation. Such a pathway in procaryotes is unusual and only a few examples are known in the literature. Among these are the maturation of cytochrome bc_1 complex from *Bradyrhizobuim japonicum* (Thony-Meyer *et al.*, 1989). formation of mature subtilisin of *Bacillus subtilis* (Ikemura *et al.*, 1987) and the derivation of two independent enzyme activities for starch cleavage in *Bacillus polymyxa* (Uozumi *et al.*, 1989).

The most studied of the maturation pathways of benzylpenicillin acylases has been that of *E. coli* ATCC 11105. Findings which have contributed to the understanding of the maturation steps have been those of Bock and his co-workers (Bock *et al.*, 1983a,b; Schumacher *et al.*, 1986 and Sizmann *et*

al., 1990),of Bolivar and coworkers (Oliver *et al* .,1985;Valle *et al.*,1986) and of Ohashi *et al.* (1987).These included the identification of a large membrane - bound polypeptide of approximately M_r 95,000 in *E. coli* which immunologically cross reacts with antibodies directed against the purified enzyme; nucleotide sequencing of the *pac* gene and correlation of the *pac* gene sequence with the primary structure of the two constituent subunits of the active enzyme. The gene/protein relationship *in vitro* was established through plasmid constructs lacking sequences coding for specific domains of the precursor protein or leading to the synthesis of mutant penicillin acylase precursors.

In summary the gene/protein relationship and *in vitro* processing pathway of *E. coli* penicillin acylase precursor indicated that the structural *pac* gene is built up of 4 domains, nucleotide positions 1 - 78 coding for a signal peptide, positions 79 - 705 coding for the α- subunit, positions 706 - 867 coding for a spacer endopeptide and positions 868 - 2538 coding for the β-subunit.

The maturation pathway of the precursor protein comprises the following steps:

(a) The precursor protein is transported across the cytoplasmic membrane into the periplasmic space followed by the removal of the signal peptide comprising 26 amino acids leaving a polypeptide chain comprising the α-subunit, spacer peptide and β-subunit domains.

(b) Proteolytic cleavage at the N-terminus of β-subunit (557

amino acids).

(c) Removal of the spacer peptide from the α-subunit (209 amino acids) by proteolytic removal of the spacer peptide in two or three steps.

The post-translational modifications apparently are not brought about by specific processing enzyme(s) as the *pac* structural gene yields active enzyme heterologously (Sizmann *et al.*,1990).

The precursor protein and its cleavage products are enzymatically inactive till the final α,β heterodimer formation. It has been suggested that the function of the spacer peptide may be for correct folding of the α and β-subunit domains to yield active enzyme (Sizmann *et al.*, 1990; Lindsay and Pain, 1990;1991).

A schematic representation of the maturation pathway of *E. coli* penicillin acylase is set out in **Fig I.3.**

pac Gene from *K. citrophila* has also been cloned and sequenced and the gene/protein relationship has been shown to resemble that of *E. coli* in the maturation steps(Garcia and Buesa,1986; Barbero *et al.*,1986) . The nucleotide sequence of both contain the four domains for signal peptide, α-subunit, spacer endopeptide and β-subunit.

In addition to the benzylpenicillin acylases,cephalosporin acylases have also been shown to be composed of two non-identical subunits derived from a single precursor polypeptide chain (Matsuda *et al.*, 1985; 1987). The precursor polypeptides of the cephalosporin acylases,however, differ from those of the

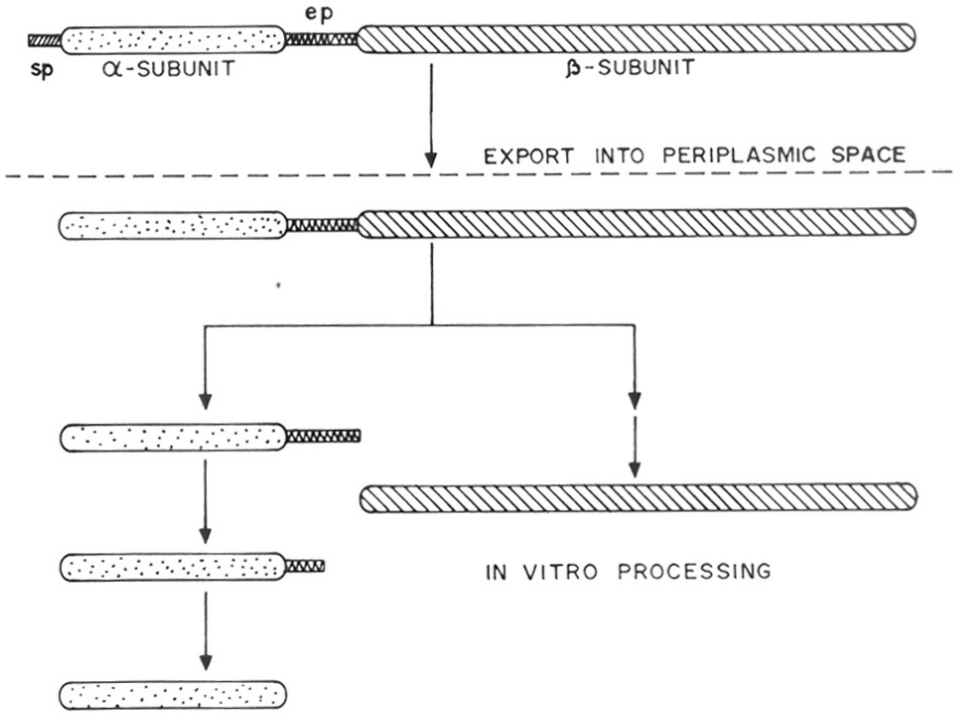


FIG. I·3

Post-translational processing of E. coli penicillin acylase precursor (adapted from Sizmann et al., 1990)

sp. (signal peptide), ep (endopeptide)

benzylpenicillin acylases of *E. coli* and *K. citrophila* in being devoid of spacer endopeptides. Further, except for the precursor of GL-7-ACA acylase of *Pseudomonas* SPGK 16, the precursors of GL-7-ACA acylase I and II of *Pseudomanas* SE83 are also devoid of the signal peptide domain.

The data on the gross features of the domains in precursor proteins of penicillin and cephalosporin acylases are summarized in **Table I.6.**

The processing of *E. coli pac* gene has been shown to be temperature sensitive, the precursor polypeptide accumulating without production of active enzyme at 42°C (Oliver *et al.*, 1985). This explains earlier observations regarding the optimum temperatures of enzyme production in *E. coli*. The post-translational processing of *Pseudomonas* SC83 GL7-ACA acylase II has also been reported to be temperature dependent (Matsuda *et al.*, 1987).

I.3.iii. Primary Structure

The amino acid sequences of the precursor polypeptides of benzylpenicillin acylases from *E. coli* (Schumacher *et al.*, 1986) and from *K. citrophila* (Garcia and Buesa, 1986; Barbero *et al.*, 1986) have been derived from the nucleotide sequences of their structural *pac* genes. Partial N-terminal amino acid sequence of the α and β-subunit of benzylpenicillin acylase from *A. viscosus* has been reported from peptide sequencing (Ohashi *et al.*, 1988).

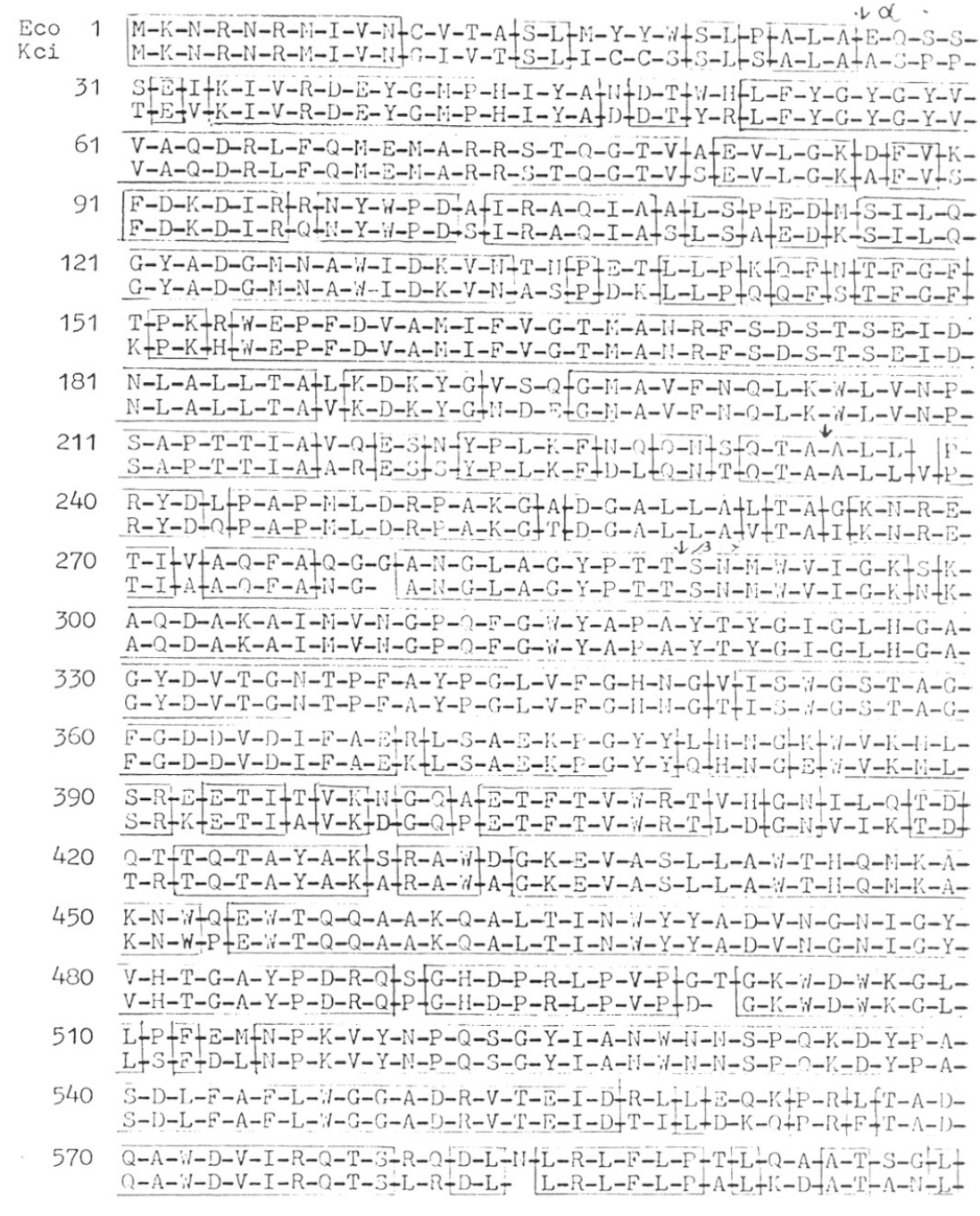
The sequence data of the precursor proteins of *E. coli* and *K.citrophila* benzylpenicillin acylases are set out in **Fig I.4.**

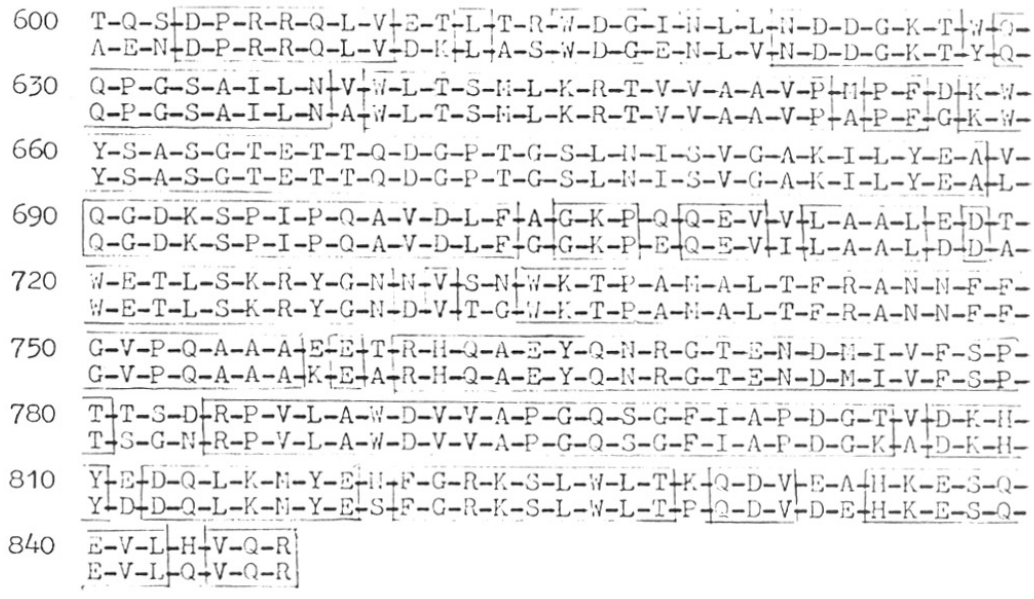
A close homology (87%) exists between the enzymes from *E.*

Table I.6 Precursor protein domains of benzylpenicillin acylases and cephalosporin acylases

Enzyme	Number of amino acid residues				Reference
	Signal peptide	α-subunit	spacer peptide	β-subunit	
BENZYLPENICILLIN ACYLASES					
E. coli	26	209	54	557	Schumacher *et al.* (1986)
K. citrophila	26	209	54	555	Barbero *et al.* (1986)
CEPHALOSPORIN ACYLASES					
GL-7 ACA acylase					
Pseudomonas sp. GK 16	29	169	absent	not available	Matsuda *et al.* (1985)
GL-7 ACA acylase I					
Pseudomonas sp. SE 83	absent	366	absent	181	Matsuda *et al.* (1987)
GL-7 ACA acylase II					
Pseudomonas sp. SE 83	absent	238	absent	535	Matsuda *et al.* (1987)

Fig. I.4 Amino acid sequences of the penicillin acylase precursor proteins of *E. coli* (Eco) and *K. citrophila* (Kci) deduced from the nucleotide sequences encoding the *pac* structural genes. Arrows (↓) indicate the protein cleavage sites during processing of the precursor polypeptide chains. Conserved residues are boxed. α and β-subunit sequences are indicated above the amino acid sequences. *E. coli* sequence data from Schumacher *et al.*, (1986) and *K. citrophila* data from Barbero *et al.* (1986).


Eco 1
M-K-N-R-N-R-M-I-V-N-C-V-T-A-S-L-M-Y-Y-W-S-L-P-A-L-A-E-Q-S-S-
Kci
M-K-N-R-N-R-M-I-V-N-C-I-V-T-S-L-I-C-C-S-S-L-S-A-L-A-A-S-P-P-
31
S-E-I-K-I-V-R-D-E-Y-G-M-P-H-I-Y-A-N-D-T-W-H-L-F-Y-G-Y-G-Y-V-
T-E-V-K-I-V-R-D-E-Y-G-M-P-H-I-Y-A-D-D-T-Y-R-L-F-Y-G-Y-G-Y-V-
61
V-A-Q-D-R-L-F-Q-M-E-M-A-R-R-S-T-Q-G-T-V-A-E-V-L-G-K-D-F-V-K-
V-A-Q-D-R-L-F-Q-M-E-M-A-R-R-S-T-Q-G-T-V-S-E-V-L-G-K-A-F-V-S-
91
F-D-K-D-I-R-R-N-Y-W-P-D-A-I-R-A-Q-I-A-A-L-S-P-E-D-M-S-I-L-Q-
F-D-K-D-I-R-Q-N-Y-W-P-D-S-I-R-A-Q-I-A-S-L-S-A-E-D-K-S-I-L-Q-
121
G-Y-A-D-G-M-N-A-W-I-D-K-V-N-T-N-P-E-T-L-L-P-K-Q-F-N-T-F-G-F-
G-Y-A-D-G-M-N-A-W-I-D-K-V-N-A-S-P-D-K-L-L-P-Q-Q-F-S-T-F-G-F-
151
T-P-K-R-W-E-P-F-D-V-A-M-I-F-V-G-T-M-A-N-R-F-S-D-S-T-S-E-I-D-
K-P-K-H-W-E-P-F-D-V-A-M-I-F-V-G-T-M-A-N-R-F-S-D-S-T-S-E-I-D-
181
N-L-A-L-L-T-A-L-K-D-K-Y-G-V-S-Q-G-M-A-V-F-N-Q-L-K-W-L-V-N-P-
N-L-A-L-L-T-A-V-K-D-K-Y-G-N-D-E-G-M-A-V-F-N-Q-L-K-W-L-V-N-P-
211
S-A-P-T-T-I-A-V-Q-E-S-N-Y-P-L-K-F-N-Q-Q-N-S-Q-T-A-A-L-L-P-
S-A-P-T-T-I-A-A-R-E-S-S-Y-P-L-K-F-D-L-Q-N-T-Q-T-A-A-L-L-V-P-
240
R-Y-D-L-P-A-P-M-L-D-R-P-A-K-G-A-D-G-A-L-L-A-L-T-A-G-K-N-R-E-
R-Y-D-Q-P-A-P-M-L-D-R-P-A-K-G-T-D-G-A-L-L-A-V-T-A-I-K-N-R-E-
270
T-I-V-A-Q-F-A-Q-G-G-A-N-G-L-A-G-Y-P-T-T-S-N-M-W-V-I-G-K-S-K-
T-I-A-A-Q-F-A-N-G- A-N-G-L-A-G-Y-P-T-T-S-N-M-W-V-I-G-K-N-K-
300
A-Q-D-A-K-A-I-M-V-N-G-P-Q-F-G-W-Y-A-P-A-Y-T-Y-G-I-G-L-H-G-A-
A-Q-D-A-K-A-I-M-V-N-G-P-Q-F-G-W-Y-A-P-A-Y-T-Y-G-I-G-L-H-G-A-
330
G-Y-D-V-T-G-N-T-P-F-A-Y-P-G-L-V-F-G-H-N-G-V-I-S-W-G-S-T-A-G-
G-Y-D-V-T-G-N-T-P-F-A-Y-P-G-L-V-F-G-H-N-G-T-I-S-W-G-S-T-A-G-
360
F-G-D-D-V-D-I-F-A-E-R-L-S-A-E-K-P-G-Y-Y-L-H-N-G-K-W-V-K-H-L-
F-G-D-D-V-D-I-F-A-E-K-L-S-A-E-K-P-G-Y-Y-Q-H-N-G-E-W-V-K-M-L-
390
S-R-E-E-T-I-T-V-K-N-G-Q-A-E-T-F-T-V-W-R-T-V-H-G-N-I-L-Q-T-D-
S-R-K-E-T-I-A-V-K-D-G-Q-P-E-T-F-T-V-W-R-T-L-D-G-N-V-I-K-T-D-
420
Q-T-T-Q-T-A-Y-A-K-S-R-A-W-D-G-K-E-V-A-S-L-L-A-W-T-H-Q-M-K-A-
T-R-T-Q-T-A-Y-A-K-A-R-A-W-A-G-K-E-V-A-S-L-L-A-W-T-H-Q-M-K-A-
450
K-N-W-Q-E-W-T-Q-Q-A-A-K-Q-A-L-T-I-N-W-Y-Y-A-D-V-N-G-N-I-G-Y-
K-N-W-P-E-W-T-Q-Q-A-A-K-Q-A-L-T-I-N-W-Y-Y-A-D-V-N-G-N-I-G-Y-
480
V-H-T-G-A-Y-P-D-R-Q-S-G-H-D-P-R-L-P-V-P-G-T-G-K-W-D-W-K-G-L-
V-H-T-G-A-Y-P-D-R-Q-P-G-H-D-P-R-L-P-V-P-D- G-K-W-D-W-K-G-L-
510
L-P-F-E-M-N-P-K-V-Y-N-P-Q-S-G-Y-I-A-N-W-N-N-S-P-Q-K-D-Y-P-A-
L-S-F-D-L-N-P-K-V-Y-N-P-Q-S-G-Y-I-A-N-W-N-N-S-P-Q-K-D-Y-P-A-
540
S-D-L-F-A-F-L-W-G-G-A-D-R-V-T-E-I-D-R-L-L-E-Q-K-P-R-L-T-A-D-
S-D-L-F-A-F-L-W-G-G-A-D-R-V-T-E-I-D-T-I-L-D-K-Q-P-R-F-T-A-D-
570
Q-A-W-D-V-I-R-Q-T-S-R-Q-D-L-N-L-R-L-F-L-P-T-L-Q-A-A-T-S-G-L-
Q-A-W-D-V-I-R-Q-T-S-L-R-D-L- L-R-L-F-L-P-A-L-K-D-A-T-A-N-L-


600
T-Q-S-D-P-R-R-Q-L-V-E-T-L-T-R-W-D-G-I-N-L-L-N-D-D-G-K-T-W-Q-
A-E-N-D-P-R-R-Q-L-V-D-K-L-A-S-W-D-G-E-N-L-V-N-D-D-G-K-T-Y-Q-
630
Q-P-G-S-A-I-L-N-V-W-L-T-S-M-L-K-R-T-V-V-A-A-V-P-M-P-F-D-K-W-
Q-P-G-S-A-I-L-N-A-W-L-T-S-M-L-K-R-T-V-V-A-A-V-P-A-P-F-G-K-W-
660
Y-S-A-S-G-T-E-T-T-Q-D-G-P-T-G-S-L-N-I-S-V-G-A-K-I-L-Y-E-A-V-
Y-S-A-S-G-T-E-T-T-Q-D-G-P-T-G-S-L-N-I-S-V-G-A-K-I-L-Y-E-A-L-
690
Q-G-D-K-S-P-I-P-Q-A-V-D-L-F-A-G-K-P-Q-Q-E-V-V-L-A-A-L-E-D-T-
Q-G-D-K-S-P-I-P-Q-A-V-D-L-F-G-G-K-P-E-Q-E-V-I-L-A-A-L-D-D-A-
720
W-E-T-L-S-K-R-Y-G-N-N-V-S-N-W-K-T-P-A-M-A-L-T-F-R-A-N-N-F-F-
W-E-T-L-S-K-R-Y-G-N-D-V-T-G-W-K-T-P-A-M-A-L-T-F-R-A-N-N-F-F-
750
G-V-P-Q-A-A-A-E-E-T-R-H-Q-A-E-Y-Q-N-R-G-T-E-N-D-M-I-V-F-S-P-
G-V-P-Q-A-A-A-K-E-A-R-H-Q-A-E-Y-Q-N-R-G-T-E-N-D-M-I-V-F-S-P-
780
T-T-S-D-R-P-V-L-A-W-D-V-V-A-P-G-Q-S-G-F-I-A-P-D-G-T-V-D-K-H-
T-S-G-N-R-P-V-L-A-W-D-V-V-A-P-G-Q-S-G-F-I-A-P-D-G-K-A-D-K-H-
810
Y-E-D-Q-L-K-M-Y-E-H-F-G-R-K-S-L-W-L-T-K-Q-D-V-E-A-H-K-E-S-Q-
Y-D-D-Q-L-K-M-Y-E-S-F-G-R-K-S-L-W-L-T-P-Q-D-V-D-E-H-K-E-S-Q-
840
E-V-L-H-V-Q-R
E-V-L-Q-V-Q-R

coli and *K. citrophila* through all the domains of their precursor proteins. Both carry a putative 26 amino acid signal peptide preceding the N-terminal of the α-subunit for translocation into the periplasmic space. The lengths of these are unusually large for a procaryotic protein (Barbero *et al.*,1986). Both signal peptides have conserved sequences at the N- and C-termini with divergences in their central cores which however are both hydrophobic. These and other sequence similarities and predicted secondary structure similarities have been discussed by Barbero *et al*,(1986).

The amino acid sequence of phenoxymethylpenicillin acylase from *B.sphaericus*, a tetramer built up of identical subunits of M_r 37,500 and 338 amino acids residues has also been derived from the nucleotide sequence of its structural gene (Olsson and Uhlen, 1986). In this case, the signal peptide sequence is not present as would be expected from its intracellular location. Further the amino acid sequence shows no homology with the sequences of the benzylpenicillin acylases.

The interrelationships and sequence homologies strongly suggest that the benzylpenicillin acylases evolved from a common ancestral protein while the phenoxymethylpenicillin acylase probably has a different phylogenetic origin.

I.3.iv. Secondary structure and Subunit Organization in Benzylpenicillin Acylase

The non-identical α- and β-subunits of all the benzylpenicillin enzymes studied to date have been shown to be held by non-covalent interactions. SDS-polyacrylamide gel and

urea polyacrylamide gel electrophoreses do not require reducing conditions for subunit separation. The *P. rettgeri* enzyme contains a single disulphide bond buried in the structure titratable with 5,5´-dithiobis-2-nitrobenzoate only after denaturation and dithiothreitol reduction (Daumy *et al.*,1985a, b). Far - UV CD spectrum of the enzyme from *K.citrophila* at neutral pH showed the presence of 11% of α- helix, 44% β-sheet, 11% β-turn and 34% random coil in the molecule (Marquez *et al.*,1988). The structural stability of the molecule has been attributed to the high content of the β -sheet structures.

The *E. coli* enzyme is devoid of cysteine and cystine residues (Schumacher *et al.* 1986). The *E. coli* holoenzyme has been characterized for it's solution conformation by UV circular dichroism, steady -state and time -resolved fluorescence spectroscopy and differential sedimentation rates (Lindsay and Pain, 1990). The enzyme was found to be spherical and stable, unfolding over a narrow range of urea concentrations with a mid-point of 4.5 M urea. The physical studies carried out by these authors indicated that the refolded separated α-peptide has a compact but asymmetric structure with more α-helix than the native enzyme and indicated the presence of a hydrophobic patch which bound 8-anilino-1- naphthalene sulphonic acid .The patch apparently was covered by the β-peptide in the native enzyme. The data indicated that the α-peptide folds first and probably plays a key role in the folding of the precursor penicillin acylase polypeptide chain. The assembly of active penicillin acylase from the isolated α and β- subunits of enzyme from *P.rettgeri*

(Daumy *et al.*, 1985 b) and from *E. coli* (Lindsay and Pain, 1991) have been studied. Recoveries of only 6 -12% of native enzyme activity were obtained on mixing unfolded α and β subunits after separation by urea-gel electrophoresis in the case of *P.rettgeri* enzyme. This has been attributed to the characteristic problem in refolding subunits that result from proteolytic processing leading to products which unlike their precursor are not in their lowest accessible free energy states (Lindsay and Pain, 1991). Factors such as pH, ionic strength and temperature in particular have been shown to have marked effect on assembly of the active enzyme from the urea unfolded α and β chains of the *E. coli* enzyme (Lindsay and Pain,1991). Under defined conditions about 60% activity could be recovered from mixing of the constituent subunits of the *E.coli* enzyme unfolded in urea.

I.4.APPLIED ASPECTS

The industrial importance of 6-APA and the currently favoured enzymatic route for the production of this key intermediate have led to intensive efforts to develop immobilized penicillin acylase systems with desirable characteristics. The main objectives have been to achieve high immobilization efficiencies with retention of activity and to enhance catalyst half-life of the immobilized system through improvements in enzyme stability and reduction of losses through leakage.

The need to use immobilized biocatalyst systems in 6-APA technology is mainly for (a) the obvious economic benefit of continuous operation or repeated reuse in batch mode, (b)

exclusion of allergenic macromolecules from the product and (c) rapid conversions with high catalyst- loading to minimize losses due to the spontaneous degradation of the unstable substrate. This would be prohibitively expensive if high levels of soluble native enzyme are used without the possibility of recovery.

The extensive literature both in journal publications and in the patent literature would put any comprehensive review outside the scope of the present **GENERAL INTRODUCTION**. For this reason, only aspects and examples relevant to the work reported in this THESIS are set out in this chapter. Some specific examples are taken up for discussion in the chapters on immobilized enzyme and whole cells and these are not repeated here.

All the strategies of immobilized biocatalyst technology have been applied for immobilization of penicillin acylase.These include

(a) immobilization of microbial whole cells in or on supports by entrapment, crosslinking and covalent binding in or on supports., (b) enzyme immobilization by adsorption on carriers, covalent binding to supports, intermolecular crosslinking, entrapment in gels and encapsulation within semipermeable membranes.

I.4.i Immobilized Microbial Whole Cells with Penicillin Acylase Activity

An advantage of an immobilized system of whole cells with cell-bound penicillin acylase activity as in *E. coli* as opposed to the immobilized isolated enzyme is that the costly processes of enzyme isolation,concentration and purification are avoided.

The main disadvantage, however, is of relatively lower activities that are generally present in whole cells compared to purified enzyme preparations entailing extended conversion periods or greater catalyst loading. This disadvantage is partially overcome with mutants or constructs with higher enzyme activities. Diffusional restrictions imposed by cell wall/membrane and of the matrix used for whole cell immobilization also need to be minimized, the former by solvent or detergent treatment to perturb the cell wall/membrane structure and the latter by use of porous carriers. Yet another problem is of side reactions caused by enzyme activities other than the desired one which imposes the need to either use mutants without the interfering activities or to differentially inactivate these.

Some of the examples reported in the literature of immobilized whole cell systems with benzylpenicillin acylase activity are summarized in **Table I.7.**

I.4.ii. Immobilized Penicillin Acylase Systems

A few illustrative examples are set out in **Table I.8.**

(a) Immobilization by Adsorption

The first industrial application of immobilized penicillin acylase had been by Squibb Inc., USA, using the extracellular *B. megaterium* enzyme adsorbed on Bentonite (Squibb´ER and sons Inc., 1969) .Adsorbed systems have the disadvantage of enzyme leakage through desorption with changes in pH and other environmental conditions.An advantage however is the possibility of recharging the depleted carrier with fresh enzyme to make up for the leakage. Adsorbed enzyme systems could be stabilized by

Table I:7 Immobilized Whole Cell Systems

	Organism	Method	Reported activity	Operational stability/half life	References
1.	*E. coli*	Polyacrylamide gel entrapment	93.6% of that of native cell	42 days at 30°C in plug flow	Sato *et al.* (1976)
2.	*E. coli*	Entrapment in porous epoxy resins	-	20 cycles	Klein *et al.* (1974)
3.	*E. coli*	Ca-alginate gel entrapment	-	30 days unchanged	Klein *et al.* (1980)
4.	*E. coli*	Gelatin crosslinked with glutaraldehyde gel entrapment	-	50 days at 85% conversion 30 mM substrate	Park *et al.* (1982)
5.	*E. coli*	Covalently bound on surface of cellulose beads	70.0%	52 cycles	Zurkova *et al.* (1983)
6.	*E. coli*	Entrapment in cellulose beads + glutaraldehyde	-	-	Linko and Linko (1987)
7.	*E. coli*	Entrapment in chitosan beads	-	10% less in 70 cycles	Vorlop and Klein (1987)

Table I.8 Immobilized penicillin acylase systems

Company	Enzyme	Carrier/activation process	Reference
IMMOBILIZATION BY ADSORPTION			
Squibb Inc.	*B. megaterium*	Bentonite	Henser *et al.* (1969)
-	-	DEAE-cellulose/succinylated enzyme	Miyazaki *et al.* (1972)
-	-	DEAE-Sephadex/succinylated enzyme	Kamogashira (1972)
IMMOBILIZATION BY COVALENT BINDING			
Beecham Group	*E. coli*	Cellulose derivatives/s-triazine derivatives	Self *et al.* (1969) Warburton *et al.* (1972)
Beecham Group	*E. coli*	EMA copolymer	US Patent 3887432/ 1975
Hindustan Antibiotics Ltd.	*E. coli* *Fusarium* sp.	Activated cellulose	SivaRaman *et al.* (1976)
BayerAG	*E. coli*	Acrylamide-maleic acid-bis-acrylamide copolymers	Hueper *et al.* (1973a)
Bayer AG	*E. coli*	Maleic anhydride-methacrylic acid tetraethylene glycol dimethacrylate copolymers	Boemer *et al.* (1973) Hueper *et al.* (1973b)
Hoechst AG	-	Phenol-formaldehyde resin	Grabley *et al.*(1985)

Company	Enzyme	Carrier/activation process	Reference
Jozo Co,	B. megaterium	Derivatized polyacrylonitrile	Matsumoto et al. (1979; 1984)
-	E. coli	Glycidyl methacrylate-ethylene dimethacrylate copolymer-1,6-diaminohexane	Drobnik et al. (1979)
-	E. coli	Water soluble polyelectrolyte complex-s-triazine (reversible immobilization)	Margolin et al. (1981)
-	K. citrophila	Silanized controlled-pore ceramics	Bodhe and SivaRaman (1987)
IMMOBILIZATION BY INTERMOLECULAR CROSSLINKING			
Novo	E. coli	Inert filler/glutaraldehyde	Amotz (1974)
Beecham Group	E. coli	DEAE-cellulose/CM-cellulose + glutaraldehyde	Savidge and Powell (1975)
Beecham Group	E. coli	Methacrylate resin + glutaraldehyde + water soluble diamine	Savidge et al. (1974)
Hindustan Antibiotics Ltd.	E. coli Fusarium sp.	Glutaraldehyde	SivaRaman et al. (1976)
-	E. coli	Macroporous methacrylate polymers + glutaraldehyde	Koillpillai et al. (1990)
-	E. coli	Alumina + glutaraldehyde	Bahulekar et al.(1991)

Table I.8 contd.

Company	Enzyme	Carrier/activation process	Reference
IMMOBILIZATION BY GEL ENTRAPMENT			
-	B. megaterium	Collagen membrane	Wang and Vieth (1973)
-	E. coli	Polyacrylamide gel	Mandel et. al. (1975)
-	E. coli	Gel entrapment of soluble enzyme complex with CNBr-activated dextrans or starch	Hueper (1974)
-	P. rettgeri	Glycidyl methacrylate-N,N'-methylenebis-acrylamide copolymer	Hamsher and Lozanov (1978)
IMMOBILIZATION BY ENCAPSULATION			
SNAM Progetti	E. coli	Cellulose triacetate fibre	Dinelli (1972) Marconi et al. (1975)
Toyo Jozo	B. megaterium	Polyacrylonitrile fibre	Poulsen (1984)

crosslinking with bi-(or multi-) functional reagents like glutaraldehyde. These are set out in Table I.8 under intermolecular crosslinking.

(b) **Immobilization by Covalent Attachment to Carriers**

Sevaral systems of covalently - attached penicillin acylase are described in the literature, particularly in patents.A few representative examples are listed in **Table I.8.**

Cellulose and cellulose derivatives activated with *s*-triazine derivatives have been patented by the Beecham group. Astra AB hold patents on enzyme immobilization on CNBr -activated agarose and CNBr-activated crosslinked dextrans. Hindustan Antibiotics Ltd. have patented a process dependent on enzyme bound covalently to activated cellulose.

Bayer AG hold patents on enzyme covalently attached to polymers of acrylamide and of methacrylic acid. A process based on ethylene- maleic anhydride (EMA) copolymer has been patented by the Beecham group. Hoechst AG has patents on phenol-formaldehyde bound enzyme. Toyo Jozo Co have used derivatized polyacrylonitrile to immobilize penicillin acylase.

Other systems described are of Drobnik *et al.*,(1979) in which the reactive oxirane group of copolymers of glycidyl methacrylate and ethylene dimethacrylates was coupled to the enzyme via 1,6-diamino hexane. An interesting system reported is that of enzyme covalently attached to a water-soluble polyelectrolyte which could be reversibly insolubilized by a slight change in pH and ionic strength (Margolin *et al.*, 1981).The water soluble polyelectrolyte complex was formed from poly (4-vinyl-N-

ethylpyridinium bromide) and poly (methacrylic acid) and was activated with *s*-triazine trichloride for coupling the enzyme. The catalytic efficiency of the soluble complex was only slightly changed compared to the native enzyme. The strategy of reversible immobilization abolishes problems of diffusional restrictions. Bodhe and SivaRaman (1987) had immobilized the enzyme on glutaraldehyde derivatives of silanized controlled - pore ceramics. The enzyme immobilized on TiO_2 showed negligible diffusional limitations and was operationally stable while the enzyme attached to SiO_2 lost activity in use due to leaching out at alkaline pH.

(c) Immobilization by Intermolecular Crosslinking

Glutaraldehyde has been used extensively for immobilization through formation of crosslinked insoluble aggregates as the reagent does not inactivate the enzyme.

Crosslinking has been effected in presence of inert fillers or after adsorption on carriers . In the former type, cellulose powder has been used as a filler before crosslinking with glutaraldehyde (Amotz,1974). Enzyme adsorbed on DEAE-cellulose or CM- cellulose has been crosslinked with glutaraldehyde (Savidge and Powell,1975).

Hindustan Antibiotics Ltd. have patented processes based on intermolecular crosslinking of the enzyme from *E. coli* and from *Fusarium* sp (SivaRaman *et al*,1976). Crosslinking of enzyme adsorbed on macroporous methacrylate polymers with glutaraldehyde has been described by Koilpillai *et al*.,(1990).

(d) Immobilization by Gel Entrapment

The enzyme from *B. megaterium* has been entrapped in collagen membrane by Wang and Vieth (1973) by a process of impregnation after the support had been pre - swollen.Mandel *et al*.,(1975) have used polyacrylamide gels for entrapment of the enzyme, but leakage of activity was observed during use of the entrapped enzyme. To overcome leakage, Hueper(1974) has entrapped the soluble enzyme complex obtained with CNBr- activated dextran or starch.

(e) Immobilization by Encapsulation

In gel entrapment, the enzyme remains in a gel matrix. In contrast, in the process of encapsulation an aqueous solution of the enzyme is retained within a polymeric semipermeable membrane or casing. Examples are the SNAM Progetti process in which the enzyme solution is encapsulated within wet - spun cellulose triacetate fibres and in the Toyo Jozo process in which the enzyme is encapsulated in polyacrylonitrile fibres.

PART II

PRODUCTION AND PURIFICATION OF BENZYLPENICILLIN ACYLASE FROM ESCHERICHIA COLI AND ISOLATION OF CONSTITUTIVE MUTANT

SUMMARY

Benzylpenicillin acylase production by *Escherichia coli* NCIM 2350 was standardized. A complete medium of the following composition (g/l): yeast extract, 2; bactopeptone, 2; tryptone, 1; beef extract, 2; corn steep liquor (0.5 g/ml), 125 ml; K_2HPO_4,3; KH_2PO_4, 0.3; NaCl, 3.5; $(NH_4)_2SO_4$, 1; $MgSO_4.7H_2O$, 0.2 and phenylacetic acid (0.1%) as inducer. pH 7.3; gave high cell-bound activity in 24 h at 28°C under shake flask conditions.

A modified purification procedure was developed for obtaining pure benzylpenicillin acylase from *E.coli* cells. The procedure involved sonication of cells for extraction of enzyme activity, removal of nucleic acids by streptomycin sulphate treatment, fractional precipitation with ammonium sulphate, DEAE-Sepharose column chromatography and gel filtration through Sephadex G-200. The homogeneity of the preparation was established by disc electrophoresis in polyacrylamide gel which showed the presence of a single protein band and by SDS-polyacrylamide gel electrophoresis which showed the presence of the two component polypeptide bands of $M_r \backsim 20,000$ (α-subunit) and $M_r \backsim 70,000$ (β-subunit). The pure enzyme had a specific activity of 27 - 36 $\mu mole.min^{-1}mg^{-1}$ in hydrolysis of benzylpenicillin at pH 7.8 and 40°C.

A mutant designated as *E. coli* UV-2 was isolated by UV-irradiation of *E. coli* NCIM 2350. Unlike the parent strain, the mutant produced benzylpenicillin acylase constitutively. The enzyme produced by the mutant remained cell-bound as in the parent strain. The mutant produced higher levels of enzyme

activity constitutively than the parent strain in presence of phenylacetic acid. Glucose (1 g l^{-1}) repressed completely the induced enzyme production by the parent strain. The mutant, however, was more resistant to repression by glucose, producing about 60% of the activity obtained in absence of glucose.

INTRODUCTION

Escherichia coli represents one of the most extensively studied of benzylpenicillin acylase-producers.The organism therefore was selected for enzyme production required for studies on some basic and applied aspects of the industrially important enzyme .

As stated in the **GENERAL INTRODUCTION**, strain improvement is a prerequisite for any cost - effective development of an industrial process .One of the major objectives of strain improvment is the isolation of constitutive mutants not under catabolite repression as this can reduce considerably the cost of production of the enzyme. Classical approaches of strain improvement continue to be relevant to bacterial penicillin acylase production, mainly because of the complexity of the maturation pathway of the enzyme and the failure of recombinant DNA technology of the structural gene to give constructs that yield the expected high levels of enzyme.

This **Part** of the **THESIS** describes the growth conditions for high enzyme production by *E.coli* NCIM 2350, and standardization of a procedure for obtaining pure benzylpenicillin acylase from the organism. The production and purification of the enzyme from *E. coli* ATCC 11105 (NCIM 2068) was similar to that from *E. coli*

NCIM 2350. The isolation of a constitutive mutant of *E. coli* NCIM 2350 through induced mutation using UV irradiation is also described in this Part of the THESIS. As further evaluation of the mutant would have to be through linkages with industry, this aspect is not reported here.

MATERIALS AND METHODS

Yeast extract, beef extract, peptone and tryptone were obtained from Hi-media, India.Corn steep liquor, benzylpenicillin and 6 -aminopenicillanic acid (6-APA) were gifts from Hindustan Antibiotics Ltd., Pune. Crystalline bovine serum albumin was obtained from Sigma Chemicals, USA. Analytical grade phenylacetic acid was obtained from Aldrich Chemicals, USA. DEAE - Sepharose CL-6B and Sephadex G- 200 were from Pharmacia fine Chemicals, Sweden.Acrylamide monomer, N,N- methylenebisacrylamide and N,N,N',N'-tetramethylethylenediamine were from Eastman Organic Chemicals, USA. Sodium dodecylsulphate (SDS) was from Sigma Chemicals, USA. *p*-Dimethylaminobenzaldehyde (PDAB) was from Loba chemicals,India.All other reagents used were of analytical grade or high purity chemicals available commercially.All buffers and reagents were prepared in glass distilled water.

Microorganisms

Escherichia coli NCIM 2350 was obtained from the National Collection of Industrial Microorganisms, NCL, Pune. *E. coli* ATCC 11105 was obtained from the American Type Culture Collection, USA. The cultures were maintained routinely on nutrient agar with phenylacetic acid (0.1%) added as an inducer. The cultures were subcultured once in a month. The general characteristics of the

organism are Gram-negative, coccobacillary rods. Colonies on nutrient agar are small, opaque and discrete.

Serratia marscecens ATCC 27117, which is resistant to benzylpenicillin and sensitive to 6 - APA (Oostendorp, 1972), was obtained from the American Type Culture Collection, USA, and was routinely maintained on nutrient agar slants. The general characteristics of the organism are Gram-negative, thin bacilli which produce red coloured colonies when incubated at 28°C- 30°C.

Fermentation Media for Benzylpenicillin Acylase Production

E. coli NCIM 2350 was grown on three different types of media (**Table II.1**). Phenylacetic acid was used as an inducer at 0.1% concentration and was added either after 8 h growth of *E. coli* cells or from the inoculum stage to check the effect of the time of phenylacetic acid supplementation on induction and on the level of enzyme production.

Inoculum was prepared by transferring the culture from the nutrient agar slant to a tube containing 10 ml medium and incubating at 28°C for 24 h. The inoculum was transferred into a 250 ml Erlenmeyer flask containing 150 ml medium for enzyme production. The flasks were incubated at 28°C for 24 h on a NBS rotary shaker at 200 rpm.

The culture was harvested after 24 h by centrifugation at 4°C in a Sorvall RC 5B refrigerated centrifuge at 6000 x g for 30 min.

Assay of Penicillin Acylase Activity

Enzyme activity measurements were made by determining the 6 - APA formed with *p*-dimethylaminobezaldehyde (PDAB). The

Table II.1. Fermentation media for benzylpenicillin acylase production by *E. coli* NCIM 2350

Component	Medium A (gl^{-1})	Medium B (gl^{-1})	Medium C (gl^{-1})
Yeast extract	1.5	1.0	2.0
Beef extract	-	1.0	3.0
Bacto peptone	1.0	2.0	2.0
Tryptone	-	-	1.0
Corn steep liquor (50% w/v)	12.5 ml	-	12.5 ml
K_2HPO_4	3.0	3.0	3.0
KH_2PO_4	0.3	0.3	0.3
NaCl	3.5	3.5	3.5
$(NH_4)_2 SO_4$	-	-	1.0
$MgSO_4 .7H_2O$	-	-	0.2
Phenylacetic acid	1.0	1.0	1.0
pH (adjusted with N NaOH)	7.3	7.3	7.3

coloured Schiff's base which was obtained with the reagent was assayed according to the procedure of Bomstein and Evans (1965). Citrate- phosphate buffer, pH 2.5 was prepared by dissolving 56 g citric acid,H_2O and 8.7 g of $Na_2HPO_4,2H_2O$ in 1 l of distilled water. The colour reagent was prepared by dissolving 1 g of PDAB in 50 ml of ethanol and adding 10 ml of 0.01% hydroquinone and 110 ml of the citrate-phosphate buffer, pH 2.5. 0.1 ml samples containing 6-APA were added to 1.9 ml citrate -phosphate buffer, mixed with an equal volume of the colour reagent and read after 3 min at 415 nm.

The modification of the Bomstein and Evans (1965) procedure described by Sudhakaran and Shewale (1990) was also used in later work. In the modified procedure the colour reagent is prepared by dissolving 1 g of PDAB in 170 ml of methanol and 0.01% hydroquinone is added as stabilizer. 0.1 ml sample containing 6-APA was mixed with 1.9 ml citrate- phosphate buffer and an equal volume of PDAB reagent in methanol and read after 2 min at 415 nm. Colour yields by both methods were identical, the advantage of the modified procedure being the better storage stability of the colour reagent.

The standard curve of 6 - APA is shown in **Fig.II.1.**

Enzyme assay was carried out at 40°C essentially as described by Balsingham *et al.*(1972). The test system contained 4% w/v potassium salt of benzylpenicillin as substrate in 0.1 M potassum phosphate buffer, pH 7.8. One unit of enzyme is defined as the amount of enzyme catalysing the hydrolysis of 1 μ mole substrate in 1 min under assay conditions.

Fig. II.1 Standard curve of 6-APA. Estimations were carried out with p-dimethylaminobenzaldehyde as described in **Text.**

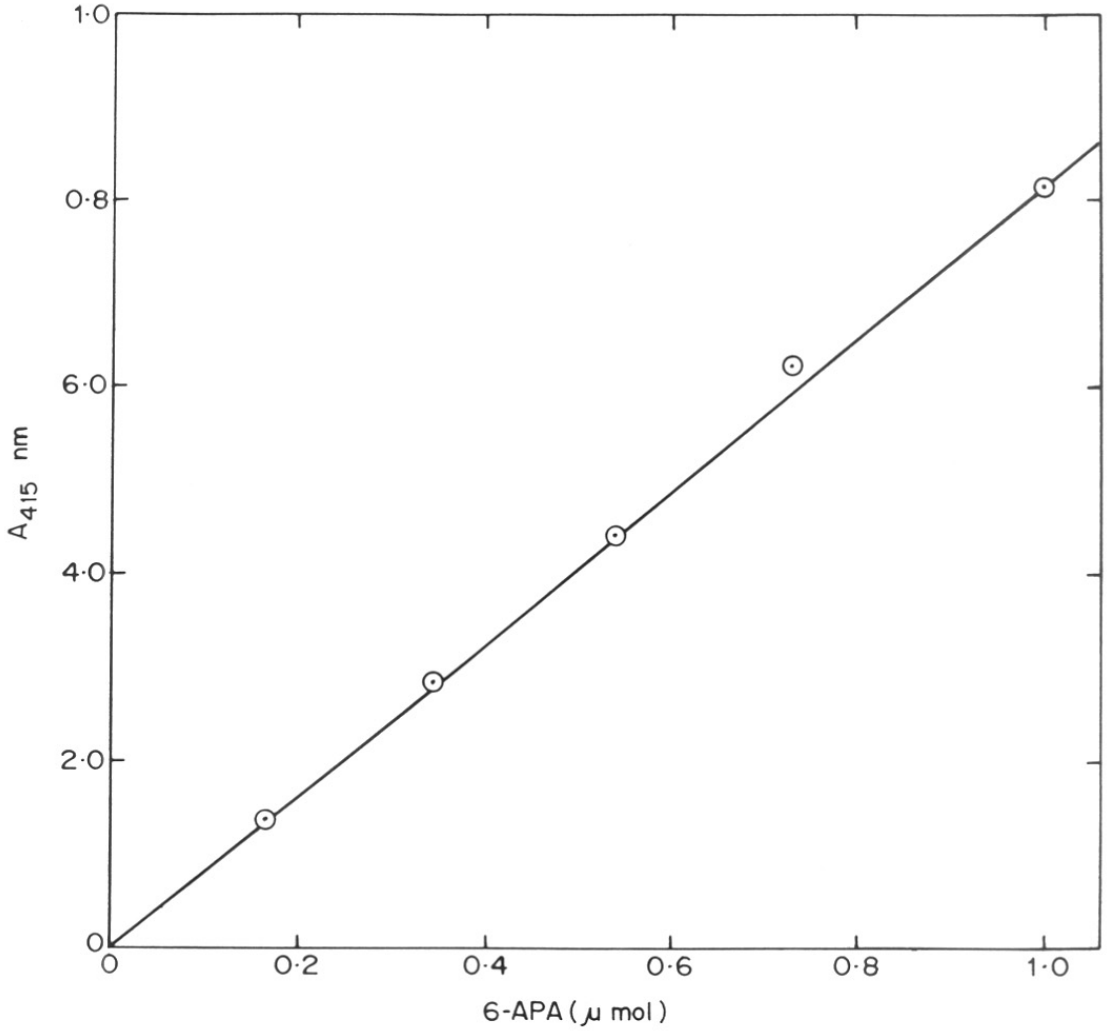


FIG. Ⅱ·1

Bioassay for Detection of Penicillin Acylase Activity

Plate assay of microorganisms producing penicillin acylase was done according to the modification of the method of Oostendorp (1972) essentially as described by Meevootisom *et al.*(1983). The test organism was point - inoculated separately on nutrient agar plates with either phenylacetic acid (0.1%) as an inducer or without the inducer. The 6-APA sensitive *S.marscecens* ATCC 27117 was simultaneously grown in nutrient broth. After 24 h, the broth containing *S.marscecens* was mixed with an equal volume of 1% agar solution containing 1% benzylpenicillin and layered on the pregrown penicillin acylase - producing test organism. The petri plates were then incubated at 28°C for 24 h and observed for inhibition against the red lawn of *S. marscecens* growth.

Ultraviolet Induced Mutation

Ultraviolet (UV) irradiation was used for inducing mutations in *E. coli* NCIM 2350 cells.10^7 -10^8 cells /ml in normal saline were transferred to a sterile petri dish and exposed to UV irradiation (Phillips UV germicidal lamp, 15 W) at a distance of 15 cm for different time intervals. The UV irradiated suspension was then plated on nutrient agar without any selection pressure and incubated at 28°C for 24 h. Exposure of 10 min which gave > 98% kill was used for obtaining isolates.

Protein Assay

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

Polyacrylamide Gel Electrophoresis

Disc electrophoresis was carried out according to Davis (1964), using 7% polyacrylamide gel. Electrophoresis runs were carried out at room temperature with a current of 4 mA/ tube applied till the bromophenol blue, tracking dye had migrated to the bottom of the gel. Protein bands were visualized by staining with Coomassie Blue R-250 in acetic acid and destained by a mixture of methanol:acetic acid:water in the proportion of 4:1:5.

SDS - Polyacrylamide Gel Electrophoresis

SDS-polyacrylamide gel electrophoresis was carried out according to the procedure of Laemmli (1970). 7.5% polyacrylamide gels were prepared containing 0.01% sodium dodecyl sulphate (SDS). Standard molecular weight markers of Sigma Chemicals were used for estimation of the M_r values of the subunits from SDS-polyacrylamide gel electrophoresis runs of the enzyme.

Electrophoresis was carried out at room temperature at 8 mA/ tube. Bromophenol blue was used as tracking dye. Gels were stained and destained as described above.

RESULTS and DISCUSSION

Benzylpenicillin acylase production by *E. coli* NCIM 2350

The media used in the present studies included complex organic sources of carbon and nitrogen, such as; corn steep liquor, yeast extract, beef extract, tryptone in various combinations and levels in addition to the inducer,phenylacetic acid.

The incubation temperature for the fermentation was maintained at 28°C as enzyme production is affected at

temperatures higher than 30°C due to the failure of the maturation pathway of the penicillin acylase precursor protein to yield active benzylpenicillin acylase (Bock *et al.*, 1983 a,b). Since high aeration rates reportedly inhibit enzyme production (Savidge and Cole, 1975), preliminary experiments were carried out at 100 - 250 rpm on the rotary shaker. 200 rpm was selected from these preliminary runs as the yield of activity was optimal at this rate of rotation.

Enzyme production on the three test media is shown in **Table II.2.** In all cases, cell and activity yields were similar both when the inducer was included in the medium from the start of the fermentation or supplemented after 8 h of growth under the conditions used. This is contrary to earlier reports, that late addition of phenylacetic acid is advantageous (Acevedo and Cooney,1973).

Highest activities were obtained on Medium C on the basis of both activity of unit weight of cells and unit volume of media (**Table II.2**).

ENZYME PURIFICATION

The procedure for purification of the enzyme was distinct from earlier methods and involved extraction by sonication of cell suspensions, streptomycin sulphate treatment for removal of nucleic acids, ammonium sulphate fractionation, chromatography on DEAE-Sepharose and Sephadex G-200 columns. Batches of 25 - 65 g wet weight of cells of *E. coli* harvested after 24 h growth at 28°C in a total volume of 5 - 10 l of Medium C were processed at a time for obtaining the pure enzyme. Details of processing are

Table II.2. Production of penicillin acylase by *E. coli* NCIM 2350 grown in test media A, B and C; 24 h growth at 28°C and 200 rpm

Medium	Yield of cells (wet weight) g l^{-1}	Penicillin acylase activity of packed μmole $min^{-1}g^{-1}$	Total activity μmole $min^{-1}l^{-1}$
A	1	3.2	3.2
B	2	4.0	8.0
C	6	7.2	43.2

described below.

Sonication of cell suspension

Freshly harvested cells were washed with 0.05 M potassium phosphate buffer, pH 7.8, the suspension centrifuged and washings discarded. The recovered cells were suspended in 0.05 M potassium phosphate buffer, pH 7.8, (4 - 5 ml/g packed cells) and disrupted by sonication (20 Kc, 300 W) for a total period of 10-12 min (2 min each exposure, 5-6 times) at 4°C on a Biosonic III (Bronwill Scientific Co., USA) sonic oscillator. The period of sonication was standardized to give > 90% cell disruption as seen microscopically. The cell debris was removed by centrifugation at 10,000 x g for 20 min at 4°C and discarded. The yield of activity was about 13 μmoles g^{-1} wet weight cells, a value comparable to that reported for *E. coli* NCIB 8743A (Balasingham *et al.*, 1972).

All subsequent steps were carried out at 0 - 4°C.

Streptomycin sulphate precipitation

The clear supernatant obtained after removal of cell debris was treated with slow addition of streptomycin sulphate (1.4% w/v) under stirring over a period of 30 min. The mixture was stirred for a further period of 1 h after which the precipitate was removed by centrifugation at 10,000 x g for 30 min and the clear supernatant which contained the activity was processed further.

Ammonium sulphate fractionation

The supernatant was fractionated with slow addition under stirring of finely ground ammonium sulphate and the fraction

which precipitated between 0.3 to 0.8 saturation was collected by centrifugation at 10,000 x g for 30 min. The supernatant was discarded and the precipitate which contained the activity was dissolved in the minimum volume of 0.01 M potassium phosphate buffer, pH 7.8 and dialysed overnight with one change against 100 volumes of buffer of the same molarity and pH.

DEAE-Sepharose column chromatography

The dialysed solution was clarified by centrifugation and loaded on a DEAE-Sepharose CL-6B column (2.5 cm x 30 cm) previously equilibrated with 0.01M potassium phosphate buffer, pH 7.8. The enzyme was eluted from the column by using a linear gradient of potassium phosphate buffer, pH 7.8, added from a mixing device. The gradient was obtained by taking 200 ml of 0.01 M of buffer solution in the mixing chamber attached to the column and addition of 0.5 M buffer solution from the reservoir. Fractions of 3 ml each were collected on an automatic fraction collector and 20 µl from each fraction was tested for enzyme activity. The enzyme eluted between 130 - 180 ml and the fractions with enzyme activity (approximately 15 ml) were pooled and precipitated at 0.8 saturation of ammonium sulphate.

Sephadex G-200 gel filtration

The ammonium sulphate precipitate obtained after the DEAE-Sepharose step was recovered by centrifugation, dissolved in the minimum volume of 0.01 M potassium phosphate buffer, pH 7.8, centrifuged and the clear supernatant (1 - 2 ml) was loaded on a Sephadex G - 200 column (2.5 x 100 cm), previously equilibrated with 0.01 M potassium phosphate buffer, pH 7.8. The enzyme was

eluted with buffer of the same composition. Fractions of 1.5 - 2 ml were collected on an automatic fraction collector and 20 µl from each fraction was tested for enzyme activity. The enzyme eluted between 60 - 80 ml and the fractions containing high enzyme activity were pooled and stored at -20°C.

The results of purification of penicillin acylase from *Escherichia coli* NCIM 2350 in a typical batch are summarized in **Table.II.3**. The specific activity of the purified enzyme varied from 27 - 36 µmoles $min^{-1}mg^{-1}$. The final recovery was about 25% of initial activity in cell extracts. Disc electrophoresis in polyacrylamide gel showed the presence of a single component (**Fig. II.2**). SDS-polyacrylamide gel electrophoresis showed two bands of proteins of M_r values of about 20,000 (α) and of about 70,000 (β) (**Fig.II.3**).

The heterodimeric structure of the *E. coli* enzyme has been well documented (Bock *et al*,1983a,b). This has been dealt with in detail in the GENERAL INTRODUCTION. The primary structures of the subunits derived from nucleotide sequencing of the *pac* gene encoding benzylpenicillin acylase of *E.coli* ATCC 11105 have established M_r values of 20,500 and 65,000 for the α and β subunits, respectively (Schumacher *et al*, 1986). All bacterial benzylpenicillin acylase obtained to date, with the exception of the enzyme from *Bacillus megaterium*, have been shown to be heterodimers built up of α and β subunits of approximately the same sizes as those from *E.coli*. The purified enzyme from *E. coli* NCIM 2350 showed the presence of a single protein band in disc electrophoresis. A similar result has been reported for the

Table II.3. Summary of purification of benzylpenicillin acylase from E. coli NCIM 2350 (30 g packed cells)

Purification step	Volume	Protein	Activity	Specific activity	Yield
	ml	$mg.ml^{-1}$	$\mu mole\ min^{-1} ml^{-1}$	$\mu mole\ min^{-1} mg^{-1}$	%
Cell-free extract	125	18.0	3.2	0.18	(100)
Streptomycin sulphate/ 0.3 - 0.8 saturation ammonium sulphate	45	30.0	7.1	0.24	78.6
DEAE-Sepharose	14	0.8	13.6	17.00	47.0
0.8 saturation amm. sulphate/Sephadex G-200	6	0.6	17.2	29.0	25.0

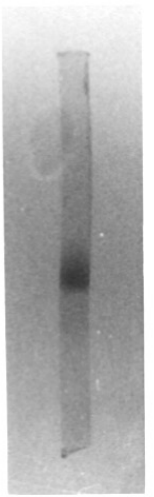


Fig. II.2 Polyacrylamide gel electrophoresis of purified penicillin acylase from Escherichia coli.

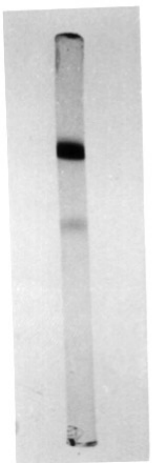

β
c

Fig. II.3 SDS-polyacrylamide gel electrophoresis of purified penicillin acylase from Escherichia coli

crystalline enzyme isolated from *E. coli* ATCC 11105 by Kutzbach and Rauenbusch (1974). However, heterogenecity has been observed on isoelectric focussing in polyacrylamide gel (Kutzbach and Rauenbusch, 1974; Kasche *et al.*, 1984) and in HPLC by hydrophobic chromatography (Kasche *et al.*, 1984), multiple components with enzyme activity being reported. The cause of the heterogenecity is not apparent. The variation in specific activity (27 - 36 μmole $min^{-1}mg^{-1}$) of the pure enzyme from different batches in the present case could possibly be due to different ratios of active components in the preparations. Enzyme purified from *E. coli* ATCC 11105 grown under the same conditions as those used for *E. coli* NCIM 2350 gave essentially similar results. The variation in specific activity values of preparations, homogeneity in disc electrophoresis and the heterodimeric character in SDS-polyacrylamide gel electrophoresis were similar in both strains.

UV Resistant Constitutive Mutant of *E.coli* NCIM 2350

Screening for constitutive mutants: 20 different colonies were selected randomly after UV irradiation of the parent strain as described under MATERIALS and METHODS. These colonies were transferred into two sets of nutrient broth tubes, one with phenylacetic acid (0.1%) as inducer and the other without any inducer to check for constitutive production of benzylpenicillin acylase activity. Tubes were incubated at 28°C for 24 h at 200 rpm, after which the tubes were centrifuged and the supernatants discarded. The sedimented pellet of cells was checked for penicillin acylase activity. The results are summarized in **Table**

II.4. It can be seen from **Table II.4** that isolate UV-2 is the only one which produces significant levels of active enzyme in the absence of the inducer, phenylacetic acid.

Screening for extracellular enzyme activity: All the isolates were also inoculated in Medium C containing phenylacetic acid (0.1%) and incubated at 28°C for 24 h at 200 rpm to check for cell-bound and/or extracellular activity. This was done since an earlier report had claimed extracellular production of benzylpenicillin acylase by an *E. coli* isolate (Vojtisek *et al.*, 1981). After 24 h, cells were harvested by centrifugation and cells and supernatant were checked separately for penicillin acylase activity. Neither the parent nor any of the mutants showed penicillin acylase activity in the supernatant, indicating that in all cases the activity remains cell-bound.

Enzyme production by the UV-2 constitutive mutant was compared with that of the parent *E. coli* NCIM 2350, both in absence of phenylacetic acid and in presence of phenylacetic acid. These experiments were carried out using the complete Medium C with phenylacetic acid (0.1%) as an inducer as well as Medium C without any phenylacetic acid. The results are summarized in **Table II.5.**

As seen from **Table II.5,** UV-2 mutant produces significantly higher penicillin acylase activity than the parent even in the presence of phenylacetic acid. Enzyme production by the mutant in the absence of the inducer was comparable if not higher than that obtained in presence of phenylacetic acid. In contrast the parent strain produced only very low levels of

Table II.4 Screening for the constitutive mutants of UV-irradiated *E. coli* NCIM 2350

Organism	Induced activity	Constitutive activity
Parent strain	+	-
E. coli UV-1	+	-
E. coli UV-2	++	++
E. coli UV-3	+	-
E. coli UV-4	+	-
E. coli UV-5	+	-
E. coli UV-6	+	-
E. coli UV-7	+	-
E. coli UV-8	+	-
E. coli UV-9	+	-
E. coli UV-10	+	-
E. coli UV-11	+	-
E. coli UV-12	+	-
E. coli UV-13	+	-
E. coli UV-14	+	-
E. coli UV-15	+	-
E. coli UV-16	+	-
E. coli UV-17	+	-
E. coli UV-18	+	-
E. coli UV-19	+	-
E. coli UV-20	+	-

Table II.5 Enzyme production by *E. coli* NCIM 2350 and *E. coli* UV-2 mutant constitutively and induced

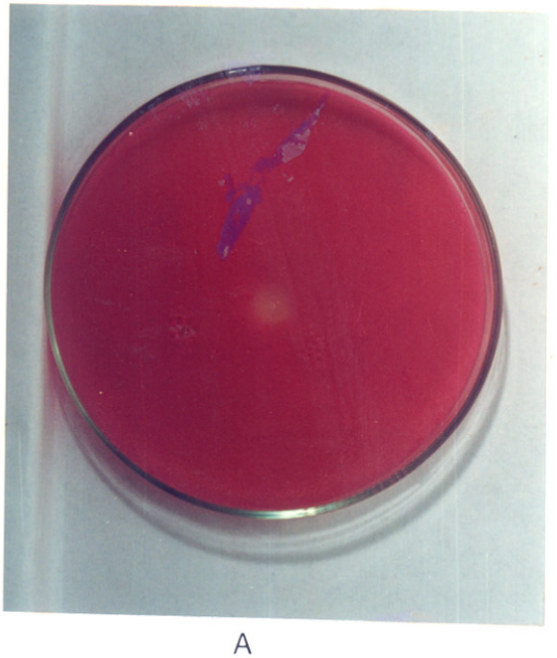
Organism	Activity ($\mu mol.min^{-1}g^{-1}$)	
	Medium C without PAA*	Medium C with PAA
E. coli NCIM 2350 (parent strain)	0.5	7.2
E. coli UV-2 (mutant)	12.6	11·6

*Phenylacetic acid

activity in the absence of the inducer. These findings were confirmed by bioassay with *S. marscecens* ATCC 27117. The parent strain showed little or no zone of inhibition in absence of the inducer. Unlike the parent strain, isolate UV-2 showed distinct inhibition under these conditions. The results are shown in Plate II.1.

Test for Catabolite Repression: The effect of D-glucose on enzyme production by *E. coli* NCIM 2350 and UV-2 mutant was tested by growing cells on Medium C supplemented with glucose (1 g l^{-1}).

Inclusion of glucose (1 g l^{-1}) abolished penicillin acylase production in the case of the parent strain grown in Medium C containing phenylacetic acid. In contrast, the mutant UV-2 was more resistant to repression by glucose, enzyme production being about 60% of that in absence of both glucose and phenylacetic acid as well as in the absence glucose but in presence of phenylacetic acid.



A

B

C

Plate II.1 Zone of inhibition of benzylpenicillin resistant and 6-APA sensitive Serratia marcescens ATCC 27117

A. Parent strain grown constitutively

B. Mutant grown constitutively

C. Mutant grown with inducer

PART III

ESSENTIAL ARGININE AND TRYPTOPHAN RESIDUES IN THE PUTATIVE SIDE CHAIN BINDING SUBUNIT OF PENICILLIN ACYLASE FROM ESCHERICHIA COLI

SUMMARY

Incubation of penicillin acylase from *Escherichia coli* with phenylglyoxal or 2,3-butanedione results in enzyme inactivation. Benzylpenicillin, phenylacetamide and phenylacetate protected the enzyme against the inactivation, indicating the presence of arginine at or near the catalytic site. The reactions follow pseudo first-order kinetics and the inactivation kinetics indicate the presence of a single essential arginine moiety.

The enzyme is also inactivated by incubation with the tryptophan-modifying reagents, N-bromosuccinimide (NBS) and 2-hydroxy-5-nitrobenzyl bromide (HNBBr). Benzylpenicillin, phenylacetate and 6-aminopenicillanic acid (6-APA) significantly lower the extent of inactivation by the tryptophan-modifying reagents. The inactivation reaction kinetics are indicative of the involvement of a single essential tryptophan residue in the hydrolysis of benzylpenicillin by penicillin acylase from *E.coli*.

Subunit-complementation experiments in which various combinations of the physically isolated α and β-subunits derived from the untreated enzyme and from the phenylglyoxal inactivated or the NBS-inactivated enzyme were assayed for recovery of enzymatic activity, indicated that both the essential arginine and the essential tryptophan residues are present on the putative substrate-binding α-subunit of the enzyme molecule. These are the first reports on the essential amino acid residues involved at the putative substrate side chain binding domain of the α-subunit of penicillin acylase.

INTRODUCTION

Benzylpenicillin acylases (EC 3.5.1.11) catalyse reversibly the hydrolysis of penicillins to the side chain and 6-aminopenicillanic acid (6-APA), benzylpenicillin being the preferred substrate among the penicillins. The enzymes, which are usually of bacterial origin, act on a wide range of derivatives of the side chains of penicillin molecules, including acylamino acids and amides. Substrate susceptibility is determined mainly by the acyl moiety and in the case of penicillin acylase from *Escherichia coli*, the most studied among these enzymes, some of the non-penicillin compounds such as phenylacetamide and phenylacetylglycine are better substrates than benzylpenicillin (Cole, 1964; 1969a,b; Kaufmann and Bauer, 1964). Benzylpenicillin acylases from *E. coli* (Bock *et al.*, 1983), *Proteus rettgeri* (Daumy *et al.*, 1985 b), *Kluyvera citrophila* (Barbero *et al.*, 1986) and *Arthrobacter viscosus* (Ohashi *et al.*, 1988) are heterodimers ($\alpha\beta$), the dissimilar subunits in all cases being polypeptides of M_r values of α about $\sim$25,000 and β of about $\sim$ 65,000.

The kinetics of the enzyme catalysed hydrolysis of benzylpenicillin have indicated that the reaction proceeds via an acylenzyme intermediate (Konecny, 1981 a,b; Konecy,1983), an hypothesis supported by the irreversible inactivation of the enzymes from *E.coli* (Kutzbach and Rauenbusch, 1974; Slade *et al.*, 1991), *P.rettgeri* (Daumy *et al.*, 1985 b) and *K.citrophila*(Martin *et al.*,1991) by an equimolar amount of phenylmethanesulfonyl fluoride (PMSF). Subunit-complementation experiments with the separated subunits from the PMSF-

inactivated and the untreated enzyme from *P.rettgeri* have shown that the larger β- subunit of the enzyme contains the essential phenylmethanesulfonyl fluoride - sensitive residue involved in catalytic activity (Daumy *et al.*, 1985b). The PMSF - sensitive residue in the enzyme from *K. citrophila* (Martin *et al.* 1991) and from *E.coli* (Slade *et al.*,1991) have been identified as a serine moiety. That the smaller subunit of the *P.rettgeri* enzyme has the domain that binds the side chain moiety of the penicillin molecule has been shown from subunit- complementation between the subunits of the enzymes from the wild type *P.rettgeri* and mutants with altered penicillin side chain specificities (Daumy *et al.*, 1985 b).

Robak and Szewczuk (1981) and Mahajan and Borkar (1983) had reported that the enzyme from *P.rettgeri* and *E.coli* are inactivated in presence of tryptophan-modifying reagents,but no kinetic analysis nor estimates of the number of residues modified were reported.

Little else is known regarding the active site residues of benzylpenicillin acylase, particularly of those involved at the putative substrate binding sites of the smaller α- subunit.

In this Part of the THESIS evidences are presented for the presence of an essential arginine and an essential tryptophan residue in the *E. coli* enzyme from chemical modification studies. Both the arginine residue and the tryptophan residue have been shown to be present in the putative substrate side chain binding subunit from subunit-complementation experiments.

Materials and Methods

Materials

Benzylpenicillin and 6-aminopenicillanic acid were gifts from Hindustan Antibiotics Ltd. Phenylacetic acid was obtained from Aldrich. Phenylglyoxal was obtained from Fluka, N-bromosuccinimide(NBS),2-hydroxy-5-nitrobenzyl bromide(HNBBr) and sodium dodecylsulfate (SDS) were from Sigma.Acrylamide, N,N´-methylene bisacrylamide,and N,N,N,´N´-tetramethylethylene diamine were from Eastman Kodak. Urea solutions were prepared fresh from recrystallysed guaranteed grade reagent. Phenylacetamide was synthesised from phenylacetic acid essentially according to the procedure described by Kao and Shao-Yuan (1930) for the preparation of benzamide from benzoic acid.

Microorganism

Escherichia coli NCIM 2350 was obtained from the National Collection of Industrial Microorganisms, Pune.

Methods

Penicillin acylase: Benzylpenicillin acylase was purified from *E. coli* as described in **Part II** of the **THESIS**. The preparation was homogeneous in disc electrophoresis and showed the presence of the constituent α-M_r $\backsim$ 20,000 and β-M_r $\backsim$ 70,000 subunits in SDS-polyacrylamide gel electrophoresis. The specific activity towards benzylpenicillin in the hydrolytic reaction was 27 - 36 μmoles $min^{-1}mg^{-1}$ at pH 7.8 and 40°C.

Activity towards phenylacetamide was determined using 0.4% w/v substrate in potassium phosphate buffer, pH 7.8 at 40°C, the ammonia released being assayed with ninhydrin (Moore and Stein, 1961; 1964).

Treatment with Arginine-modifying Reagents

Purified benzylpenicillin acylase from *E. coli* was incubated at 25°C with either phenylglyoxal (2.5 mM to 20 mM) in 0.05 M potassium phosphate buffer, pH 8.0 or 2,3-butanedione (10 mM to 40 mM) in 0.05 M sodium borate buffer, pH 8.0. The reaction system was shielded from light when 2,3-butanedione was used as the arginine modifying reagent to prevent any photochemical effect since 2,3-butanedione is known to be prone to photosensitization (Riordan, 1973; Ryang and Wang, 1978). Aliquots were withdrawn at different time intervals for assay of enzymatic activity.

Enzyme incubated with buffers in absence of the modifying reagent, served as control.

Treatment with Tryptophan-modifying Reagents

N-Bromosuccinimide (NBS) and 2-hydroxy-5-nitrobenzyl bromide (HNBBr)were used as modifying reagents. The former reagent oxidizes both tryptophan and cysteine residues and the absence of cysteine moieties in benzylpenicillin acylase from *E. coli* (Schumacher *et al.*, 1986) obviates ambiguity about the modified amino acid residues.

Purified benzylpenicillin acylase from *E. coli* was incubated at 25°C with either NBS (10 - 100 μM) in 0.05 M sodium acetate buffer, pH 5.0 or HNBBr (10 - 40 mM) in 0.05 M sodium acetate buffer at pH 5.0 Aliquots were withdrawn at regular interval of time for the assay of enzymatic activity.

Enzyme incubated in the buffer in absence of the modifying reagents served as control.

Kinetics of Inactivation

The apparent first-order rate constant of inactivation, K_{app}, depends upon the concentration of the modifier according to the following relationship

$$K_{app} = K (M)^n$$

$$\text{or } \log K_{app} = \log K + n \log (M)$$

where K is the second-order rate constant, (M) the concentration of the modifier and n = the average order of the reaction with respect to concentration of the modifier. K_{app} can be calculated from a semi-logarithmic plot of the enzyme activity remaining as a function of time. The order of the reaction (n) can be experimentally estimated by determining K_{app} at a number of different concentrations of the modifier. A plot of log K_{app} against log (M) should give a straight line with a slope equal to n, where n is the number of molecules of the modifier reacting with each active unit of the enzyme to produce an enzyme-inhibitor complex (Levy *et al.*, 1963; Ramakrishna and Benjamin, 1981; Marcus *et al.*, 1976).

Protection from inactivation: Protection of the enzyme against inactivation by the arginine reagents were tested with the substrates benzylpenicillin-K salt and phenyacetamide and the product, phenylacetic acid. The compounds were tested at 50 or 30 mM final concentration and pH 8.0 and were added immediately before the addition of phenylglyoxal (20 mM final concentration) or 2,3-butanedione (40 mM) final concentration. Protection of penicillin acylase against inactivation by the tryptophan reagents was tested with benzylpenicillin, 6-APA and phenylacetic acid. The compounds were tested at pH 5.0 and were added

immediately before the addition of NBS (100 μM final concentration) or HNBBr (40 mM final concentration).

Titration of Tryptophan Residues by NBS

The tryptophan-modifying reagent is a potent oxidizing agent which oxidizes the indole moiety of the amino acid, a chromophore absorbing strongly at 280 nm, to oxyindole, a much weaker chromophore at this wavelength. The titration of accessible tryptophan residues in penicillin acylase with NBS was followed spectrophotometrically at 280 nm. The control cuvette had 50 mM sodium acetate buffer, pH 5.0 and the experimental had penicillin acylase (500 μg) in a total volume of 1 ml of 50 mM sodium acetate buffer. Successive 10 μl aliquots of the 50 μM NBS were added to both control and experimental cuvettes and changes in absorbance at 280 nm recorded. The number of tryptophan residue oxidized (x) / mole enzyme after each addition of NBS was calculated as described by Witkop (1961) and Spande and Witkop (1967) using the equation:

$$\Delta x = \frac{1.31 \times \Delta A_{280}}{5500 \times \text{molarity of enzyme}}$$

where A_{280} is the decrease in absorbance at 280 nm, 1.31 is an empirical factor based on oxidation of model tryptophan – containing peptides (Patchornik *et al.*, 1958) and 5500 is the molar absorption coefficient of tryptophan at 280 nm.

Enzyme (500 μg) treated separately with the modifying agent was also tested for residual activity after each addition of 10 μl of 50 μM NBS.

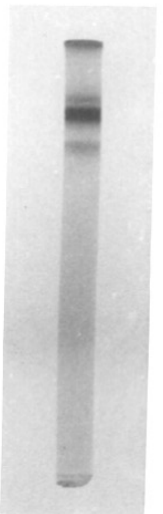
Subunit-Complementation Experiments

Subunit-complementation experiments were carried with combinations of subunits separated by polyacrylamide gel disc electrophoresis in presence of 5 M urea from phenylglyoxal-inactivated enzyme and untreated enzyme and with combinations of subunits separated from the NBS-inactivated enzyme and untreated enzyme. In all experiments subunits separated from untreated enzyme by disc electrophoresis in polyacrylamide gel containing 5 M urea and reconstituted by mixing served as controls (Plate III.1).

For subunit-complementation experiments involving phenylglyoxal-inactivated enzyme, the enzyme was modified by treatment with 20 mM phenylglyoxal for 1 h in 0.05 M potassium phosphate buffer, pH 8.0 after which the pH was adjusted to 5 with sodium acetate buffer and the solution was dialysed extensively against 100 volumes of 0.05 M sodium acetate buffer of pH 5 at 4°C for 1 h with one change.

For subunit-complementation experiments involving NBS-inactivated enzyme, the enzyme was modified by treatment with 100 µM NBS in 0.05 M sodium acetate buffer, pH 5.0 for 40 min followed by dialysis at 4°C against 100 volumes buffer of the same composition for 1 h with one change.

The experiments were carried out essentially as described by Daumy *et al.* (1985 b). Subunits of the native and the chemically modified enzyme were individually separated by polyacrylamide gel disc electrophoresis in presence of 5 M urea after unfolding in 9 M urea. Combinations of the isolated subunits from untreated and the chemically modified enzyme were mixed without removal of



→ β
→ α

1

2

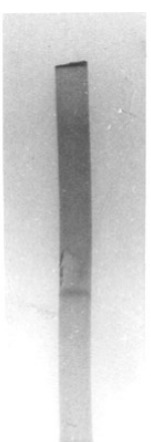


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Fig.III. Resolution of *E. coli* penicillin acylase into α- and β-subunits:

Lane 1: Purified enzyme resolved into two bands in disc electrophoresis in presence of 5 M urea.

Lane 2: SDS-polyacrylamide gel electrophoresis of β-subunit isolated from urea-polyacrylamide gel.

Lane 3: SDS-polyacrylamide gel electrophoresis of α-subunit isolated from urea-polyacrylamide gel.

urea and allowed to reassociate by dialysing against urea-free 0.1 M potassium phosphate buffer, pH 7.8 before assaying for enzyme activity.

Sets of 400 µg each of the chemically modified and dialyzed enzyme and native untreated enzyme were dissociated separately in 9 M urea in 50 mM potassium phosphate buffer, pH 7.8, for 24 h at 20°C. The urea concentration used was that recommended by Lindsay and Pain (1990; 1991). Native enzyme was dissociated under similar conditions for subunit-complementation with the phenylglyoxal- and the NBS-modified enzymes.

Subunits were then separated from aliquots by the Davis (1964) procedure of disc electrophoresis in 7.5% polyacrylamide gel containing 5 M urea as described by Daumy *et al.* (1985b). Electrophoresis of each sample was carried out in duplicate and one of each set stained with Coomassie Blue G-250 instant staining reagent (Reisner *et al.*, 1975). The reagent comprised Coomassie Brilliant Blue G-250 (100 mg) dissolved in 7 ml of 70% perchloric acid and diluted with addion of 100 ml of glass distilled water, the solution being filtered before use. The unstained gels were aligned next to the duplicates stained with the Coomassie Blue reagent. The regions of the unstained gels comprising the subunits of both unmodified and modifed enzyme were separately excised and various combinations of the gel slices were macerated together with 0.1 M potassium phosphate buffer, pH 7.8, and dialysed overnight against the urea-free buffer at 4°C. The dialysed combinations were assayed for penicillin acylase activity towards benzylpenicillin.

The identity of the protein components separated by disc electrophoresis in presence of 5 M urea was checked by SDS-polyacrylamide gel electrophoresis (Plate III 1).

RESULT

Activity of *E. coli* benzylpenicillin acylase towards phenylacetamide

The purified enzyme hydrolysed 30 mM phenylacetamide at pH 8.0 and 40°C more rapidly than benzylpenicillin under similar conditions, the relative initial rate of hydrolysis of phenylacetamide being about 150% compared to that of benzylpenicillin. This is in agreement with the earlier report of Kutzbach and Rauenbusch (1974).

Inactivation of *E. coli* penicillin acylase by arginine-modifying reagents

E. coli penicillin acylase inactivated rapidly on incubation at pH 8.0 with phenylglyoxal or 2,3-butanedione, the former being a more potent inactivator (**Fig. III.1**). The rates of inactivation in both cases followed pseudo first-order kinetics, plots of the logarithm of the residual activity *versus* time of contact with the reagents being linear throughout the test period of 1 h. The residual enzyme activity at the end of this period was less than 5% initial activity, in presence of 20 mM phenylglyoxal, the corresponding value in presence of 40 mM 2,3-butanedione being 26%. The enzyme activity remained unchanged in the absence of the modifiers. The reaction order (n) with respect to the arginine-modifying reagent was determined from the plot of the logarithm of the apparent first-order rate constant, K_{app} *versus* the logarithm of the reagent concentrations (**Fig.**

Fig. III.1 Inactivation of penicillin acylase from *E. coli* by -carbonyl reagents. The enzyme (60 ug/ml) was incubated at 25°C in 50 mM potassium phosphate buffer (pH 8.0) and varying concentrations of phenylglyoxal or in 50 mM sodium borate buffer (pH 8.0) and varying concentrations of 2,3-butanedione

(A) Phenylglyoxal 0 mM (-●-), 2.5 mM (-o-), 5 mM (-■-), 10 mM (-□-) and 20 mM (-Δ-).

(B) 2,3-butanedione 0 mM (-●-), 10 mM (-o-), 20 mM (-■-), 30 mM (-□-) and 40 mM (-Δ-).

At the indicated periods of time aliquots were withdrawn for assay of enzyme activity.

Insets: Determination of the order of the reaction with respect to phenylgloxal (A) and 2,3-butanedione (B)

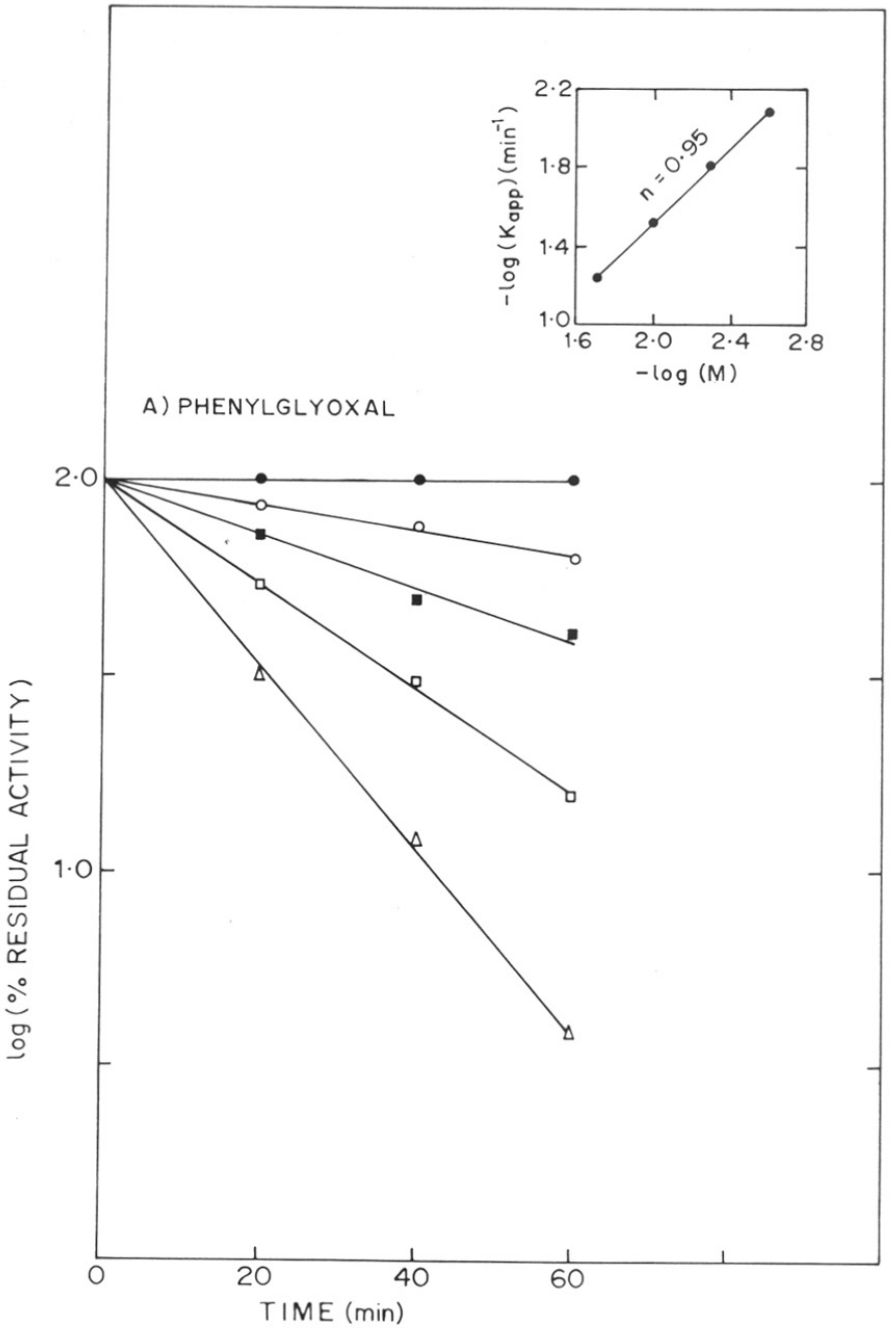

A) PHENYLGLYOXAL
log (% RESIDUAL ACTIVITY)
2·0
1·0
0
20
40
60
TIME (min)
-log (Kapp) (min-1)
2·2
1·8
1·4
1·0
n = 0.95
1·6
2·0
2·4
2·8
-log (M)

FIG. Ⅲ·1 (A)

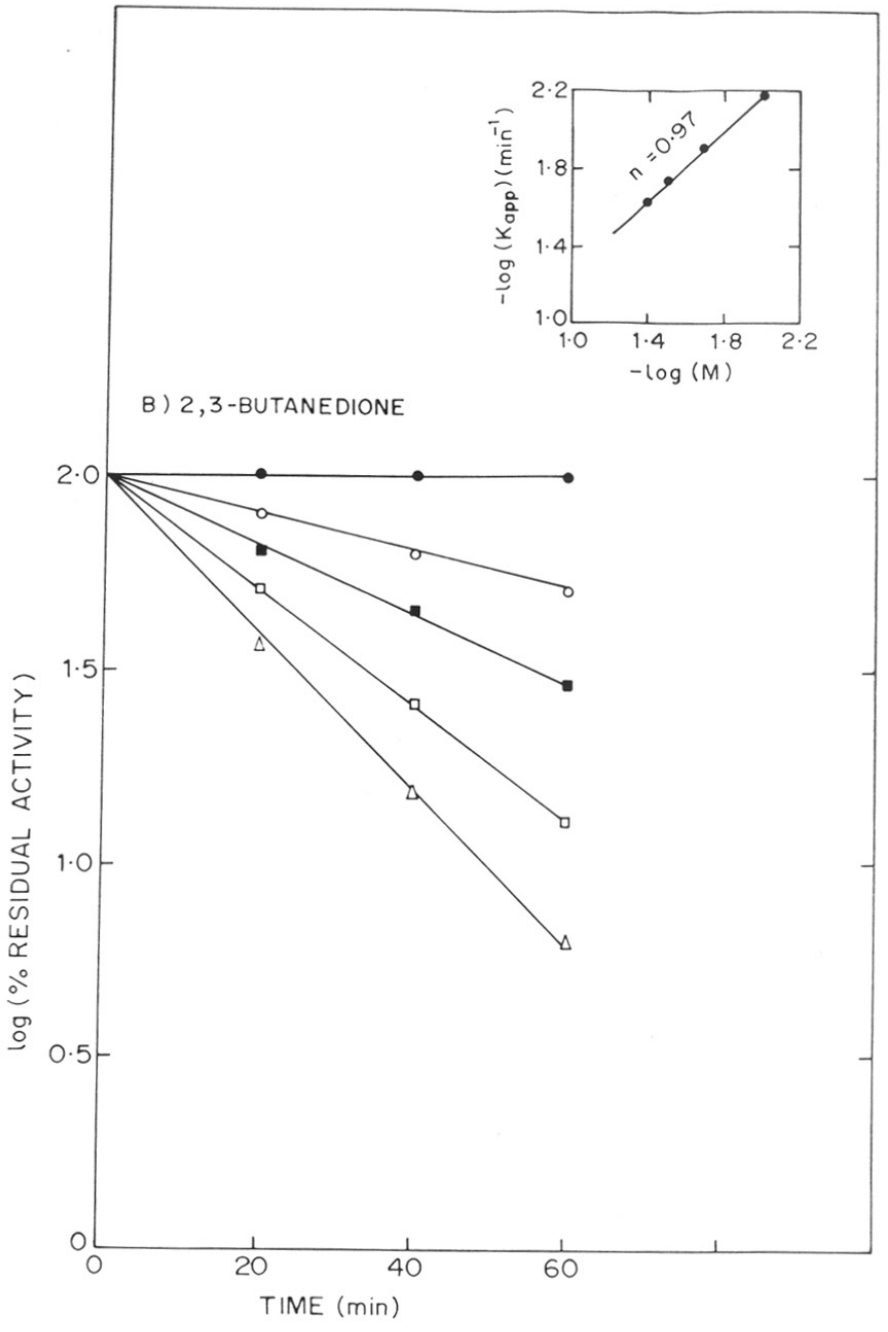

B) 2,3-BUTANEDIONE
log (% RESIDUAL ACTIVITY)
2·0
1·5
1·0
0·5
0
0
20
40
60
TIME (min)
-log (Kapp) (min⁻¹)
2·2
1·8
1·4
1·0
n = 0·97
1·0
1·4
1·8
2·2
-log (M)

FIG. Ⅲ·1 (B)

III.1 inset) and the value of n = 0.95 for phenylglyoxal and of n = 0.97 for 2,3-butanedione indicate that loss of enzyme activity results from the reaction of one arginine per mole penicillin acylase.

Protection from inactivation by arginine reagents

Table III.1 summarizes the data on protection of penicillin acylase from *E. coli* against inactivation by phenylglyoxal and 2,3-butanedione in the presence of benzylpenicillin, phenylacetamide or phenylacetate.

Benzylpenicillin (50 mM) protected the enzyme against inactivation by both 20 mM phenylglyoxal and 40 mM 2,3-butanedione, 95% and 98% respectively of the initial activity being retained at the end of the test period of 1 h, compared to 4% and 26% respectively in the absence of the substrate. The corresponding values for protection by phenylacetate (50 mM) were 60% against inactivation of the enzyme by phenylglyoxal and 92% against inactivation by 2,3-butanedione. The neutral substrate, phenylacetamide, which was tested at only 30 mM concentration on account of solubility problems, protected the enzyme to the extent of 90% against inactivation by phenylglyoxal.

These results would indicate that the reactive arginine residue is located at or near the active site of the penicillin acylase molecule as substrates and products protect the enzyme against inactivation by chemical modification by arginine-specific reagents.

Inactivation of *E. coli* penicillin acylase by tryptophan-modifying reagents

Table III.1 Protection of *E. coli* penicillin acylase against inactivation by arginine specific reagents

Treatment[a]	Enzyme activity[b] (% initial activity)
None	100
Phenylglyoxal (20 mM)	4
Benzylpenicillin (50 mM) + phenylglyoxal (20 mM)	95
Phenylacetate (50 mM) + phenylglyoxal (20 mM)	60
Phenylacetamide (30 mM) + phenylglyoxal (20 mM)	90
2,3-Butanedione (40 mM)	26
Benzylpenicillin (50 mM) + 2,3-butanedione (40 mM)	98
Phenylacetate (50 mM) + 2,3-butanedione (40 mM)	92

[a] Test compounds when used were added immediately before the α-carbonyl reagent. Enzyme (60 µg/ml) was treated at 25°C as indicated in the Table and samples were withdrawn periodically for assay of enzyme activity.

[b] Values obtained after 1 h.

Both NBS and HNBBr inactivated the enzyme rapidly at pH 5.0, the latter inactivating the enzyme more rapidly (**Fig.III.2**). The enzyme activity in the presence of 100 μM NBS was 5% at the end of the 40 min test period, while residual enzyme activity in the presence of 30 mM HNBBr was 6% at the end of 30 min of incubation. The plots of logarithm residual activity *versus* time of incubation were linear upto the end of the test periods, indicating pseudo first-order kinetics of inactivation by both the reagents.

The reaction order (n) with respect to the tryptophan modifying reagents determined from the plots of log K_{app} *versus* log reagent concentration gave values of n = 1 for NBS and n = 0.97 for HNBBr (**Fig. III.2 inset**). These values would indicate that the modification of a single tryptophan residue results in the inactivation of a mole of the enzyme.

Protection against inactivation by tryptophan reagents

The protective action of substrate and products of penicillin acylase-catalysed reaction on the inactivation of the enzyme by NBS and HNBBr is shown in **Table III.2**.

In presence of benzylpenicillin (50 mM) residual activity was 60% after 40 min incubation with NBS (100 μM)and 64% after 30 min incubation with HNBBr (30 mM), compared with 6% in both cases in the absence of the substrate. Phenylacetate (50 mM) gave corresponding values of 50% and 65% activity, respectively, 6APA (50 mM) protected the enzyme almost completely (97%) against inactivation of the enzyme by NBS (100 μM) in 30 min. Under the conditions of the experiment, NBS (100 μM) had no

Fig.III.2 Inactivation of penicillin acylase from E. coli by tryptophan modifying reagents.

(A) N-Bromosuccinimide 0 μM (-□-), 10 μM (-●-). 20 μM (-Δ-), 50 μM (-○-) and 100 μM (-▲-)

(B) 2-Hydroxy-5-nitrobenzylbromide 0 mM (-□-), 10 mM (-●-), 20 mM (-Δ-), 30 mM (-○-) and 40 mM (-▲-).

The enzyme 100 μg/ml was incubated at 25°C in 50 mM acetate buffer, pH 5.0 and varying concentrations of the inhibitor.

Inset: Determination of the order of the reaction with respect to N-bromosuccinimide (A) and 2-hydroxy,5-nitrobenzylbromide (B).

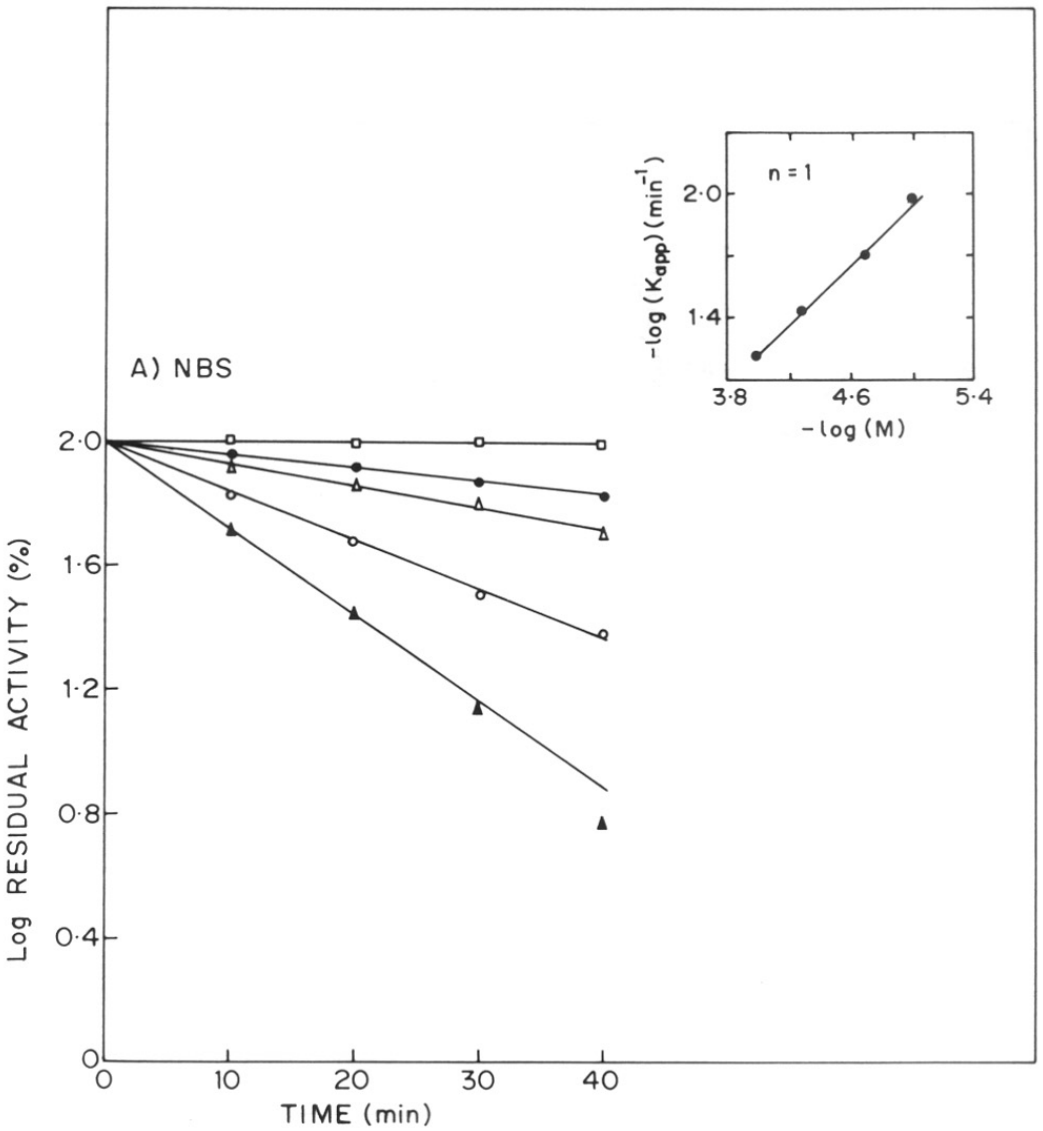

A) NBS
Log RESIDUAL ACTIVITY (%)
2·0
1·6
1·2
0·8
0·4
0
0
10
20
30
40
TIME (min)
n = 1
-log (Kapp) (min-1)
2·0
1·4
3·8
4·6
5·4
-log (M)

FIG. Ⅲ·2(A)

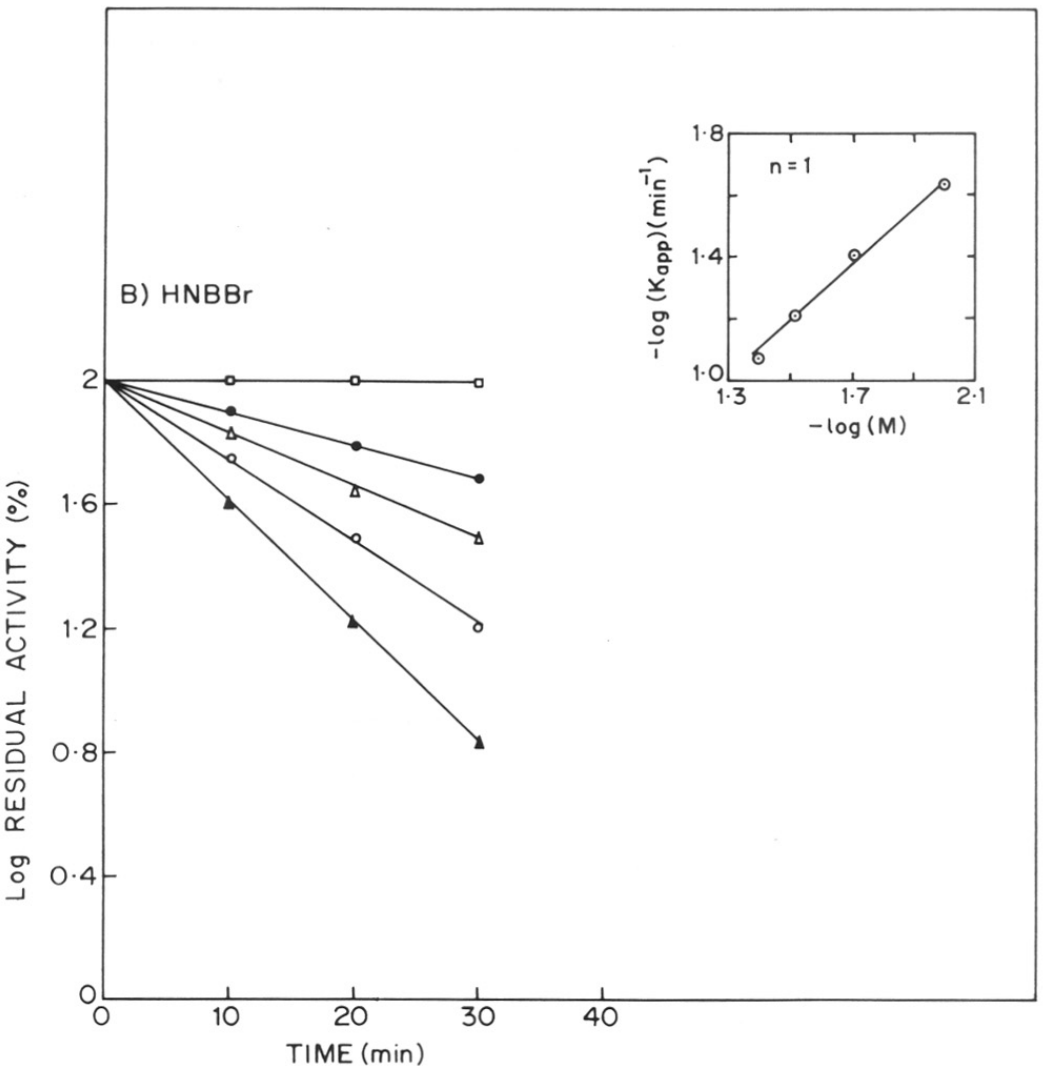

B) HNBBr
Log RESIDUAL ACTIVITY (%)
2
1·6
1·2
0·8
0·4
0
0
10
20
30
40
TIME (min)
n = 1
-log (Kapp)(min-1)
1·8
1·4
1·0
1·3
1·7
2·1
-log (M)

FIG. Ⅲ·2(B)

Table III.2 Protection of *E. coli* penicillin acylase against inactivation by tryptophan specific reagents

Treatment[a]	Enzyme activity[b] (% initial activity)
None	100
N-Bromosuccinimide (100 μM)	5
Benzylpenicillin (50 mM) + NBS (100 μM)	60
Phenylacetic acid (50 mM) + NBS (100 μM)	50
6-aminopenicillanic acid (50 mM) +NBS (100 μM)	97
2-Hydroxy.5-nitrobenzyl bromide (40 mM)	6
Benzylpenicillin (50 mM) + HNBBr (40 mM)	64
Phenylacetic acid (50 mM) +HNBBr (40 mM)	65

[a] Test compounds when used were added immediately before addition of tryptophan-modifying reagent. Enzyme (100 μg/ml) was treated at 25°C as indicated in the Table and samples were withdrawn periodically for assay of enzyme activity.

[b] Values obtained with NBS after 40 min and with HNBBr after 30 min.

action on 6-APA (50 mM) when the two were incubated at 25°C in 0.05 M sodium acetate buffer, pH 5.0, the assay of 6-APA showing quantitative recovery at the end of the 40 min incubation period.

The kinetic evidence for the involvement of a single tryptophan residue and the protective action of the substrate and products against inactivation of *E. coli* penicillin acylase by the tryptophan-modifying reagent are indicative of the presence of the tryptophan moiety at or near the active site of the enzyme.

Titration with NBS of accessible tryptophan residues in *E. coli* penicillin acylase

The tryptophan residues in *E. coli* penicillin acylase were oxidized with stepwise addition of NBS as described in **MATERIALS AND METHODS**. The number of tryptophan residues oxidized was determined from A_{280} nm and the molar ratio calculated assuming M_r value of 85,500 for the enzyme (Schumacher *et al.*, 1986). **Figure** III.3 shows the effect of NBS on enzyme activity after each addition of NBS. There was a progressive decrease in absorption at 280 nm, as well as increase in extent of inactivation. By extrapolating the initial linear portion of the plot (**Fig. III.3**) to zero activity, the number of tryptophan moieties oxidized per mole of enzyme was found to be about 4. This usually gives the number of residues modified when the enzyme is completely inactivated, but does not give the number of residues essential for activity. The total number of residues in *E. coli* penicillin acylase is 28, of which 5 are in the smaller α-subunit (Schumacher *et al.*, 1986).

Fig.III.3 Titration of accessible tryptophan residues in E. coli penicillin acylase.

The tryptophan residues of E. coli penicillin-acylase were oxidized with stepwise addition of NBS as described in **MATERIALS AND METHODS.**

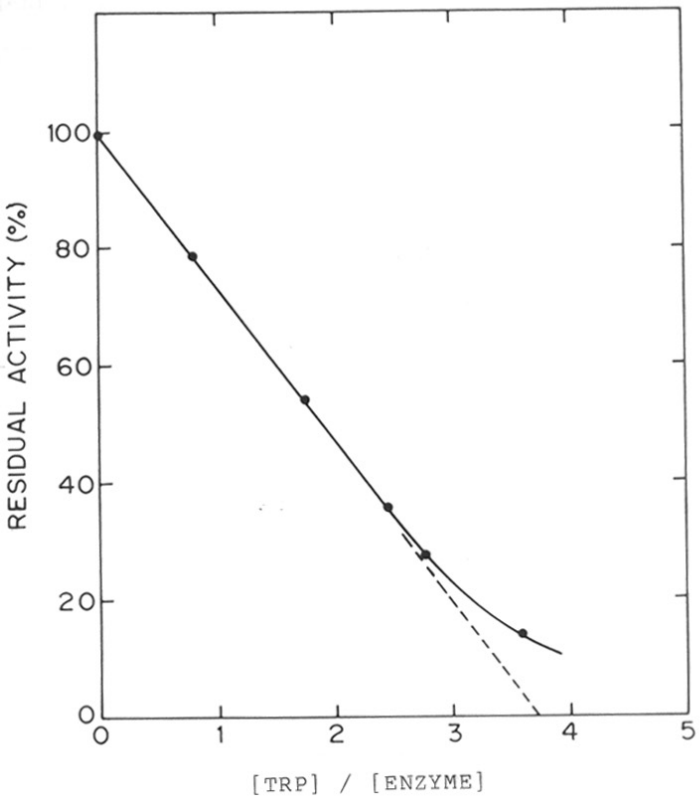


FIG. Ⅲ·3

Subunit complementation experiments

Combinations of subunits separated from the untreated *E. coli* penicillin acylase and the enzyme inactivated either with phenylglyoxal or with NBS were reassociated as described in the **MATERIALS AND METHODS**.

The results of the experiments are summarized in **Table III.3**.

It can be seen from **Table III.3** that enzyme activity comparable to that in the control was recovered with β-subunit obtained from the phenylglyoxal-inactivated enzyme + the α-subunit derived from untreated enzyme. A combination of the α-subunit obtained from the phenylglyoxal-inactivated enzyme + β-subunit from untreated enzyme showed markedly lower enzymatic activity. The low activity (10% of the control) obtained with the subunits combination of α- (phenylglyoxal treated) + β- (untreated) could have been due to the partial reversibility of phenylglyoxal modification of arginine on prolonged dialysis at an alkaline pH which has been reported in the case of bovine pancreatic ribonuclease A by Takahashi (1968). The present findings would indicate that the essential arginine residue of *E. coli* benzylpenicillin acylase is present in the α-subunit, the putative subunit that has the side chain binding domain.

Similar results were obtained with combinations of subunits derived from the NBS-treated enzyme and from the untreated enzyme. Only a combination of α-subunit derived from untreated enzyme and the β-subunit obtained from the NBS-modified enzyme showed activity (60% of control), while the α-subunit from NBS-

Table III.3 Recovery of activity of *E. coli* benzylpenicillin acylase after subunit complementation

In the controls the untreated enzyme was unfolded with 9 M urea, the subunits physically separated after disc electrophoresis in presence of 5 M urea and then mixed and allowed to renature during extensive dialysis against 50 mM potassium phosphate buffer, pH 7.8.

In the test systems, the subunits from the untreated enzyme and the chemically modified enzyme were unfolded, physically separated and mixed as described under subunit combinations.

Treatment	Subunit combinations	Acylase activity (% control)
Control (untreated)	α-untreated + β-untreated	(100)
Phenylglyoxal (20 mM)	α-untreated + β-treated	100
	β-untreated + α-treated	10
NBS (100 μM)	α-untreated + β-treated	60
	β-untreated + α-treated	0

inactivated enzyme + β-subunit of untreated enzyme was without activity. This result again is indicative of the presence of the essential tryptophan residue on the smaller α-subunit of the enzyme molecule.

DISCUSSION

Besides evidence for the presence of an essential serine residue in the catalytically active, large β subunits of benzylpenicillin acylases from *E coli* (Slade *et al.*, 1991) and *K. citrophila* (Martin *et al.*, 1991) and of an essential hydroxyamino acid residue in the enzyme from *P. rettgeri* (Daumy *et al.*, 1985b) little is known about the amino acid residue(s) involved at the putative substrate-binding domain of the small α- subunit. Ampicillin acylase from *Pseudomonas melanogenum* which is a homodimer of M_r 146,000 has been shown to have two histidine residues per subunit which are essential for it's catalytic activity (Kim and Byun, 1990).

The present data indicate for the first time the involvement an essential tryptophan and an essential arginine residue in the putative substrate binding α -subunit of benzylpenicillin acylase from *E.coli*. The involvment of an hydrophobic residue like tryptophan is in keeping with the results from other lines of investigations on the role of hydrophobic interactions in the hydrolytic activity of benzylpenicillin acylases. These include the report on the inhibitory effect of aliphatic alcohols on the activity of the enzyme from *E.coli*, the inhibitory effect varying with the hydrophobicity of the alcohols (Klyosov *et al.*, 1977a; b). More recent studies using circular dichroism have shown that the

binding of benzylpenicillin sulfoxide, a substrate analogue and a competitive inhibitor,results in changes in the dichroism of aromatic residues of the *E.coli* enzyme (Marquez *et al.*, 1988). Chemical modification studies have indicated that specific hydrophobic interactions may be a prerequisite for access to the catalytic site of benzylpenicillin acylases.The formation of an acylenzyme intermediate had been postulated from the kinetics of benzylpenicillin hydrolysis catalysed by the *E.coli* enzyme (Konecny, 1981) and the presence of an essential serine residue had been established by titration with phenylmethane sulfonyl fluoride, the serine reagent inactivating completely equimolar amounts of the enzymes from *E.coli* (Kutzbach and Rauenbusch, 1974; Slade *et al.*, 1991), *P.rettgeri* (Daumy *et al.*, 1985 a) and *K.citrophila* (Martin *et al.* ,1991), the β subunits in the enzymes containing the catalytically active serine. The *E.coli* enzyme, however, has been shown to be recalcitrant to inactivation by diisopropyl fluorophosphate (Kutzbach and Rauenbusch, 1974). The difference in effects of the two serine reagents has been attributed to the active site of the enzyme comprising hydrophobic domains which require a reagent structurally related to the phenylacetyl moiety for gaining access to the catalytically reactive serine residue (Martin *et al.*, 1990). The essential tryptophan residue apparently is at the hydrophobic substrate binding domain of the subunit,the interaction contributing to the subsequent acylation of the reactive serine of the β-subunit.An intriguing finding has been the location of the active-site serine residue at the amino

terminal positions of β-subunit of both the *E.coli* (Slade *et al.*, 1991) and *K.citrophila* enzymes (Martin *et al.*, 1991) by site-directed chemical conversion to cysteine. The importance of topography of the hydrophobic domain on the catalytic activity of benzylpenicillin acylases of *E.coli* and *K. citrophila* has been indicated by site-directed mutagenesis of a methionine residue in the α-chains. The Met-(168)-->Ala mutant has been shown to have altered substrate specificity, benzylpenicillin being hydrolysed at markedly lower rates while 6-bromohexyl-6-APA is hydrolysed at a 4-fold faster rate than the Met (168) enzyme (Martin *et al.*, 1990; Williams and Zuzel, 1985). The altered specificities might reflect conformational changes in the mutants resulting in changes in active-site geometry .

The role of an essential arginine residue in the substrate binding domain of the α-subunit is unclear. The arginine residue apparently is not involved merely in binding of an anionic substrate such as benzylpenicillin since the chemical modification of the arginine residue also inhibits enzyme catalysed hydrolysis of a neutral substrate such as phenylacetamide. Kinetic studies on the mechanism of the penicillin acylase-catalysed synthesis of benzylpenicillin have indicated that the binding site for 6-APA of the acyl enzyme intermadiate is positively charged (Kasche *et al.*, 1984). Confirmatory evidences for the involvement of an arginine and a tryptophan residue and the 3-dimensional location of these residues in the α-subunit relative to the catalytically active serine moiety in the β-subunit of the heterodimer would require X-ray crystallographic analysis.

PART IV

IMMOBILIZATION OF PERMEABILIZED ESCHERICHIA COLI CELLS WITH BENZYLPENICILLIN ACYLASE ACTIVITY

SUMMARY

Escherichia coli cells with penicillin acylase activity were sequentially treated at pH 7.8 with aqueous solutions of N-cetyl-N,N,N-trimethylammonium bromide and glutaraldehyde and then immobilized within porous polyacrylamide beads. The immobilized whole cells showed enhanced hydrolysis rates in the conversion of benzylpenicillin to 6-APA compared to untreated cells immobilized and used under identical conditions.

The immobilized system showed no apparent loss in enzyme activity when used repeatedly over 90 cycles for 6-APA production from 4% benzylpenicillin.

INTRODUCTION

Penicillin acylases (EC 3.5.1.11), in particular benzylpenicillin acylases, find wide application in the manufacture of 6-APA, the key intermediate in the production of semisynthetic penicillins. *Escherichia coli*, in which the activity remains cell-bound, has been one of the most widely used sources of this class of industrial enzymes.

The use of immobilized systems of both isolated penicillin acylase and of microbial whole cells with cell-bound activity have been reported extensively in the literature, examples of which have been reviewed in the GENERAL INTRODUCTION to this THESIS.

An advantage in the use of immobilized microbial whole cell systems with cell-bound activity is that this obviates the need for enzyme extraction and the removal of the unwanted macromolecules released during the extraction process, steps

which can be both tedious and expensive.

A disadvantage of using whole cell systems, however, is that of diffusional restrictions. This difficulty could be alleviated through cell permeabilization (Felix, 1982). Subsequent retention of cell-bound activity would, however, require fixation of cell-bound enzyme activity by chemical crosslinking (Joshi *et al.*, 1987), entrapment of such cells within suitable polymer matrices or by a combination of these approaches.

This Part of the THESIS deals with the advantage of permeabilization of *E. coli* cells containing penicillin acylase activity by treatment with N-cetyl-N,N,N-trimethylammonium bromide (CTAB), crosslinking of the cell-bound penicillin acylase with glutaraldehyde and immobilization of the CTAB-glutaraldehyde treated cells in porous polyacrylamide beads.

MATERIALS AND METHODS

High purity grade, commercially available chemicals were used as already described in Part II of this THESIS. Sodium alginate was Protonal grade LF 120 from Protan A/S, Drammen, Norway. N-cetyl-N,N,N-trimethylammonium bromide (CTAB) was obtain from Loba Chemie, India. Glutaraldehyde (50% in water) was obtained from Fluka AG, Switzerland.

Microorganisms

Escherichi coli NCIM 2350 was obtained from the National Collection of Industrial Microorganisms, NCL, Pune, India and was routinely maintained on nutrient agar slants containing 0.1% phenylacetic acid as inducer and was grown in Medium C in shake

flasks as described in PART II of this THESIS. Cells were harvested by centrifugation at 4 °C for 30 min at 6,000 x g and washed with 0.01 M potassium phosphate buffer, pH 7.8.

Assay of Penicillin Acylase Activity

Activity in Extracts and Cell Suspensions: Penicillin acylase activity in cell extracts and cell suspensions were assayed as described in PART II of this THESIS by measuring the amount of 6-APA produced at 40°C using 4% benzylpenicillin K-salt as substrate in 0.1 M potassium phosphate buffer, pH 7.8. The 6-APA produced was estimated with *p*-dimethylaminobenzaldehyde (PDAB) (Bomstein and Evans, 1965).

Activity in Immobilized Preparations: Immobilized cell systems were assayed for penicillin acylase activity in stirred, water-jacketed vessels maintained at 40°C. The assay medium was similar to that used for cell extracts and cell suspensions. Aliquots (10 µl) were withdrwan at intervals and the 6-APA formed was determined with PDAB.

For the sake of comparison, activities of cell preparations are expressed as percentage of the total activity in cells determined after sonication of free cells for a total period of 12 min and estimation of activities in cell extract and cell debris.

Standardization of Conditions for Permeabilization of *E.coli* Cells with CTAB

Effect of CTAB concentration: Cells were suspended in cold 0.05 M potassium phosphate buffer, pH 7.8 (10 ml/g packed cells) and gently stirred intermittently for 30 min at 4°C. Treated cells

were recovered by centrifugation at 6,000 x g for 30 min at 4°C and assayed for penicillin acylase activity.

Effect of Temperature: CTAB-treatment was also carried out as above, but at 30°C.

Crosslinking of CTAB-treated Cells with Glutaraldehyde

CTAB-treated cells were suspended in 0.05 M potassium phosphate buffer, pH 7.8 (5 ml/g packed cells) at 25°C and treated with an equal volume of 2% glutaraldehyde prepared in 0.05 M potassium phosphate buffer, pH 7.8. Cells were recovered after an hour by centrifugation and washed free of the reagent with 0.05 M potassium phosphate buffer, pH 7.8.

Entrapment of *E. coli* Cells in Porous Polyacrylamide Beads

E. coli cells were entrapped in the porous polyacrylamide bead system developed by us earlier for the entrapment of yeast cells Pundle et al.(1988).The steps of the process are as follows: rapid dispersal of cells at 4°C in an aqueous solution containing sodium alginate and acrylamide-N,N´-methylenebisacrylamide monomers, dropping the suspension immediately into a solution of calcium formate to give calcium alginate coated beads and allowing the polyacrylamide to gel within the bead. The calcium alginate is then leached out of the composite bead with potassium phosphate buffer solution.

Both CTAB-glutaraldehyde treated as well as untreated *E. coli* cells were separately entrapped in the polyacrylamide bead system.

The set up used for entrapment process is represented schematically in Fig. IV.1.

An aqueous solution (25 ml) containing acrylamide monomer

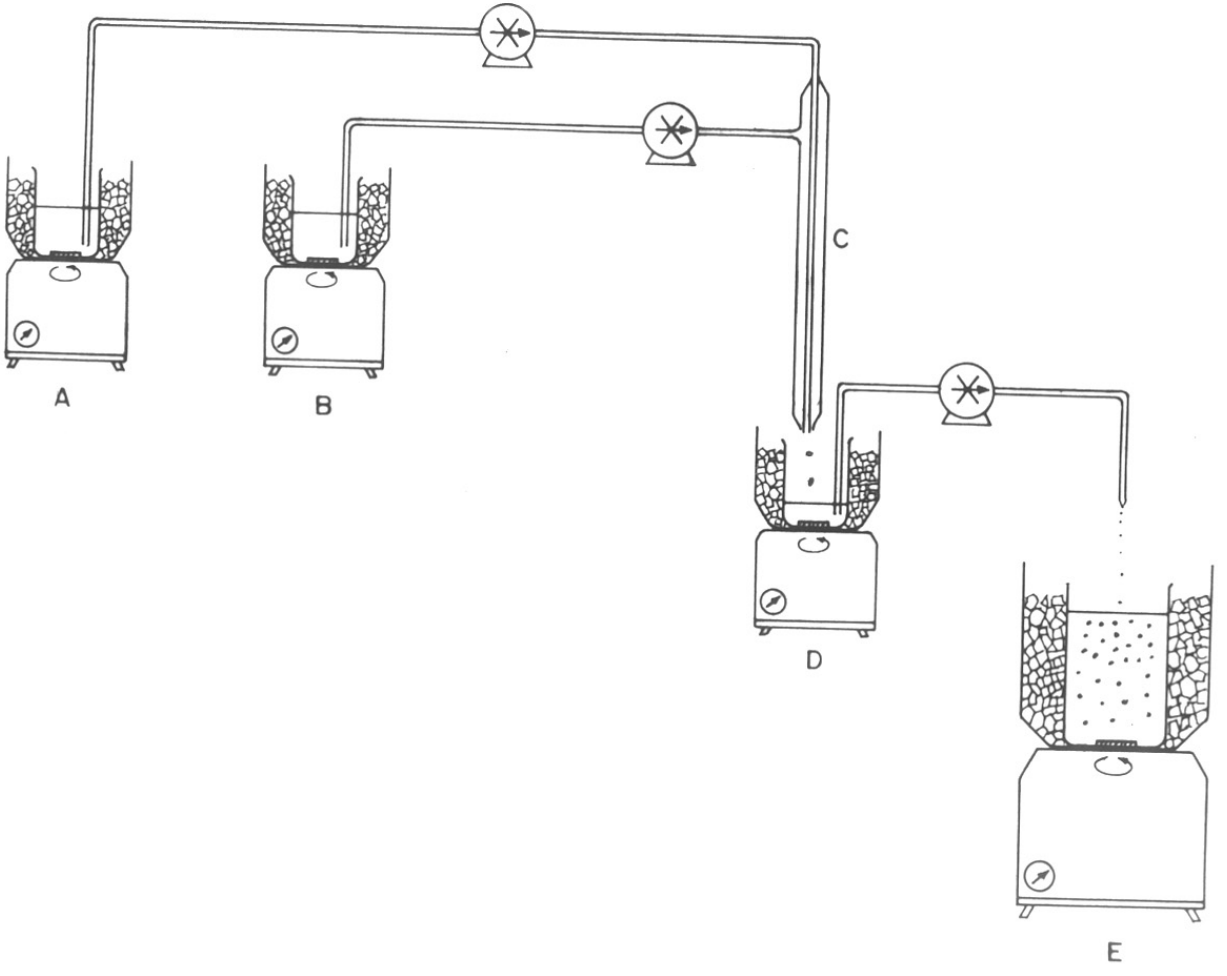


FIG. Ⅳ·1

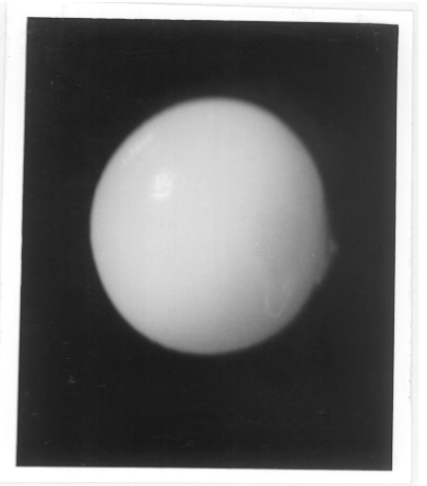
(15% w/v), N,N'-methylenebisacrylamide (0.8% w/v) and N,N,N',N'-tetramethylethylene diamine (1% v/v) and a suspension (25 ml) of *E. coli* cells (5 g packed cells) in an aqueous solution of sodium alginate (0.5% w/v) were cooled separately in ice baths (Reservoirs A, B) and pumped at equal flow rates into a cooled mixing chamber (D) through a double-walled tubular device (C), the cell suspension being conveyed along the internal tube and the acrylamide solution through the outer annulus. The cells and monomers were mixed rapidly and remained in contact only briefly before passing into a gently stirred calcium formate solution (3%) containing ammonium persulphate (0.5% w/v) cooled to 4°C (E). Calcium alignate coated beads were left in the calcium formate solution for 1 h for polymerization of the acrylamide and then leached with several changes of 0.05 M potassium phosphate buffer, pH 7.8 to remove the alginate component. The beads were uniform and about 1.5 - 2.0 mm in diameter (Plate IV.1).

RESULTS

Effect of CTAB concentration on Permeabilization of *E.coli* cells

Table. IV.1. summarizes the data on effect of CTAB concentration on penicillin acylase activity expressed by *E. coli* cells.

As seen from Table IV.1, treatment with 0.1% CTAB gives the highest expression of total activity contained in the *E. coli* cells. Treatment at 30°C did not significantly affect the results. 0.1% CTAB concentration was therefore used at 4°C before crosslinking with glutaraldehyde.



P. 1

Plate IV.1 Spherical porous polyacrylamide bead (2 mm diameter) with entrapped *E. coli* cells.

Table IV.1 Effect of CTAB concentration on permeabilization of *E. coli* cells

CTAB concentration (% w/v)	Cell-bound penicillin acylase activity (% of total activity)
0.05	50
0.075	60
0.1	74
0.15	50

Table IV.2 summarizes the data on effect of CTAB-treatment (0.1%) of *E. coli* cells on initial rate of hydrolysis of benzylpenicillin at pH 7.8 and 40°C. Cell-bound activity is calculated both in units of μmoles $h^{-1}g^{-1}$ packed cells and as percentage expressed of the total activity obtained on sonication of untreated cells. Treatment of cells with CTAB (0.1%) or with CTAB (0.1%) followed by glutaraldehyde (1%) resulted in enhanced hydrolytic rates, more than 70% of the activity present in the cells being expressed by the treated cells compared to about 55% expressed by the untreated cells.

The effect of glutaraldehyde-treatment on the retention of cell-bound penicillin acylase activity in CTAB-treated *E. coli* cells is shown in Fig. IV.2. Reuse of free cells treated with CTAB resulted in rapid loss of cell-bound penicillin acylase activity, the residual activity after the third use being only about 10% of the initial value. However, incubation of CTAB-treated cells with 1% glutaraldehyde, effectively prevented the leakage of activity from the cells.

The progress curves of hydrolysis of benzylpenicillin with the untreated *E. coli* cells and with CTAB-glutaraldehyde treated cells immobilized in porous polyacrylamide gel beads are shown in Fig. IV.3. As can be seen from the Fig. IV.3, CTAB-glutaraldehyde treated cells bring about almost quantitative hydrolysis of the substrate in approximately 65% of the time taken by an equivalent amount of the untreated whole cells.

Operational stability:

The CTAB-glutaraldehyde treated *Escherichia coli* cells

Table IV.2 Effect of CTAB treatment of *E. coli* cells on initial rates of hydrolysis of benzylpenicillin at pH 7.8 and 40°C

Treatment	Cell-bound penicillin acylase activity	
	μmoles $h^{-1}g^{-1}$	% expressed of total
None	368	55
CTAB (0.1%)	496	74
CTAB (0.1%) + glutaraldehyde (1%)	472	71

Fig. IV.2 Effect of glutaraldehyde treatment on operational stability of CTAB treated cells of *E. coli*. Cells were treated with 0.1% CTAB in 0.05 M potassium phosphate buffer (pH 7.8), subsequent treatment was with glutaraldehyde (1%). Cell suspensions (1 g packed cells) were used for the hydrolysis of 4% benzylpenicillin, K-salt (10 ml) at 40°C and pH 7.8.

(o) CTAB treated cells.

(•) CTAB-glutaraldehyde treated cells.

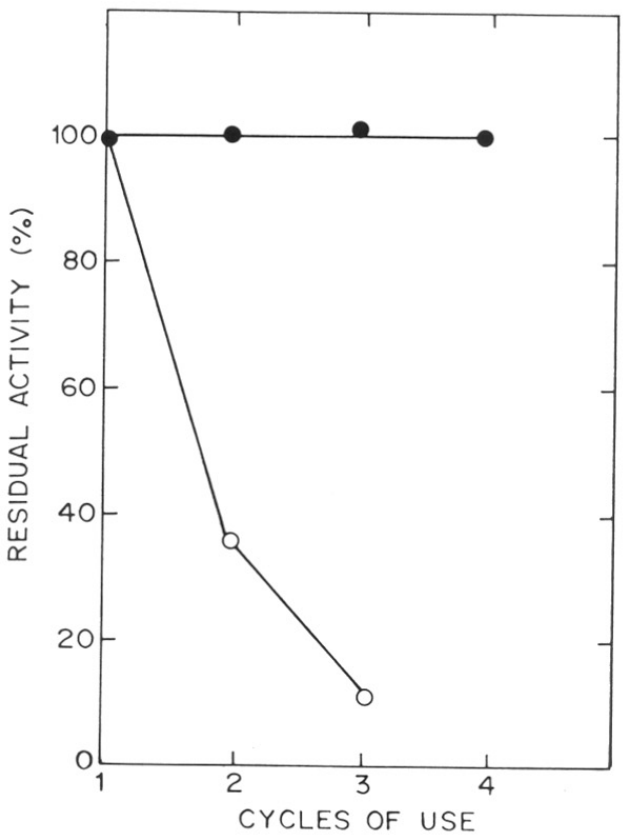


FIG. Ⅳ·2

Fig. IV.3 Progress curves of hydrolysis of 4% benzylpenicillin K-salt at 40°C at pH 7.8 by *E. coli* cells entrapped in porous polyacrylamide beads.

(o) Untreated cells

(●) CTAB-glutaraldehyde treated cells.

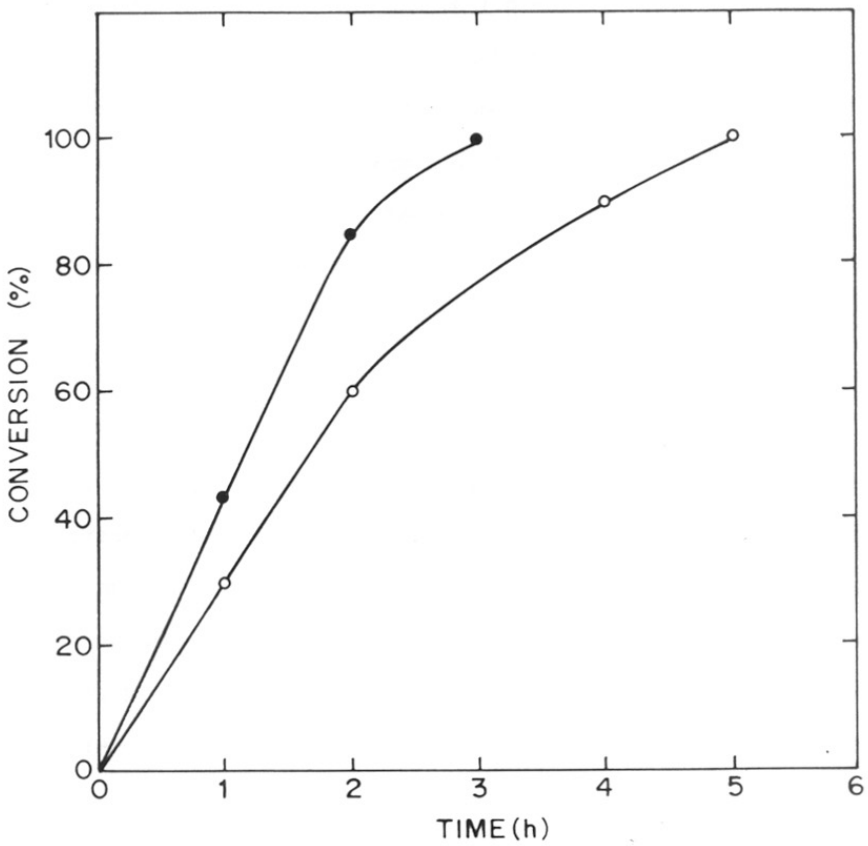


FIG. Ⅳ·3

entrapped in porous polyacrylamide beads showed a high operational stability when tested for hydrolysis of 4% (w/v) benzylpenicillin K- salt at pH 7.8 and 40°C. The beads were reused in successive cycles of batch operations after completion of 95-100% hydrolysis of the substrate and the retention of enzyme activity was determined from the initial rates of hydrolysis of substrate. The immobilized whole system showed no apparent loss of enzyme activity over 90 cycles of use.

DISCUSSION

Penicillin acylase produced by *E. coli* cells is cell-bound and present in the periplasmic space (Bock *et al*,1983a; b). The periplasmic space is a feature of Gram-negative eubacteria and comprises the region between the outer face of the plasma membrane and and the inner face of the outer membrane (Beveridge, 1981; Graham and Beveridge, 1991). The penicillin acylase activity thus remains cell-bound, enveloped within the two membranes and isolated from the external medium by the outer membrane and cell wall. Earlier workers have reported the application of immobilized systems of intact whole cells of *E.coli* with penicillin acylase activity for the hydrolysis of benzylpenicillin to 6-APA.These include cells entrapped in polyacrylamide particles (Sato *et al*1976), in gelatin matrix crosslinked with glutaraldehyde (Park *et al*., 1982) and in beads of chitosan (Vorlop,.1987). *E. coli* cells covalently attached to modified macroporous copolymers of glycidyl methacrylate with ethylenedimethacrylate and of methacrylaldehyde with divinylbenzene have also been used for the hydrolysis of

benzylpenicillin to 6-APA (Zurkova et al., 1983).

The immobilized cells have been reported to retain upto about 94% of the penicillin acylase activity of the free cells when mild conditions are used as in the case of gel entrapment in polyacrylamide. A strategy for enhancing the catalytic effectiveness of cell bound enzymes is through cell permeabilization for reducing the diffusional restrictions imposed by the cell membrane and the cell wall (Felix, 1982).

The present investigations report for the first time the advantage of such an approach for the enhancement of the penicillin acylase activity of *E. coli* cells. Treatment of cells with a permeabilizing agent such as CTAB enhances the release of the enzyme from the cell-bound state. Fixing of activity by a subsequent treatment with bifunctional crosslinking reagents such as glutaraldehyde prevents the leakage of enzyme activity through the pores of the CTAB-treated cells during the progress of hydrolysis of substrate.

The activity expressed by cells after permeabilization with CTAB and crosslinking of the enzyme within the cells with glutaraldehyde is about 30% higher than that of untreated free cells indicating lower diffusional restrictions.

CTAB-glutaraldehyde treated *E. coli* cells entrapped in porous polyacrylamide gels also catalyse the hydrolysis of benzylpenicillin at significantly higher rates than untreated cells immobilized under similar conditions.

The immobilized treated cells show good operational stability. The immobilization of CTAB-glutaraldehyde treated *E.*

coli cells with penicillin acylase activity thus provides a convenient and efficient system for the production of 6-APA from benzylpenicillin.

PART V

IMMOBILIZATION OF PENICILLIN ACYLASE IN POROUS BEADS OF POLYACRYLAMIDE GEL

SUMMARY

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

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

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

INTRODUCTION

Penicillin acylase is an important industrial enzyme which is applied extensively for the

manufacture of 6-APA, the key intermediate in the production of semisynthetic penicillins. This aspect has been discussed in the GENERAL INTRODUCTION. Current industrial applications of the enzyme are as immobilized systems which permit repeated reuse, and the use of high biocatalyst concentrations for rapid conversion of the labile penicillin substrate. Further, leakage of allergenic macromolecules and consequent contamination of the final pharmaceutical product is avoided.

The several approaches that have been used for immobilization of penicillin acylase have also been reviewed in the GENERAL INTRODUCTION. Among these, entrapment in hydrophilic gels has attractive features. These are mainly the high enzyme loading that the system permits and the relatively mild conditions used for enzyme entrapment. The disadvantages are the problems of diffusional restrictions in the substrate entering and the products leaving the gel. This can be minimised with particle sizes in the micron range. Such sizes would entail difficulties of losses through abrasion and during discharge of the reaction system at the completion of a batch process or during continuous operations at high flow rates.

The present work has adopted an alternative approach of using a porous matrix for enzyme entrapment in order to minimise the diffusional problem. A synthetic polymer has been used as it combines high rigidity with resistance to degradation through abrasion when operated in stirred, batch-type reactors. The spherical bead form is also ideally suited both to packed bed and free fluidised bed operations. The porous spherical bead

polymer selected for development was the polyacrylamide system which we had originally developed for the immobilization of yeast cells for ethanolic fermentation (Pundle *et al.*,1988). The procedure depends on the *in situ* polymerization of cooled acrylamide-N,N´-methylene-bis -acrylamide monomers within calcium alginate-coated beads containing pre-grown yeast cells and the subsequent differential leaching out of the calcium alginate.

In the present work a modification of this procedure is described for the entrapment of penicillin acylase from *E.coli*.

MATERIALS AND METHODS

MATERIALS

Acrylamide and bis-acrylamide were obtained from Eastman Organic Chemicals Ltd. and N,N,N´-N´-tetramethylethylenediamine (TEMED) were obtained from Eastman Kodak Co., Rochester, USA. Sodium alginate, Protonal type LF 120 was obtained from Protan A/S Drammen, Norway. Glutaraldehyde (50% in water) was obtained from Fluka AG, Switzerland. Methanol (AR) was obtained from S.D. Fine Chemicals, *p*-dimethylaminobenzaldehyde was from LOBA Fine Chemicals. All other chemicals used for this work were of high purity available commercially. Tris (hydroxymethyl) aminomethane hydrochloride (Tris-HCl) was obtained from Sigma, USA.

MICROORGANISMS

Escherichia coli ATCC 11105 (NCIM 2068) was obtained from the National Collection of Industrial Microorganisms, Pune, India and was maintained routinely on nutrient agar slants containing 0.1% phenylacetic acid as an inducer.

METHODS

Penicillin acylase assay: The assay was carried out as described earlier (**PART** II of this **THESIS**). Enzyme was assayed at 40°C with a 4% benzylpenicillin solution in 0.1 M potassium phosphate buffer, pH 7.8. Amount of 6-aminopenicillanic acid (6-APA) formed was estimated using *p*-dimethylaminopenzaldehyde by the method of Sudhakaran and Shewale (1990).

Growth and extraction of enzyme from Escherichia coli

Growth: *Escherichia coli* NCIM 2068 was grown in a Medium C as described for *E. coli* NCIM 2350 (**PART II** of this **THESIS**). Enzyme was also extracted from the cells as described earlier, cells being sonicated to give >90% disintegration.

Partial Purification of Enzyme: The cell extracts were processed as described earlier in **PART II** of this **THESIS** upto the DEAE-Sepharose step. The enzyme preparations had a specific activity of about 15 μmole $min^{-1}mg^{-1}$.

Enzyme Immobilization

An aqueous solution containing acrylamide 15% (w/v), N-N´-methylene-bisacrylamide 0.8% (w/v) and TEMED 1% (v/v) and a solution of the enzyme (50 U/ml) and sodium alginate 0.5% (w/v) were separately cooled in an ice bath and pumped at equal flow rates into a cooled mixing chamber through a double-walled tubular device. The enzyme and acrylamide monomer solutions were mixed rapidly and remained in contact with each other for a very short period of time before dropping into a gently stirred solution containing calcium formate (3% w/v) and ammonium per-

sulphate (0.5% w/v) buffered with 0.05 M sodium tetraborate buffer, pH 8.5 at 5 - 10°C. The calcium alginate coated beads which formed were left for hardening in the buffered calcium formate solution for 1 h in which time the acrylamide polymerises. (Fig. IV.1).

After polymerization has taken place, the calcium formate solution is drained off and the calcium alginate is leached out with 0.1 M potassium phosphate buffer, pH 7.8 till the washings are clear. The beads are now washed thoroughly with 0.01 M potassium phosphate buffer and are ready for use. Beads thus obtained are uniform in size (~2 mm dia.) and spherical.

Crosslinking of entrapped enzyme: Conditions were optimised for the crosslinking of enzyme entrapped in polyacrylamide gel such that no enzyme was lost by leaching out of the calcium alginate. Various concentrations of glutaraldehyde ranging from 0.2% - 2.0% prepared in saline were used and time of contact with the beads was kept constant at 1 h.

Assay of immobilized system: Immobilized enzyme preparation were assayed for penicillin acylase activity in stirred, water-jacketed vessels maintained at 40°C. The assay of the system was similar to that used for the soluble enzyme. The final volume of the mixture was 10 ml. Aliquots (0.01 ml) were withdrawn at regular time intervals (15 min) and the 6-APA formed was determined with *p*-dimethylaminobenzaldehyde.

Operational stability: Beads with immobilized penicillin acylase (50U) was suspended in a final volume of 50 ml of 0.1 M potassium phosphate buffer, pH 7.8 containing 4%(w/v) benzylpenicillin

sodium salt and stirred in a water - jacketed reaction vessel maintained at 40°C. Aqueous ammonia (1.2 N) was added from a microburette to maintain pH at 7.8. After every complete conversion (95 - 100%) the beads were thoroughly washed with distilled water, till free of substrate and products. 6-APA was determined in the filtrate and washings. The beads were used repeatedly and the initial rates of hydrolysis were used as a measure of residual activity.

Optimum pH of Hydrolytic Reaction : The pH dependence of penicillin acylase activity of the native and of the immobilized enzyme systems was determined in 0.1M buffers (citrate-phosphate,phosphate and Tris) of various pHs ranging from 5 -10.

Kinetics:

The Km for benzylpenicillin was calculated from the double-reciprocal plots.

RESULTS Modification of native enzyme with glutaraldehyde: Preliminary experiments were carried out to determine the effect of glutaraldehyde on the native enzyme. An aqueous solution of the enzyme (10 U/ml) was treated with glutaraldehyde (1% w/v in 1 ml final volume) for 1 h at 25°C and the excess reagent removed by exhaustive dialysis. The resulting pale yellow opalescent solution of modified enzyme showed a slightly enhanced hydrolytic activity toward 0.05 M benzylpenicillin at pH 7.8 compared to the native enzyme (**Table V.1**). This finding is in keeping with an earlier report. Carleysmith *et al.* (1980) had observed that treatment of penicillin acylase from *E. coli* with glutaraldehyde yields a modified enzyme that shows a shift in the substrate -

Table V.1. Comparative kinetics of *E. coli* penicillin acylase preparations

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

hydrolytic activity profile toward higher concentrations of benzylpenicillin such that at pH 8.0 and benzylpenicillin concentrations above 0.01 M, the modified enzyme showed slight enhancement in activity compared to the native enzyme.

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

Kinetic Properties of Immobilized Enzyme:

Table V.1 compares some of the kinetic properties of penicillin acylase entrapped and fixed by glutaraldehyde treatment in porous polyacrylamide gel beads with those of the native enzyme and the soluble enzyme modified with glutaraldehyde. As stated earlier, the V_{max} values of both glutaraldehyde-modified soluble enzyme and of the modified enzyme entrapped in polyacrylamide gel are about 10% higher than that of the native enzyme preparation. The K_m value toward benzylpenicillin at pH 7.8 in presence of 0.1 M potassium phosphate is 30.1 μM in the case of the native enzyme, compared to corresponding values of 18.9 μM and 20 μM for the glutaraldehyde-modified soluble enzyme and the modified enzyme entrapped in porous polyacrylamide gel. Carleysmith *et al.* (1980), however, had reported enhancement in the K_m value of glutaraldehyde-modified enzyme compared to the native enzyme.

Although both native and entrapped enzyme have an optimum pH of 7.8

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

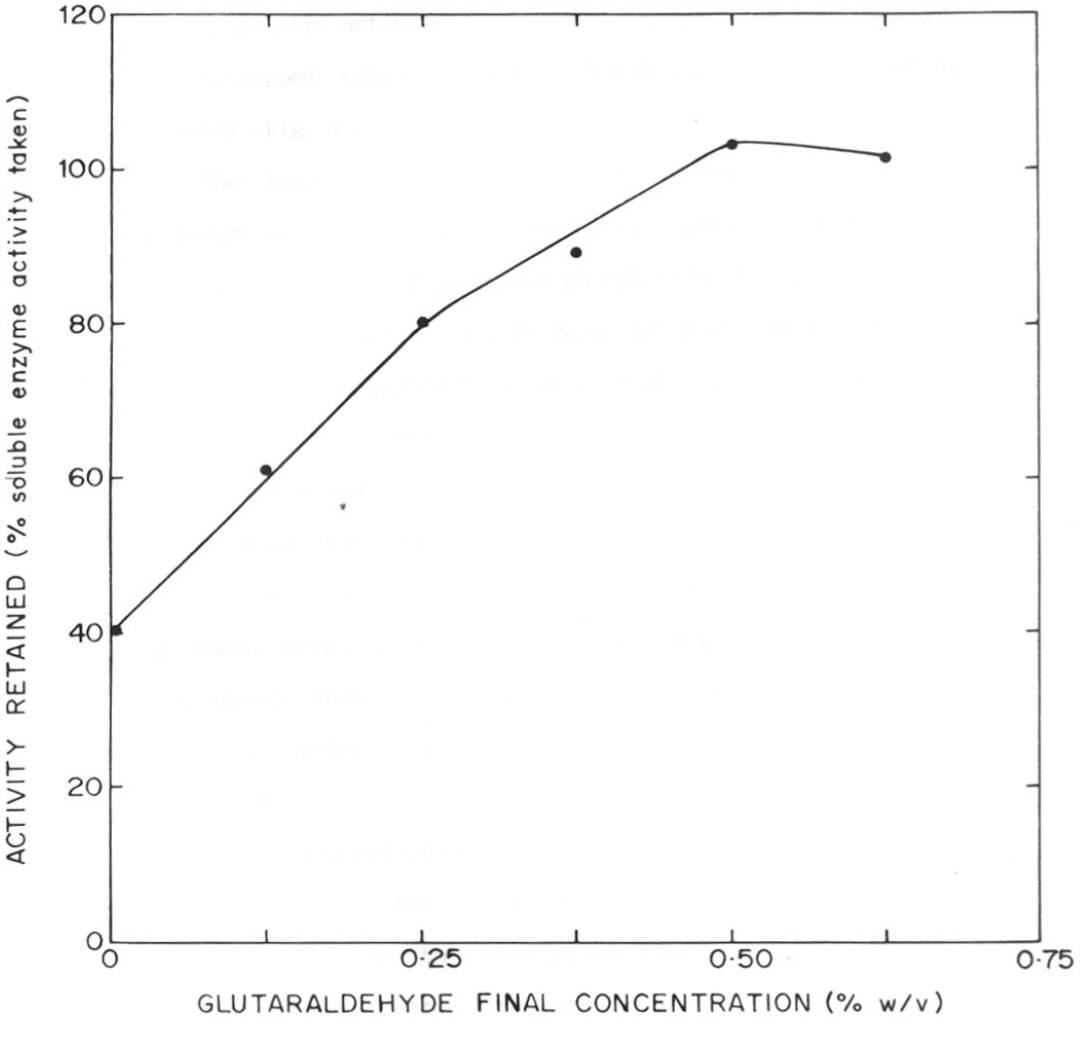

120
100
80
60
40
20
0
0
0·25
0·50
0·75
ACTIVITY RETAINED (% soluble enzyme activity taken)
GLUTARALDEHYDE FINAL CONCENTRATION (% w/v)

FIG. V·1

for hydrolytic activity, the pH activity profiles are distinct, the entrapped enzyme showing a broadening near its optimum activity (**Fig. V.2**).

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

Operational Stability

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

DISCUSSION

The entrapment of enzymes within gel particles of relatively large size has the attendant disadvantage of diffusional limitation despite the ease in handling and recovering such systems. The effect of particle size of Amberlite XAD7, a macroporous resin carrier, on the efficiency of benzylpenicillin acylase covalently bound to the carrier with glutaraldehyde has been described by Carleysmith *et al.* (1980). The rate of deacylation of benzylpenicillin catalyzed by the immobilized enzyme was shown to increase when the support was ground from its original 1 mm diameter spherical form to particles of 15 μm mean diameter, the ground preparation showing the same efficiency as

Fig. V.2 Effect of pH on initial rates of hydrolytic activity of *E. coli* penicillin acylase toward 0.05 M benzylpenicillin at 37°C. 0.1 M citrate-phosphate buffer (pH 4 - 6), 0.1 M potassium phosphate buffer (pH 7 - 8), 0.1 M Tris glycine buffer (pH 10).

(-●-) Native enzyme;

(-o-) Enzyme entrapped in porous polyacrylamide gel and modified with glutaraldehyde

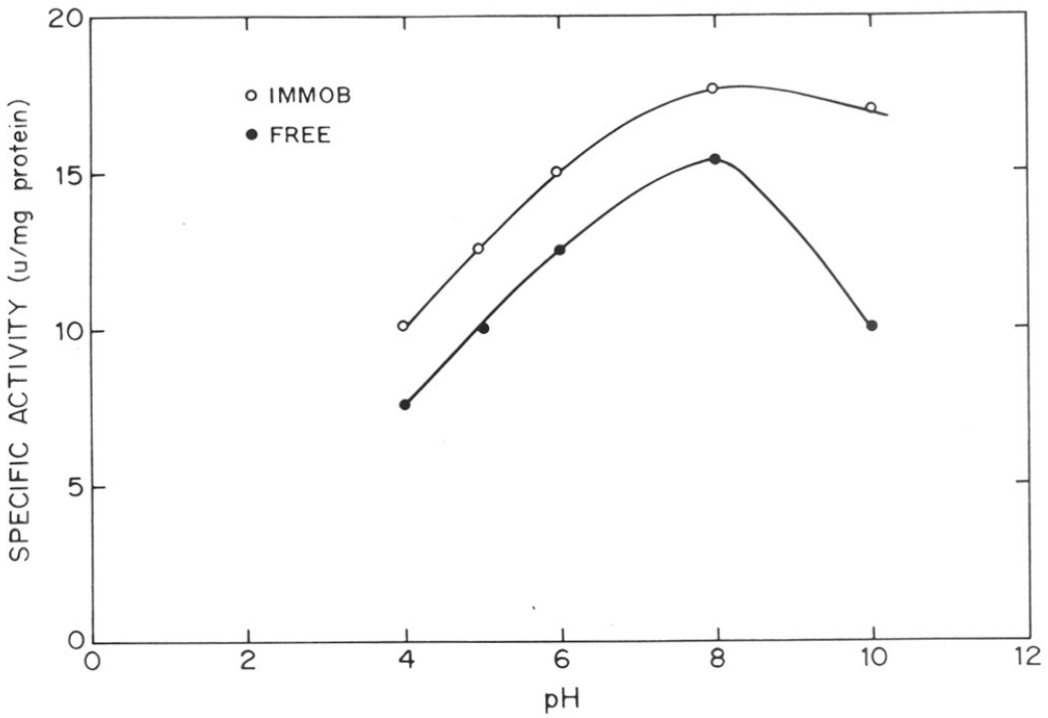

20
15
10
5
0
0
2
4
6
8
10
12
SPECIFIC ACTIVITY (u/mg protein)
pH
o IMMOB
• FREE

FIG. V·2

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

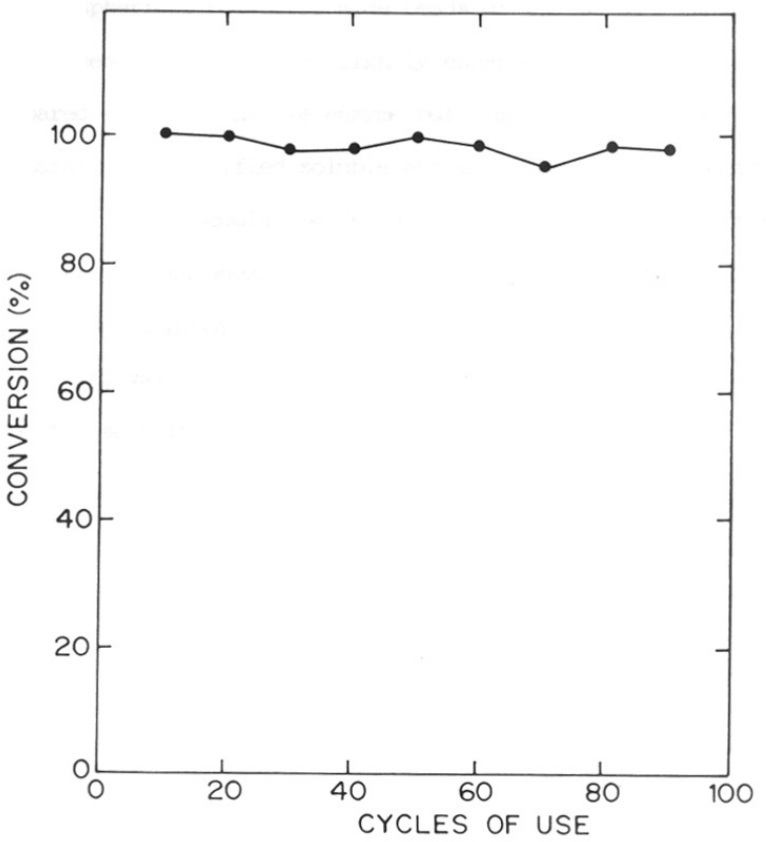


FIG. V·3

the native enzyme. In the present studies benzylpenicillin acylase entrapped and crosslinked with glutaraldehyde within porous spherical polyacrylamide beads of 1.5 mm to 2 mm diameter have been found to have slightly enhanced hydrolytic activity compared to the native enzyme but comparable to that of the glutaraldehyde-modified soluble enzyme. The porous structure of the gel beads could be expected to alleviate diffusional limitation. An unexpected finding has been that the masking of primary amino groups of the enzyme through chemical modification lowers K_m value significantly and causes an enhancement of it's specific activity.

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LIST OF PUBLICATIONS AND PATENTS

1. Immobilisation of Saccharomyces uvarum cells in porous beads of polyacrylamide gel for ethanolic fermentation.
   Applied Microbiology & Biochemistry. Vol. 29,1988,p426-429.
   A.V. Pundle, A.A. Prabhune & H. Sivaraman.

2. Immobilisation of Penicillin acylase in porous beads of polyacrylamide gel.
   Applied Biochemistry & Biotechnology 1991,Vol.30(3),265-272
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5. Immobilisation of permeabilised Escherichia coli cells with Penicillin acylase activity. Enzyme Microbiol Technol, Vol 14, No.2, 1992, 161-163.
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Patents.

1. H. Sivaraman,A.V. Pundle,A.A. Prabhune,B.S. Rao & S.M.Kotwal
   Ind. pat. appl. no. 1234/DEL/90.
   A process for obtaining porous spherical beads of polyacrylamide gel with entrapped enzymes and cells and other biologolically active macromolecules and organelles.

2. Bahulekar,R.V., Prabhune,A.A., Pundle,A.V., Gadgil,J.M., Rajan,C.R., Ponrathnam, S., & Sivaraman H.
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   An improved process for the production of immobolised Penicillin G acylase using crosslinked spherical macroporous hydroxyethyl methacrylateterpolymer beads useful for the preparation of 6-aminopenicillanic acid (6-APA).

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Ind. pat. no. 1208/DEL/90.
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4. A.A. Prabhune & H. Sivaraman.
A process for enhancing all bound penicillin acylase activity through permeabilisation & subsequent stabilisation with a crosslinking reagent.
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