SYNTHETIC POLYMER BASED AFFINITY MATRICES: SEPARATION OF PENICILLIN G ACYLASE AND PENICILLINASE

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Dedicated to my parents

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Certificate

Certified that the work incorporated in this thesis "Synthetic polymer based affinity matrices: separation of penicillin G acylase and penicillinase" submitted by Ms. Varsha B. Ghadge for the degree of Doctor of Philosophy, University of Pune, Pune was carried out by the candidate under my supervision at Polymer Science and Engineering Unit, Chemical Engineering Division, National Chemical Laboratory, Pune 411 008. Such material obtained from other sources has been duly acknowledged in this thesis.

Dr. Surendra Ponrathnam

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ABSTRACT

Separation and purification of biologically functional molecules (eg: proteins, enzymes, peptides, antibodies, antigens, nucleic acids, hormones etc.) is of fundamental importance to biotechnology. Affinity chromatography is one of many adsorption techniques used in protein purification. Although the antecedents of affinity chromatography reach back to the beginning of the twentieth century, only a handful of papers cite biospecificity as a means of purification before 1968 when the term affinity chromatography was coined. Since 1970 there have been widespread developments in all aspects of the technique.

Among the very many enzymes, penicillin acylases have received considerable attention owing to the commercial implications associated with production of 6-aminopenicillanic acid (6-APA), an important intermediate for semisynthetic penicillins such as methicillin, ampicillin, amoxycillin etc. The enzyme penicillin acylase selectively hydrolyses the side chain linear amide bond in penicillin G (Pen G) or penicillin V (pen v) molecules producing the β-lactam nucleus, 6-APA and the corresponding side chain acid. The 6-APA production by enzymatic route using immobilised penicillin G acylase is recognised as a great success in biotechnology. The interest in penicillin G acylase continues to grow with the world production of 6-APA, which is estimated to have reached 7000 tons in the year 2000. The enzyme on large scale is obtained from Escherichia coli, Bacillus megaterium, Bovista plumbea and Achrombacter sp.

For preparation of immobilised penicillin G acylase it is necessary to first obtain the soluble enzyme in fairly purified form characterised by specific activity greater then 10 IU mg^{-1} protein. This high specific activity of penicillin acylase solutions plays a fundamental role in the immobilisation of this enzyme. The production cost of 6-APA not only depends on the substrate price (e.g. benzyl penicillin), but also on enzyme production and immobilisation costs. One way to minimise the costs is to obtain an enzyme preparation in partially purified form by employing a minimum of purification steps. Affinity chromatography is the best suited technique in the purification process.

Penicillinase (β-Lactamase EC 3.5.2.6), a member of β-lactamase group of enzymes,

hydrolyses cyclic amide bond present in penicillins to form corresponding penicilloic acid. β-lactamases have gained importance since these β-lactam ring opened molecules are physiologically inactive and is the mechanism by which pathogenic bacteria develop resistance to β-lactam antibiotics. Simple decolourisation as an end point for detection of penicillinase has promoted its use as a marker in ELISA based diagnostic kits. Penicillinase is being used in detection of amoebiasis, esterone, 3-glucaronide, human choronic gonadotropin, mycoplasmosis, nongonococcal urethritis, toxoplasmomis etc. Thus penicillinase is an important industrial enzyme. For purification of enzyme on large scale, adsorption and elution using affinity gels offer advantages of specific interactions with target enzyme.

This thesis aims at the synthesis of new affinity matrices having desired physical and chemical properties and investigating their effectiveness for the purification of enzymes penicillin G acylase and penicillinase. The objectives of this work are:

(i) To develop a reproducible synthesis methodology for generating rigid, spherical base copolymers of allyl glycidyl ether of desired particle size and distribution.

(ii) To synthesis allyl glycidyl ether copolymers differing in hydrophilicity by replacing ethylene glycol dimethacrylate with hydrophobic divinyl benzene, hydrophilic pentaerythritol triacrylate, very moderately hydrophobic trimethylolpropane trimethacrylate and trimethylolpropane triacrylate.

(iii) To prepare suitable affinity matrices for purification of penicillin G acylase by derivatising the above said epoxy polymers with affinity ligands of specific structures like butyl amine, hexyl amine, octyl amine, dodecyl amine, benzyl amine etc.

(iv) To synthesis metal chelate and triazine dye ligand supports for the separation of penicillin G acylase.

(v) To synthesis matrices for the isolation of penicillinase by modifying the copolymers of allyl glycidyl ether with different ligands such as ampicillin, amoxycillin, cephalexin, cephalosporin C etc.

(vi) Study of affinity matrices thus developed for the purification of commercially pertinent enzymes such as penicillin G acylase and penicillinase.

The thesis comprises of three chapters:

Chapter 1 deals with the general introduction on affinity chromatography, types and properties of various affinity matrices, general properties of affinity ligands, and variants of affinity chromatography. It also covers the variety of procedures used for activation of supports and immobilisation of ligands. The chapter gives a brief introduction on suspension polymerisation methodology. It also deals with the scope of present study.

Chapter 2 discusses the synthesis of copolymers of allyl glycidyl either differing in millimoles of reactive (epoxy) groups, swelling factors, hydrophicity/ hydrophobicity, pore size, particle size and its distribution. Methods for the preparation of suitable matrices of both penicillin G acylase and penicillinase as well as their binding studies are also discussed. The chapter also deals with the methods to determine the degree of functionalisation of support, estimation of protein and enzyme activity for penicillin G acylase and penicillinase.

Chapter 3 is concerned with results and discussion on effect of stirrer type, stirring speed, protective colloid concentration on particle size distribution of allyl glycidyl ether copolymers. It describes the adsorption and elution behaviour of pure enzyme penicillin G acylase on different amine matrices in order to establish the nature of interaction between the matrix and enzyme, evaluation of selected matrices for the purification of crude enzyme, effect of crosslink density, hydrophilicity of copolymers and ligand concentration on degree of purification of enzyme. The chapter discusses the effectiveness of different triazine dye matrices and copper chelate matrices for the separation of penicillin G acylase. The chapter also deals with the study of binding profile of various matrices prepared for penicillinase. Cephalexin was identified as the ligand of choice for the purification of penicillinase. The copolymers differing in hydrophilicity were converted into cephalexine matrices under optimised conditions and the binding of penicillinase was evaluated.

1 INTRODUCTION

Separation and purification of biologically functional molecules (e.g.: proteins, enzymes, peptides, antibodies, antigens, nucleic acids, hormones etc.) is of fundamental importance to biotechnology. Affinity chromatography is one of many adsorption techniques used in protein purification. Although the antecedents of affinity chromatography reach back to the beginning of the twentieth century,[1] only a handful of papers cite biospecificity as a means of purification before 1968 when the term 'affinity chromatography' was coined.[2] Since 1970 there have been widespread developments in all aspects of the technique.

1.1 Principle of affinity chromatography

Affinity chromatography is a type of adsorption chromatography in which the bed material has biological affinity for the substance to be isolated. Each enzyme or other biomolecule that one wishes to purify has a biologically significant selective attraction for other, usually called ligand or affinant. The specific adsorptive properties of the bed material are obtained by covalent coupling of an appropriate binding ligand or affinant to an insoluble matrix. If a crude cell extract containing the biologically active products to be isolated is passed through a column of such affinant, then all compounds which, under the given experimental conditions, have no affinity for the affinant, will pass through unretarded, where as products that show an affinity for the ligand are retained on the column as a complex with the attached affinant. The product then can be released from the complex in the pure state by changing parameters which affect binding such as the pH, ionic strength, temperature etc.[3] Column procedure of an affinity chromatography is shown in Figure 1.1.

1.2 History of affinity chromatography

Starkenstein[4] in 1910 isolated α-amylase by using an insoluble starch. The principle of affinity chromatography using affinants covalently bound to a solid matrix has been known for more than 40 years. Campbell et al.[5] were the first to use this principle in 1951 for the isolation of antibodies on a column of cellulose with covalently bound antigen. The first enzyme to be purified with the use of a synthetic immobilised bioligand was tyrosinase, which was purified by Lerman[6] in 1953 using azo dyes immobilised on a column of cellulose.

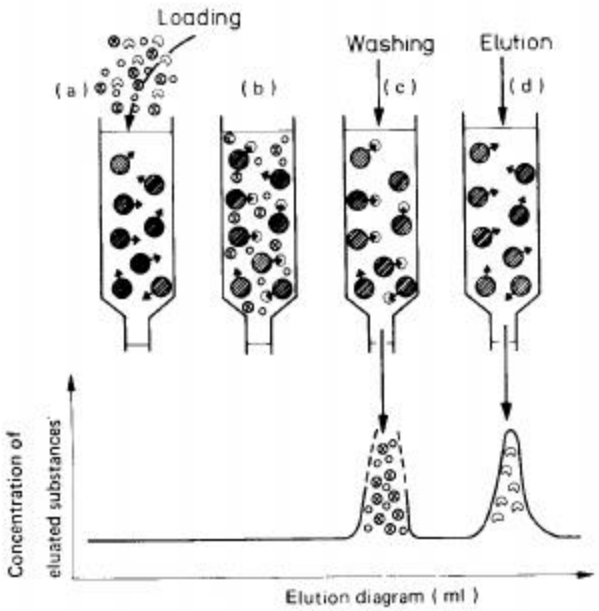


FIGURE 1.1 Column procedure of an affinity chromatography. (a) The column is filled with the matrix-bound ligand ( ) and loaded with the sample containing the bioaffine component and other substances ( ). (b) The bioaffine component contacts the ligand immobilized to the matrix particles ( ). (c) The nonbound substances are removed by washing, and in the elution diagram a more or less sharp peak appears. (d) The bioaffine component is eluted by change of pH, ionic strength or addition of detergents or protein unfolding agents.

The complex organic chemistry required for the synthesis of a reliable matrix and for covalent attachment of ligands prevented this technique from becoming generally established in biological laboratories. However, in 1967 Porath et al.[7,8] reported that molecules containing primary amino groups could be coupled to polysaccharide matrices activated by cyanogen bromide. This was a milestone in the development of affinity chromatography as a routine separation technique. This process was further promoted as Cuatrecasas et al.[9] in 1968 succeeded in solving the problems of steric accessibility by interposition of a spacer arm between the ligand and solid support.

1.3 Matrix (Support)

A "matrix" is any material to which a biospecific ligand may be covalently attached.[10] Usually, the material to be used as an affinity matrix is insoluble in the system in which the target molecule is found. The matrix is the most important

component of an affinity chromatographic medium as it composes, for the most part, the largest volume of the adsorbent.

At present a wide range of high quality, high performance and economical matrices are commercially available. Inspite of that, a carrier that fulfils all possible requirements for every application is not yet available. Hence it is often necessary to synthesise an appropriate matrix.

1.3.1 General properties of solid matrix support

Matrix should possess the following properties to be an ideal support for successful application in affinity chromatography:

1. Hydrophilic character: Hydrophilic character of matrix is essential for minimising nonspecific interactions and inactivation of bound enzyme. Hydrophobic character of matrix can decrease the stability of enzyme on the basis of denaturation analogous to that produced by organic solvent.

2. Chemically stable but easily chemically modifiable: The matrix should be chemically stable over a wide range of pH (2-12) and ionic strengths. It should be stable at elevated temperature and in organic solvents, which are essential conditions for the modification of matrix. On the other hand, support should possess a sufficient number of chemical groups that can be easily modified to introduce affinity ligands or enzyme on it.

3. Resistant to microbial and enzymatic degradation: The matrix should not be attacked by microorganisms and enzymes.

4. Large surface area with no nonspecific adsorption: Support should have the greatest possible surface area and at the same time be completely inert, have no nonspecific interactions with the substance to be purified and the contaminants.

5. High rigidity and suitable form of particles: The flow characteristics of a matrix depend largely on rigidity, particle size and particle shape of support material. Good flow properties are important for the success of affinity chromatography. The eluent should penetrate the support column at a sufficient rate even when the affinate is bound onto it. Matrix must be highly rigid to withstand the pressure of packing and solvent flow during elution and washing. Irregularly shaped particles lead to unequal path lengths for the substances to be

separated, which ultimately leads to band broadening. Generally, the suitable shape is spherical. However, spherical beads may not be essential if a very rigid matrix is available. Excellent results have been obtained with irregular particles.[11] Increasing particle size reduces flow resistance and separation power whereas very small particles have too high a flow resistance and soon become clogged.[12] A narrow particle size distribution is not a critical parameter for affinity chromatography as in case of ion-exchange or reverse phase techniques. However, narrow particle size range will result in better and more efficient column capacity because of less frequent column channeling and greater concentration of final and eluted product. This is due to reduced void volume.

6. Macroporous: Larger pores allow all areas of the matrix to be available to most of the biomolecules.

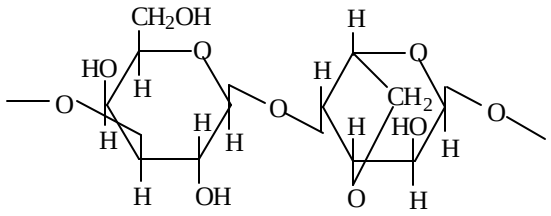
7. Low cost: Matrix costs are the total cost of entire system including labour costs and product value. If the affinity system uses an expensive ligand such as a monoclonal antibody, then an expensive matrix may be acceptable.[13]

1.3.2 Natural supports (biopolymers)

Hydrophilic biopolymer has been widely used as supporting materials for affinity chromatography. Most commonly used biopolymers are agarose, dextran, cellulose, and to a lesser extent starch.

1.3.2.1 Agarose

Agarose is the most commonly used carrier in affinity chromatography. Cuatrecasas et al.[2] introduced agarose into affinity chromatography as a support material. Agarose is a linear water soluble polysaccharide consisting of alternating 1,3-linked β-D-galactose and 1,4-linked 3,6-anhydro-α-L-galactose units.[14] Partial structure of agarose is shown below:

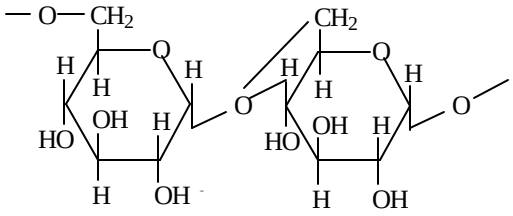


Along with agaropectin, agarose is a component of agar, which may be isolated from different species of Rhodophyceae family of red seawater algae.[15]

The agarose matrices are very hydrophilic, relatively stable mechanically and readily chemically modifiable via primary and secondary hydroxyl groups. It is stable in the pH range 4-9. It is resistant to high salt concentrations, even stable at room temperature when exposed to 0.1 M sodium hydroxide solution and 1 M hydrochloric acid for 2-3 hours. However, agarose also has considerable disadvantages. Below 0°C freezing causes irreversible damage to the bead structure and results in smaller pores with poor chromatographic behaviour. It cannot be heat sterilised. Agarose beads are solubilised when heated above 40°C, so one can not work at elevated temperatures or with organic solvents, which are frequently needed for modification to couple affinity ligand. The chemical and mechanical stability of agarose matrix gel can be increased by crosslinking with epichlorohydrin,[16,17] 2,3-dibromo propanol[17] or divinyl sulphone,[17,18] especially in aqueous medium, in both acidic and alkaline regions (pH 3-14). Crosslinking also increases stability of matrix in organic solvents such as ethanol, dimethylformamide, tetrahydrofuran, acetone, chloroform, dimethyl sulphoxide, dichloromethane and dichloroethane. Agarose is also used in combination with synthetic polymers as a supporting material. The Ultragel AcA (LKB-Producer AB, Bromma, Sweden), for example, consists of a three dimensional polyacrylamide lattice and an interstitial agarose gel of a composition different from that of both partners. The acrylamide-agarose ratio may vary from 1:1 to 10:1. The main producers of agarose are Pharmacia (Uppsala, Sweden), under the trade name Sepharose, and Bio-Rad Labs. (Richmond, Calif., USA), under the trade name BioGel A.

1.3.2.2 Dextran

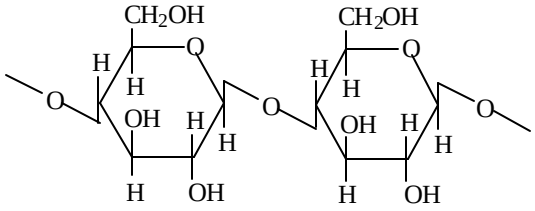
Dextran is a branched-chain glucose polysaccharide produced by microorganism of genus *Leuconostoc mesenteroides*. It is composed of 1,6-linked α-D-glucose units (more than 90%) and can be branched out 1,2; 1,3 or 1,4 binding. Partial structure of dextran is shown below:



Dextran based gels have abundant hydroxyl groups for activation and coupling of ligand. They are widely used without any modification as specific sorbents for isolation of a series of lectins.[19] Two types of gels, Sephadex and Sephacryl, are mainly used in the chromatography of biomolecules. Dextran does not form a gel in its natural state but it is used as a base for formation of gel via covalent crosslinks. Crosslinking of dextran with epichlorohydrin gives Sephadex. The most important producer of Sephadex is Pharmacia (Uppsala, Sweden). The Sephadex gels are very stable to chemical attack. They are stable in water, salt solution, organic media and in alkaline solution. The glucosidic bond in dextran is sensitive to hydrolysis at low pH. Strong acids split the glycosidic bonds, although they are stable for 5 months in 0.02 M hydrochloric acid, or for 1-2 hour in 0.1 M hydrochloric acid or 88 % formic acid without appreciable degradation.[20] Drying and swelling of gel is completely reversible. Oxidants cause degradation of the matrix. Sephacryl gel is prepared by covalent crosslinking of allyl dextran with N,N'-methylene-bis-acrylamide.[21] It is porous and yet mechanically stable. The main advantage of this matrix is the excellent flow rate, because of its exceptional rigidity. This gel contains a large number of hydroxyl groups and should be an ideal matrix material. However, presence of small number of carboxyl groups gives rise to nonspecific interactions with proteins.

1.3.2.3 Cellulose

Cellulose has been widely used as a matrix in industrial processes because of its easy availability and it is inexpensive. Cellulose, a vegetable polysaccharide, is a linear polymer of 1,4-β-D-glucose units:



Among the bioligands that have been successfully immobilised as bioselective adsorbents with cellulose as the matrix are soyabean trypsin inhibitor, used to purify trypsin[22] and acrosin,[23] and chitin used to purify lysozyme.[24] In many ways cellulose would be an ideal matrix. It has little nonspecific adsorption. It is relatively stable against physical and chemical influences. The glycosidic

bonds are hydrolysable only under extremely acidic conditions. Cellulose can withstand denaturing and mild oxidation conditions and also a wide range of solvents. Cellulose is more stable under acidic conditions than alkaline conditions. Working pH range should be maintained between 3-10.

Unfortunately, cellulose possesses very small pores that are not uniformly distributed throughout the matrix. The absence of macroporosity prevents good penetration of macromolecules and leads to a relatively low ligand density and flow rate.[25,26] The linear polymeric chains in cellulose are aggregated to form micro-heterogeneous fibres, with crystalline region interrupted by amorphous region. Chemical modifications preferentially occur in amorphous regions. This may result in undesirable non-uniform distribution of ligands.

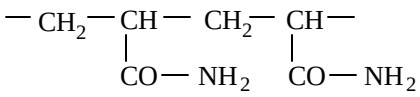
There are two other basic problems that diminish the usefulness of cellulose as a matrix.[27] First, because of its fibrous nature, it is easily clogged by particulate material and easily compressed by the application of even moderate pressure. Thus, it is of limited use in rapid, high-pressure applications.

1.3.3 Synthetic polymers

Besides biopolymers, synthetic polymers have been used extensively as support materials for affinity chromatography. They offer some advantages over biopolymers. They typically have superior chemical and physical durability. Most polymeric matrices can withstand changes in ionic strength or buffer composition. Many are stable in organic solvents and can tolerate extremes in pH without decomposition. An additional advantage is that reactive groups can be easily incorporated onto polymer during the polymerisation process itself. Incorporation of monomers with suitable functional groups can provide activation sites on these supports for ligand immobilisation. Usually monomers containing primary and secondary hydroxyl groups are used to prepare synthetic supports. Presence of hydroxyl groups offers both suitable coupling chemistries as well as hydrophilicity to the matrix. Desired properties can be obtained by proper choice of monomers and polymerisation conditions. The most widely used synthetic polymers are vinyl (acrylic) polymers. Some important synthetic polymers are presented here.

1.3.3.1 Polyacrylamide

Polyacrylamide was first used for protein chromatography in 1962.[28,29] It is made from a copolymerisation of acrylamide and a crosslinking agent, usually N,N'-methylene bisacrylamide containing a hydrocarbon framework with carboxamide side chain:

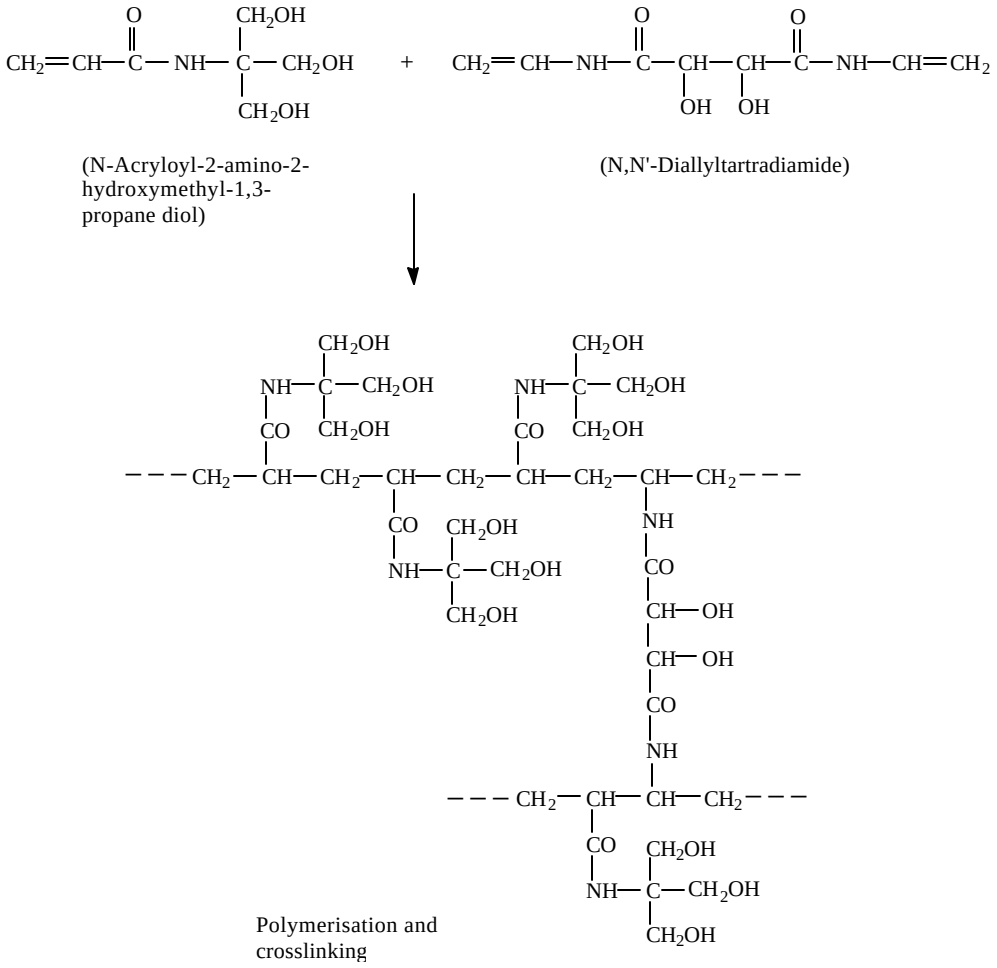


The main producer of polyacrylamide gels is Bio-Rad Labs., under the trade name Bio-Gel p. This support can be prepared with varying range of pore sizes, from Bio-Gel p-2, with a molecular weight exclusion limit of 1800, up to Bio-Gel p-300, with a molecular weight exclusion limit of 4,00,000. The polyacrylamide gels are hydrophilic, stable in pH range 1-10. As these are synthetic polymers, they are not attacked by microorganisms. They have excellent chemical stability due to a polyethylene backbone and show low nonspecific binding characteristics. Polyacrylamide gels, however, suffer from some disadvantages, which have prevented them from becoming popular support in affinity chromatography. These gel particles strongly adhere to clean glass surfaces, so use of siliconised glass or polyethylene vessels are recommended. They have poor mechanical stability and have low flow rates. Polyacrylamide has a tendency to shrink or swell in various solvents or buffer compositions.

Polyacrylamide gels have been improved upon in last few years. Copolymerisation of acrylamide with suitably reactive monomers provides synthesis of acrylamide copolymers with wide range of groups functionally active to enzyme binding. Several supports have been commercialised under the trade name Enzacryl.[30,31]

1.3.3.2 Trisacryl

Trisacryl is based on a derivative of an acrylamide monomer. It is produced by copolymerisation of an unique trihydroxyl derivative of acrylamide and the cross-linking agent N,N'-diallyltartradiamide[10] (Scheme 1.1).

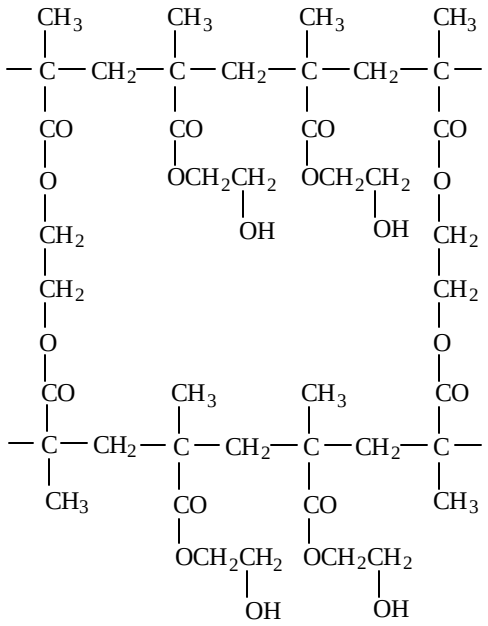


Scheme 1.1 Trisacryl supports made by the copolymerisation of an unique trihydroxyl derivative of acrylamide and the cross-linking agent N,N'-diallyltartradiamide

This matrix is rigid and macroporous, with very low nonspecific binding for biological molecules. Trisacryl is a hydrophilic synthetic polymer with almost no hydrophobic sites.[32] Unlike basic polyacrylamide beads, these polymer derivatives have much better mechanical stability and flow rate. They can tolerate pH from 1 to 11. More basic pH environments can degrade the matrix due to break down of amide bonds. Trisacryl matrices are stable to temperatures in the range -20°C to 121°C and are also stable in organic solvents and detergents.

1.3.3.3 Hydroxyalkylmethacrylate gel

In the early 1970s this type of synthetic support was synthesised by Coupek and coworkers[33] and introduced to affinity chromatography by investigations of Turkova.[34] They are marketed under the trade names Spheron (Lachema Brno) and Separon (Laboratory Instruments Works). Spheron and Separon are prepared by heterogeneous suspension copolymerisation of hydroxyalkylmethacrylate and ethylenedimethacrylate in aqueous solution in the presence of inert solvents. This gel (Spheron and Separon) has the following structure:[35]



Gel is chemically and mechanically more stable than polyacrylamide gel. They do not change their structures after heating in 1 M sodium glycolate solution at 150°C for 8 hours or even after boiling in 20 % hydrochloric acid for 24 hours. These properties allow their use in large-scale operations and in industries. Unlike other hydrophilic matrices, macroporous structure of hydroxyalkyl-methacrylate gels is kept even in dry state. There is no change in volume of gel with change in pH or organic solvents. More hydrophobic nature of gel is not suitable for many applications. However, this property can be used to study hydrophobic interaction chromatography of proteins and peptides.[36]

1.3.3.4 Other supports

Many carriers other than those discussed above such as polystyrene, vinyl copolymers, copolymers of ethylene and maleic anhydride, maleic anhydride and butanediol-divinylether or acrylamide and methacrylic anhydride are used as support in affinity chromatography.[12]

Porous polystyrene matrices are prepared by copolymerisation of styrene and divinyl benzene. They are commercially available under the trade names Poragel, Styragel and Aquapak from Waters Assoc. (Milford, Mass., USA) and BioBeads from Bio-Rad Labs. (Richmond, Calif., USA). Since the base matrix is crosslinked polystyrene, it gives good chemical and physical stability to the support. It is stable over the pH range 1-14. However, the material is not suitable for broad application in affinity chromatography because its hydrophobic nature can denature the adsorbed or covalently bound protein molecules.

Hydrophilic vinyl copolymers (Toyopearl, Toyo Soda, Japan; Fractogel, Merck, Germany) are remarkably stable but are of low working volume. The achievable ligand concentration is up to 10 times higher than that of agarose but protein-binding capacity is low.[37]

1.3.4 Inorganic supports

When extreme rigidity of support is needed, as for HPLC and large-scale industrial application, inorganic materials are used. The frequently used inorganic materials are porous glass and porous silica. These support materials have high mechanical and chemical stability. Because of their high rigidity and mechanical strength, they show resistance to bed compression. Porous inorganics are easy to handle and can be stored as dry material when not in use. However, they suffer from several disadvantages. These include the solubility of glass and other silica based material at pH >8, which restricts activation and coupling chemistry. Alkaline solubility can be minimised by extensive derivatisation of the silanol groups on their surface with functional groups, which shield the surface from interaction with hydroxyl ions in the mobile phase.[38] Their stability can also be enhanced by coating them with zirconium[39,40] or with an inert polymer.[41]

Glass and silica show non-specific adsorption of protein due to charged surface silanol groups.[42,43] This effect can be reduced by surface treatment with

hydrophilic silanes, such as ϒ-aminopropyl triethoxy silane[42,44] and glycidoxy propyl trimethoxy silane.[45]

Most workers use the same affinity column repeatedly. Regeneration of the column is usually achieved by flushing column with 0.5 M sodium hydroxide (NaOH) and 4 M urea.[46] Such a procedure cannot be applied to silica-based materials because of their solubility at alkaline pH.[13]

A special type of inorganic support is the magneto-gel. Iron oxide particles could be directly loaded with protein, enzyme or polysaccharide.[47] It is difficult to obtain magnetite particles that are chemically stable and are free from ferric ion leakage.

1.4 Affinity ligand

Affinity chromatography is based on the exceptional ability of biologically active substance to bind specifically and reversibly other substances, generally called ligands, affinity ligands or affinants.[48] Ligands used in affinity purifications are very many. They must be chosen with care. Affinity ligands should possess the following properties:

1. Ligands should interact strongly but reversibly with the biomolecules to be isolated. Weaker interactions may not give proper binding and sometimes binding may not occur at all, whereas very strong interactions would require harsh conditions to recover the product.

2. Ligands should be specific for the molecule or group of molecules to be purified.

3. Ligands should be stable during coupling and should not leach out during various chromatographic operations.

1.4.1 Ligand density

Ligand density is the quantity of ligand coupled per millilitre or gram of the support. The concentration of the immobilised ligand has a distinct influence on the performance of affinity matrix. Selection of optimal ligand density range often leads to effective separation or isolation of biomolecules. Too low a density of ligand molecules may produce a weakly interacting affinity matrix. Too high a ligand concentration may increase the nonspecific interactions and the binding

strength, which may create conditions under which the elution of target molecules is difficult. The elution of adsorbed macromolecules from highly substituted gel or from gel with a ligand of very high affinity is often possible only under denaturing conditions. High ligand density can also decrease porosity, which may affect the loading capacity.[38] In case of systems with low affinity interactions between ligand and target molecule, a high ligand loading may actually give better performance. Conversely, a strong affinity pairing may respond well to low or average ligand loading.[49]

High concentrations of small ligands can block some active sites on the matrix, causing a lower binding efficiency. Similarly, larger ligands can also block the adjacent sites, again resulting in lower binding efficiency.[50] A matrix with an optimal immobilised affinity ligand can be produced by choosing proper chemistry of activation of base matrix and coupling of ligand onto it. Ligand concentration and affinity of the partners have an influence on the column geometry.[51] When both of these are very high, column length is not important. Adsorption takes place at the head of the column and dilute solution can thus be concentrated simultaneously. For systems with low binding capacity, the column length is of decisive importance.

1.5 Activation and coupling

Activation is the process of chemically modifying a matrix such that the product of the process will react/couple with a ligand via covalent linkages. A good ligand that is badly coupled to a good matrix will result in the failure of affinity chromatography purification. Following factors must be considered during chemical modification of supports and immobilisation of affinity ligands:

1. Ligand attachment should be via stable leak-resistant links.

2. Activation as well as coupling chemistry should not alter the structure and stability of both ligand and matrix.

3. Coupling should be rapid and efficient, with no side reactions that lead to the introduction of unwanted groups into matrix.

4. Excess of active groups should be easily and completely blocked to avoid any nonspecific effects.

5. Reactants should be readily available, inexpensive, nontoxic and non-inflammable. Activation procedure should be easily scaled up without difficulty.

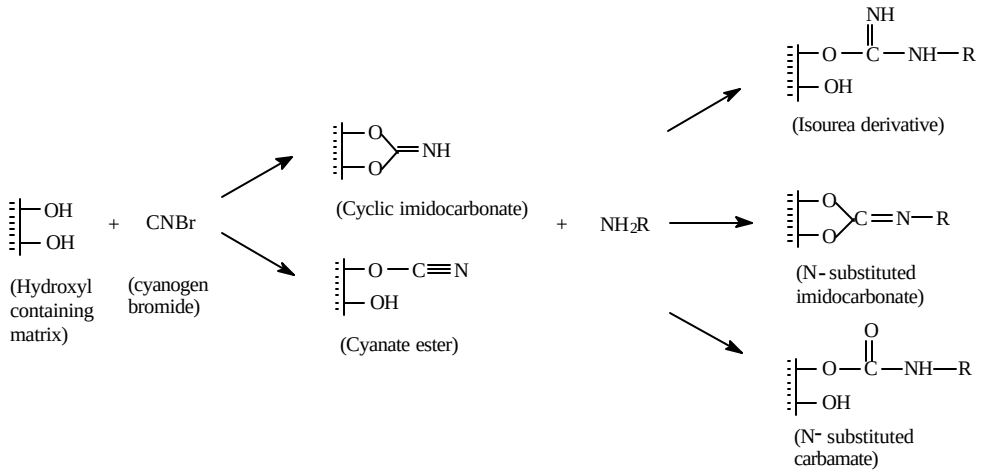
6. The activation and coupling chemistry should be reproducible.

1.5.1 General reactions

A variety of procedures have been used to activate supports and to immobilise ligands. Few commonly used procedures are discussed here.

1.5.1.1 Cyanogen bromide (CNBr)

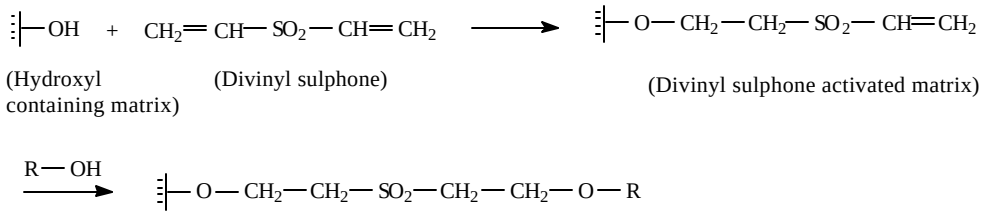
Cyanogen bromide is one of the first methods used to activate solid supports.[7] It is suitable for all support materials with hydroxyl groups. Besides the polysaccharides, synthetic polymers with accessible hydroxyl groups, such as Spheron or Separon, can also be activated by this procedure.[52] Cynogen bromide activated supports can be used to couple smaller ligands as well as high molecular weight biopolymers with quaternary structure such as haemoglobin.[53] The activation procedure is relatively simple to carry out and is reproducible. Reaction is carried out either in aqueous media or in an acetone-water mixture using trimethylamine as catalyst[54] (i.e. cyano transfer reagent to enhance the electrophilicity of CNBr). Cyanogen bromide in aqueous alkaline media introduces cyanate esters and imidocarbonates into matrix by reacting with endogenous hydroxyl groups. Depending upon the type of polysaccharide matrix used, the relative amounts of the two active species vary. The predominant species on agarose is cyanate ester (70-85 % of total coupling capacity) while cyclic imidocarbonates predominate on activated crosslinked dextrans (Sephadex) and cellulose. In polymers lacking vicinal hydroxyl groups (e.g. Sepharose), the cyanate esters are hydrolysed rapidly at high pH to insert carbamate groups. In contrast, cyanate esters are stable at low pH whereas cyclic imidocarbonates are hydrolysed rapidly. The mechanism of activation of polysaccharides by CNBr is presented in Scheme 1.2. Although CNBr activation is effective and used widely, it is associated with several problems. CNBr is volatile and highly toxic. There is a small but continuous leakage of coupled ligand.[55,56] This is primarily due to instability of isourea bridging group between polymer and ligand.



Scheme 1.2 Matrix activation with cyanogen bromide and subsequent ligand immobilisation

1.5.1.2 Divinyl sulphone

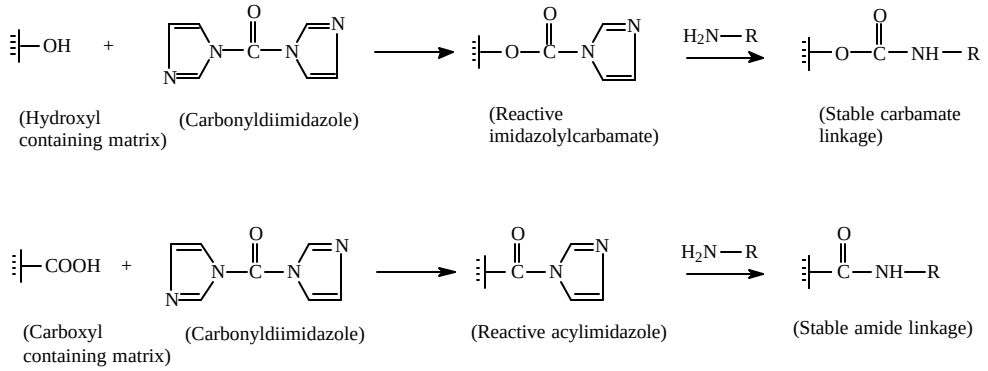
Divinyl sulphone (DVS) is used with alkali resistant non-shrinking hydroxyl containing polymers.[57] DVS introduces vinyl groups into the matrix (Scheme 1.3) that will couple to alcohols, amines, phenols and sulphdryls. The vinyl groups introduced into the matrix are more reactive than the oxirane groups. Therefore, the coupling takes place rapidly and completely at lower temperatures and at lower pH than in the oxirane activated matrices. DVS activated agarose provides extreme rigidity and can be used for HPLC. However, immobilised ligands prepared by the DVS method are unstable in alkaline solution. The amino linked matrices are unstable above pH 8 and the hydroxyl linked matrices are unstable at about pH 9 or 10. DVS is toxic and expensive.



Scheme 1.3 Mechanism of activation of hydroxylic matrices by divinyl sulphone and subsequent coupling to hydroxyl containing ligands

1.5.1.3 Carbonyldiimidazole

N,N'-Carbonyldiimidazole (CDI) activation method can be used to activate both carboxyl and hydroxyl containing matrices that can withstand non-aqueous conditions.[58-60] The activation of hydroxyl groups with CDI forms an intermediate imidazolyl carbamate that can react with amines to give stable carbamate linkage. The hydroxyl containing matrix activated by CDI is stable in nonaqueous solution for years. Carboxyl-containing matrices can be activated with CDI to give highly reactive acylimidazole. Amine containing ligands will rapidly couple to these reactive groups to yield stable amide linkages between the matrix and ligand. CDI is extremely unstable in aqueous environments. It breaks down to carbon dioxide and imidazole in presence of water. Mechanism of activation of hydroxyl as well as carboxyl containing matrices by carbonyldiimidazole and subsequent coupling to amine containing ligand is presented in Scheme 1.4.

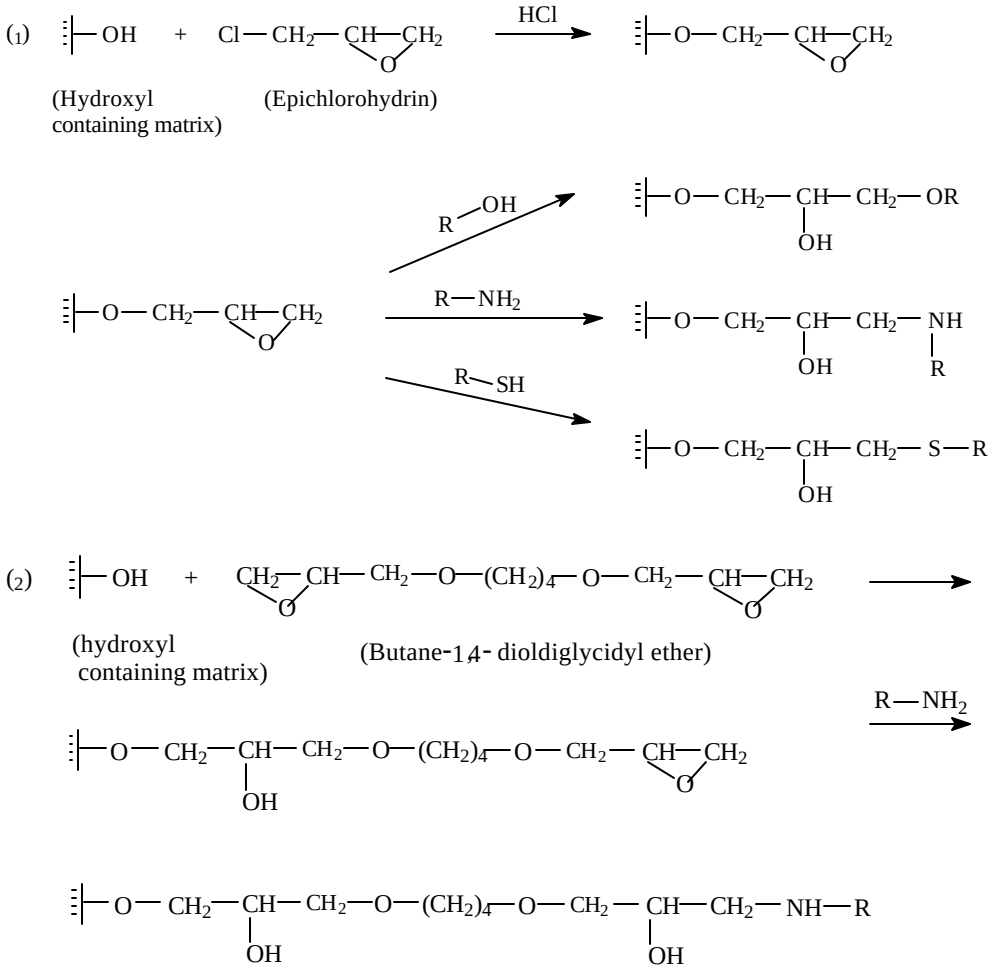


Scheme 1.4 Matrix activation with carbonyldiimidazole and subsequent ligand immobilisation

1.5.1.4 Epoxy (bisoxirane) activation

Reaction of -OH groups of hydroxylic polymer with epichlorohydrin leads to activated support with oxirane groups. Bisoxirane such as 1,4-bis(2,3-epoxypropoxy) butane are also used for the introduction of the oxirane group on the support material. During activation with bisoxirane, one of the oxirane groups of the reagent is allowed to react with hydroxyl groups in the polymer matrix, leaving other group free to couple with the ligand. This method introduces a long chain hydrophilic spacer arm with a reactive oxirane group at the end. The spacer arm places the ligand at a considerable distance from the polysaccharide chains.

All amino-, hydroxyl-, and thiol- containing compounds can be immobilised on epoxy activated matrix, so this is one of the most universal of all activation methods. The linkage between the matrix and ligand is extremely stable. Epoxide activated materials are mechanically and chemically stable. Epoxide activation is widely used for a range of polymers such as agarose,[61] polyacrylamide,[62] polymethacrylate,[63,64] cellulose,[65] porous glass[45] and silica.[66] Mechanism of activation of hydroxylic matrices by epichlorohydrin / bisoxirane and subsequent ligand coupling is presented in Scheme 1.5.



Scheme 1.5 **Matrix activation with epichlorohydrin / bisoxirane and subsequent ligand immobilisation**

1.6 Variants of affinity chromatography

Several variations within affinity chromatography have evolved. These arise from the introduction of ligands which induce a specific type of interaction to bring about affinity towards the biomolecule to be separated. A simplified representation illustrating the different modes of interation between immobilised and affine components is presented in Figure 1.2. The subclasses are presented below:

1.6.1 Biospecific affinity chromatography (bioadsorption)

This technique involves the covalent binding of biomolecules as ligands. The separation arises from biospecific recognition of the ligand towards the biomolecule to be separated.[2] Biologically active substances, viruses, cells, nucleic acids, antibodies etc. are purified by this procedure.

The natural ability of enzymes to recognise their substrates, effectors (activators and inhibitors), cofactors have been used successfully in the last few years to separate these substances from biological material of differing origin. The affinity chromatography of chymotrypsin on Spheron 300, with an attached trypsin inhibitor, is an example of bioadsorption.[34] Vice versa, enzymes are also used as affinants for the isolation of inhibitors. Potato chymotrypsin inhibitor was separated on a column of hydroxyalkyl methacrylate gel with attached chymotrypsin.[34] In both cases elution was done by a change in pH.

Immobilised natural polysaccharides are used for the purification of a wide variety of proteins, the most powerful tool being matrix bound heparin. Immobilised heparin has been used in affinity chromatography of RNA polymerase,[67,68] lipoprotein lipase,[69] collagenase[70] etc.

Lectins from seeds of *Vicia ervilia* have been purified on D-mannose-Sepharose 6B column. Desorption of lectin took place on passage of 0.5 M glucose in 6 M phosphate buffered saline of pH 7.0.[71] Immobilised lectins are used for the separation and purification of glycoproteins and glycoprotein conjugates. For example, immobilised concanavalin A has been used to purify immunoglobulins, brain and lysosomal hydrolases, α-feroprotein, human alkaline and acid phosphatase.[72] Lectins of *Vicia ervilia* seeds bound on Sepharose 2B column can be applied for the separation of intact influenza viruses but not for

glycoprotein after virus solubilisation. On the contrary, Crotolaria juncea lectin columns are very useful tool for the purification of bovine viral diarrhea virus and for the separation of influenza A virus glycoconjugates after solubilisation.[73]

Immobilised nucleic acids are used both as general ligands for all DNA-binding proteins and for sequence-recognising molecules. Among the general use, DNA-cellulose is widely used. Isolation of protein kinase,[74] DNA polymerase,[75] RNA polymerase,[76] RNA ligase[77] can be achieved by using immobilised DNA adsorbents.

Separation of cells can also be achieved on the basis of their interaction with bioligands. Eshhar et al. have isolated thymocytes on anti-thymocyte globulin bound Sepharose 4B matrix.[78] Adipose cells can be isolated on insulin incorporated Sepharose 4B column.[79] Matrix bound antigens and antibodies are used for the separation of complementary immuno-substances. For example, Cuatrecasas prepared a column of insulin-Sepharose and demonstrated the adsorption of insulin antibody to it at pH 8.8 and its elution with hydrochloric acid.[80] Vice versa, the antialbumin antibodies hooked to Sepharose may serve as an affinant for the isolation of albumin.[81]

1.6.2 Dye ligand chromatography

The use of dye molecules as affinity ligands introduced a pseudo-affinity chromatography in which structure of dye molecule resembles a biospecific ligand. The most widely used reactive dyes in protein purification are the triazine dyes. The interaction of proteins with the triazine dyes was first observed in 1968. It was shown that the dye c*ibacron blue F3G-A* could bind to some enzymes.[82,83] Until then a dextran conjugate of the triazine dye, c*ibacron blue F3G-A* had been used as a void volume marker in gel filteration.[84] Reactive triazine dyes were developed originally at Imperial Chemical Industry (ICI) in 1954, primarily for use in textile industries. These dyes are called so because they are based on the chemistry of cyanuric chloride (1,3,5-trichlorotriazine).[85] Procion range of reactive dyes are composed of various polysulphonated chromophores linked either to reactive dichlorotriazinyl functional groups by an amino ether bridge (Procion MX dyes) or to less reactive monochlorotriazinyl group (Procion H, HE or P dyes).[86] c*ibacron blue F3G-A* is the most important representative of this class of chromatography. Other triazine dyes such as *Procion Red H-E3B,*

Procion Red H-8BN, Procion Yellow MX-8G, Procion Scarlet MX-G, Procion Green H-4G, Procion Brown MX-5BR etc. have been used as suitable ligands for the purification of large number of proteins and enzymes.

Chlorotriazine dyes have been covalently attached to a variety of support materials including agarose, Sephadex, beaded cellulose, metal oxides, polyacrylamide, Sephacryl S-200, Ultragel (agarose-polyacrylamide copolymers).[86] Immobilisation of dye is carried out in presence of salt and alkali. The salt step is essential to "salt" the dye onto the matrix, which corresponds to faster reaction time, while alkaline conditions facilitate the formation of ether linkages between dye and support. The covalent binding of the dye can be achieved in different ways. It can be accomplished by nucleophilic exchange of the chlorine atom at the triazine ring with the formation of ether bond. Binding can also be by the direct covalent attachment of the dye to the matrix by cyanogen bromide method or by periodate oxidation of the support. The binding in both cases is accomplished via the $-NH_2$ group at the anthroquinone ring of dye molecule.[87] An immobilised dye can bind to a sizable number of different proteins. Selective elution of protein of interest is achieved using a competitive ligand, a salt gradient or chaotropic agents such as thiocyanate, urea, ethylene glycol etc.

The use of dye ligand as chromatographic media offers several significant advantages over the more conventional immobilised biological ligands.[88] For example, the protein binding capacity of immobilised dye adsorbents exceed those of natural biological media by 10-100 fold. Low capital cost, ready availability and ease of coupling to solid supports offer prospects for large scale affinity chromatography.[89-91] In addition, dye columns are readily reusable (up to 40 times for a single column)[85] which enhances the attractiveness for industrial applications. Synthetic dyes are chemically and biologically stable. The characteristic spectral properties of the dye permit ready monitoring of ligand concentrations and identification of column material. Dye bound columns can be operated at high flow rates and in presence of detergents. Hazardous cyanogen bromide activation of the support can be avoided since triazine ring of the dye reacts rapidly with hydroxyl groups on the matrix.

Group specific nature of dyes allows the use of these columns for many different applications. For example, biomolecules like dehydrogenases, kinases, albumins, interferons, blood clotting factors, can be purified without a separate ligand synthesis for each protein.[85] Immobilised dyes appear to be especially effective adsorbents for the purification of oxido-reductase, phosphokinases and other co-enzyme-dependent enzymes, hydrolases, transferases, oligo- and polynucleotides-dependent enzymes, synthetases, hydroxylases, esterases, decarboxylases, glycolytic enzymes and blood proteins.

1.6.3 Metal chelate affinity chromatography

Porath et al.[92] introduced chelones (carboxymethylated amine) immobilised on agarose as ligands for metals and demonstrated the separation of several serum proteins on chelated Zn^{2+} and Cu^{2+}. Metal chelate affinity chromatography is widely recognised as a popular technique for the purification of proteins.[93] This method is based on the ability of various metal ions to form co-ordinate compounds with high- or low- molecular weight ligands.[94] The affinity adsorbents are prepared by introduction of chelate forming groups (having multiple points of attachment) into a common gel followed by co-ordination of a suitable metal ion. Metal ion should be strongly fixed onto the chelating matrix so as to form the stable adsorption centre. To form useful affinity supports, these metal ion complexes must have some free or weakly associated and exchangable co-ordination sites (eg., H_2O, NH_3). These exchangable sites can then form complexes with co-ordination sites on proteins or other biomolecules. Metal ions are highly solvated in an aqueous solution as a result of co-ordination of water molecule.[95] Usually, weakly co-ordinated water molecules can undergo ligand exchange with a suitable electron donor grouping on biomolecules. In addition, metal ion must has a much higher affinity for the gel than for the substance to be isolated, so that it will not create problem during elution. Biomolecules are usually eluted by one or combination of the following: (1) decreasing pH, (2) raising the salt strength, or (3) using competitive ligands such as ethylenediamine tetraacetic acid (EDTA).

Amino acids form stable chelates with metal ions. Proteins bind metal ions mainly via amino acid residues which have electron donating side chains. In peptides, peptide (amide) nitrogens play an important role in binding metal ions.[96]

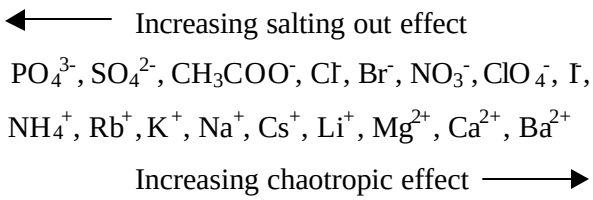
The interaction of proteins or peptides with metal chelate support is mainly based on the formation of stable complexes with cysteine and histidine residues. These amino acids form stable complexes with immobilised Zn^{2+} and Cu^{2+} ions in neutral aqueous solution. In addition to these ions, cadmium, cobalt, nickel and mercury also form co-ordination compounds with histidine and cysteine.

Matrix bound iminodiacetic acid is a well known metal chelate affinity support.[92,97] In addition, immobilised tris(carboxymethyl) ethylene-diamine (TED),[97] EDTA[98] and hydroxamic acids[99] have also been used. Porath effectively used Fe^{3+} biscarboxymethylamine-Sephadex G 25 adsorbents in the purification of tyrosine-containing peptides. Elution was achieved in 100 mM ammonium acetate at pH 5.[100] Two enzymes, glycogen phosphate and lactate dehydrogenase, were purified on Fe^{3+}-Sephadex column.[101] Human fibroblast interferon has been purified on Zn^{2+}-biscarboxymethyl amino-Sepharose 6B matrix.[102]

1.6.4 Hydrophobic interaction chromatography

The formation of contacts between apolar groups in aqueous solution forms the the basis of this mode of separation and purification of proteins and nucleic acids.[103] Hydrophobic interaction chromatography takes advantage of the hydrophobic domains or binding sites on proteins and other biomolecules. In native biologically functional conformations proteins are folded so as to bury maximum possible of their hydrophobic side chains in the interior and to expose maximum possible of their polar, charged side chains to the exterior for interaction with water.[104] Still, complete burying of all hydrophobic groups is generally not achieved. Some of the hydrophobic groups remain exposed at the surface of the molecules which may act as binding sites for the apolar (hydrophobic) groups implanted on the inert matrix. The association energy involved in this interaction is a function of the ordered structure of water molecules surrounding the apolar regions. Structure forming agents, such as certain electrolytes, tend to stabilise the water structure and thus promote the association of apolar groups,[105,106] whereas structure breaking electrolytes have a reverse effect on the water structure.[107] Various hydrogels, substituted by apolar side chains of different structure and length (eg. amino alkane groups), may be used successfully as ligands for separation of a number of proteins and nucleic acids.

Neutral salts may exert a strong influence on the adsorption-elution chromatography of bimolecules in hydrophobic interaction chromatography. On the basis of salt action, Jennissen distinguished two types of chromatography.[108] The first is "salting in chromatography" in which adsorption of bimolecules takes place at low salt concentration and elution is at an increasing gradient chosen from the Hofmeister series.[109] Phosphorylase kinase adsorbed to an ethylamine-Sepharose matrix is an example of salting in chromatography. In the second type, "salting out chromatography", biomolecules are adsorbed at high ionic strength (over 1 M) and eluted at decreasing salt concentration. If phosphorylase b is applied to a methylamine-Sepharose column in the absense of ammonium sulphate, the enzyme is excluded from the gel. Addition of 1.1 M ammonium sulphate to the buffer does not precipitate enzyme but leads to adsorption. A decrease in salt concentration elutes the enzyme. The effectiveness of different salts in promoting hydrophobic interactions vary depending on the chemical nature of ions present. Both cations and anions can be arranged in order from those that are particularly effective in increasing hydrophobic interactions to those that show a tendency to disrupt the structure of water and lead to a relative decrease in the strength of hydrophobic interactions.



Therefore, salts consisting of ions chosen from the leftside of the above series will enhance the structure of water and strengthen hydrophobic interactions, whereas those from the right side will act as powerful chaotropic agents.

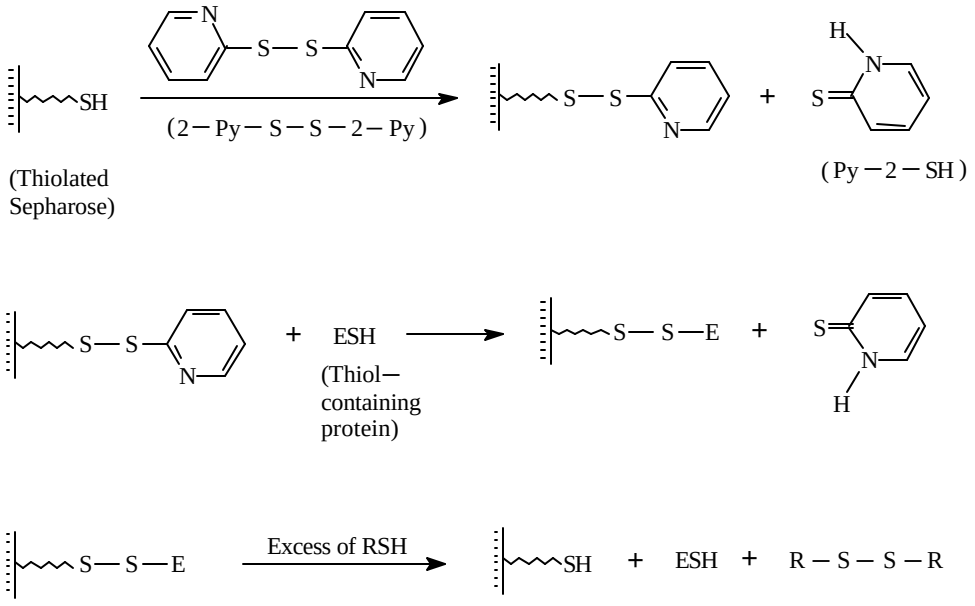
1.6.5 Charge transfer adsorption chromatography

Charge transfer adsorption chromatography utilises interaction between electron donating and electron accepting groups. These groups may be present on the ligand and on the biomolecule.[100,110] Amino acids, peptides and nucleotides can be purified by this method. For the separation of any compound with electron donor or acceptor groups a selected nonionisable compound with contrasting properties is chemically introduced in the matrix material.[111] Dextran (Sephadex) or agarose (Sepharose) are the most suitable supports in charge transfer adsorption

chromatography. The most commonly used ligands are acriflavine, acridine yellow, trityl group, pentachlorophenol and malachite green. Immobilised acriflavine is used in separation of nucleotides, oligonucleotides, single chain nucleic acid from double chain nucleic acid and a variety of aromatic compounds.[37] Egly et al. have studied the chromatographic behaviour of adenosine and adenosin monophosphate (AMP) on Sephadex G-25 epichlorohydrin support coupled with acriflavin.

1.6.6 Covalent affinity chromatography

This technique leads to the formation of covalent bonds between ligand and biomolecules to be isolated. The covalent bonds should be sliceable under mild conditions without affecting the biomolecule. The procedure is selective in the purification of proteins and peptides with thiol group.[112] Brocklehurst et al. employed polymer with 2,2-dipyridyl sulphide for the isolation of papain, chymopapin, ficin, propapain, creatine, phosphokinase and phosphofructokinase.[113] The isolation of protein (ESH) containing a thiol group takes place according to Scheme 1.6:



Scheme 1.6 General run of covalent chromatography

In the adsorption step, the biomolecule to be separated is covalently bond to the activated thiolated support formed by thiol-disulphide exchange reaction.

After washing away the unbound substances, elution is by the reduction of disulphide bonds with an excess of low molar mass thiol (RSH).

Blumber and Strominger[114,115] applied covalent affinity chromatography to the isolation of penicillin-binding components present in *Bacillus subtilis* membranes. The penicillin binding components were covalently linked to a Sepharose column containing 6-aminopenicillanic acid and subsequently eluted with hydroxylamine.

Another example of covalent chromatography is the isolation of acetyl-cholinesterase. Ashari and Wilson[116] allowed acetylcholinesterase to react with a column containing a bound "irreversible" cholinesterase inhibitor, 2-aminoethyl-p-nitrophenyl methyl phosphonate and subsequently eluted the enzyme with 2-(hydroxyiminomethyl)-1-methyl pyridinium iodide (2PAM) or 1,1'-trimethylene bis(4-hydroxyiminomethyl pyridinium)dibromide (TMB_4).

1.7 Suspension polymerisation

Beaded polymer supports and gels, both organic and inorganic, are the widely used materials for various chromatographic techniques[13,46,117-122] and a number of other applications such as solid phase peptide synthesis,[123-125] hydrometallurgy,[126] chemical catalysis[127] etc. The well-established route to synthesise beaded, porous polymer is by suspension polymerisation technique.[128,129] In suspension process, polymerisation occurs in a monomer containing phase that is dispersed in a continuous liquid phase. Controlled agitation and the presence of drop stabilisers are necessary to maintain the suspension. In most cases the continuous phase is aqueous and polymerisation occurs by free radical mechanism. For example, beaded polystyrene resins are produced by oil-in-water (o/w) suspension polymerisation. Preparation of beaded polymers from water-soluble monomers (e.g. acrylamide) is similar, except that an aqueous solution of the monomer is dispersed in an oil to form a water-in-oil (w/o) droplet suspension. The process is often referred to as inverse suspension polymerisation. The following discussion is mainly based on the o/w suspension polymerisation. Thus, suspension process can be defined as a two-phase system in which liquid monomer droplets are converted to the corresponding solid microbeads.

A key problem is the formation of uniform suspension of monomer droplets in the aqueous phase and reduction of coalescence of the droplets during polymerisation. Droplet formation in an oil-water mixture is achieved by mechanical stirring. In the initial stage of particle formation a liquid-liquid dispersion exists, the liquid monomer is dispersed in small droplets and stabilised by the combined action of the stirrer and the suspending agents. At moderate conversions (between 25 and 75% conversion), when droplets comprise of polymer and monomer, the droplet surface is tacky and particles can agglomerate during collision. This stage of polymerisation is called sticky period. The droplets break up by the impeller's shear stress and coalesce back after collision with each other. At the end of the sticky period, as the monomer is consumed further, particles become solid like and they do not stick any more. After this point, which is called particle identity point (PIP), the tendency of particle to agglomerate is reduced and their diameter remains constant.[130]

The weight ratio of the discontinuous monomer phase to the continuous water phase varies from 1:1 to 1:4 in most commercial processes. Lower ratios are not limited but are seldom practical for economical production. Use of higher ratios is excluded, by the proportion of water being insufficient to fill the volume between the monomer droplets.[129] In most cases bulk viscosity of the slurry is near that of water, which allows good mixing of the reactor contents, thereby improving the heat transfer in polymerisation reactor.

1.7.1 Porosity and surface area

Matrix porosity is the basis of support characteristics in gel permeation chromatography. Porosity and surface area play important role in other applications such as ion exchange, affinity chromatography, polymer supported catalysis and solid phase synthesis. Porous matrices have large surface area and volume. This allows binding of large amount of enzyme and hence increases the commercial value. Extensive study of macroporous morphology and formation of porous texture have been conducted for beaded, crosslinked styrene-divinyl benzene resins.[131-132]

The internal structure of the resin beads can be controlled by different parameters in the polymerisation process, such as amount of crosslinking monomer used, type and volume of diluent/ porogen/ pore generating solvent

added to the monomer phase. Three main classes of porogens are known: (i) solvents for the polymer structure (e.g. toluene in case of styrene-DVB resins); (ii) non-solvents for the polymer (e.g. aliphatic hydrocarbons, organic alcohols in case of styrene-divinyl benzene resins) and (iii) polymer soluble in monomer. The last gives only large pores. The molar mass of the porogen is then an important parameter. The pore volume is large (up to 1 mL/g) when the molar mass is high.[133] When solvating diluent is used, large amounts of diluent and crosslinking agent are necessary to get permanent porosity. In such conditions high surface areas, in excess of 500 m^2/g and a narrow distribution of very small pores (10 nm), are obtained together with a limited pore volume (around 0.3 mL/g) of large-size pores.[134] The most complex and often studied case is where a non-solvating diluent is used as porogen. Then, as initially proposed by Kun and Kunin[135] and later on experimentally observed by Jacobelli and coworkers,[131] the bead contains large agglomerates of microspheres (100-200 nm), which look like cauliflowers, and each microsphere shows smaller nuclei (10-30 nm) more or less fused together (Figure 1.3). In between the nuclei, there is a first family of very small pores (5-15 nm), which are mainly responsible for the high surface area of these materials. In between the microspheres, a second family of intermediate pores (mesopores) is observed (20-50 nm) which may account for moderate surface areas (up to 100 m^2/g). A third family of large pores (50-1000 nm) is located between the agglomerates. This family is responsible for the high pore volumes (up to 3 mL/g), which can be achieved, mainly when the amount of diluent is high. This structure of macroporous resin is illustrated in Figure 1.3.[134]

1.7.2 Particle size

In chromatographic processes, flow properties of column are governed by the particle size, particle size distribution and rigidity of the column material. There is no exact theory that can be used to predict and control the size and size distribution of the particles for the suspension polymerisation process. This is mainly because of two reasons. First, the design of reactor and stirrer plays a very important role in determining the shear distribution at various locations inside the reactor. Secondly, the characteristics of the system change during the polymerisation because of the very large increase in the viscosity inside the discontinuous phase.

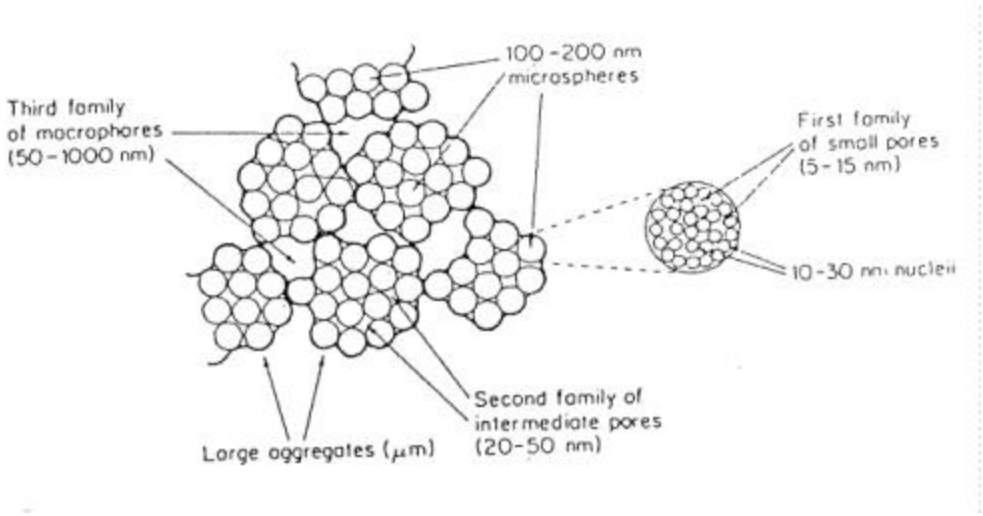


Figure 1.3 Detailed structure of macroporous resin

There are a number of factors that determine particle size. For example, small particles are obtained by increasing the water /monomer ratio or diluting the organic phase with a solvent for the polymer to be produced. Increasing amount of crosslinker has the opposite effect. However, the two most important factors that control this are the choice of the suspending agent and the stirring process. A typical water-insoluble organic monomer has a lower surface tension than water. When such a monomer is mixed continuously as a dispersed phase in a continuous phase of water with no surfactants present, an unstable dispersion forms due to the continuous breakup and coalescence of monomer droplets. If the agitation stops, the monomer - water system will separate into its two phases. Two types of suspending agent (stabiliser) known are: (i) Water soluble suspending agent and (ii) Water insoluble suspending agent. Water-soluble stabilisers include organic polymers, both natural and synthetic polymers. Organic polymers, which are insoluble in monomer droplets and have relatively low solubility in the suspension medium, are highly effective as droplet stabilisers. Among the most commonly used organic stabilisers for o/w suspension system are poly(vinylpyrrolidone) and poly(vinyl alcohol). A wide range of other water -soluble polymers such as methylcellulose, gelatin and natural gums are also used.[136] As the polymeric stabiliser dissolves in the aqueous phase, it acts in two ways. First, it decreases the interfacial tension between the monomer droplet and water to promote the dispersion of droplets. Second, the stabiliser molecules are adsorbed on the surface of the monomer droplets to produce a thin layer, which prevents

coalescence when a collision occurs. Insoluble suspending agents are mostly inorganic powders, which include talc, bentonite, calcium sulphate etc. In general a small concentration of surfactant is sufficient. Large addition of surface-active agents prevents formation of polymer beads by leading to emulsion polymerisation and latex formation. Another important processing factor, the stirring process is very effective, especially during the sticky period of polymerisation. Agitation must be sufficient to redisperse droplet pairs and clusters. It must also be sufficient to prevent separation of the dispersion because of differences in specific gravity between two phases. Generally, particle size decreases with increasing rate of agitation, in a given system.

1.8 Scope of work

Among the very many enzymes, penicillin acylases have received considerable attention owing to the commercial implications associated with production of 6-aminopenicillanic acid (6-APA), an important intermediate for semi-synthetic penicillins such as methicillin, ampicillin, amoxycillin etc.[137] The enzyme penicillin acylase selectively hydrolyses the side chain linear amide bond in penicillin G (Pen G) or penicillin V (Pen V) molecules producing the β-lactam nucleus, 6-APA and the corresponding side chain acid.[138] Initially, attempts were made to accumulate 6-APA in the fermentation broth.[139,140] This approach was abandoned as the titres obtained were unrealistically low. Alternate routes starting with biosynthetic penicillins and cleavage of the side chain, either by chemical or by enzymatic methods, were therefore developed and at one time both approaches were used for the industrial production of 6-APA.[137] However, economic, environmental and operational advantages of the enzymatic process over the chemical route has been realised with immobilised preparation of purified enzyme. The interest in penicillin G acylase continues to grow with the world production of 6-APA, which is estimated to have reached 7000 tons in the year 2000.[141] The enzyme is obtained on large scale either from *Escherichia coli, Bacillus megaterium, Bovista plumbea and Achrombacter* sp.[142]

For preparation of immobilised penicillin G acylase it is necessary to first obtain the soluble enzyme in fairly purified form characterised by specific activity greater then 10 IU mg^{-1} protein.[143] This high specific activity of penicillin acylase solutions plays a fundamental role in its immobilisation. The production cost of 6-

APA depends not only on the substrate price (e.g. benzyl penicillin), but also on cost of enzyme production and immobilisation. One way to minimise the costs is to obtain an enzyme preparation in partially purified form by employing a minimum of purification steps.[143] Affinity chromatography is the best-suited technique in the purification process.

Penicillinase (β-Lactamase EC 3.5.2.6), a member of β-lactamase group of enzymes, hydrolyses cyclic amide bond present in penicillins to form corresponding penicilloic acid. β-Lactamases have gained importance since these β-lactam ring opened molecules are physiologically inactive and this is the mechanism by which pathogenic bacteria develop resistance to β-lactam antibiotics.[144] Simple decolouration as an end point for detection of penicillinase has promoted its use as a marker in ELISA based diagnostic kits.[145] Penicillinase is being used in detection of amoebiasis, esterone, 3-glucaronide, human chronic gonadotropin, mycoplasmosis, nongonococcal urethritis, toxoplasmomis etc. Thus, penicillinase is an important industrial enzyme.

Affinity matrices commercially available in the international market are based on agarose, cellulose, polyacrylamide, polystyrene, poly(hydroxy ethyl methacrylate), controlled pore glass, iron oxide, agarose-polyacrylamide etc. These widely used matrices suffer from one or more disadvantages, as presented in Section 1.3. The objective of the present work is to develop new affinity chromatography matrices having desired physical and chemical properties to overcome these shortcomings. The present study aims at investigating effectiveness of polymer supports of different characteristics for the purification of enzymes, penicillin G acylase and penicillinase.

The study involves developing a reproducible synthesis methodology for generating rigid, spherical base copolymers. The following parameters were varied to generate polymers differing in millimoles of reactive (epoxy) groups, swelling factors, hydrophilicity / hydrophobicity, pore size, particle size and its distribution: (1) feed ratio of monomers, (2) crosslinking monomers, (3) stirrer type and stirring speed, (4) protective colloid concentration. The spherical beaded copolymers were synthesised by suspension polymerisation. The base copolymer chosen for optimisation of particle size was based on allyl glycidyl ether (AGE) and ethylene glycol dimethacrylate (EGDM). In addition, EGDM was replaced by

divinyl benzene (DVB), pentaerythritol triacrylate (PETA), trimethylolpropane triacrylate (TMPTA) and trimethylolpropane trimethacrylate (TMPTMA) to vary the hydrophilic-hydrophobic balance in the matrices. Particles of controlled size distribution were obtained by close sieving.

Once copolymers of controlled particle size were synthesised, a particular copolymer was derivatised with an excess of ligands to form suitable matrices for penicillase and penicillin G acylase. Matrices for penicillin G acylase were synthesised by derivatising with ethylamine, butylamine, hexylamine, octylamine, dodecylamine, benzylamine and phenethylamine. Copper chelated copolymers were prepared for penicillin G acylase by derivatising with excess of 2-picolylamine, 3-picolylamine and iminodiacetic acid and then binding copper. Dye ligand matrices were synthesised by modifying copolymers with different triazine dyes such *as cibacron blue F3G-A, basilen blue E-3G, reactive red 120* and r*eactive green 5.* The modified copolymers were characterised for degree of derivatisation.

Probable affinity matrices were synthesised for penicillinase by derivatising copolymers with ampicillin, amoxycillin, 6-aminopenicillanic acid, cephalosporin C, cephalexin, cephadroxyl, 7-aminodesacetoxy cephalosporanic acid, 7-aminocephalosporanic acid and DL-valine. Ligands showing best performance were choosen and further studies were carried out by derivatising the base copolymer, differing in hydrophobicity, with selected ligands. Effect of ligand concentration and crosslinking agents were studied in the purification of penicillin G acylase.

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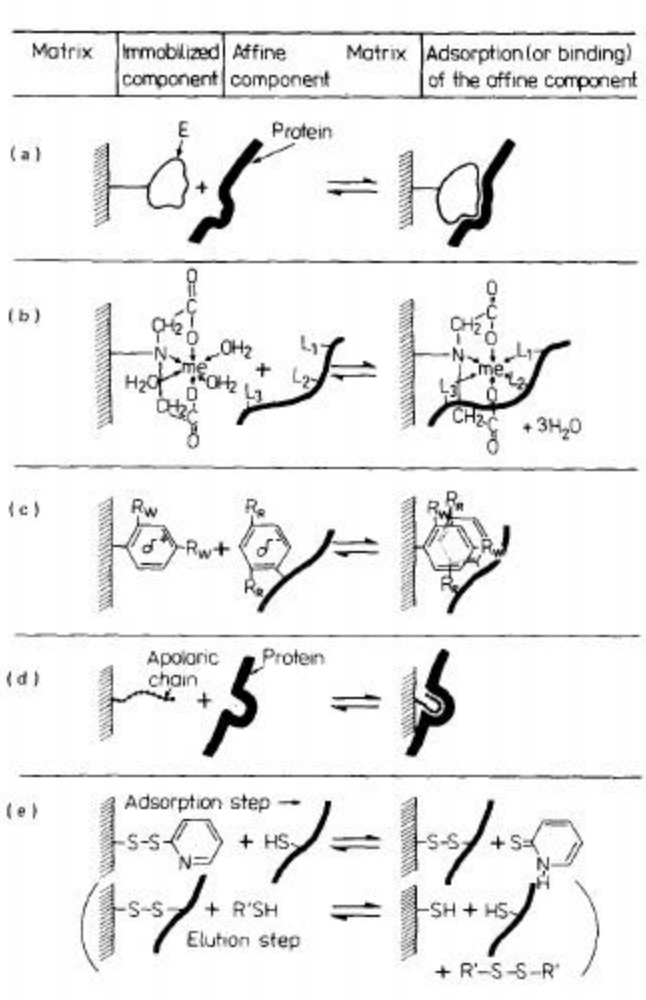


Figure 1.2 **Variants of affinity chromatography. Biospecific affinity chromatography (biosorption), (b) metal chelate chromatography, (c) charge transfer adsorption chromatography, (d) hydrophobic interaction chromatography and (e) covalent chromatography (chemisorption). Abbreviations: E = enzyme, L = amino acid group, me = metal ion, R_w = electron-withdrawing substituent, R_R = electron-donating substituent. The thick lines symbolise the protein.**

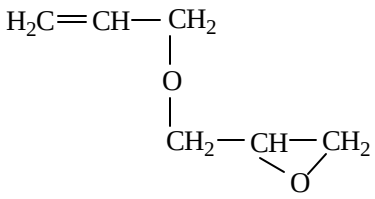
2 EXPERIMENTAL

Experimental procedures followed in this research are presented in this chapter.

2.1 Materials

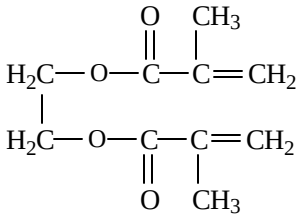
Number of chemicals has been used for this study. Physical constants, source and application of the particular chemical are mentioned below.

2.1.1 Allyl glycidyl ether

Empirical formula	$C_6H_{10}O_2$
Molecular weight	114.14
Density	0.962 g cm^{-3}
Chemical structure	

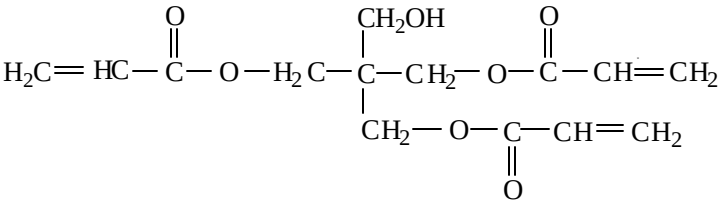
Allyl glycidyl ether (AGE), also known as 1-allyloxy-2,3-epoxypropane, was obtained from Aldrich Chemical Co. (USA). It was used in the preparation of polymer supports for purification of enzymes.

2.1.2 Ethylene glycol dimethacrylate

Empirical formula	$C_{10}H_{14}O_4$
Molecular weight	198.22
Density	1.051 g cm^{-3}
Chemical structure	

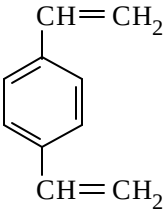
Ethylene glycol dimethacrylate (EGDM), also known as 1,2-ethane diol dimethacrylate, was obtained from Aldrich Chemical Co. (USA). It was used as crosslinking agent in the preparation of support for enzyme purifications.

2.1.3 Pentaerythritol triacrylate

Empirical formula	$C_{14}H_{18}O_7$
Molecular weight	298.30
Density	1.180 g cm^{-3}
Chemical structure	

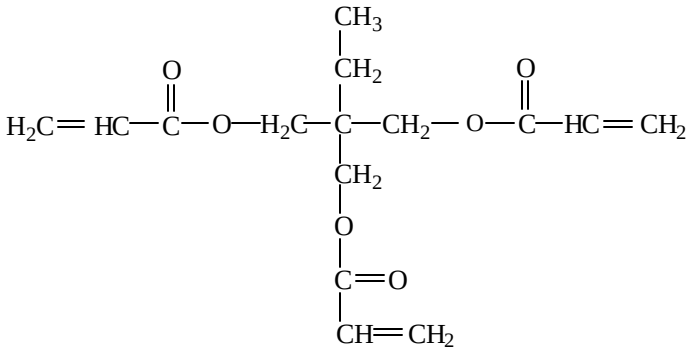
Pentaerythritol triacrylate (PETA) was obtained from Aldrich Chemical Co. (USA). It was used as crosslinking agent in the preparation of polymer supports for enzyme purifications.

2.1.4 Divinyl benzene

Empirical formula	$C_{10}H_{10}$
Molecular weight	130.19
Density	0.914 g cm^{-3}
Chemical structure	

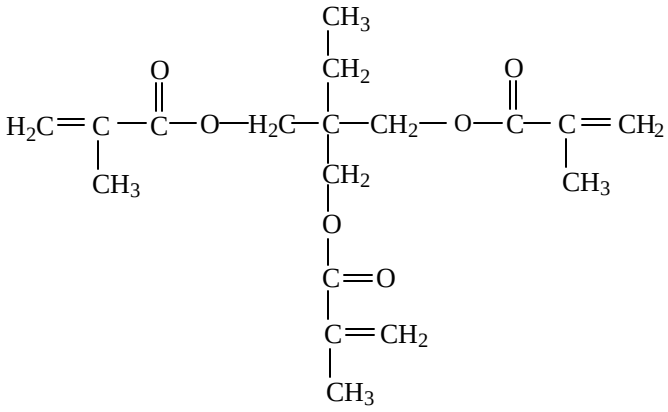
Divinyl benzene (DVB) was obtained as a mixture of the three isomers from Fluka A/G (Switzerland). The active concentration of DVB was 60 weight percent. It was used as crosslinking agent in the preparation of polymer supports for purification of enzymes.

2.1.5 Trimethylolpropane triacrylate

Empirical formula	$C_{15}H_{20}O_6$
Molecular weight	296.32
Density	1.100 g cm^{-3}
Chemical structure	

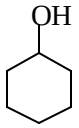
Trimethylolpropane triacrylate (TMPTA), also known as 2-ethyl-2-(hydroxymethyl)-1,3-propanediol triacrylate, was obtained from Aldrich Chemical Co. (USA). It was used as crosslinking agent in the preparation of polymer supports for enzyme purifications.

2.1.6 Trimethylolpropane trimethacrylate

Empirical formula	$C_{18}H_{26}O_6$
Molecular weight	338.40
Density	1.060 g cm^{-3}
Chemical structure	

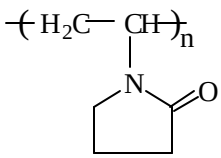
Trimethylolpropane trimethacrylate (TMPTMA), also known as 2-ethyl-2-(hydroxy)-1,3-propanediol trimethacrylate, was obtained from Aldrich Chemical Co. (USA). It was used as crosslinking agent in the preparation of polymer supports for enzyme purifications.

2.1.7 Cyclohexanol

Empirical formula	$C_6H_{12}O$
Molecular weight	100.16
Density	0.948 g cm^{-3}
Chemical structure	

Cyclohexanol was obtained from Aldrich Chemical Co. (USA). It was used as pore-generating solvent in the synthesis of macroporous beaded polymer supports by suspension polymerisation.

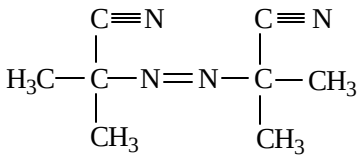
2.1.8 Poly(vinyl pyrrolidone)

Empirical formula	$(C_6H_9NO)_n$
Chemical structure	

Poly (vinyl pyrrolidone) (PVP), was obtained from Polysciences (USA). It was used as protective colloid in the preparation of macroporous polymer supports.

2.1.9 2,2'-Azo bis(isobutyronitrile)

Empirical formula	$C_8H_{12}N_4$
Molecular weight	164.21
Melting point	103-105^oC

Chemical structure 

2,2'-Azo bis(isobutyronitrile) (AIBN), also known as 2,2'-azobis(2-methylpropionitrile), was obtained from Sisco (India). It was used as a free radical initiator for suspension polymerisation to generate macroporous supports.

2.1.10 Ethyl amine

Empirical formula	C_2H_7N
Molecular weight	45.09
Density	0.810 g cm^{-3}
Chemical structure	$CH_3CH_2NH_2$

70 % solution of ethyl amine was obtained from S. D. Fine-Chem. Ltd. (India). It was used as a ligand for the preparation of affinity matrices for purification of penicillin G acylase.

2.1.11 Butyl amine

Empirical formula	$C_4H_{11}N$
Molecular weight	73.14
Density	0.740 g cm^{-3}
Chemical structure	$CH_3(CH_2)_3NH_2$

2.1.12 Hexyl amine

Empirical formula	$C_6H_{15}N$
Molecular weight	101.19
Density	0.766 g cm^{-3}
Chemical structure	$CH_3(CH_2)_5NH_2$

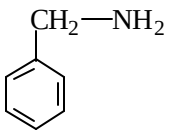
2.1.13 Octyl amine

Empirical formula	$C_8H_{19}N$
Molecular weight	129.25
Density	0.782 g cm^{-3}
Chemical structure	$CH_3(CH_2)_7NH_2$

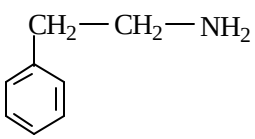
2.1.14 Dodecyl amine

Empirical formula	$C_{12}H_{27}N$
Molecular weight	185.36
Density	0.806 g cm^{-3}
Chemical structure	$CH_3(CH_2)_{11}NH_2$

2.1.15 Benzyl amine

Empirical formula	C_7H_9N
Molecular weight	107.16
Density	0.981 g cm^{-3}
Chemical structure	

2.1.16 Phenethyl amine

Empirical formula	$C_8H_{11}N$
Molecular weight	121.18
Density	0.965 g cm^{-3}
Chemical structure	

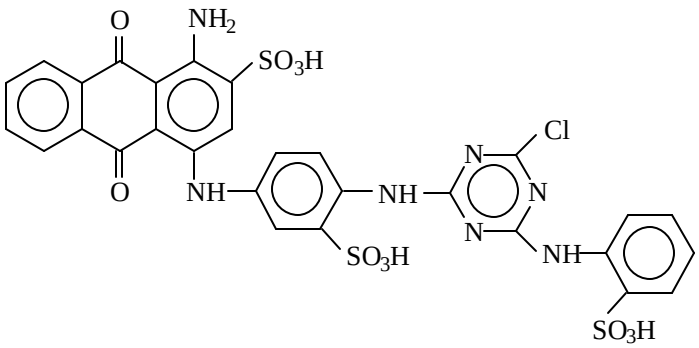
Butyl amine, hexyl amine, octyl amine, dodecyl amine, benzyl amine and phenethyl amine were obtained from Aldrich Chemical Co. (USA). These were used as ligands for preparation of affinity matrices for purification of penicillin G acylase.

2.1.17 Cibacron blue F3G-A

Empirical formula $C_{29}H_{20}ClN_7O_{11}S_3$

Molecular weight 774.20

Chemical structure

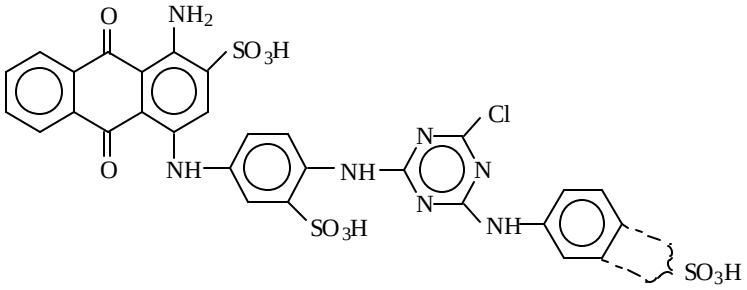


2.1.18 Basilen blue E-3G

Empirical formula $C_{29}H_{20}ClN_7O_{11}S_3$

Molecular weight 774.20

Chemical structure

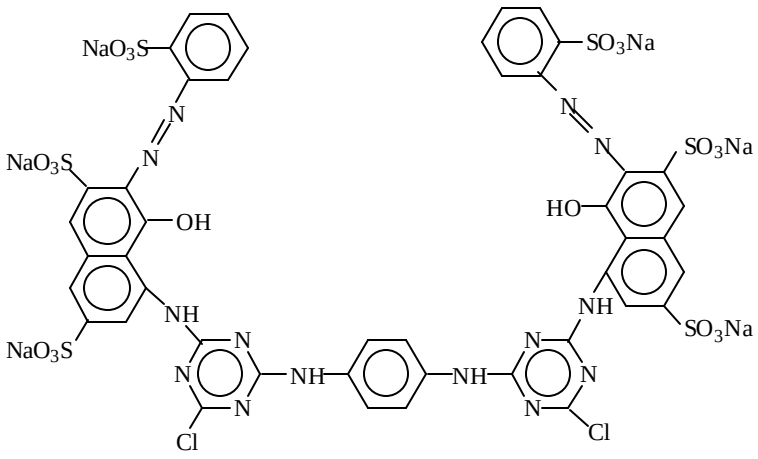


2.1.19 Reactive red 120 (Procion red HE-3B)

Empirical formula $C_{44}H_{24}Cl_2N_{14}Na_6O_{20}S_6$

Molecular weight 1470.00

Chemical structure

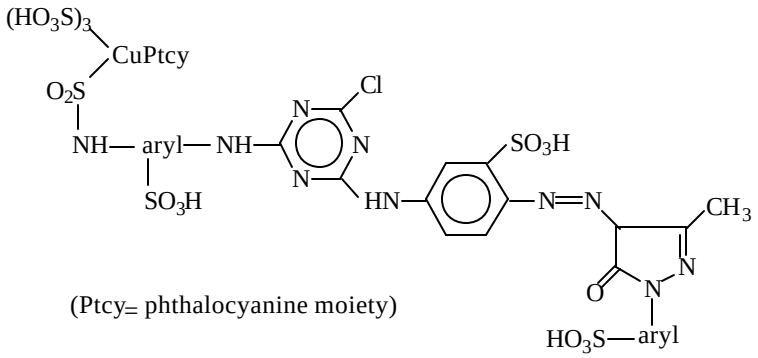


2.1.20 Reactive green 5

Empirical formula $C_{57}H_{41}ClCuN_{18}O_{21}S_7$

Molecular weight 1637.27

Chemical structure



Cibacron blue F3G-A, Basilen blue E-3G, Reactive red 120 and Reactive green 5 were obtained from Sigma Chemical Co. (USA). They were used as ligands in preparation of dye ligand affinity matrices for purification of penicillin G acylase.

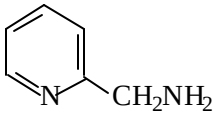
2.1.21 2-Picolylamine

Empirical formula $C_6H_8N_2$

Molecular weight 108.14

Density 1.049 g cm^{-3}

Chemical structure



2-Picolylamine, also known as 2-(aminomethyl) pyridine, was obtained from Aldrich Chemical Co. (USA). It was used as ligand for preparation of metal chelate affinity matrices for penicillin G acylase purification.

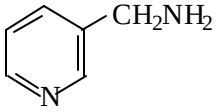
2.1.22 3-Picolylamine

Empirical formula $C_6H_8N_2$

Molecular weight 108.14

Density 1.062 g cm^{-3}

Chemical structure



3-Picolylamine, also known as 3-(aminomethyl) pyridine, was obtained from Aldrich Chemical Co. (USA). It was used as ligand for preparation of metal chelate affinity matrices for penicillin G acylase purification.

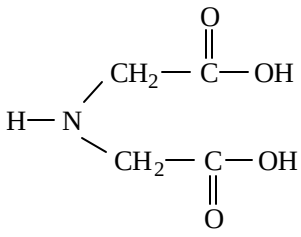
2.1.23 Iminodiacetic acid

Empirical formula $C_4H_7NO_4$

Molecular weight 133.10

Melting point 243^oC

Chemical structure



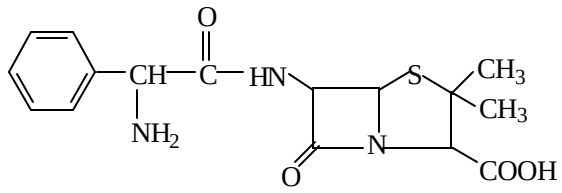
Iminodiacetic acid was obtained from Aldrich Chemical Co. (USA). This was used as a ligand for preparation of metal chelate affinity matrices for penicillin G acylase purification.

2.1.24 Ampicillin

Empirical formula $C_{16}H_{19}N_3O_4S$

Molecular weight 349.41

Chemical structure

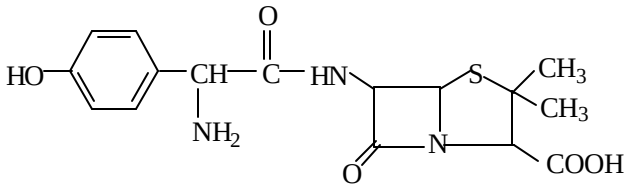


2.1.25 Amoxycillin

Empirical formula $C_{16}H_{19}N_3O_5S$

Molecular weight 365.41

Chemical structure

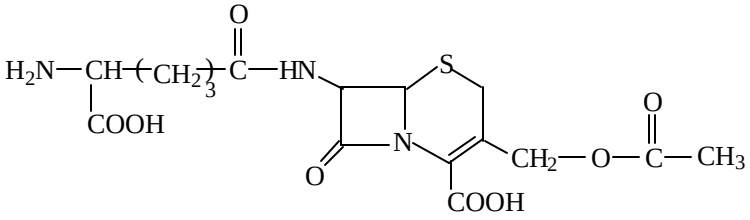


2.1.26 Cephalosporin C

Empirical formula $C_{16}H_{21}N_3O_8S$

Molecular weight 415.12

Chemical structure

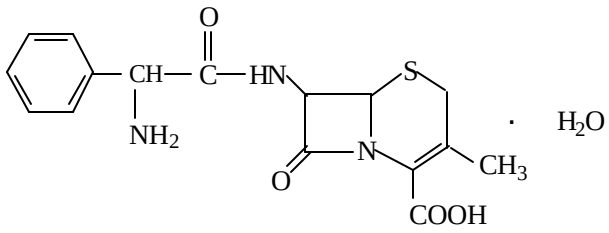


2.1.27 Cephalexin monohydrate

Empirical formula $C_{16}H_{17}N_3O_4S \cdot H_2O$

Molecular weight 365.39

Chemical structure

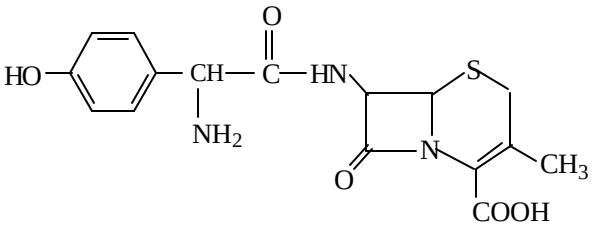


2.1.28 Cephadroxyl

Empirical formula $C_{16}H_{17}N_3O_5S$

Molecular weight 363.33

Chemical structure

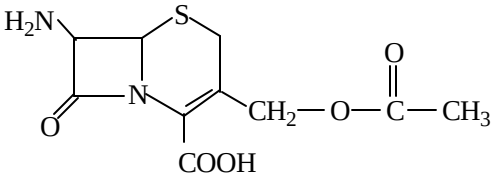


2.1.29 7-Aminocephalosporanic acid (7-ACA)

Empirical formula $C_{10}H_{12}N_2O_5S$

Molecular weight 272.28

Chemical structure

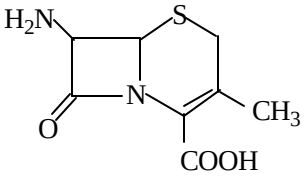


2.1.30 7-Aminodesacetoxy cephalosporanic acid (7-ADCA)

Empirical formula $C_8H_{10}N_2O_3S$

Molecular weight 214.21

Chemical structure



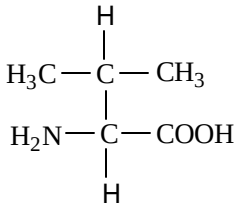
Ampicillin, amoxycillin, cephalosporin C, cephalexin monohydrate, cephadroxyl, 7-aminocephalosporanic acid (7-ACA) and 7-aminodesacetoxy cephalosporanic acid (7-ADCA) were obtained from the production line of M/S Hindustan Antibiotics Ltd. Pimpri (India). They were used as affinity ligands for purification of penicillinase.

2.1.31 DL-Valine

Empirical formula $C_5H_{11}NO_2$

Molecular weight 117.15

Chemical structure



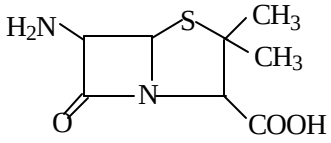
DL-Valine was obtained from S. D. Fine-Chem. Ltd. (India). It was used as affinity ligand for purification of penicillinase.

2.1.32 6-Aminopenicillanic acid

Empirical formula $C_8H_{12}N_2O_3S$

Molecular weight 216.26

Chemical structure



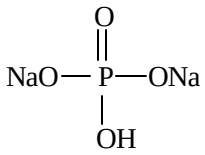
6-Aminopenicillanic acid was obtained from the production line of M/S Hindustan Antibiotics Ltd., Pimpri (India). It was used as affinity ligand for purification of penicillinase. It was also used in the enzyme assay to estimate the activity of crude and purified penicillin G acylase.

2.1.33 Disodium hydrogen phosphate

Empirical formula Na_2HPO_4

Molecular weight 141.96

Chemical structure



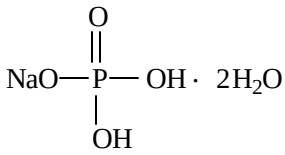
Disodium hydrogen phosphate was obtained from S. D. Fine-Chem. Ltd. (India). It was used in the preparation of buffer to assay the activity of crude and pure penicillin G acylase and also to bind penicillin G acylase and penicillinase on the polymer supports.

2.1.34 Sodium dihydrogen phosphate dihydrate

Empirical formula $NaH_2PO_4 . 2H_2O$

Molecular weight 156.01

Chemical structure



Sodium dihydrogen phosphate was obtained from S. D. Fine-Chem. Ltd. (India). It was used in the preparation of buffer used to assay the activity of crude and pure penicillin G acylase and also to bind penicillin G acylase and penicillinase on the polymer supports.

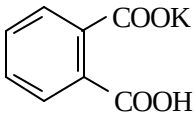
2.1.35 Potassium hydrogen phthalate

Empirical formula $C_8H_5KO_4$

Molecular weight 204.23

Melting point 295-300^oC

Chemical structure



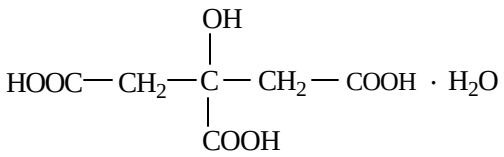
Potassium hydrogen phthalate was obtained from S. D. Fine-Chem. Ltd. (India). It was used as the primary standard to estimate the normality of sodium hydroxide used in the wet analysis to evaluate degree of functionalisation of amine matrices. It was also used in the preparation of buffer solution used in the chelation experiments.

2.1.36 Citric acid monohydrate

Empirical formula $C_6H_8O_7.H_2O$

Molecular weight 210.14

Chemical structure



Citric acid monohydrate was obtained from S. D. Fine-Chem. Ltd. (India). It was used in the preparation of buffer used to quench the enzymatic conversion of penicillin G to 6-aminopenicillanic acid.

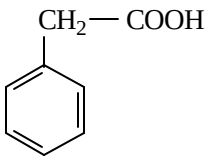
2.1.37 Phenyl acetic acid

Empirical formula $C_8H_8O_2$

Molecular weight 136.15

Melting point 77-78.5^oC

Chemical structure



Phenyl acetic acid (PAA) was obtained from Aldrich Chemical Co. (USA). 0.5 M solution of PAA in 0.05 M phosphate buffer, pH 7.6 was used to elute penicillin G acylase.

2.1.38 Ammonium sulphate

Empirical formula $(NH_4)_2SO_4$

Molecular weight 132.14

Ammonium sulphate was obtained from Loba Chemie (India). This was used to study pseudoaffinity interactions of matrices with penicillin G acylase.

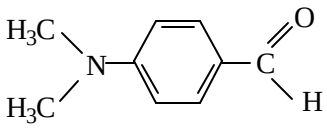
2.1.39 4-(Dimethylamino) benzaldehyde

Empirical formula $C_9H_{11}NO$

Molecular weight 149.19

Melting point 73-75^oC

Chemical structure



4-(Dimethylamino) benzaldehyde (PDAB) was obtained from Aldrich Chemical Co. (USA). It was used as reagent in the assay to estimate activity of penicillin G acylase.

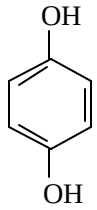
2.1.40 Hydroquinone

Empirical formula $C_6H_6O_2$

Molecular weight 110.11

Melting point 172-175^oC

Chemical structure



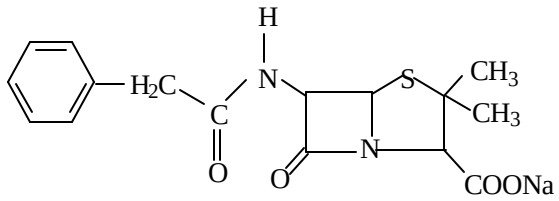
Hydroquinone was obtained from Loba Chemie (India). It was employed as stabiliser for 4-(dimethylamino) benzaldehyde, used to estimate the activity of penicillin G acylase.

2.1.41 Penicillin G sodium salt

Empirical formula $C_{16}H_{17}N_2O_4SNa$

Molecular weight 356.38

Chemical structure



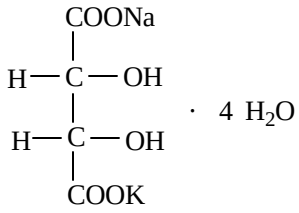
Penicillin G sodium salt was obtained from the production line of M/S Hindustan Antibiotics Ltd., Pimpri (India). It was used as substrate to estimate the activity of penicillin G acylase and penicillinase. Penicillin G potassium salt was also used in some cases.

2.1.42 Sodium potassium tartrate tetrahydrate (Rochelle salt)

Empirical formula $C_4H_4O_6NaK.4H_2O$

Molecular weight 282.22

Chemical structure



Sodium potassium tartrate was obtained from S. D. Fine-Chem. Ltd. (India). It was used as 2 weight percent aqueous solution in the protein estimation.

2.1.43 Copper sulphate pentahydrate

Empirical formula $CuSO_4. 5H_2O$

Molecular weight 249.68

Copper sulphate pentahydrate was obtained from S. D. Fine-Chem. Ltd. (India). It was used as 1 weight percent aqueous solution in the protein estimation. The buffered metal ion solution was used for the chelation experiment.

2.1.44 Folin and Ciocalteu's phenol reagent

Folin and Ciocalteu's phenol reagent was obtained from S. D. Fine-Chem. Ltd. (India). It was used for protein estimation by diluting in 1:1 proportion with distilled water.

2.1.45 Bovine serum albumin

Crystallised bovine serum albumin (BSA) was obtained from Sigma Chemical Co. (USA). It was used to construct the standard plot for protein estimation.

2.1.46 Iodine

Empirical formula I_2

Molecular weight 253.81

Melting point 113^oC

Iodine was obtained from Aldrich Chemical Co. (USA). It was used as reagent for the estimation of activity of penicillinase.

2.1.47 Potassium iodide

Empirical formula KI

Molecular weight 166.01

Potassium iodide was obtained from S. D. Fine-Chem. Ltd. (India). 0.1 N solution of iodine was prepared in 0.1 M potassium iodide and used to estimate the activity of penicillinase.

2.1.48 Gelatin

Gelatin was obtained from E. Merck (India) Ltd. A 1 wt % solution of gelatin in 0.1 M phosphate buffer, pH 7 was used to prevent the sticking of penicillinase to glass during activity estimation.

2.1.49 Starch

Starch was obtained from E. Merck (India) Ltd. It was used as indicator for the estimation of penicillinase activity.

2.1.50 Penicillin G acylase

The crude penicillin G acylase enzyme (penicillin amidase or penicillin amidohydrolase EC 3.5.1.11) from *Escherichia coli* NCIM 2400 was obtained from the production line of M/S Hindustan Antibiotics Ltd., Pimpri (India).

2.1.51 Penicillinase

The crude penicillinase enzyme (β-Lactamase EC 3.5.2.6) from *Bacillus cereus* 5/B NCTC 9946 was obtained from the production line of M/S Hindustan Antibiotics Ltd., Pimpri (India).

All other chemicals e.g. sodium hydroxide, hydrochloric acid, acetic acid, sodium chloride, sodium carbonate, potassium carbonate, potassium bromide, ethanol, methanol etc. were of analytical grade and were obtained from local suppliers.

2.2 Base copolymers

The base copolymer matrices of controlled size and porosity, having functional epoxy groups, were synthesised by suspension polymerisation. The synthesis of base copolymers was conducted in 500 mL double walled cylindrical reactor of 7.5 cm diameter and 15 cm height (Fig. 2.1). The continuous phase comprised of an aqueous solution of poly(vinyl pyrrolidone) (PVP). The discontinuous phase consisted of allyl glycidyl ether (AGE), crosslinking monomer, and polymerisation initiator, azo bis(isobutyronitrile) (AIBN). The ratio of aqueous phase to organic phase was 4:1. The discontinuous organic phase was introduced into the aqueous phase under stirring. The reaction was conducted isothermally at 70^{o}C under a nitrogen overlay for 3 hours. The copolymer beads obtained were then thoroughly washed with water and methanol. The beads were dried and then sieved.

The epoxy content of the copolymer was calculated from the mole fraction of allyl glycidyl ether.

The effect of synthesis variables such as stirring rates, number of agitator blades, the protective colloid concentrations on the particle size and its distribution were examined so as to design optimal system for preparing affinity matrices.

The base copolymer chosen for optimisation of particle size distribution was based on allyl glycidyl ether and ethylene glycol dimethacrylate (AGE-EGDM) with 50 mole % cross-link density (CLD). Here, the mole percent of the crosslinking monomer relative to the moles of comonomer is termed as crosslink density. Conditions used for optimising particle size distribution are presented in Table 2.1.

Once the suspension methodology was standardised, four copolymers were prepared under optimised conditions by varying the mole percent of AGE and EGDM. These are termed as AE-1, AE-2, AE-3 and AE-4. The data is presented in Table 2.2.

Similarly, copolymers differing in hydrophilicity were synthesised by replacing ethylene glycol dimethacrylate with hydrophobic divinyl benzene (DVB), hydrophilic pentaerythritol triacrylate (PETA), very moderately hydrophobic trimethylolpropane trimethacrylate (TMPTMA), and trimethylolpropane triacrylate (TMPTA). The prepared copolymers are termed as AD-1, AP-1, AT-1 and ATa-1 respectively. The details of composition are presented in Table 2.3.

2.3 Affinity matrices

Copolymer beads passing through 140 mesh screen and retained on 270 mesh screen were used for preparing affinity matrices.

2.3.1 Matrices for penicillin G acylase

2.3.1.1 Amine matrices

The reaction of the epoxide group with amines is a nucleophilic addition, in which the nitrogen atom attacks as a rule the least protected carbon atom of the epoxide ring, particularly in neutral and basic medium.[1]

Table 2.1 **Compositions of allyl glycidyl ether (AGE) - ethylene glycol dimethacrylate (EGDM) copolymers synthesised.**

Polymer No.	AGE		EGDM		RPM	No. of blades of agitator[a]	PVP Wt. %[b]
	Volume, mL	Mole	Volume, mL	Mole			
AM-1	9.6	0.0808	7.6	0.0404	100	6	1
AM-2	9.6	0.0808	7.6	0.0404	200	6	1
AM-3	9.6	0.0808	7.6	0.0404	300	6	1
AM-4	9.6	0.0808	7.6	0.0404	400	6	1
AM-5	9.6	0.0808	7.6	0.0404	500	6	1
AM-6	9.6	0.0808	7.6	0.0404	600	6	1
AM-7	9.6	0.0808	7.6	0.0404	400	8	1
AM-8	9.6	0.0808	7.6	0.0404	500	8	1
AM-9	9.6	0.0808	7.6	0.0404	600	8	1
AM-10	9.6	0.0808	7.6	0.0404	400	8	2
AM-11	9.6	0.0808	7.6	0.0404	500	8	2
AM-12	9.6	0.0808	7.6	0.0404	600	8	2
AM-13	9.6	0.0808	7.6	0.0404	400	6	2
AM-14	9.6	0.0808	7.6	0.0404	500	6	2
AM-15	9.6	0.0808	7.6	0.0404	600	6	2
AM-16	9.6	0.0808	7.6	0.0404	400	8	5
AM-17	9.6	0.0808	7.6	0.0404	500	8	5
AM-18	9.6	0.0808	7.6	0.0404	600	8	5

RPM = rotations per minute; PVP = poly (N-vinyl pyrrolidone); a = Ruston turbine; b = weight percent of aqueous solution.

Polymerisation temperature = 70^{o}C; polymerisation time = 3 hours; water = 160 mL; AIBN = 0.6 g; porogen (cyclohexanol) = 22.8 mL.

Table 2.2 Compositions of allyl glycidyl ether (AGE) - ethylene glycol dimethacrylate (EGDM) copolymers synthesised.

Polymer No.	AGE		EGDM		CLD %	Epoxy content Mmole/g
	Volume, mL	Mole	Volume, mL	Mole		
AE-1	9.6	0.0809	7.6	0.0403	50	4.6979
AE-2	6.6	0.0556	10.6	0.0562	100	3.1805
AE-3	5.1	0.0430	12.1	0.0642	150	2.4390
AE-4	4.1	0.0346	13.1	0.0695	200	1.9509

CLD = Crosslink density.

Polymerisation temperature = 70^{o}C; polymerisation time = 3 hours; water = 160 mL; AIBN = 0.6 g; porogen (cyclohexanol) = 22.8 mL; PVP = 3.2 g; RPM = 600; Agitator = Ruston turbine, 8 bladed.

Table 2.3 Compositions of AGE-DVB, AGE-PETA, AGE-TMPTMA, AGE-TMPTA co polymers synthesised.

Polymer No	AGE		CLA	CLA used		CLD, %	Epoxy content, Mmole/ g
	Volume, mL	Mole		Volume, mL	Mole		
AD-1	8.9	0.0750	DVB	8.3	0.0583	50	4.6452
AP-1	10.1	0.0851	PETA	7.1	0.0281	50	4.7046
AT-1	9.1	0.0767	TMPTMA	8.1	0.0254	50	4.4231
ATa-1	9.8	0.0826	TMPTA	7.4	0.0275	50	4.7017

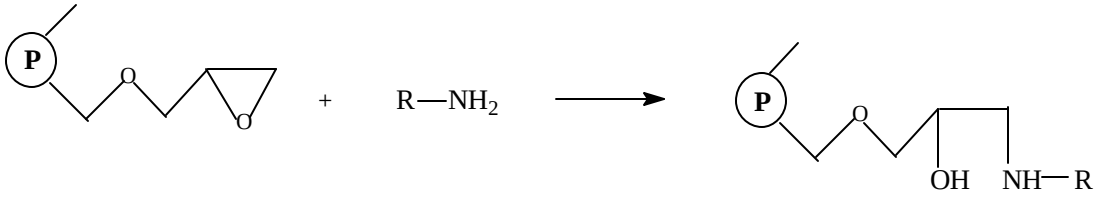
CLA = crosslinking agent; CLD = crosslink density.

Polymerisation temperature = 70^{o}C; polymerisation time = 3 hours; water = 160 mL; AIBN = 0.6 g; porogen (cyclohexanol) = 22.8 mL; PVP = 3.2 g; RPM = 600; Agitator = Ruston turbine, 8 bladed.

2.3.1.1.1 AGE-EGDM (AE-1) amine matrices

2 g (9.3958 mmole epoxy unit) of AE-1 copolymer beads, taken in a 250 mL conical flask, were contacted with different aliphatic and aromatic amines (as in

Table 2.4) in 20 mL ethanol. The flasks were incubated at 25^{o}C for 48 hours. The mole ratio of amine:epoxy group of polymer was 5:1. The resultant amine incorporated beads were filtered, washed with ethanol, distilled water till neutral and dried at 40^{o}C under vacuum. The reaction of amine with epoxy polymer is presented in Scheme 2.1.



Scheme 2.1 Synthesis of amine matrix

The degree of modification of base copolymer was estimated by wet analysis (Section 2.6.1.1) as well as from elemental analysis for nitrogen (Section 2.6.1.2).

Table 2.4 Derivatisation of epoxy groups in AE-1 copolymer with amines.

Polymer matrix code	Amine type	Amine mass 'g'
AE-1-BuA	butyl amine	3.44
AE-1-HA	hexyl amine	4.75
AE-1-OA	octyl amine	6.10
AE-1-DDA	dodecyl amine	8.71
AE-1-BzA	benzyl amine	5.03
AE-1-PhEA	Phenethyl amine	5.70

AE-1 copolymer = 2 g (9.3958 mmole epoxy unit); Reaction temperature = 25^{o}C; Reaction time = 48 hours; Solvent (ethanol) = 20 mL.

2.3.1.1.2 Butyl amine matrices

(i) Hydrophilicity: Butyl amine was covalently bound to various copolymers differing in hydrophilicity as presented in Table 2.3, using the same modification conditions as in Section 2.3.1.1.1. The data for amount of butyl amine used for the reactions is presented in Table 2.5.

(ii) Ligand concentration: The AE-1 copolymer was modified by incubating 2 g of polymer beads (having 9.3958 mmole epoxy units) with 20 mL of an ethanolic solution of butyl amine for 48 hours at 25^{o}C. The mole ratio of butyl amine:epoxy group in polymer were varied as 2.5:1, 5:1, 10:1, 20:1. The resultant butyl amine incorporated beads were filtered, washed with ethanol, distilled water till neutral and dried at 40^{o}C under vacuum. Table 2.6 gives the data for the amount of butyl amine used for the reactions.

Table 2.5 Modification of epoxy groups in AE-1, AD-1, AP-1, AT-1, ATa-1 copolymers with butyl amine.

Polymer matrix code	Base copolymer	Amount of butyl amine used, g
AE-1-BuA	AE-1	3.44
AD-1-BuA	AD-1	3.40
AP-1-BuA	AP-1	3.44
AT-1-BuA	AT-1	3.24
ATa-1-BuA	ATa-1	3.44

Base copolymer = 2 g; Reaction temperature = 25^{o}C; Reaction time = 48 hours; Solvent (ethanol) = 20 mL.

Table 2.6 Butyl amine used for reaction with epoxy groups in AE-1 copolymers.

Polymer matrix code	Ratio of amine:epoxy group in polymer	Butyl amine used, g
AE-1-BuA-2.5	2.5:1	1.72
AE-1-BuA-5.0	5:1	3.44
AE-1-BuA-10	10:1	6.87
AE-1-BuA-20	20:1	13.74

AE-1 copolymer = 2 g (9.3958 mmole epoxy unit); Reaction temperature = 25^{o}C; Reaction time = 48 hours; Solvent (ethanol) = 20 mL.

(iii) Cross-link density: 2 g of AGE-EGDM copolymer beads of differing cross-link density were modified with butyl amine by procedure presented in Section 2.3.1.1.1. The mole ratio of butyl amine:epoxy group in polymer was set at 5:1. The detailed data for modification conditions is shown in Table 2.7.

2.3.1.1.3 Benzyl amine matrices

(i) Hydrophilicity: 2 g of each AE-1, AD-1, AP-1, AT-1, ATa-1 copolymer beads differing in hydrophilicity were modified with benzyl amine by procedure described in Section 2.3.1.1.1. The details of reactions are summarised in Table 2.8.

(ii) Ligand concentration: 2 g (8.8462 mmole epoxy units) of allyl glycidyl ether - trimethylolpropane trimethacrylate copolymer with 50% cross-link density (AT-1 copolymer) were treated with 20 mL of an ethanolic solution containing differing moles of benzyl amine for 48 hours. The mole ratios of benzyl amine:epoxy units in polymer was varied as 1:1, 2.5:1, 5:1 and 10:1. The modified beads were filtered, washed with ethanol, distilled water till neutral and dried at 40^{o}C under vacuum. The detailed data is represented in Table 2.9.

Table 2.7 Butyl amine used for reaction with epoxy groups in AE-1, AE-2, AE-3 and AE-4 copolymer beads.

Polymer matrix code	Base copolymer used	Butyl amine used, g
AE-1-BuA	AE-1	3.44
AE-2-BuA	AE-2	2.33
AE-3-BuA	AE-3	1.78
AE-4-BuA	AE-4	1.43

Base copolymer = 2 g; Reaction temperature = 25^{o}C; Reaction time = 48 hours; Solvent (ethanol) = 20 mL.

2.3.1.1.4 Ethyl amine matrices

2 g of polymer beads (AE-1, AD-1, AP-1, AT-1 and ATa-1) were suspended in 20 mL ethanolic solution of ethyl amine. The amine to epoxy mole ratio was set at 5:1. The flasks were set at ambient for 48 hours. The beads were filtered, washed with ethanol, distilled water till free from amine and dried. The detailed data is presented in Table 2.10.

Table 2.8 Benzyl amine used for reaction with epoxy groups in AE-1, AD-1, AP-1, AT-1 and ATa-1 copolymer beads.

Polymer matrix code	Base copolymer used	Benzyl amine used, g
AE-1-BzA	AE-1	5.03
AD-1-BzA	AD-1	4.98
AP-1-BzA	AP-1	5.04
AT-1-BzA	AT-1	4.74
ATa-1-BzA	ATa-1	5.04

Base copolymer = 2 g; Reaction temperature = 25^{o}C; Reaction time = 48 hours; Solvent (ethanol) = 20 mL.

Table 2.9 Benzyl amine used for reaction with epoxy groups in AT-1 copolymer.

Polymer matrix code	Ratio of amine:epoxy group in polymer	Benzyl amine used, g
AT-BzA-1	1:1	0.95
AT-BzA-2.5	2.5:1	2.37
AT-BzA-5	5:1	4.74
AT-BzA-10	10:1	9.48

AT-1 copolymer = 2 g (8.8462 mmole epoxy unit); Reaction temperature = 25^{o}C; Reaction time = 48 hours; Solvent (ethanol) = 20 mL.

Table 2.10 Ethyl amine used for reaction with epoxy groups in AE-1, AD-1, AP-1, AT-1, ATa-1 copolymer beads.

Polymer matrix code	Base copolymer used	Ethyl amine [a] g
AE-1-EA	AE-1	3.03
AD-1-EA	AD-1	3.00
AP-1-EA	AP-1	3.03
AT-1-EA	AT-1	2.85
ATa-1-EA	ATa-1	3.03

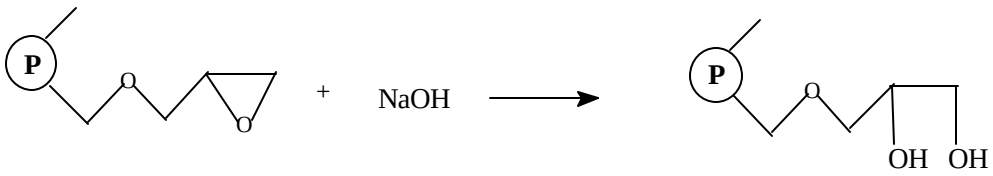
[a] 70 % solution of ethyl amine was used for the modification.
Base copolymer = 2 g; Reaction temperature = 25^{o}C; Reaction time = 48 hours; Solvent (ethanol) = 20 mL.

2.3.1.1.5 Blocking unreacted epoxy groups in amine matrices

1 g of all modified copolymer beads were taken in a 250 mL stoppered conical flask and treated with 50 mL 0.1 M sodium hydroxide solution. The flasks were incubated at 25^{o}C for 24 hours. The beads were filtered and washed thoroughly with distilled water till neutral and dried at 40^{o}C under vacuum.

2.3.1.2 Hydroxyl matrices (gem diol matrices)

The base epoxy copolymers presented in Table 2.3 were hydrolysed to convert the epoxy to gem diol by treatment with alkali. 2 g of beads (AE-1, AD-1, AP-1, AT-1 and ATa-1) taken in 250 mL conical flask were contacted with 100 mL 0.1 N sodium hydroxide solution and left aside at ambient for 48 hours. The beads were filtered, washed with distilled water till neutral and dried in vacuum at 40^{o}C. The reaction is presented in Scheme 2.2.



(Gem diol matrix)

Scheme 2.2 Synthesis of hydroxyl matrix (gem diol matrix)

2.3.1.3 Triazine dye matrices

2 g of AE-1 copolymer beads were suspended in 15 mL distilled water in a water bath at 60^{o}C. A solution of 40 mg of c*ibacron blue F3G-A* in 5 mL distilled water was slowly added to the bead suspension and maintained isothermally at 60^{o}C for 30 minutes. 1.8 g of sodium chloride was added and reaction mixture was kept for 1 hour at 60^{o}C. Temperature was raised to 80^{o}C. 0.18 g of sodium carbonate was added and the mixture was incubated for 2 hours. Reaction mixture was then cooled

to room temperature and filtered. Modified beads were extensively washed with hot water (hot water often speeds this washing process) until the washings were colourless. Finally, beads were washed with 1 M sodium chloride solution and then with water.

Similarly, AE-1 copolymer beads were modified with *reactive green-5, basilen blue E-3G* and *reactive red 120* by procedure presented above. The base copolymer based on allyl glycidyl ether and ethylene glycol dimethacrylate with 200% CLD (AE-4 copolymer) was derivatised with b*asilen blue E-3G* by same procedure. The resulting dye bound polymer is termed as AE-4-BB copolymer.

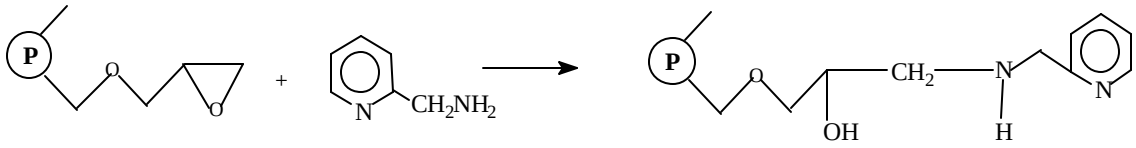
The concentration of *cibacron blue F3G-A, basilen blue E-3G* and *reactive red 120* bound to AE-1 copolymer were determined by hydrolysis[2] of corresponding column material in 6 M hydrochloric acid at 80^{o}C for 90 minutes, followed by spectrophotometric measurement of the released dye as described in Section 2.6.1.3.1. Similarly, concentration of the immobilised *reactive green 5* was estimated by hydrolysis of modified matrix in 50% (v/v) acetic acid[3] at 80^{o}C for 90 minutes. The detailed procedure is described in Section 2.6.1.3.2.

2.3.1.4 Metal chelate affinity matrices

The following chelating copolymers were synthesised by modification of AGE-EGDM copolymers: (i) 2-picolylamine copolymer; (ii) 3-picolylamine copolymer and (iii) Iminodiacetic acid copolymer.

2.3.1.4.1 2-Picolylamine copolymer

An ethanolic solution containing 12.7 g (117.5 mmole) 2-picolylamine was refluxed with 5 g of allyl glycidyl ether-ethylene glycol dimethacrylate (AE-1) copolymer beads (equivalent to 23.5 mmole epoxy unit) for 24 hours. The modified beads were filtered out, washed with ethanol and water. The mole ratio of 2-picolylamine:epoxy group in polymer was 5:1. The modified polymer is termed as AE-1-2PA copolymer. The reaction is presented in Scheme 2.3.



(2-Picolylamine copolymer)

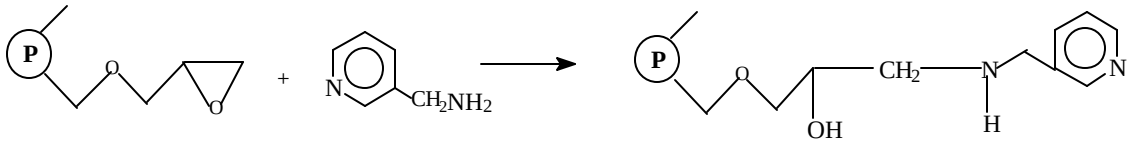
Scheme 2.3 Synthesis of 2-picolylamine copolymer

The elemental analysis: C=60.16%; H=7.37%; N=1.68%. The nitrogen estimation indicated that 1 g of derivatised polymer contains 0.6 mmole of 2-picolylamine, which corresponds approximately to 13% ligand loading with respect to the epoxy content of the copolymer.

Chemical titration: The chemical titration of the protonated form of the chelating copolymer, AE-1-2PA with 0.1 N sodium hydroxide showed 0.9123 mmole of ligand per gram of copolymer, which corresponds approximately 19.42% ligand loading.

2.3.1.4.2 3-Picolylamine copolymer

The 3-picolylamine copolymer (AE-1-3PA) was synthesised by reacting the base copolymer with 3-picolylamine by the same procedure presented in Section 2.3.1.4.1. The reaction is presented in Scheme 2.4.



(3-Picolylamine copolymer)

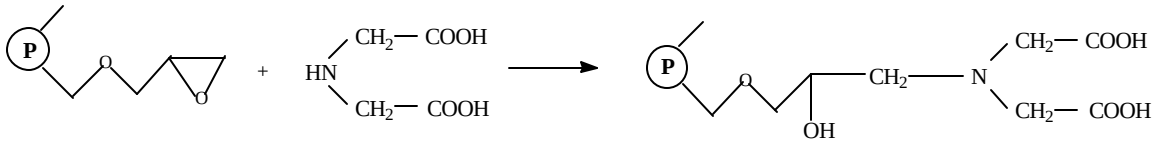
Scheme 2.4 Synthesis of 3-picolylamine copolymer

The elemental analysis: C=53.09%; H=5.53%; N=1.27%. As calculated from nitrogen estimation, AE-1-3PA copolymer contains 0.4535 mmole of 3-picolylamine per gram of copolymer beads, which corresponds to 9.65% ligand loading.

Chemical titration: Chemical titration of AE-1-3PA copolymer showed 15.19% ligand loading, which corresponds to 0.7135 mmole of 3-picolylamine hooked on to 1 g of polymer beads.

2.3.1.4.3 Iminodiacetic acid copolymer

The AGE-EGDM copolymer was modified by refluxing beads of copolymer AE-1 (5.0 g; 23.5 mmole epoxy group) with 50 mL 2 N potassium carbonate solution containing 15.64 g (117.5 mmole) iminodiacetic acid (IDA) for 24 hours. The mole ratio of IDA:epoxy group was 5:1. The resultant IDA incorporated beads (AE-1-IDA) were washed with distilled water, 0.1 N hydrochloric acid, 0.2 N sodium hydroxide solution and finally with distilled water. The reaction of IDA with epoxy polymer is presented in Scheme 2.5.



(Iminodiacetic acid copolymer)

Scheme 2.5 Synthesis of iminodiacetic acid copolymer

Elemental analysis: C=55.71%; H=6.56%; N=0.40%. Amount of the ligand loaded per gram of copolymer was 0.2857 mmole, corresponding to 6.10% ligand density.

Chemical titration: Protonated form of AE-1-IDA copolymer titrated with 0.1 N sodium hydroxide showed 0.3387 mmole of ligand per gram of copolymer, which corresponds approximately to 7.21% ligand loading.

2.3.1.4.4 Blocking unreacted epoxy groups on the chelating copolymer

Unreacted reactive groups on all chelating copolymers were blocked by reacting the derivatised copolymers with 0.1 N sodium hydroxide solution by procedure presented in Section 2.3.1.1.5.

2.3.1.4.5 Chelation experiment

Potassium hydrogen phthalate and sodium hydroxide of analytical grade were used to prepare pH 7 buffer. Copper sulphate pentahydrate, ($CuSO_4.\ 5H_2O$) of analytical reagent grade was used to prepare the stock solution by dissolving 0.1 g of copper salt in the pH 7 buffer and making up to 100 mL in standard volumetric flask with buffer solution. This buffered metal ion solution was used for chelation experiments.

The metal complexation experiments were conducted in batch process. 1 g of chelating copolymer beads was shaken with 100 mL buffered metal ion solution for 24 hours at 25^{o}C. The initial and equilibrium metal ion concentration were determined by atomic absorption spectroscopy (AAS). The amount of coordinated metal ions was calculated from the change in the concentration of metal ion in the supernatant aqueous solution prior to and after chelation.

2.3.2 Matrices for penicillinase

2 g (9.3958 mmole epoxy units) of AE-1 copolymer beads were suspended in 0.1 M phosphate buffer, pH 8.5 having 23.5 mmole of the ligands in a flask (data is presented in Table 2.11) and incubated at room temperature for 48 hours under constant shaking.[4] The copolymer beads were separated by decantation and suspended in 100 mL distilled water, pH of which was adjusted to 10.5 with sodium hydroxide and incubated at room temperature for 2 hours. The beads were washed till neutral and stored in 0.1 M phosphate buffer, pH 7.5 at 4^{o}C.

Similarly, copolymer beads (2 g each) differing in hydrophilicity (i.e. AE-1, AD-1, AP-1, AT-1 and ATa-1) were converted into the cephalexin matrices by procedure described in Section 2.3.2. The data is shown in Table 2.12.

Table 2.11 Ligands used for reaction with epoxy groups in AE-1 copolymer beads.

Polymer matrix code	Ligand	Ligand used, g	0.1 M phosphate buffer, pH 8.5 mL
AE-1-ampi	Ampicilloic acid (Ampicillin)[a]	8.21	600
AE-1-amox	Amoxycilloic acid (Amoxycillin)[a]	8.59	1200
AE-1-apa	6-Aminopenicilloic acid (6-Aminopenicillanic acid)[a]	5.10	400
AE-1-adca	7-Aminodesacetoxy cephalosporoic acid (7-Aminodesacetoxy cephalosporanic acid)[a]	5.03	500
AE-1-aca	7-Aminocephalosporoic acid (7-Aminocephalosporanic acid)[a]	6.40	500
AE-1-cephc	Cephalosporoic acid C (Cephalosporin C)[a]	9.76	600
AE-1-cephl	7-phenylglycinamido cephalosporoic acid (Cephalexin)[a]	8.59	450
AE-1-cephd	7-p-hydroxy phenylglycinamido cephalosporoic acid (Cephadroxyl)[a]	8.54	500
AE-1-val	DL-Valine	2.75	200

[a] Parenthesis gives the parent compound used for binding.
AE-1 copolymer = 2 g (9.3958 mmole epoxy unit); Reaction temperature = 25^{o}C; Reaction time = 48 hours.

Table 2.12 Reaction of cephalexin with AE-1, AD-1, AP-1, AT-1, ATa-1 copolymer beads.

Polymer matrix code	Base copolymer	Cephalexin used, g
AE-cephl	AE-1	8.59
AD-cephl	AD-1	8.49
AP-cephl	AP-1	8.60
AT-cephl	AT-1	8.08
ATa-cephl	ATa-1	8.59

Base copolymer = 2 g; Reaction temperature = 25^{o}C; Reaction time = 48 hours; 0.1 M phosphate buffer, pH 8.5, volume = 450 mL.

2.4 Binding studies

Binding of penicillin G acylase and penicillinase on different matrices were studied.

2.4.1 Matrices for penicillin G acylase

Various matrices such as amine, hydroxyl, triazine dye and metal chelate were used to carry out the binding study of penicillin G acylase.

2.4.1.1 Amine matrices

Column packing: Plastic columns (0.7 cm x 5.5 cm) were used to pack the amine matrices (Section 2.3.1.1). A porous disk was placed at the bottom of the column to hold the matrix. The slurry of the matrix (~ 1 g) prepared in a binding buffer, was packed and equilibrated with the same buffer. Air bubbles were removed from the slurry by gently swirling the gel and tapping the column. Two types of adsorption and elution conditions were performed at room temperature.

Affinity conditions: After the equilibration of column, enzyme extract was loaded on to the column followed by washing with binding buffer, 0.05 M phosphate buffer, pH 7.6. The penicillin G acylase desorption was achieved by 0.5 M phenylacetic acid in 0.05 M phosphate buffer, pH 7.6. Fractions were collected and assayed for protein and enzyme activity.

Pseudoaffinity conditions: The penicillin G acylase extracts were adjusted to an ammonium sulphate concentration of 30% (wt/v) and were clarified through a filter paper before the adsorption step. The affinants were equilibrated with 0.05 M phosphate buffer containing 30% (wt/v) ammonium sulphate. The penicillin G acylase was eluted by 0.05 M phosphate buffer, pH 7.6 devoid of ammonium sulphate.

2.4.1.2 Hydroxyl matrices (gem diol matrices)

The gem diol matrices were packed in a column (0.7 cm x 5.5 cm) and equilibrated with 0.05 M phosphate buffer, pH 7.6. 115 IU of crude, dialysed penicillin G acylase was loaded onto the column. The column was washed with equilibration buffer and eluted with 0.05 M phosphate buffer, pH 7.6 containing 0.5 M phenylacetic acid. Fractions were collected and assayed for protein and enzyme activity.

2.4.1.3 Triazine dye matrices

The column packing of triazine dye matrices was performed by procedure presented in Section 2.4.1.1. The adsorption and elution studies of the penicillin G acylase were carried out under both affinity as well as pseudoaffinity conditions (as described for amine matrices, Section 2.4.1.1).

2.4.1.4 Metal chelate affinity matrices

A slurry of matrix was prepared in a binding buffer (0.025 M phosphate buffer of pH 7.0) and packed in a column by the procedure presented in Section 2.4.1.1. The column was equilibrated with 20 times bed volume of binding buffer and loaded successively with 2 mL of crude penicillin G acylase and 5 mL of 0.025 M phosphate buffer, pH 7.0. The elution of enzyme was achieved by washing the column first with 5 mL of 0.5 M phosphate buffer, pH 7.0 and then with 0.025 M phosphate buffer containing 2 M sodium chloride. The fractions were collected and assayed for protein and enzyme activity.

2.4.2 Matrices for penicillinase

Affinity matrices synthesised as per Section 2.3.2 were used to prepare slurries in the binding buffer, 0.1 M phosphate buffer pH 7.5, packed in a plastic column (0.7 cm x 5.5 cm), equilibrated and then loaded with crude penicillinase. The column was washed with the equilibration buffer and eluted with 0.1 M phosphate buffer, pH 7.5, containing 10% (wt/v) sodium chloride. Fractions were collected and assayed.

2.5 Penicillin G acylase binding capacity of a support

1 g of a matrix was packed in a column according to the procedure described in Section 2.4.1.1. The column was equilibrated with a suitable binding buffer. The enzyme was then added to the support in a definite number of aliquots. Each fraction was collected in a new test tube after each lot of added enzyme had completely entered the gel. Addition of enzyme was continued until the concentration of target molecule in the column effluent equaled its concentration in the original sample. The matrix was washed with binding buffer to remove unbound substances from the column. Elution of the bound enzyme was achieved with a suitable elution buffer. Pooled fractions were assayed for enzyme activity.

Regeneration of matrices: Some of the matrices were regenerated by washing the column successively with elution buffer, distilled water and equilibration (binding) buffer. As a special case, basilen blue E-3G matrix (AE-4-BB) was regenerated for twelve times by washing the column material with elution buffer, 0.1 N NaOH containing 1 M NaCl followed by washing with binding buffer.

2.6 Analysis

The quantitative estimation of various ligands incorporated onto the matrix is described. Similarly methods followed for the estimation of protein and enzyme activity of penicillin G acylase and penicillinase are explained.

2.6.1 Degree of functionalisation

2.6.1.1 Wet analysis (chemical titration) for amine matrices

The chemical titrations[5] were performed to estimate the extent of modification in case of amine and chelated matrices. 0.2 g of the derivatised copolymer beads were converted into the protonated (acid) form by equilibrating with 50 mL 2 N hydrochloric acid. The protonated beads were filtered and thoroughly washed with distilled water to remove adhering acid. The beads were then shaken with 20 mL 0.1 N sodium hydroxide solution at room temperature for 24 hours. The beads were filtered out and washed with distilled water till neutral. The resultant sodium hydroxide solution was titrated with 0.1 N hydrochloric acid solution. The mmole of sodium hydroxide consumed by the protonated chelating copolymers was determined by back titration with hydrochloric acid, which corresponds to the amount of ligand loaded relative to epoxy group on the copolymer.

2.6.1.2 Elemental analysis

The amount of the ligand loaded onto the copolymer was also calculated from the nitrogen content of the modified copolymers. The mmole of ligand / g of copolymer was calculated as:

$$\frac{N \times 10}{n \times 14}$$

Where ‘n’ is the number of nitrogen atoms in the chelating ligand and ‘N’ is the wt % of nitrogen in the copolymer obtained from elemental analysis.

2.6.1.3 Ligand concentration assay for triazine dye matrices

2.6.1.3.1 *Cibacron blue F3G-A, basilen blue E-3G* and *reactive red 120* matrices

The concentration of dyes bound to polymer beads was determined by hydrolysis of column material in 6 M hydrochloric acid at 80^{o}C followed by spectrophotometric determination of the dye released.[6] The procedure was modified slightly. Standard solutions containing 0-10 µg/mL of dyes were prepared by diluting

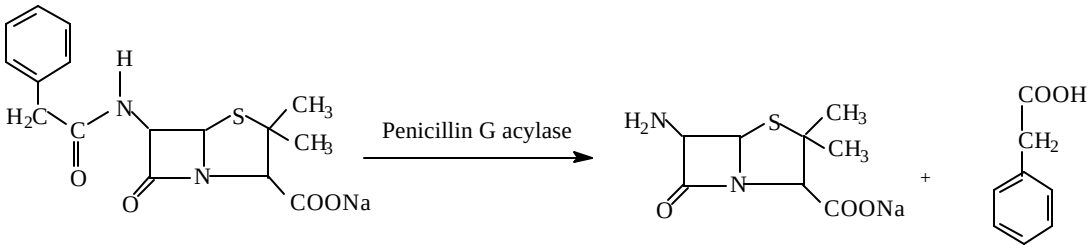
appropriate aliquots of 200 µg/mL solutions of *cibacron blue F3G-A, basilen blue E-3G* and *reactive red 120* in distilled water (water is necessary because of low solubility of dyes in 6 M hydrochloric acid)[7] to a final volume of 10 mL in 6 M hydrochloric acid. All solutions were incubated in water bath at 80°C. Standards were removed after 90 minutes and absorbance at 503 nm, 501 nm and 527 nm for *cibacron blue F3G-A, basilen blue E-3G* and *reactive red 120* respectively were recorded. Same procedure was repeated for the dye bound polymer sample. 10 mg of both blue and red dye polymer beads were incubated with 5 mL 6 M hydrochloric acid at 80°C for 90 minutes. A blank was run using 10 mg of base copolymer instead of blue and red polymer beads.

2.6.1.3.2 *Reactive green-5* matrix

The immobilised ligand concentration was determined by hydrolysis of the green dye matrix in 50% (v/v) acetic acid at 80°C for 1 hour.[3] Standard solutions containing 0-10 µg/mL of dyes were prepared by diluting appropriate aliquots of a 200 µg/mL solution of *reactive green-5* in water to a final volume of 10 mL in 50% (v/v) acetic acid. The standards were incubated in water bath at 80°C for 1 hour. The solutions were removed and absorbance at 689 nm was measured. Same procedure was applied for the green dye copolymer. 10 mg of dye incorporated beads were incubated with 5 mL 50% (v/v) acetic acid at 80°C for 1 hour. The beads were filtered. The absorbance of the resulting solution was measured at the λ_{max}. The blank sample was prepared by the same procedure by using 10 mg of base copolymer in place of dye matrix.

2.6.2 Enzyme activity estimation for penicillin G acylase

Penicillin G acylase catalyses the cleavage of the side chain linear amide bond in penicillin G molecules to produce 6-amino penicillanic acid (6-APA). The reaction is shown in Scheme 2.6.



(6-aminopenicillanic acid)

Scheme 2.6 Cleavage of penicillin G molecule by penicillin G acylase

6-APA produced under assay conditions reacts with p-dimethylamino benzaldehyde to form yellow coloured complex at low pH (2.5). This complex has an absorbance maximum at 415 nm.[8] One unit of enzymatic activity is defined as the amount of enzyme required to produce 1 μmole of 6-APA per minute.

2.6.2.1 Reagents for activity estimation of penicillin G acylase

(i) 0.1 M phosphate buffer, pH 7.0; (ii) Citrate buffer: Citrate buffer was prepared by dissolving 50.5 g of anhydrous citric acid and 6.95 g of anhydrous disodium hydrogen phosphate in 1 litre of distilled water; (iii) Colour reagent: The colour reagent was prepared by dissolving 4.0 g of p-dimethylamino benzaldehyde in 240 mL of absolute ethanol. 0.004 g of hydroquinone was added to impart stability to the reagent; (iv) Working colour reagent: The working colour reagent was prepared by adding 3.5 mL of colour reagent in 6.5 mL citrate buffer; (v) Penicillin G potassium salt (substrate): The solution of substrate was prepared by dissolving 0.372 g penicillin G potassium salt in 10 mL 0.1 M phosphate buffer, pH 7.0.

2.6.2.2 Standard plot of 6-aminopenicillanic acid (6-APA)

Stock solution of 6-APA was prepared by dissolving 0.125 g 6-APA in 0.1 M phosphate buffer of pH 7.0, and used for further studies. Volume of 6-APA was varied from 0.1 mL (0.125 g) to 1 mL (1.250 g) to which the corresponding volume of citrate buffer was added to make the total volume to 4.0 mL in each case. 1 mL of working colour reagent was then added to each sample. The absorbance was read at 415 nm after exactly 3 minutes against the blank prepared under identical conditions

by mixing 1 mL of distilled water with 3.0 mL of citrate buffer and 1 mL of working colour reagent. The standard plot is shown in Figure 2.2.

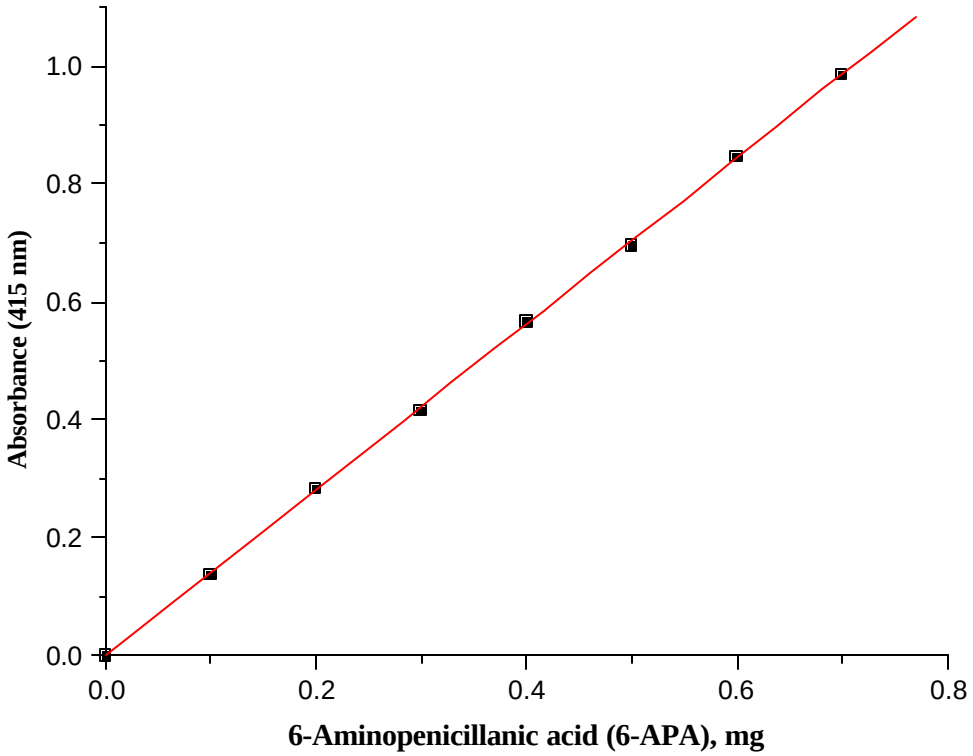


Figure 2.2 Standard plot of 6-Aminopenicillanic acid (6-APA)

2.6.2.3 Penicillin G acylase activity estimation

To 0.2 mL of eluent, in which the penicillin G acylase activity is to be determined, 0.8 mL of 0.1 M phosphate buffer, pH 7.0 was added. This solution (1 mL) was incubated at 40^{o}C for 1 hour with 1 mL of penicillin G potassium substrate solution. Then, 0.2 mL aliquot was withdrawn, added to 3.8 mL of citrate buffer, 1 mL of working colour reagent was added and absorbance against distilled water was measured at 415 nm after 3 minutes. The blank was prepared under identical conditions except for the initial inactivation of the eluent in a boiling water bath for 2 minutes.

2.6.3 Enzyme activity estimation for penicillinase

A modified iodometric method[9] used for the estimation of penicillinase is based on the enzymatic decomposition of penicillin to penicilloic acid, which reacts stoichiometrically with iodine to give a compound that does not produce any colour with starch. The time required to decolourise a known quantity of iodine in presence of sufficient substrate is measured.

2.6.3.1 Reagents for activity estimation of penicillinase

(i) Iodine solution: 0.01 N iodine solution was prepared by dissolving 0.2538 g iodine in 1000 mL 0.1 M potassium iodide; (ii) Sodium penicillin G (substrate) solution: 0.1506 g of sodium penicillin G was dissolved in 0.1 M phosphate buffer, pH 7.0; (iii) Gelatin solution: 0.250 g gelatin was dissolved in 25 mL 0.1 M phosphate buffer; (iv) Starch solution: 1% starch solution was prepared by dissolving 1 g of starch powder in 100 mL distilled water.

One unit of penicillinase is defined as the amount of enzyme that will hydrolyse 1 μmole of sodium penicillin G in one hour at pH 7.0 at 30^{o}C.

2.6.3.2 Penicillinase activity estimation

All reagents were equilibrated at 30^{o}C in a water bath prior to use. To 2 mL of gelatin solution, 0.1 mL of the eluent (in which the penicillinase activity is to be determined) and 1 drop of starch solution were added. 1 mL of sodium penicillin G substrate solution was then added and 2 mL of iodine solution was added within 10-15 seconds. The time to decolourise iodine was recorded with a stopwatch from the time of addition of penicillin solution, which was blown into the reaction mixture from 1 mL pipette. The blank was prepared under identical conditions using water in place of enzyme solution.

2.6.4 Protein estimation

Protein concentration was measured according to Lowry et al. by using bovine serum albumin as a standard. [10]

2.6.4.1 Reagents for protein estimation

(i) Solution A: 2 % wt/v sodium carbonate in 0.1 N sodium hydroxide solution; (ii) Solution B: 1 % wt/v copper sulphate solution; (iii) Solution C: 2 % wt/v sodium potassium tartrate solution; (iv) Solution D: Solution D was prepared by adding 0.5 mL of solution B and solution C each to 50 mL of solution A; (v) Solution E: Solution E was prepared by mixing 1 mL of water with 1 mL of Folin and Ciocalteu's phenol reagent.

2.6.4.2 Standard plot of bovine serum albumin (BSA) for protein estimation

Stock solutions of various concentrations (mg/mL) of BSA were prepared in distilled water. 1 mL of BSA solution was mixed with 5 mL of solution D at room temperature. After 10 minutes, 0.5 mL of solution E was added and after 30 minutes absorbance was read at 660 nm. The standard plot of BSA is shown in Figure 2.3.

2.6.4.3 Protein estimation of the samples

The eluent (0.2 mL) in which the protein estimation was to be carried out was mixed at room temperature with 0.8 mL water and 5 mL of solution D. After 10 minutes, 0.5 mL of solution E was added and after 30 minutes absorbance was measured at 660 nm. A blank was prepared under identical conditions by using 1 mL of water instead of sample.

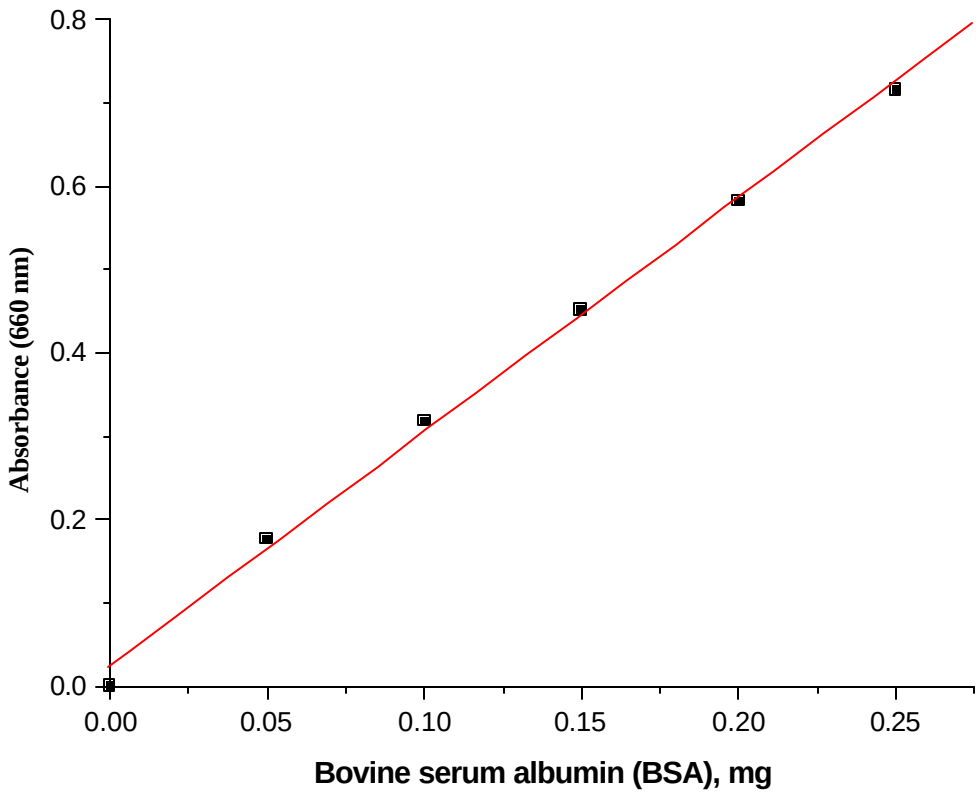


Figure 2.3 Standard plot for protein estimation

2.7 References

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3 RESULTS AND DISCUSSION

3.1 Development of base copolymer (Polymer matrix)

3.1.1 Introduction

One of the most important factors in the development of affinity chromatography is the development of solid supports. This development is the controlling factor of whether and when it will be possible to introduce into practice, procedures that have mainly been used in laboratories so far. At present a wide range of high quality, high performance and economical matrices are available. Still, no perfect matrix material for every application has been established. The general properties include adequate particle size, particle size distribution, particle shape, porosity, sufficient stability (both mechanical and chemical) etc. For large-scale operations good flow properties are paramount, which are highly dependent on particle size, particle size distribution and rigidity. A narrow particle size distribution offers better column capacity. Similarly, spherical particles with high compression resistance impart good flow properties.

Beaded porous polymer particles are widely used as ion exchange resins or packing materials for chromatography. Uniformity of particle size is an important factor in performance in terms of reproducible separation. Therefore, great concern has been taken for the production of single-size (monodisperse) polymer particles. Generally, polymer particles are made by the process of suspension polymerisation, in which fine droplets of monomers are suspended in a dispersion medium with a stabiliser. The most important issue in the practical operation of suspension polymerisation is the control of the average diameter and preferably the size distribution of the product particle. This is due to the fact that there is no way of modifying the particle size after completion of the reaction.[1] The present work deals with the standardisation of particle size and it's distribution based on allyl glycidyl ether-ethylene glycol dimethacrylate (AGE-EGDM) copolymer system with 50 mole % cross-link density (CLD).

3.1.2 AGE-EGDM copolymers

Allyl glycidyl ether (AGE) containing epoxy side groups was chosen as comonomer. Polymers containing epoxy groups are of technical interest as they provide means for the introduction of a variety of reagents into the polymer network.[2-4] The porous AGE-EGDM copolymers were synthesised by suspension polymerisation. Experiments were performed in a 500 mL double walled cylindrical glass reactor thermostated by circulating constant temperature water kept at 70^{o}C. The reactor was fitted with a stirrer, reflux condenser and nitrogen inlet. The continuous phase consisted of 160 mL of aqueous solution of poly(vinyl pyrrolidone). The discontinuous organic phase comprised of 17.2 mL of AGE and EGDM along with 22.8 mL of cyclohexanol. This pore generating solvent is dispersed uniformly together with monomers in a discontinuous phase. During chain growth the pore generating solvent is expelled as droplets within the discontinuous phase. Homopolymerisation in the presence of pore-generating solvent would initially generate pores. These would collapse in the absence of supporting bridges provided by the crosslinking agent in nonporous beads.[5] To ensure an inert atmosphere and to prevent inhibition effects from oxygen, nitrogen was purged continuously in the reactor at least half an hour before and during the course of reaction. Polymerisation was initiated by using 0.6 g of free radical initiator, azobis(isobutyronitrile).

For AGE-EGDM copolymers of a specific composition (crosslink density), we have investigated the effect of three experimental variables: (i) stirring speed, (ii) type of stirrer and (iii) concentration of suspending stabiliser (protective colloid) on the particle size and it's distribution. The variables maintained at constant levels were: relative volumes of the continuous (water) and discontinuous (organic) phase, volume of pore generating solvent, the initiator concentration, reaction temperature and time. The particle size of the gel generally used for affinity chromatography should not exceed 200 μm and should not be less than 5 μm.[6] In the present study it was decided to optimise the reaction variables in such a fashion to get the maximum particles in the range 53-105 μm.

3.1.3 Effect of stirring speed

Among various factors influencing particle size, stirring speed (or more generally, the power of mixing) provides a relatively convenient means of particle size control for most practical purposes. A series of copolymers AM-1 to AM-6 (Table 2.1) were prepared by varying the stirring speed from 100 to 600 rpm. These experiments were carried out at a constant protective colloid concentration of 1 % wt/v. The agitation was performed by 6-bladed Ruston turbine stirrer. Particle size distributions of the beads were estimated by sieving and recording the weight of each size fraction. Data for the particle size distribution is presented in Table 3.1 and graphically shown in Figure 3.1.

Table 3.1 Particle size distribution[a] in AGE-EGDM copolymers AM-1 to AM-6[b]

Polymer code	RPM[c]	weight percent distribution							
		< 20 mesh	20-45 mesh	45-60 mesh	60-80 mesh	80-100 mesh	100-140 mesh	140-270 mesh	> 270 mesh
AM-1	100	41.54	4.40	7.79	2.07	1.66	1.25	0.00	0.00
AM-2	200	17.87	58.05	18.26	1.87	2.34	1.69	0.00	0.00
AM-3	300	4.21	75.18	11.57	2.10	3.15	3.79	0.00	0.00
AM-4	400	0.00	23.74	65.59	2.62	3.66	2.51	1.88	0.00
AM-5	500	0.59	0.70	15.85	8.69	22.30	33.10	18.78	0.00
AM-6	600	0.12	0.37	7.40	4.44	21.33	34.28	30.82	1.23

Experiments were with 1 wt% aqueous poly(vinyl pyrolidone) as continuous phase; a = agitator was 6-bladed Ruston turbine; b = Experimental conditions are in Table 2.1; c = rotations per minute.

It has been observed that the particle size of the copolymer beads decreased with increase in the impeller speed. However, there are limits within which particle size can be controlled by the adjustment of the stirring speed. These limits depend on the size and configuration of polymerisation reactor (including its stirring arrangement). For laboratory preparation involving a total volume of 200 mL, the stirring speed was varied between 100 to 600 rpm. Lower stirring speed may not be sufficient to establish a steady state droplet size distribution, whereas too vigorous a stirring may exceed the shear tolerance of the whole set up.[7] At a stirring speed of 100 rpm, maximum beaded copolymer particles were in diameter range exceeding

850 μm (<20 mesh). The maxima shifted to the range 850-355 μm (20-45 mesh) at a constant stirring speed of 200 and 300 rpm. At 400 rpm, 65% of the particles were obtained in the range of 355-250 μm (45-60 mesh). With further increase in the agitation i.e. at 500 and 600 rpm, maxima shifted to a range 150-105 μm (100-140 mesh). From Figure 3.1 it is clear that larger particles were obtained at a stirring speed of 100 to 300 rpm while an increase in the constant speed from 400 to 600 rpm results in the formation of smaller particles. So for further study, the experiments were conducted by maintaining the speed constant at 400 to 600 rpm.

3.1.4 Effect of stirrer type and suspending stabiliser

The effect of variance in stirrer type on particle size distribution was then studied (Table 3.2 and Figure 3.2). A set of AGE–EGDM copolymers were synthesised by using 8-bladed Ruston turbine stirrer. In a set, three copolymers, AM-7, AM-8 and AM-9 were prepared at the stirring speeds 400, 500 and 600 rpm, respectively. Concentration of suspending stabiliser was kept constant at 1 % wt/v. At a stirring speed of 400 rpm, 36% of the copolymer beads were obtained in the range 180 to 150 μm (80-100 mesh). With an increase in the stirring speed, the maxima shifted to the range 150-105 μm (100-140 mesh). Approximately 50% of the particles were obtained in this range. From Tables 3.1 and 3.2 it is clear that at 500 and 600 rpm, there is no change in the maxima, only percent of copolymer beads in the size range 150-105 μm increased from 30% to about 50% with an increase in the number of agitator blades from 6 to 8. The object was to obtain a maximum of copolymer beads in the diameter range 53-105 μm. Change in the stirrer type did not make any significant influence on particle size distribution at a given set of experimental conditions. So the role of concentration of suspending stabiliser was investigated in the next series of experiments.

The aqueous solution of protective colloid, poly(N-vinyl pyrrolidone) (PVP), forms the continuous phase for the distribution of discontinuous phase comprising the monomers, porogen and polymerisation initiator as nearly uniform droplets. Thus, at a specific agitation rate (using a particular agitator) the formation and distribution of droplets and in turn the bead formation is influenced by the viscosity of the

continuous phase. This in turn is related to the molecular weight and concentration of PVP. By changing the concentration of suspending agent, the bead size can be varied to a large extent. Copolymer beads at different stirring speed (eg. 400, 500 and 600 rpm) were prepared using a continuous phase consisting of 1, 2 and 5 % wt/v of PVP. Agitation was performed by using 8-bladed Ruston turbine stirrer.

Table 3.2 Particle size distribution[a] in AGE-EGDM copolymers AM-7 to AM-9[b]

Polymer code	RPM[a]	weight percent distribution							
		< 20 mesh	20-45 mesh	45-60 mesh	60-80 mesh	80-100 mesh	100-140 mesh	140-270 mesh	> 270 mesh
AM-7	400	0.51	1.44	15.95	28.09	36.52	12.35	5.15	0.00
AM-8	500	1.65	1.65	1.78	1.90	19.42	52.66	20.94	0.00
AM-9	600	0.12	1.05	1.87	1.75	7.49	46.08	40.47	1.17

Experiments were with 1 wt% aqueous poly(vinyl pyrolidone) as continuous phase; a = agitator was 8-bladed Ruston turbine; b = Experimental conditions are in Table 2.1; c = rotations per minute.

Results obtained by using 1 % wt/v PVP solution were discussed in Section 3.1.3. Table 3.3 presents the data for the particle size distribution obtained for a set of experiments carried out using 2 % wt/v aqueous solution of PVP at stirring speeds of 400, 500 and 600 rpm. The data is graphically presented in Figure 3.3. Polymer prepared at 400 rpm (AM-10) showed maxima in the range between 180-150 μm (80-100 mesh). This range was shifted to 105-53 μm (140-270 mesh) when the copolymer beads were synthesised at 500 and 600 rpm (copolymers AM-11 and AM-12 respectively). The fraction of fine particles (less than 105 μm) was seen to increase as the PVP concentration was increased to 2%. At higher stirring speed of 500 rpm (i.e. copolymer AM-11), 55% of the copolymer beads were obtained in the range between 105-53 μm. This percentage increased to 71% at 600 rpm. Thus, with an increase in the stabiliser concentration, coalescence was reduced and the particle size decreased with an increase in stirring speed from 500 to 600 rpm. More uniform particle size was observed with an increase in stirring speed.[8] To support the results obtained from the above set of experiments, another series of copolymers (copolymers AM-13, AM-14, AM-15) were synthesised by using the same PVP concentration (2 % wt/v), but

agitation was performed using a 6-bladed Ruston turbine stirrer. Data obtained for particle size distribution is summarised in Table 3.4 and Figure 3.4. Reaction carried out at 400 rpm produced maximum copolymer particles in the diameter range 180-150 μm (80-100 mesh). At 500 rpm, the percent of copolymer beads ranging between 105-53 μm was 38%. No significant change in particle size distribution was observed when stirring speed was increased from 500 to 600 rpm. With 6-bladed stirrer, even though the maxima was in the same range, the percent of particles was less than that obtained using 8-bladed Ruston turbine stirrer.

Table 3.3 Particle size distribution[a] in AGE-EGDM copolymers AM-10 to AM-12[b]

Polymer code	RPM[a]	Weight percent distribution							
		< 20 mesh	20-45 mesh	45-60 mesh	60-80 mesh	80-100 mesh	100-140 mesh	140-270 mesh	> 270 mesh
AM-10	400	0.11	0.11	14.75	23.28	35.40	14.54	11.36	0.44
AM-11	500	0.00	1.05	3.27	1.29	4.79	30.37	55.14	4.09
AM-12	600	0.12	0.23	1.63	1.63	6.41	15.61	70.87	3.5

Experiments were with 2 wt% aqueous poly(vinyl pyrolidone) as continuous phase; a = agitator was 8-bladed Ruston turbine; b = Experimental conditions are in Table 2.1; c = rotations per minute.

To see the effect of further increase in protective colloid concentration on particle size distribution, copolymerisation experiments were repeated using 5 % wt/v aqueous solution of PVP as the continuous phase. Stirring was performed by 8-bladed Ruston turbine stirrer. The effect of speed was studied in the 400-600 rpm range. Respective copolymers were termed AM-16, AM-17 and AM-18. Data for the particle size distribution is given in Table 3.5 and graphically shown in Figure 3.5. All these copolymers showed the maxima in the same range (105-53 μm) as was observed for the corresponding set of experiments carried out using 2 % wt/v of protective colloid (Table 3.3). However, the fraction of particles obtained in this range, on using 5 % wt/v PVP, is lower than what was achieved with 2 % wt/v PVP. Thus among the various reaction conditions investigated for the standardisation of particle size, the tailor made conditions for synthesising AGE-EGDM copolymer with 50 % CLD, with maximum particles having diameter ranging between 105-53 μm,

are stirring at 600 rpm using 8-bladed Ruston turbine stirrer with 2 % wt/v of protective colloid. Once the reaction conditions were optimised, a variety of copolymers were synthesised under the standardised conditions eg. copolymers AE-1, AE-2, AE-3, AE-4, AD-1, AP-1, AT-1 and ATa-1.

Table 3.4 Particle size distribution[a] in AGE-EGDM copolymers AM-13 to AM-15[b]

Polymer code	RPM[a]	weight percent distribution							
		< 20 mesh	20-45 mesh	45-60 mesh	60-80 mesh	80-100 mesh	100-140 mesh	140-270 mesh	> 270 mesh
AM-13	400	7.56	9.44	6.82	6.61	30.43	26.76	12.07	0.31
AM-14	500	0.31	3.87	5.64	2.51	18.50	29.26	38.24	1.67
AM-15	600	0.11	0.90	8.70	3.28	17.63	27.68	38.87	2.82

Experiments were with 2 wt% aqueous poly(vinyl pyrolidone) as continuous phase; a = agitator was 6-bladed Ruston turbine; b = Experimental conditions are in Table 2.1; c = rotations per minute.

Table 3.5 Particle size distribution[a] in AGE-EGDM copolymers AM-16 to AM-18[b]

Polymer code	RPM[a]	weight percent distribution							
		< 20 mesh	20-45 mesh	45-60 mesh	60-80 mesh	80-100 mesh	100-140 mesh	140-270 mesh	> 270 mesh
AM-16	400	3.97	3.27	2.38	1.39	5.36	25.79	52.57	5.26
AM-17	500	6.84	4.15	2.81	0.98	2.44	8.67	63.74	8.46
AM-18	600	2.49	5.07	4.28	1.69	4.18	13.33	34.03	31.54

Experiments were with 5 wt% aqueous poly(vinyl pyrolidone) as continuous phase; a = agitator was 8-bladed Ruston turbine; b = Experimental conditions are in Table 2.1; c = rotations per minute.

3.2 Penicillin G acylase

6-Aminopenicillanic acid (6-APA) is used in the production of semisynthetic penicillins such as methicillin, ampicillin, amoxycillin, etc. by acylating the molecule. It is produced from penicillin G (Pen G) or penicillin V (Pen V), either chemically or enzymatically. However, economic, environmental and operational advantages of enzymatic process over the chemical route have now been realised using immobilised preparations of purified penicillin G acylase (PA) which catalyses the degradation of

Pen G or Pen V in almost quantitative yields under appropriate conditions of pH.[9] 6-APA production by enzymatic route using immobilised penicillin acylase is recognised as a great success in biotechnology.[10] For the preparation of immobilised PA, it is necessary to first obtain the soluble enzyme in a fairly purified and concentrated form, employing a minimum number of treatment steps. In this process, the production cost of soluble enzyme alone accounts to >70% of the total cost.[11]

Cuatrecasas et al.[12] developed techniques for protein purification using affinity concept, i.e. biospecific recognition of the target protein. The affinity chromatography is one of these biospecific techniques implemented in some industrial applications.[13] Affinity chromatography is subdivided into different classes according to the kinds of affinity ligands and interactions between the biomolecules. A limited number of attempts have been made to use affinity chromatography methods to purify penicillin G acylase. Adsorption and elution using hydrophobic gels and affinity gels have proved to be very beneficial. Various hydrophobic gels used includes leucine-, octyl-, aniline-, hydroxyaniline-, 2,4-dinitroaniline, phenylglycine-, tyrosine-, tryptophan-, ampicillin-, amoxycillin-Sepharose.[14,15] On the other hand, the ligands used in affinity chromatography are 6-APA, penicillin V, p-aminophenyl acetic acid, phenyl acetyl chloride etc.[16]

The present study aims at investigating the effectiveness of polymer matrices of differing characteristics for the separation (isolation) of enzyme penicillin G acylase from *E. coli* NCIM 2400. Allyl glycidyl ether (AGE) containing epoxy side groups, was chosen as comonomer. A number of copolymers were synthesised under controlled experimental conditions by altering dependent variables such as crosslink density and crosslinking monomers. Various ligands (aliphatic amines, aromatic amines, triazine dyes, etc.) were attached to different base copolymers to form the suitable affinity matrices for penicillin G acylase. The effects of following variables on the purification profile of penicillin G acylase were investigated: (i) crosslink density, (ii) hydrophilicity of copolymers and (iii) ligand concentration.

3.2.1 Amine matrices

Amine matrices for penicillin G acylase were synthesised by derivatising the allyl glycidyl ether copolymers with a molar excess of different aliphatic and aromatic amines.

3.2.1.1 AGE-EGDM amine matrices

The porous AGE-EGDM copolymer beads of 50 mole % cross-link density (AE-1 copolymer), with particle size between 53-105 µm, were used as base matrix for the initial screening of amine ligands. The copolymer preparation is presented in Table 2.2. Particles prepared were spherical, as seen from the scanning electron micrograph in Figure 3.6.

5 molar excess of ethanolic solution of different aliphatic and aromatic amines were treated with sieved copolymer beads at 25ºC for 24 hours, the unreacted oxirane groups were blocked by reacting with 0.1 N sodium hydroxide solution at 25ºC for 24 hours and washed with water till neutral to pH. The degree of modification was estimated by chemical titration method[17] as well as from elemental analysis for nitrogen. Table 3.6 presents the amounts of each ligand coupled with matrices.

The amine matrices were tested with partially pure penicillin G acylase to establish whether the interaction between the matrix and the enzyme was due to affinity or hydrophobic interaction. Binding of an enzyme on affinity matrix is due to its interactions with substrate, product, inhibitor, cofactor or their analogues that are ligands covalently linked to an inert solid matrix where as hydrophobic interactions of an enzyme with the hydrophobic matrices are determined by hydrophobic character of enzyme.[16] Separation of the enzyme from other proteins is possible since the hydrophobicity of different proteins like other gross properties, varies.

The binding of penicillin G acylase was studied in presence and absence of 30 % wt/v ammonium sulphate. The elution was achieved either with buffer devoid of

ammonium sulphate [$(NH_4)_2SO_4$] or with buffer containing phenyl acetic acid (PAA). All operations were carried out at the ambient temperature.

Table 3.6 The degree of modification of amine matrices estimated by wet and elemental analysis

Polymer matrix code	Amine type	Elemental analysis			Degree of modification	
		C %	H %	N %	Elemental analysis, %	Wet analysis, %
AE-1-BuA	Butyl amine	63.46	9.20	1.39	21	21
AE-1-HA	Hexyl amine	60.83	7.82	0.95	14	15
AE-1-OA	Octyl amine	56.72	7.38	0.93	14	12
AE-1-DDA	Dodecyl amine	59.45	7.85	1.08	16	19
AE-1-BzA	Benzyl amine	60.67	7.32	0.85	13	11
AE-1-PEA	Phenethyl amine	60.74	6.10	0.72	11	14

The quantitative binding (100%) of penicillin G acylase was achieved on butyl amine matrix (AE-1-BuA), (Table 3.7) in a binding buffer, 0.05M phosphate buffer, pH 7.6 saturated with 30% wt/v ammonium sulphate. 88% of the adsorbed enzyme was eluted with buffer devoid of ammonium sulphate. Only 10% 0f the enzyme was adsorbed in 0.05 M phosphate buffer devoid of ammonium sulphate. Thus, the interaction of the enzyme with AE-1-BuA was totally due to hydrophobic character. This observation can be supported by the following two reasons:

(i) Protein and enzyme molecules are known to undergo conformational changes in presence of salts.[18] Therefore it is possible that in presence of ammonium sulphate, penicillin amidase molecule undergoes a conformational change and the side chain binding site forms a hydrophobic core which interacts with the butyl amine.

(ii) It is well known that structure forming agents such as certain electrolytes e.g. ammonium sulphate [$(NH_4)_2SO_4$], ammonium phosphate [$(NH_4)_2PO_4$] tend to

stabilise the water structure and promote the association of apolar groups, there by strengthening hydrophobic interactions.[19]

Table 3.7 Hydrophobic interaction of penicillin G acylase with butyl amine matrix

Polymer matrix code	30% saturated $(NH_4)_2SO_4$ in binding buffer, 0.05 M PB, pH 7.6	Adsorption %	Eluted with 0.05 M PB, pH 7.6 devoid of $(NH_4)_2SO_4$	Eluted with 0.05 M PB, pH 7.6 containing 0.5 M PAA
			Elution %	Elution %
AE-1-BuA	+	100	88	ND
	-	10	NA	ND

AE-1-BuA: AGE-EGDM copolymer (AE-1) with butyl amine; PB: Phosphate buffer; PAA: Phenylacetic acid; ND: Not determined; NA: Not applicable.

Hexyl amine matrix showed 100% adsorption of penicillin G acylase in presence of ammonium sulphate but only 24% was eluted with the buffer devoid of ammonium sulphate, where as incorporation of 0.5 M PAA in 0.05 M phosphate buffer pH 7.6 results in the elution of 75% of the adsorbed enzyme. Thus overall 99% elution was achieved by applying combination of both elution strategies. In the absence of ammonium sulphate, 76% of the enzyme was adsorbed on the hexyl amine matrix and 90% was eluted with buffer containing 0.5 M PAA. The correct choice of elution conditions to break an affinity interaction is often as important to successful purification as correct choice of binding conditions. The most common elution conditions employ a shift in the composition of mobile phase so that the optimal binding environment created by the binding buffer is lost. The basic approach involves displacement of the bound material by competition with counter ligand.[20] The counter ligand can be the same ligand as is immobilised on the matrix or it can be the substrate, product, inhibitor, cofactor or their analogues of the binding enzyme. Penicillin G acylase catalyses the hydrolysis of penicillin G to 6-amino penicillanic acid (6-APA). Phenyl acetic acid (PAA) is a by-product of this reaction. Therefore when PAA is added to the elution buffer it will effectively compete for the binding sites on the adsorbed molecule and will break the affinity interactions there by eluting

the bound enzyme. With hexyl amine matrix (AE-1-HA) even though the adsorption of penicillin G acylase was 100% in presence of ammonium sulphate, the elution achieved under hydrophobic condition (i.e. in absence of ammonium sulphate) was only 24%. Further elution of bound enzyme was possible under affinity conditions (in presence of PAA). In the absence of ammonium sulphate, 76% of the enzyme molecule was bound to the matrix and 90% of this could be eluted in a binding buffer containing 0.5 M PAA. This clearly indicates that the nature of interaction of enzyme with hexyl amine matrix is 24% hydrophobic and 76% affinity. The results are summarised in Table 3.8.

Table 3.8 Hydrophobic and affinity interaction of penicillin G acylase with hexyl amine matrix

Polymer matrix code	30% saturated $(NH_4)_2SO_4$ in binding buffer, 0.05 M PB, pH 7.6	Adsorption %	Eluted with 0.05 M PB, pH 7.6 devoid of $(NH_4)_2SO_4$	Eluted with 0.05 M PB, pH 7.6 containing 0.5 M PAA
			Elution %	Elution %
AE-1-HA	+	100	24	75
	-	76	NA	90

AE-1-HA: AGE-EGDM copolymer (AE-1) with hexyl amine; PB: Phosphate buffer; PAA: Phenylacetic acid; NA: Not applicable.

Penicillin G acylase was bound to octyl amine matrix (AE-1-OA) quantitatively (i.e. 100%) in presence of ammonium sulphate but no elution was achieved in the absence of ammonium sulphate. As against this, 71% of the adsorbed enzyme was eluted with buffer containing 0.5 M PAA. Even the binding of enzyme was 100% in the absence of ammonium sulphate. About 87% of the bound penicillin G acylase was eluted with buffer containing PAA. Thus the binding of enzyme with octyl amine matrix was totally due to affinity interactions. The results obtained with dodecyl amine matrix (AE-1-DDA) are almost same as for octyl amine matrix. All the units of enzyme loaded on column of dodecyl amine were adsorbed in presence as well as in absence of ammonium sulphate. But the elution was obtained only under affinity conditions. 83% of the adsorbed enzyme was eluted with PAA when

adsorption of enzyme was carried out in a binding buffer with out ammonium sulphate. The adsorption and elution behaviour of penicillin G acylase on these matrices is presented in Table 3.9.

Table 3.9 Affinity interaction of penicillin G acylase with octyl amine and dodecyl amine matrices

Polymer matrix code	30% saturated $(NH_4)_2SO_4$ in binding buffer, 0.05 M PB, pH 7.6	Adsorption %	Eluted with 0.05 M PB, pH 7.6 devoid of $(NH_4)_2SO_4$	Eluted with 0.05 M PB, pH 7.6 containing 0.5 M PAA
			Elution %	Elution %
AE-1-OA	+	100	0	71
	-	100	NA	87
AE-1-DDA	+	100	0	71
	-	100	NA	83

AE-1-OA: AGE-EGDM copolymer (AE-1) with octyl amine; AE-1-DDA: AGE-EGDM copolymer (AE-1) with dodecyl amine; PB: Phosphate buffer; PAA: Phenylacetic acid; NA: Not applicable.

Benzyl amine matrix (AE-1-BzA) containing phenyl acetyl group, which is analogous to the side chain of penicillin G molecule, showed 100% binding of enzyme in absence of ammonium sulphate. Elution was possible with phenyl acetic acid (96%). When the enzyme was saturated with 30% ammonium sulphate, binding remained to the same extent but only 8% of the adsorbed penicillin G acylase was eluted with buffer devoid of ammonium sulphate indicating that interactions are not mainly due to hydrophobic character. Further, 84% of the elution was obtained by addition of appropriate concentration (0.5 M) of phenyl acetic acid in 0.05 M phosphate buffer, pH 7.6. The binding here, was due to affinity of the enzyme towards phenyl acetyl group.

Interactions of enzyme with phenethyl amine matrix (AE-1-PEA) was similar to that with benzyl amine matrix except that the binding efficiencies were marginally lower in absence of ammonium sulphate. This may be due to increase in the spacer arm by CH_2 group. A decrease in affinity binding is known to occur with an increase

in length of spacer arm.[21] In case of phenethyl amine matrix, binding of enzyme was 100% in presence of ammonium sulphate. Enzyme could not be eluted under the hydrophobic conditions (in absence of ammonium sulphate), but the elution was possible under affinity condition brought forth by the incorporation phenyl acetic acid in buffer. In the absence of ammonium sulphate, binding was almost quantitative (97%). The amount of enzyme eluted with PAA was 88%. Table 3.10 gives the data for the adsorption and elution of penicillin G acylase on benzyl amine and phenethyl amine matrices.

Table 3.10 Affinity interaction of penicillin G acylase with benzyl amine and phenethyl amine matrices

Polymer matrix code	30% saturated $(NH_4)_2SO_4$ in binding buffer, 0.05 M PB, pH 7.6	Adsorption %	Eluted with 0.05 M PB, pH 7.6 devoid of $(NH_4)_2SO_4$	Eluted with 0.05 M PB, pH 7.6 containing 0.5 M PAA
			Elution %	Elution %
AE-1-BzA	+	100	8	84
	-	100	NA	96
AE-1-PEA	+	100	0	74
	-	97	NA	88

AE-1-BzA: AGE-EGDM copolymer (AE-1) with benzyl amine; AE-1-PEA: AGE-EGDM copolymer (AE-1) with phenethyl amine; PB: Phosphate buffer; PAA: Phenylacetic acid; NA: Not applicable.

From the results presented in Tables 3.9 and 3.10 it is observed that behaviour of enzyme with amine matrices carrying more than 6 carbon atoms i.e. octyl amine and dodecyl amine is the same as was observed with matrices prepared by coupling with aromatic amines i.e. benzyl amine and phenethyl amine. All these matrices have shown affinity interactions with penicillin G acylase. Aliphatic amines having carbon atom more than six may form a loop that has a net effect equivalent to phenyl ring. Thus, their behaviour shows a resemblance to aromatic system. The interaction of the enzyme with butyl amine matrix with 4 methylene units was totally hydrophobic in character. The enzyme interaction with matrix having 6 methylene units in the ligand

i.e. hexyl amine was neither exclusively affinity nor hydrophobic. Interaction of penicillin G acylase with hexyl amine matrix is 24% hydrophobic and 76% affinity. Adsorption-elution behaviour of penicillin G acylase on all amine matrices are summarised in Fig. 3.7.

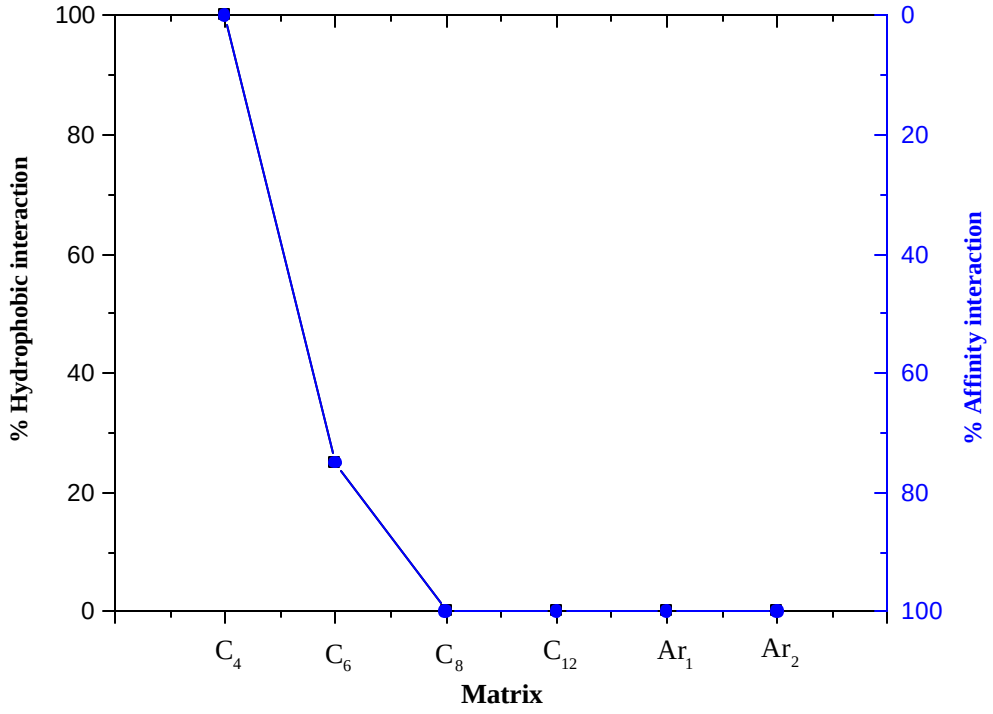


Figure 3.7 **Net behaviour of amine matrices with penicillin G acylase.**

Here, C_4 = AGE-EGDM copolymer with n-butyl amine; C_6 = AGE-EGDM copolymer with n-hexyl amine; C_8 = AGE-EGDM copolymer with n-octylamine; C_{12} = AGE-EGDM copolymer with n-dodecyl amine; Ar_1 = AGE-EGDM copolymer with benzyl amine; Ar_2 = AGE-EGDM copolymer with phenethyl amine

To summarise, among these butyl amine matrix had the best hydrophobic interaction with penicillin G acylase where as benzyl amine matrix showed the best affinity interaction with the enzyme. Hence, these two ligands were covalently bound to the different copolymers and the binding characteristics were investigated towards penicillin G acylase. The binding of these matrices against the different loading concentration of ligands was also studied. The experiments were repeated at least thrice with reproducible results.

3.2.1.2 Butyl amine matrices for hydrophobic interaction

3.2.1.2.1 Hydrophilicity of copolymer

The influence of variance in the hydrophilic/hydrophobic character of the copolymers due to differing cross-linking comonomer on separation of penicillin G acylase was investigated. Series of copolymers were prepared by replacing ethylene glycol dimethacrylate (EGDM) with hydrophobic divinyl benzene (DVB), hydrophilic pentaerythritol triacrylate (PETA), very moderately hydrophobic trimethylolpropane triacrylate (TMPTA) and trimethylolpropane trimethacrylate (TMPTMA) as cross-linking agents for allyl glycidyl ether (AGE). The effect of change of hydrophilic/hydrophobic character on the binding and elution profile of penicillin G acylase was investigated. The copolymer composition was kept invariant. The copolymers were prepared by the same methodology as was standarised for the synthesis of AGE-EGDM copolymers (Section 3.1.2). The compositions of copolymers synthesised are presented in Table 2.3. The copolymer beads obtained in the range 53 μm (270 mesh) to 105 μm (140 mesh) were used for further study. The butyl amine matrices were prepared by repeating procedure indicated in Section 3.2.1.1. The data on the modification of derivatised copolymer beads, as estimated by wet and elemental analysis, is presented in Table 3.11. IR spectra of the base copolymer (AE-1) and butyl amine attached copolymer were recorded (Shimadzu IR-470). 0.001 g of each AGE-EGDM copolymer (AE-1) and the corresponding butyl amine copolymer beads (AE-1-BuA) were separately mixed with 0.1 g of potassium bromide and pressed to form a pellet. The spectra of the pellet were recorded (Figure 3.8). IR Spectra of base copolymer, AE-1 showed a distinct peak at about 910 cm^{-1}, which emanated from the epoxy group,[22] where as spectra of butyl amine matrix (AE-1-BuA) does not show any peak at 910 cm^{-1}, indicating the opening of epoxy groups.

The prepared butyl amine matrices were packed in plastic columns and equilibrated with 0.05 M phosphate buffer, pH 7.6 containing 30% ammonium sulphate. The enzyme was dialysed against 0.05 M phosphate buffer, pH 7.6 in order to remove the low molar mass impurities. The crude dialysed extract with 5.07 IU/mL activity, 1.5 mg/mL protein and 3.38 IU/mg specific activity was made to 30%

saturation with ammonium sulphate. This extract was loaded onto the butyl amine column. The enzyme was desorbed from the column by treatment with 0.05 M phosphate buffer, pH 7.6 devoid of ammonium sulphate. The purification profile of penicillin G acylase on butyl amine matrices is presented in Table 3.12.

Table 3.11 The degree of modification of AE-1, AD-1, AP-1, AT-1 and ATa-1 copolymers with butyl amine

Polymer matrix code	Base copolymer	Cross-linker	Degree of modification	
			Elemental analysis, %	Wet analysis, %
AE-1-BuA	AE-1	EGDM	21	21
AD-1-BuA	AD-1	DVB	11	11
AP-1-BuA	AP-1	PETA	10	9
AT-1-BuA	AT-1	TMPTMA	14	15
ATa-1-BuA	ATa-1	TMPTA	19	17

EGDM= ethylene glycol dimethacrylate; DVB= divinyl benzene; PETA= pentaerythritol triacrylate; TMPTMA= trimethylolpropane trimethacrylate; TMPTA= trimethylolpropane triacrylate.

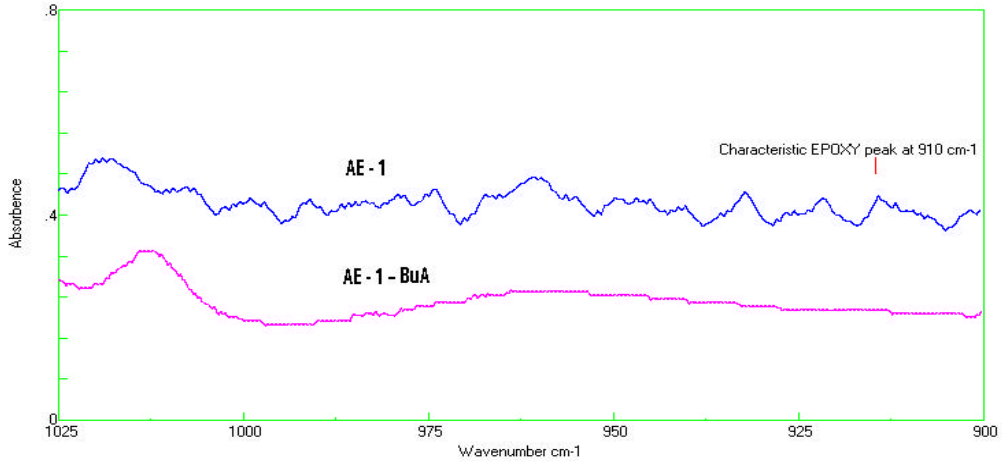


Figure 3.8 Comparative infra-red spectra of AGE-EGDM base copolymer (AE-1) and butyl amine copolymer (AE-1-BuA)

Table 3.12 The purification profile of penicillin G acylase on butyl amine matrices

Polymer matrix code	Adsorption %	Elution %	Recovery %	Specific* activity IU/mg	Fold purification
AE-1-BuA	100.00	80.00	80.00	13.69	4.05
AD-1-BuA	14.70	35.51	5.55	-	-
AP-1-BuA	27.89	91.80	25.6	-	-
AT-1-BuA	100.00	76.00	76.00	7.88	2.33
ATa-1-BuA	17.00	88.00	17.00	-	-

* Crude penicillin G acylase with 5.07 IU/mL activity, 1.5 mg/mL protein and 3.38 IU/mg specific activity was used.

Penicillin G acylase is reported to have a hydrophobic domain at or near the active site.[23] The enzyme prefers hydrophobic environment and binds to a greater extent on hydrophobic matrices. However, with AD-1-BuA matrix, prepared by covalent binding of butyl amine to AGE-DVB copolymer, enzyme just showed 14.70% adsorption, 35.51% elution and 5.55% recovery. The incorporation of DVB in AGE-DVB copolymer increases the hydrophobicity of the polymer, due to the aromatic groups, to the extent of disrupting tertiary structure of the enzyme[5] that inhibits the interaction of enzyme with the matrix. The matrix AP-1-BuA prepared by modifying AGE-PETA copolymer with butyl amine has low adsorption-desorption results for penicillin G acylase. Pentaerythritol triacrylate (PETA) possesses a hydroxyl group. The hydrophilicity of AGE-PETA polymer is the greatest in this series of matrices. The more hydrophilic environment provided by PETA is found to depress the adsorption by hindering the interaction of penicillin G acylase with the polymer.

The adsorption of penicillin G acylase on AE-1-BuA and AT-1-BuA matrices was quantitative. The elution and recovery of the adsorbed enzyme achieved from these columns were 80% and 76% respectively. In case of AT-1-BuA matrix (based on AGE-TMPTMA copolymer) the fold purification of adsorbed enzyme was slightly lower (2.33) relative to that on the corresponding AE-1-BuA matrix having EGDM as crosslinking monomer (4.05). This means that the binding of penicillin G acylase is

not highly selective on AT-1-BuA. Other biomolecules are also being bound on this matrix along with penicillin G acylase. The elution profiles of penicillin G acylase on AE-1-BuA and AT-1-BuA columns are shown in Figures 3.9 and 3.10 respectively.

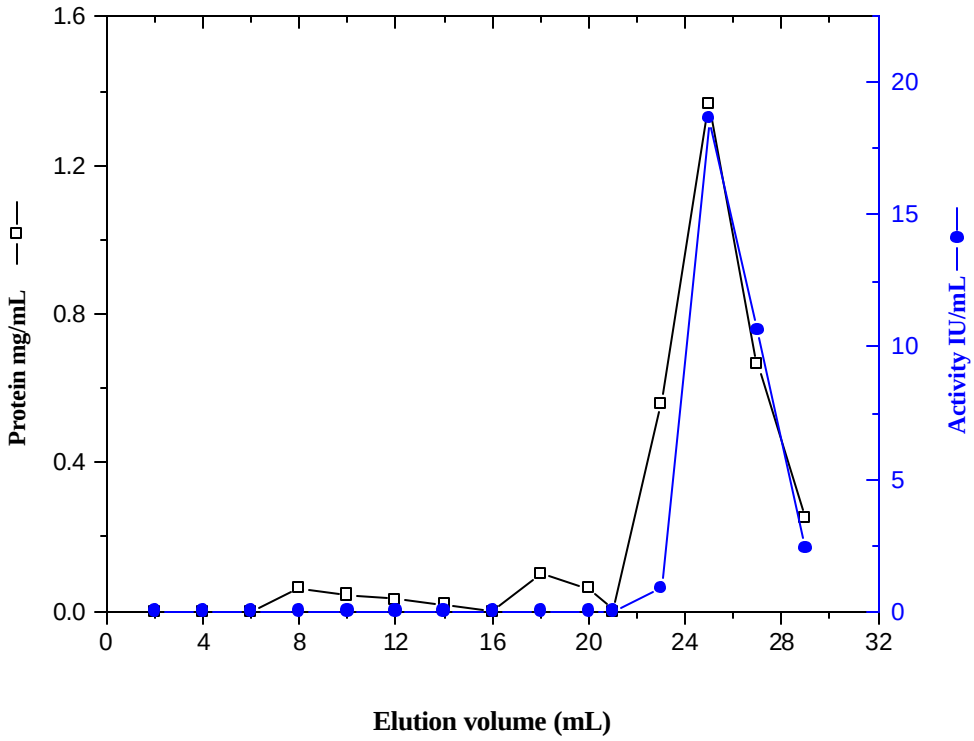


Figure 3.9 **Elution profile of penicillin G acylase from *E. coli* on AE-1-BuA column**

The capacity of the ATa-1-BuA matrix was very low (17% adsorption with 17% recovery). AGE-TMPTA is the base copolymer used to synthesise the above matrix. TMPTA is more hydrophilic than TMPTMA but is less hydrophilic than PETA. TMPTA differs from TMPTMA in the absence of an α-methyl group which is known to contribute to hydrophobic character. This marginal difference dramatically influences the binding characteristics towards penicillin G acylase. Thus it bears out that in the absence of methyl groups penicillin G acylase does not bind to the matrix. The best results are obtained with moderately hydrophobic AGE-EGDM matrices. Hence, ethylene glycol dimethacrylate was identified as the optimal cross-linking

agent for the purification of penicillin G acylase, based on hydrophobic interactions of enzyme with matrices.

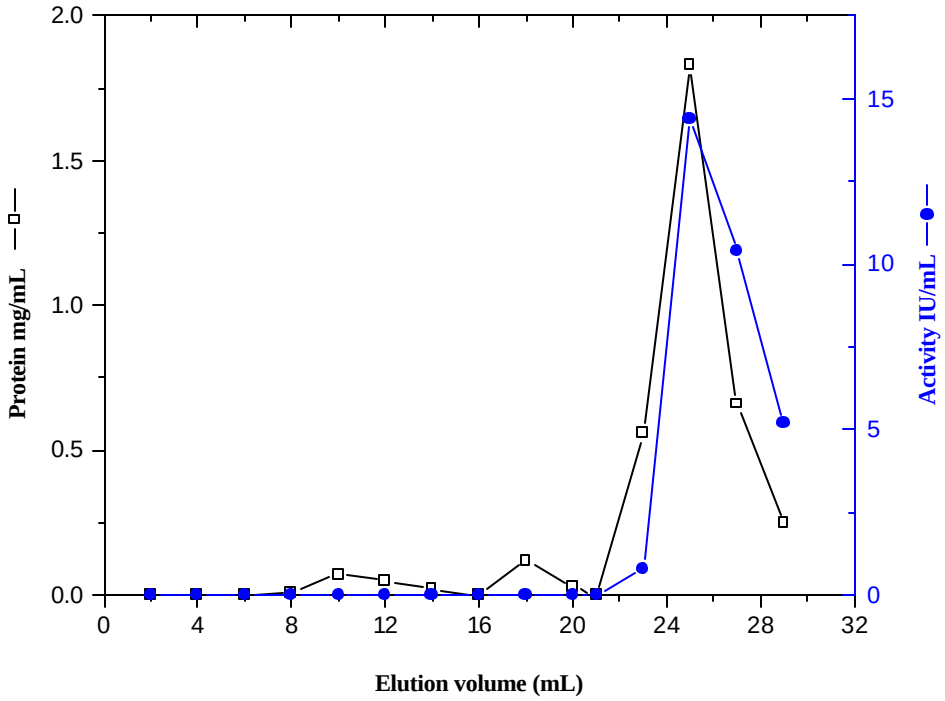


Figure 3.10 Elution profile of penicillin G acylase from *E. coli* on AT-1-BuA column

3.2.1.2.2 Ligand concentration

This study was directed at optimising the ligand binding. Allyl glycidyl ether-ethylene glycol dimethacrylate copolymers having CLD 50 mole % (AE-1 copolymers), prepared by suspension polymerisation (Section 3.2.1.1), were used to couple butyl amine ligand. The study was conducted with a large excess of ligand relative to the active oxirane groups present in the copolymer. The mole ratio of butyl amine:epoxy group were varied as 2.5:1, 5:1, 10:1 and 20:1. The resulting matrices were termed as AE-1-BuA-2.5, AE-1-BuA, AE-1-BuA-10 and AE-1-BuA-20 respectively. The medium used for the reaction was ethanol. The covalent coupling reaction was conducted isothermally at 25ºC for 24 hours. After the coupling reaction some of the active groups that could have remained unreacted were blocked by treating 1 g of amine modified beads with 50 mL 0.1 N NaOH at 25ºC for 24 hours.

Sodium hydroxide is known to open epoxy groups. The beads were washed with water till neutral. Table 3.13 summarises the relative modification of epoxy groups in copolymer by ligand. The highest ligand loading was achieved (21%) at a mole ratio of 5:1. The slurry of the modified gel prepared in 0.05 M phosphate buffer, pH 7.6 containing 30% ammonium sulphate was transferred into a plastic column (0.7cm x 5.5cm) and equilibrated with the same. The crude enzyme was brought to 30% saturation with ammonium sulphate and loaded onto the column. Elution of the enzyme took place in the same buffer without ammonium sulphate. The adsorption-elution profile of penicillin G acylase on butyl amine matrix is given in Table 3.14. From Table 3.13 it is clear that the highest ligand loading was achieved on AE-1-BuA matrix. This matrix manifests the maximum fold purification (4.05), with a specific activity of 13.32 IU/mg. Greater loading of ligand did not increase the percent covalent attachment of ligand since most epoxy groups are not accessible. The trend was also reflected in purification characteristics as seen from Table 3.14.

Table 3.13 The degree of modification of AGE-EGDM (AE-1) copolymers with butyl amine at different ligand concentrations

Polymer matrix code	Ratio of butyl amine:epoxy group in copolymers	Degree of modification	
		Elemental analysis, %	Wet analysis, %
AE-1-BuA-2.5	2.5:1	13	15
AE-1-BuA	5:1	21	21
AE-1-BuA-10	10:1	16	17
AE-1-BuA-20	20:1	15	14

Quantitative adsorptions (100%) with good recoveries were noted with all matrices. Even though the fold purification with AE-1-BuA matrix is higher, it is only marginally better than that with other matrices. Butyl amine is not a bulky ligand. Variation from 13 to 21% (i.e. 8%) in extent of epoxy group modification does not greatly increase the number of enzyme that adsorb on the matrices. The penicillin G acylase and other proteinous impurities are of high molecular weight. There is a

marginal influence of ligand concentration on purification of the enzyme. The hydrophobic interaction chromatography of penicilline G acylase on butyl amine matrices at four concentrations of the bound affinant is graphically presented in Figure 3.11.

Table 3.14 Effect of ligand concentration (butyl amine) on purification of penicillin G acylase

Polymer matrix code	Adsorption %	Elution %	Recovery %	Specific* activity IU/mg	Fold purification
AE-1-BuA-2.5	100	76	76	10.86	3.30
AE-1-BuA	100	80	80	13.32	4.05
AE-1-BuA-10	100	76	76	10.86	3.30
AE-1-BuA-20	100	76	76	12.17	3.70

* Crude penicillin G acylase with 4.67 IU/mL activity, 1.42 mg/mL protein and 3.29 IU/mg specific activity was used.

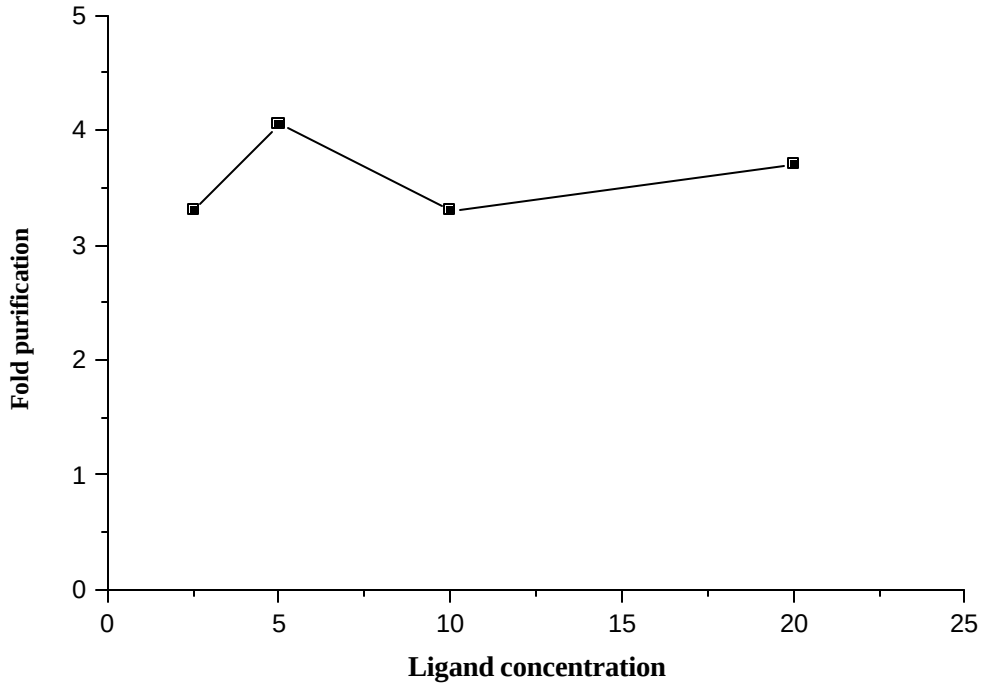


Figure 3.11 Effect of ligand concentration on the purification of penicillin G acylase from *E. coli* on AE-1 butyl amine matrices

3.2.1.2.3 Crosslink density

Series of allyl glycidyl ether-ethylene glycol dimethacrylae copolymers of differing crosslink density (CLD) were synthesised under optimised conditions (Section 3.2.1.1). The mole percent of the crosslinking monomer, EGDM relative to the mole of reactive functional comonomer AGE was varied as 50%, 100%, 150% and 200%. The respective copolymers were termed as AE-1, AE-2, AE-3 and AE-4 copolymers (Table 2.2). Since all the copolymerisation were taken to 100% conversion, the average CLD in all polymers are effectively the relative mole ratio of the crosslinking monomer in feed. All copolymers were coupled with butyl amine ligand by identical procedure (Section 3.2.1.1). The mole ratio of butyl amine:epoxy group in copolymers was set at 5:1. The data for the degree of modification is presented in Table 3.15.

Table 3.15 The degree of modification of AGE-EGDM copolymers of differing cross-link density with butyl amine

Polymer matrix code	Cross-link density %	Degree of modification	
		Elemental analysis, %	Wet analysis, %
AE-1-BuA	50	21	21
AE-2-BuA	100	18	19
AE-3-BuA	150	19	17
AE-4-BuA	200	20	20

Immobilized butyl amine matrices were packed in columns and equilibrated with 0.05 M phosphate buffer, pH 7.6 containing 30% ammonium sulphate (binding buffer). The crude enzyme of activity 4.8 IU/mL, protein 1.67 mg/mL and specific activity 2.87 IU/mg, saturated with 30% ammonium sulphate were loaded on to the columns. The bound enzyme was eluted with the same buffer but without ammonium sulphate. Table 3.16 represents the adsorption and elution behaviour of penicillin G acylase on these matrices.

Table 3.16 Effect on variation in cross-link density on the purification of penicillin G acylase

Polymer matrix code	Adsorption %	Elution %	Recovery %	Specific* activity IU/mg	Fold purification
AE-1-BuA	100	80	80	11.65	4.06
AE-2-BuA	100	81	81	9.14	3.18
AE-3-BuA	100	77	77	8.75	3.05
AE-4-BuA	100	78	78	10.10	3.52

* Crude penicillin G acylase with 4.8 IU/mL activity, 1.67 mg/mL protein and 2.87 IU/mg specific activity was used.

100% adsorption with almost 80% recovery of the penicillin G acylase were recorded with all matrices differing only in CLDs. Higher fold purification was obtained with AE-1-BuA (50% CLD) matrix. Change in the cross-link density, did not alter the purification dramatically. This is an important advantage of the system from commercial perspective. Polymer matrices of higher CLDs are very easy to handle due to greater rigidity. These matrices can withstand higher hydrodynamic pressure of industrial columns and reactors. This enhances the applicability of these matrices to commercial processes. Purification data of penicillin G acylase on butyl amine matrices of four different CLD is presented in Figure 3.12.

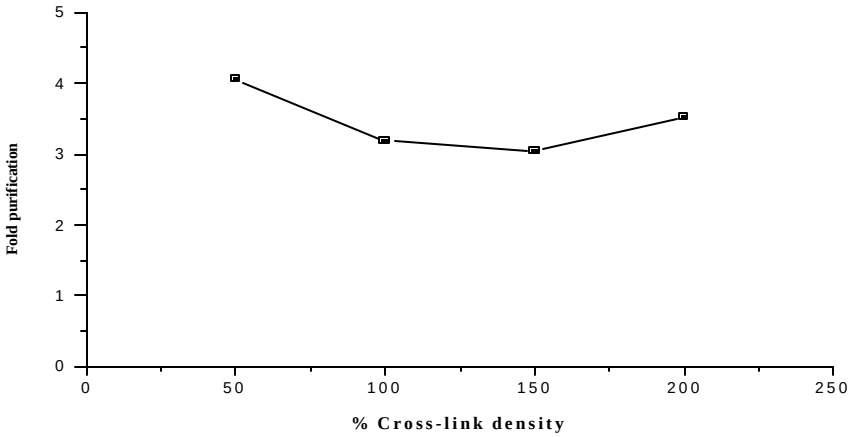


Figure 3.12 Effect of cross-link density of butyl amine modified AGE-EGDM copolymers on the purification of penicillin G acylase from *E. coli*.

3.2.1.2.4 Binding capacity

The word "capacity" is commonly used to mean the extent to which a target molecule can be selectively bound per unit volume or mass of support material. The total binding capacity of an affinity support is an important parameter to determine how much sample can be processed in a given time. Capacity measurements are of critical importance to establish usefulness of any affinity support, not only those meant for purification purposes, but also supports used in catalysis, modification or analytical applications.

AE-1-BuA (0.5 g) matrix prepared as described in Section 3.2.1 was packed in a column. The equilibration of the column material was performed in a binding buffer, 0.05 M phosphate buffer, pH 7.6 containing 30% ammonium sulphate. Crude dialysed enzyme saturated with 30% ammonium sulphate having activity 5.33 IU/mL was loaded in fractions. Each fraction was collected in separate test tube and assayed for enzyme activity. Volumes of enzyme loaded on the column and the eluant were identical, since the loaded fractions were collected separately. The column was washed with binding buffer and elution of enzyme, with 76% recovery, was achieved in 0.05 M phosphate buffer, pH 7.6 devoid of ammonium sulphate. In case of AE-1-BuA matrix, column was fully saturated with enzyme on loading 27 mL of crude enzyme. The enzyme then started to elute out with the same activity. The column was then washed with binding buffer. Excess enzyme adhering to the column was washed out. The elution profile of penicillin G acylase on butyl amine matrix, AE-1-BuA is graphically presented in Figure 3.13.

From graph it is clear that quantitative adsorption of penicillin G acylase occurred upto 22 mL of enzyme loading. After that enzyme started to appear partially in the eluant. The column was completely saturated at a enzyme loading of 27 mL, since the enzyme in the eluant equaled its concentration in the solution being loaded. Loading of enzyme was continued upto 30 mL followed by washing with binding buffer to eliminate non-bound enzyme molecules from the column. The binding capacity achieved per gram dry weight of matrix was 240.6 IU. While measuring the capacity of the support it is important to over-load the matrix significantly with target

molecule to negate the flow rate dependency. In general, the faster the flow through an affinity support, the lower the apparent binding capacity for a particular target molecule.[12] Flow rate through an affinity matrix has a significant effect on its capacity. When sample containing target molecules is applied to a column, the faster the flow of the sample through the column the less efficient is the capture of target molecules. As linear flow rate increases, the diffusion rate and contact time limitations permit fewer target molecules to find and bind to the immobilised affinity ligand. Thus, the faster the flow the less efficient is the removal of the target molecule from solution resulting in a drop in the apparent binding capacity of support material. Hence, it is necessary to overload the column to nullify the effect of flow rate on binding capacity.

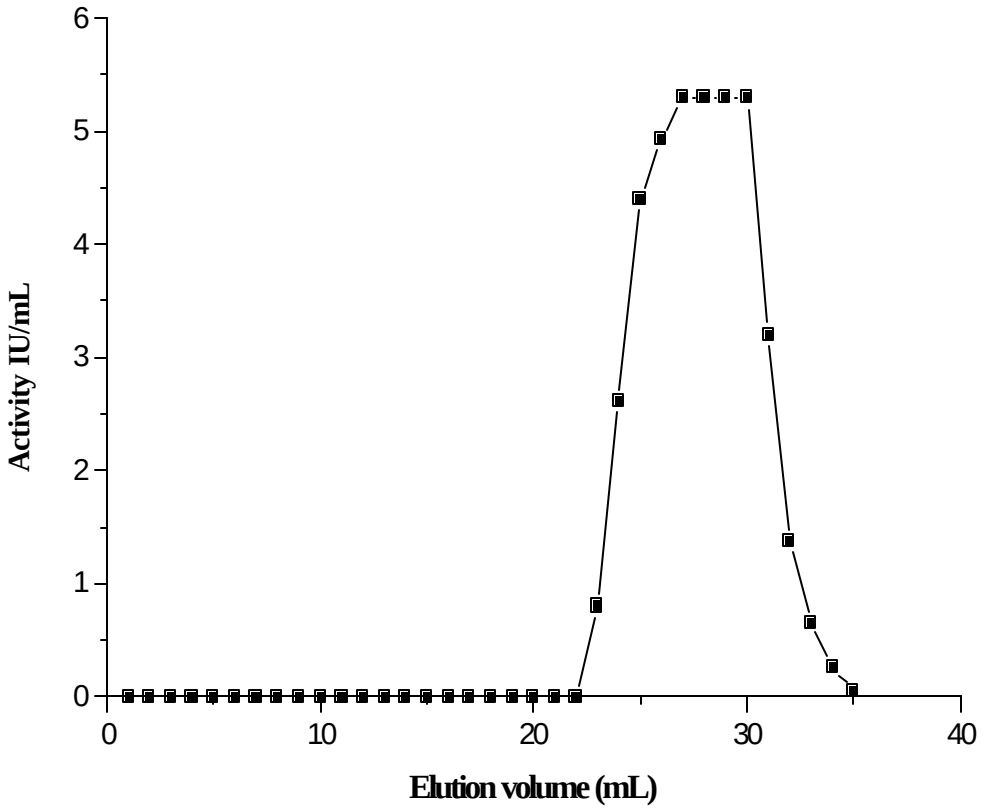


Figure 3.13 **Elution profile of penicillin G acylase from *E. coli* on AE-1-BuA column for estimating its binding capacity**

3.2.1.3 Benzyl amine matrices for affinity interactions

3.2.1.3.1 Hydrophilicity of copolymer

Macroporous beaded copolymers of allyl glycidyl ether differing in hydrophilicity were tested for their interaction with penicillin G acylase. Five sets of copolymers were prepared with various crosslinkers such as ethylene glycol dimethacrylate (EGDM), divinyl benzene (DVB), pentaerythritol triacrylate (PETA), trimethylol propane trimethacrylate (TMPTMA) and trimethylol propane triacrylate (TMPTA). The respective copolymers were termed as AE-1, AD-1, AP-1, AT-1, and ATa-1. The copolymers with 50% CLD were prepared by suspension polymerisation technique as described earlier (Section 3.2.1.2.1). The polymer beads with particle size ranging between 53-105 μm, obtained after sieving, were used in the synthesis of benzyl amine matrices. The coupling reactions were carried out at room temperature as described in Section 3.2.1.1. The loading ratio of benzyl amine to epoxy groups in polymers was set at 5:1. Table 3.17 summarises the data on benzyl amine coupled copolymers.

Table 3.17 The degree of modification of AE-1, AD-1, AP-1, AT-1 and ATa-1 copolymers with benzyl amine

Polymer matrix code	Base copolymer	Crosslinker	Degree of modification	
			Elemental analysis, %	Wet analysis, %
AE-1-BzA	AE-1	EGDM	12	11
AD-1-BzA	AD-1	DVB	10	11
AP-1-BzA	AP-1	PETA	11	9
AT-1-BzA	AT-1	TMPTMA	14	15
ATa-1-BzA	ATa-1	TMPTA	15	17

Affinants were packed in plastic columns (0.7 cm x 5.5 cm). The equilibration of a column was performed in a binding buffer, 0.05 M phosphate buffer, pH 7.6. Partially pure enzyme with activity 16 IU/mL was loaded to study the adsorption-

elution behaviour of enzyme with the affinant. The column was washed with binding buffer and the elution was effected with 0.05 M phosphate buffer containing 0.5 M phenyl acetic acid. All operations were carried out at room temperature. Table 3.18 gives the data for the adsorption and elution pattern of penicillin G acylase on benzyl amine matrices.

Table 3.18 The adsorption-elution profile of penicillin G acylase on benzyl amine matrices with different hydrophilicity

Polymer matrix code	Adsorption %	Elution %	Recovery %
AE-1-BzA	87	83	73
AD-1-BzA	7	-	-
AP-1-BzA	-	-	-
AT-1-BzA	100	82	82
ATa-1-BzA	-	-	-

Penicillin G acylase prefers a hydrophobic environment.[23] DVB is highly hydrophobic. Hence, there is a great tendency towards the disruption of the tertiary structure of penicillin G acylase upon interaction with AGE-DVB copolymer linked to benzyl amine (AD-1-BzA matrix). Obviously the adsorption capacity of AD-1-BzA matrix is very low. The cross-linker pentaerythritol triacrylate (PETA) is hydrophilic in nature. The greater hydrophilicity of AGE-PETA copolymer would presents a partition problem for the interaction with enzyme, which is ultimately reflected in the adsorption of enzyme on the AP-1-BzA matrix (0%). Benzyl amine bound on AGE-TMPTA copolymer (termed as ATa-1-BzA matrix), also did not interact with penicillin G acylase due to partitioning effects. On the other hand quantitative adsorption of enzyme was achieved with AE-1-BzA and AT-1-BzA matrices. These matrices were prepared by the covalent coupling of benzyl amine to epoxy group of allyl glycidyl ether copolymers cross-linked with marginally hydrophobic EGDM and TMPTMA respectively. EGDM is more hydrophobic than PETA and TMPTA but is less hydrophobic than DVB. The hydrophobicity of TMPTMA differs only marginally from that of EGDM, but is greater than TMPTA.

The results obtained from the above study very clearly indicate that the range of hydrophobicity provided by AGE-EGDM and AGE-TMPTMA copolymers incorporated with benzyl amine are optimal for interaction with partially pure enzyme. These two affinity matrices were investigated for the purification of crude penicillin G acylase. Column procedure used for the adsorption and elution of the enzyme was same as discussed earlier. Crude enzyme used for purification had specific activity of 2.38 IU/mg. Data obtained in typical experiments are presented in Table 3.19. On passing the crude enzyme through AT-1-BzA column, the specific activity of penicillin G acylase increased to 12.05 IU/mg. The enzyme recovered in this 5.06 fold purification was 72.61%. The performance of AE-1-BzA matrix was rather poor. Only 23.63% adsorption was noted when treated with crude penicillin G acylase as against 87% adsorption when treated with partially purified enzyme (Table 3.18). This points to a preferential adsorption of impurities present in crude enzyme. Further enzyme purification studies were conducted with AGE-TMPTMA copolymer coupled to benzyl amine (AT-1-BzA). Chromatography of crude extract of penicillin G acylase from *E coli* on this affinity matrix is illustrated in Figure 3.14.

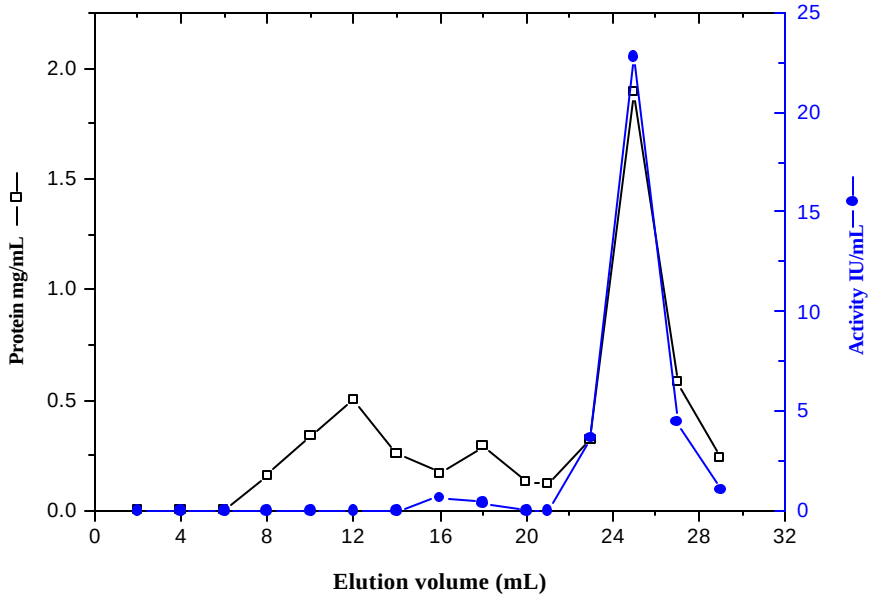


Figure 3.14 **Elution profile of penicillin G acylase from *E. coli* on AT-1-BzA column**

Table 3.19 The purification profile of penicillin G acylase on AGE-EGDM and AGE-TMPTMA-benzyl amine matrices

Polymer matrix code	Adsorption %	Elution %	Recovery %	Specific* activity IU/mg	Fold purification
AE-1-BzA	23.63	76.92	18.18	-	-
AT-1-BzA	91.57	79.37	72.61	12.05	5.06

Crude penicillin G acylase with 6.14 IU/mL activity, 2.58 mg/mL protein and 2.38 IU/mg specific activity was used.

3.2.1.3.2 Ligand concentration

Macroporous beaded copolymers of allyl glycidyl ether-trimethylolpropane trimethacrylate with 50% CLD (AT-1 copolymer) were covalently linked with benzyl amine. The ethanolic solution of benzyl amine was treated with oxirane copolymers at 25^{o}C for 24 hours. The mole ratios of benzyl amine:epoxy group in polymer was varied as 1:1, 2.5:1, 5:1 and 10:1. After the coupling reaction, unreacted epoxy groups were blocked by treating the amine incorporated beads with 0.1 N sodium hydroxide for 24 hours at 25^{o}C. The beads were thoroughly washed with distilled water till neutral. Table 3.20 presents the data on the coupling of benzyl amine to the matrix.

Table 3.20 Modification of AGE-TMPTMA (AT-1) copolymers with benzyl amine at different ligand concentrations

Polymer matrix code	Ratio of benzyl amine:epoxy group	Degree of modification	
		Elemental analysis, %	Wet analysis, %
AT-1-BzA-1	1:1	9	10
AT-1-BzA-2.5	2.5:1	10	11
AT-1-BzA	5:1	14	15
AT-1-BzA-10	10:1	12	13

Immobilised benzyl amine matrices were packed in a column. The column was equilibrated by washing with 0.05 M phosphate buffer, pH 7.6 (binding buffer).

Crude enzyme (2.38 IU/mg), was applied to the column, followed by washing with the binding buffer. The adsorbed enzyme was eluted with 0.05 M phosphate buffer, pH 7.6 containing 0.5 M phenyl acetic acid. The adsorption-elution data of penicillin G acylase is presented in Table 3.21. AT-1-BzA-1 and AT-1-BzA-2.5 matrices were prepared at molar ratios of benzyl amine:epoxy as 1:1 and 2.5:1 respectively. The concentration of benzyl amine immobilised on these gels were in the range 9% to 11%. Adsorption of penicillin G acylase on both matrices were low (50.63% and 39.36% respectively). Lower values of adsorption indicate poor enzyme-matrix interaction due to lower ligand density. Maximum ligand loading was noted with AT-1-BzA matrix (5:1 = benzyl amine:epoxy group in copolymer). The specific activity of eluted enzyme increased from 2.38 IU/mg to 12.05 IU/mg, resulting in 5.06 fold enzyme purification. However, a further increase in ligand loading (10:1 = benzyl amine:epoxy group, AT-1-BzA-10 matrix) had an adverse effect on adsorption-elution profile of enzyme. With an increase in the mole ratio of benzyl amine relative to the epoxy groups present in the matrix, the degree of modification was found to decrease. With an increase in the relative concentration of benzyl amine, the aromatic ring of benzyl amine may cause steric hindrance, which results in poor interaction of the ligand with active epoxy group and lowers covalent binding of ligand.

Table 3.21 Effect of ligand concentration (benzyl amine) on purification of penicillin G acylase

Polymer matrix code	Adsorption %	Elution %	Recovery %	Specific* activity IU/mg	Fold purification
AT-1-BzA-1	50.63	91.26	46.21	-	-
AT-1-BzA-2.5	39.36	100.00	39.36	-	-
AT-1-BzA	91.50	79.37	72.61	12.05	5.06
AT-1-BzA-10	64.54	95.66	61.75	-	-

* Crude penicillin G acylase with 6.14 IU/mL activity, 2.58 mg/mL protein and 2.38 IU/mg specific activity was used.

3.2.1.3.3 Binding capacity

Elution profile of penicillin G acylase on AT-1-BzA matrix, for estimating the binding capacity is graphically presented in Figure 3.15. Experiment was performed as presented in Section 3.2.1.2.4. Adsorption of penicillin G acylase was 100% till 24 mL of enzyme loading. Continuation of enzyme loading resulted in a partial binding of enzyme. After saturation of column, matrix was washed away with the binding buffer. Binding capacity was found to be 248.2 IU per gram of dry matrix.

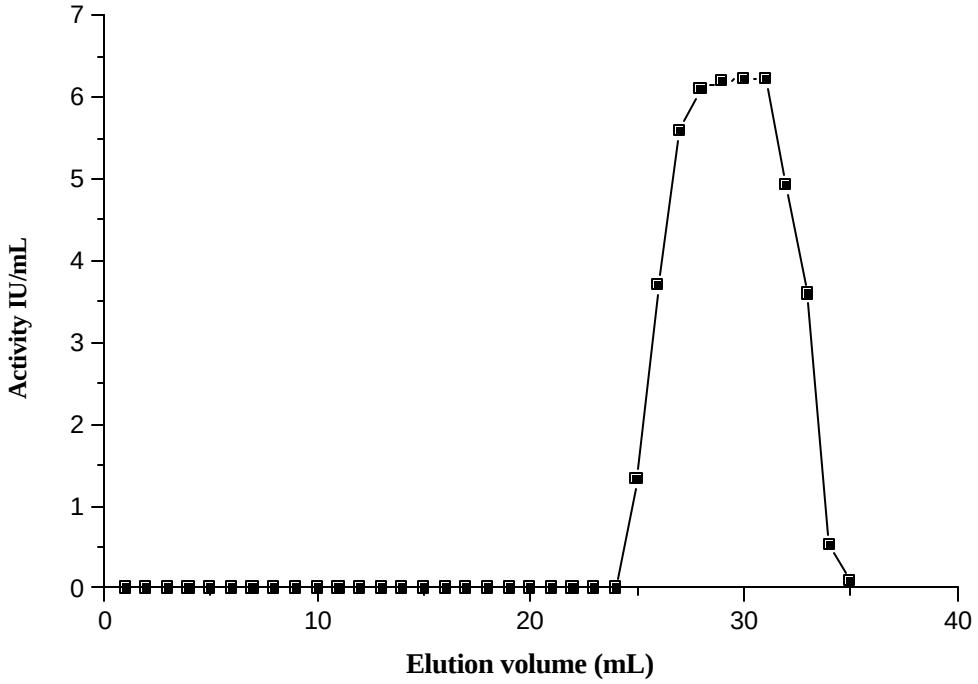


Figure 3.15 Elution profile of penicillin G acylase from *E. coli* on AT-1-BzA column for estimating its binding capacity

3.2.1.4 Ethyl amine matrix

Macroporous beaded copolymers, differing in their hydrophilicity (copolymers AE-1, AD-1, AP-1, AT-1 and ATa-1, detailed composition is presented in Tables 2.2 and 2.3), were treated with ethyl amine to convert the base copolymer to the ethyl amine matrix (Section 2.3.1.1.4). The degree of modification of copolymer was estimated from wet as well as elemental analysis. Data is presented in Table 3.22. Slurry of matrices prepared in 0.05 M phosphate buffer, pH 7.6 were packed in plastic columns. Binding studies were carried out in the same buffer both in the

presence and absence of ammonium sulphate. No binding and hence no elution of penicillin G acylase were observed on these matrices.

Table 3.22 The degree of modification of AE-1, AD-1, AP-1, AT-1 and ATa-1 copolymers with ethyl amine

Polymer matrix code	Base copolymer	Cross-linker	Degree of modification	
			Elemental analysis, %	Wet analysis, %
AE-1-EA	AE-1	EGDM	16	15
AD-1- EA	AD-1	DVB	12	13
AP-1- EA	AP-1	PETA	17	15
AT-1- EA	AT-1	TMPTMA	14	15
ATa-1- EA	ATa-1	TMPTA	21	19

3.2.1.5 Hydroxyl matrices (gem diol matrices)

Oxirane copolymers were converted to corresponding amine matrices by treating with various amines, as discussed in Sections 3.2.1.2 and 3.2.1.3, resulting in approximately 9% to 21% modification of the base copolymer. To avoid irreversible reaction of enzyme with remaining unreacted oxirane groups (91% to 79%), matrices were further treated with 0.1 M sodium hydroxide solution to convert all epoxides to their respective dihydroxy compounds. To establish the effect of gem hydroxy compound as affinity matrix blank experiments were conducted in which copolymers of allyl glycidyl ether with varying crosslinking agents (copolymers AE-1, AD-1, AP-1, AT-1 and ATa-1) were treated with 0.1 M sodium hydroxide. It was observed that matrices treated with sodium hydroxide did not show any interaction with penicillin G acylase. This clearly indicates that the blocking of amine matrices with sodium hydroxide has no effect on adsorption of enzyme, as it modified the matrix to the corresponding gem diol. Thus, the adsorption and hence the purification of penicillin G acylase was only due to the presence of amine groups on the matrix.

3.2.2 Dye ligand chromatography

Dye ligand chromatography has proved to be a very useful technique in small- and large- scale purification of enzymes and proteins.[24,25] The use of natural ligands in group specific affinity chromatographic media is now being replaced to a larger extent by the multi-coloured poly-aromatic sulphonated triazine dyes, commercially available as Cibacron (from Ciba-Geigy) or Procion (from ICI) dyes.[26] This is because many of the disadvantages associated with developing an affinity matrix can be circumvented by the use of dyes. For example, coupling dyes to support material is easy. Dyes are cheap, widely available, easily adaptable to use in large-scale purification. Dye bound matrices are reusable over very many application cycles. Synthetic dyes are susceptible to microbial attack. As against this, preparation of group specific matrices requires considerable chemical synthesis and analysis of adsorbent. Ligands are expensive. This limited their use in large-scale purification. They are prone to biodegradation.

In the last few years the applicability of the triazine dye *cibacron blue F3G-A* as a group specific ligand for the affinity chromatography of a wide range of proteins has been amply documented.[27,28] However, a series of other textile dyes have also been tested for their application as affinity ligands.[29,30] These sulphonated polyaromatic triazine molecules have proved to be effective in the purification of NAD^+-dependent dehydrogenase, kinases, glycolytic enzymes, blood proteins and a number of other enzymes and proteins.[31] The growing number of applications of triazine dyes in preparative and analytical biochemistry has prompted the initiation of a number of studies to establish the basis for the mode of interaction between proteins and triazine dyes. The reactive dyes are multifunctional ligands, which contain both hydrophobic and charged groups.[32] Hence, a dye-ligand adsorbent presents the protein with a number of binding opportunities. Consequently protein interactions with adsorbents are likely to be both heterogeneous and multivalent. It has been suggested that most proteins are not bound to dye-ligand adsorbents by a single biospecific interaction, but rather by a combination of specific and nonspecific interactions between protein and several ligand molecules. Some protein molecules

are bound only nonspecifically by a complex combination of electrostatic, hydrophobic, hydrogen bonding and charge-transfer interactions, all of which are possible considering the structural complexity of dyes.

In the present study it was decided to estimate the nature of interaction and degree of purification of penicillin G acylase by using various triazine dye matrices. A number of reactive triazine dyes including *cibacron blue F3G-A, basilen blue E-3G, reactive red 120 and reactive green 5* were covalently attached to macroporous beaded AGE-EGDM (53-105 μm) copolymer with 50 mole % cross-link density and studied for their suitability to the purification of penicillin G acylase by affinity chromatography. The reproducibility of the results was confirmed by repeating experiments for three times.

3.2.2.1 Interaction of penicillin G acylase with *cibacron blue F3G-A* matrix

2 g of exhaustively washed allyl glycidyl ether-ethylene glycol dimethacrylate (AE-1) beads were treated with a solution of 40 mg of *cibacron blue F3G-A* in water. The reaction was carried out in presence of salt (sodium chloride) and alkali (sodium carbonate). Detailed modification procedure is presented in Section 2.3.1.3. Addition of the salt into the reaction mixture leads to the 'salting' of the dye into the matrix. Immobilised dye concentration was determined by hydrolysis of the modified beads in 6 M hydrochloric acid at 80^{o}C (Section 2.6.1.3.1). The spectrophotometric determination of the dye released indicates ligand loading of 2.0 μmole/g of the matrix. C*ibacron blue F3G-A*-AGE-EGDM conjugates (copolymer AE-1-CB) were packed in a plastic column (0.7 cm x 5.5 cm). The equilibration was performed in a binding buffer, 0.05 M phosphate buffer, pH 7.6. The crude dialysed enzyme with 5.33 IU/mL activity, 1.04 mg/mL protein and 5.13 IU/mg specific activity was applied to the column. Non-adsorbed protein was immediately washed off the column with binding buffer and elution was subsequently performed with a 0.05 M phosphate buffer, pH 7.6 containing 0.5 M phenyl acetic acid. All chromatographic procedures were carried out at room temperature. No binding of penicillin G acylase on *cibacron blue F3G-A* column was observed under the given experimental conditions. This suggests that there is no affinity between the adsorbent and enzyme.

Hydrophobic binding of proteins to immobilised *cibacron blue F3G-A* column has been observed in several cases.[33-36] The forces of attraction between these dyes and proteins were extensively investigated by Glazer[37], who suggested that the adsorption of dyes to proteins occurs predominantly in area that overlap the binding sites for protein ligands in preference to other surface areas of the protein molecule. In such binding, generally there is a marginal similarity between dyes and protein ligands. However, it was proposed that one reason for the binding could be the relative hydrophobicity of these sites compared to the rest of protein. Penicillin G acylase has a hydrophobic domain at or near the active site.[23] Therefore, binding studies were carried out to check specifically whether hydrophobic forces are involved in the binding of enzyme to the dye adsorbent. Slurry of *cibacron blue F3G-A* adsorbent was poured into a column and equilibrated with 0.05 M phosphate buffer, pH 7.6 containing 30% ammonium sulphate. The column was then loaded with crude dialysed enzyme with specific activity of 5.13 IU/mg. Non-bound proteins were removed from the column by washing with binding buffer. The bound protein was eluted with 0.05 M phosphate buffer, pH 7.6 devoid of ammonium sulphate. Penicillin G acylase activity was measured in the eluted fractions. Protein content was determined by Lowry method (Section 2.6.4). A 100% adsorption with recovery of 86% and purification factor of 2.52 were achieved using *cibacron blue F3G-A* adsorbent in a single step. Buffer containing a high concentration of ammonium sulphate enhances binding of the enzyme to *cibacron blue F3G-A*-bound AGE-EGDM copolymer where as binding buffer devoid of salt serves as an effective eluent. This indicates that hydrophobic interactions play a major role in binding proteins to *cibacron blue F3G-A* adsorbent. In contrast to affinity interactions, hydrophobic interactions are enhanced by high concentrations of those anions of the Hofmeister series, such as sulphate and phosphate, which tend to inhibit denaturation.[38] The dye *cibacron blue F3G-A* possesses both hydrophobic and anionic character. The highly aromatic nature of *cibacron blue F3G-A* may be responsible for the hydrophobic interactions with penicillin G acylase. From these preliminary experiments it was apparent that careful selection of the binding buffer (0.05 M phosphate buffer, pH 7.6 containing 30% ammonium sulphate) was

necessary for the retention of the enzyme on the dye affinity supports. Chromatography of crude dialysed penicillin G acylase from *E. coli* on AGE-EGDM-*cibacron blue* column is illustrated in Figure 3.16.

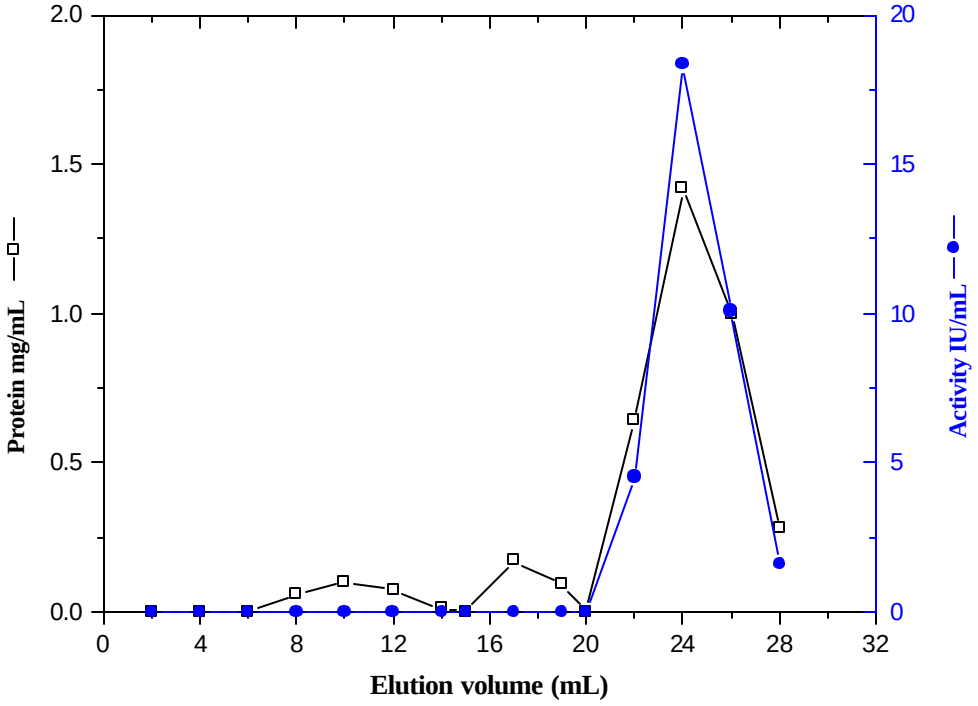


Figure 3.16 Elution of penicillin G acylase from *E. coli* on AE-1-CB column

3.2.2.2 Affinity between penicillin G acylase and triazine dye matrices

Penicillin G acylase was chromatogrammed on *basilen blue E-3G*- (Figure 3.17), *reactive green 5*- (Figure 3.18) and *reactive red 120*- (Figure 3.19) adsorbent. The immobilised triazine dye matrices were prepared by covalent anchoring of dyes to epoxy groups of copolymer by the procedure described in Section 2.3.1.3. Data for the degree of modification of dye bound matrices obtained by acid hydrolysis (Sections 2.6.1.3.1 and 2.6.1.3.2) is presented in Table 3.23.

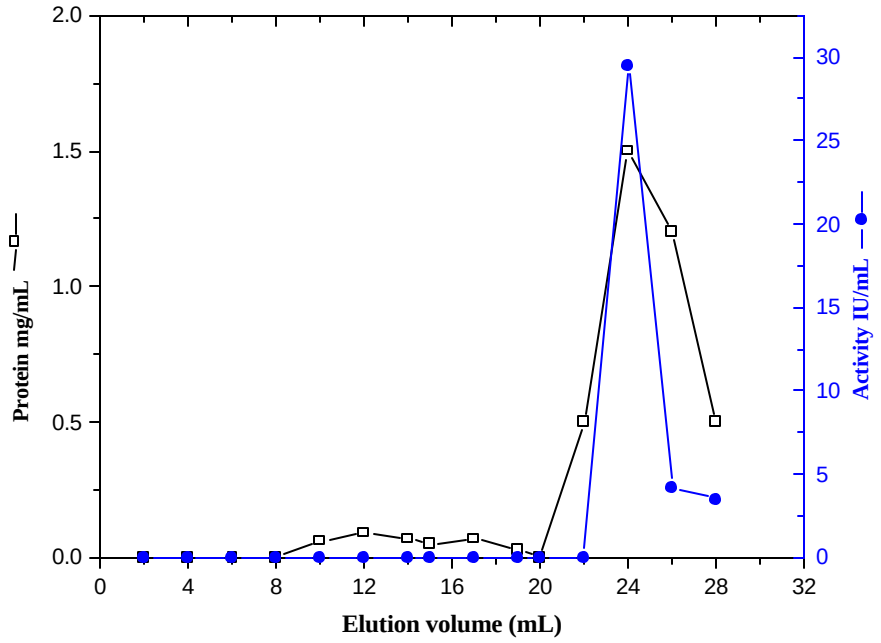


Figure 3.17 **Elution profile of penicillin G acylase from *E. coli* on AE-1-BB column**

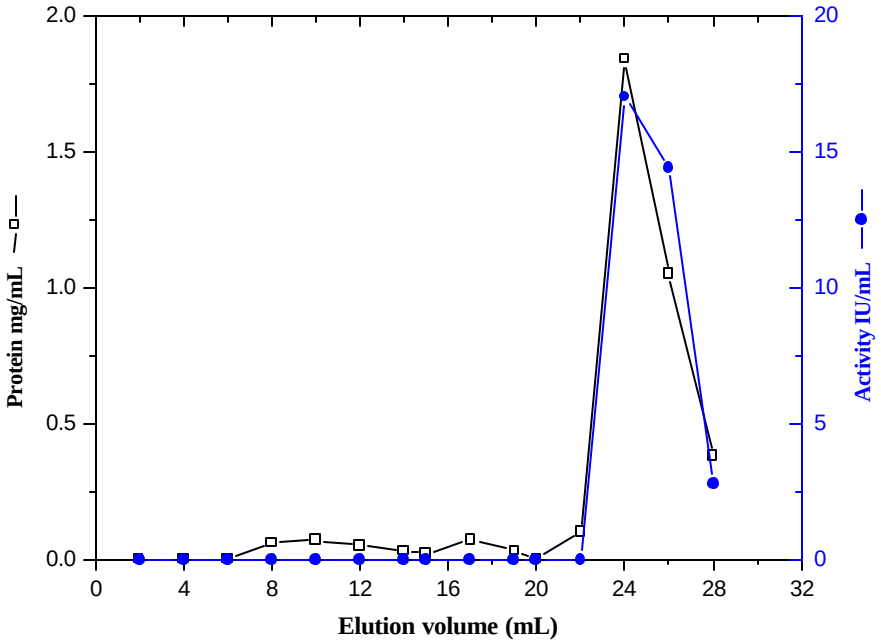


Figure 3.18 **Elution profile of penicillin G acylase from *E. coli* on AE-1-RG column**

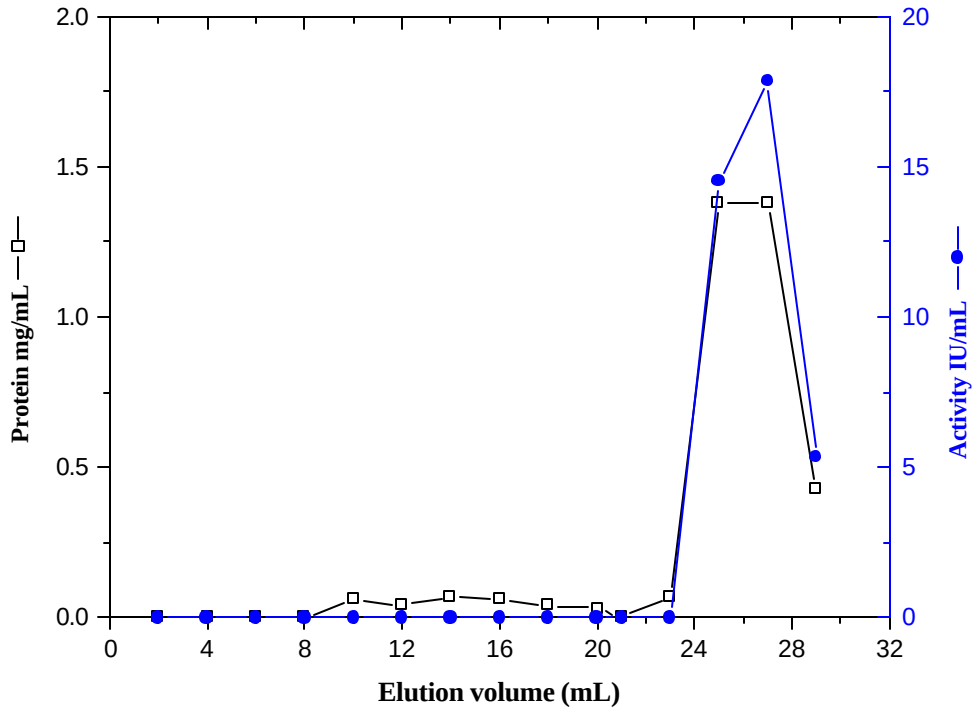


Figure 3.19 **Elution profile of penicillin G acylase from *E. coli* on AE-1-RR column**

Table 3.23 **The degree of modification of AGE-EGDM (AE-1) copolymers with triazine dye ligand**

Polymer matrix code	Dye used	Modification mmole/g
AE-1-BB	*basilen blue E-3G*	2.6
AE-1-RG	*reactive green 5*	1.2
AE-1-RR	*reactive red 120*	4.2

Plastic columns containing dye bound matrices were equilibrated with 0.05 M phosphate buffer, pH 7.6 containing 30% ammonium sulphate. The crude dialysed penicillin G acylase having activity 5.33 IU/mL, protein 0.93 mg/mL and specific activity 5.73 IU/mg was applied to the column and eluted quantitatively with the 0.05 M phosphate buffer, pH 7.6 devoid of ammonium sulphate. The enzyme activity and

protein concentration were determined in the pooled fractions. Table 3.24 gives the data for the purification of penicillin G acylase on various dye adsorbents studied.

Table 3.24 The purification profile of penicillin G acylase on triazine dye matrices

Polymer matrix code	Adsorption %	Elution %	Recovery %	Specific* activity IU/mg	Fold purification
AE-1-BB	100	92.93	92.93	19.65	3.43
AE-1-RG	100	85.53	85.53	13.71	2.39
AE-1-RR	100	78.56	78.56	12.95	2.26

* Crude penicillin G acylase with 5.33 IU/mL activity, 0.93 mg/mL protein and 5.73 IU/mg specific activity was used.

Quantitative binding of penicillin G acylase was achieved on all dye matrices. Adsorption of enzyme in presence of ammonium sulphate and desorption by buffer devoid of ammonium sulphate suggests that the binding between the enzyme and dye adsorbents is due to hydrophobic interactions. Salts are known to increase the hydrophobic interactions. In the present system hydrophobic interactions may be attributed to the interaction of aromatic components of reactive triazine dyes with the hydrophobic domain of penicillin G acylase. A recovery of about 93% and a purification factor of 3.43 were achieved using *basilen blue E-3G* adsorbent (AE-1-BB) in a single chromatographic step. Performance of both *cibacron blue F3G-A* and *basilen blue E-3G* matrices in respect of percentage recovery were almost identical. This is obvious, as these molecules are structural isomers. In *cibacron blue F3G-A* terminal benzene ring carries sulphonyl groups at ortho position where as in *basilen blue E-3G*, sulphonyl group either is present at meta or para position or is a mixture of both. However, the fold purification achieved with *basilen blue E-3G* adsorbent (3.43) was slightly greater than that with the corresponding *cibacron blue F3G-A* adsorbent (2.52). Figure 3.20 exhibits the comparative infra-red spectra of base copolymer (AE-1) and *basilen blue E-3G*-copolymer. Disappearance of characteristic epoxy peak at 910 cm^{-1} in *basilen blue E-3G-adsorbent* attributes to the opening of epoxy ring by the corresponding dye ligand.

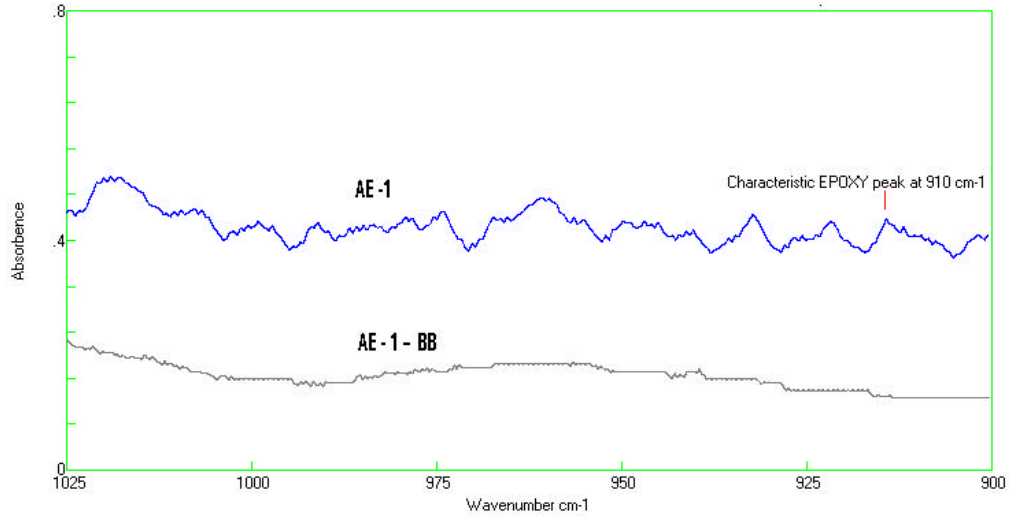


Figure 3.20 Comparative infra-red spectra of AGE-EGDM base copolymer (AE-1) and *basilen blue E-3G* copolymer (AE-1-BB)

Reactive green 5 is a copper phthalocyanine containing monochloro triazinyl dye molecule. Using this dye adsorbent (copolymer AE-1-RG), a fold purification of 2.39 and recovery of about 86% were achieved. Even though the concentration of *reactive green 5* immobilised on to the matrix was slightly towards the lower side (1.2 μmole/g), the adsorption of penicillin G acylase obtained was 100%. This suggests that the lower concentration of *reactive green 5* does not make a difference in their adsorption performance.

For immobilised *reactive red 120* adsorbent fold purification of 2.26 and recovery of about 79% was obtained. The amount of the dye bound to the matrix was 4.2 μmole/g, which is marginally greater than the other dyes. Immobilised dye concentration of the adsorbent is an important factor in the development of dye-ligand process.[39,40] It has generally been observed that increasing the dye concentration typically enhances the binding capacity of the matrix.[28] However, proteins adsorbed on the more highly substituted gels are more difficult to elute. This is the probable reason for the observed lower recovery and fold purification achieved with *reactive red 120* matrix (AE-1-RR). The performance of *basilen blue E-3G* matrix was superior to the remaining dye matrices. Hence, it was the adsorbent of choice for further study of purification of penicillin G acylase.

3.2.2.3 *Basilen blue E-3G* matrix

Studies included an estimation of binding capacity of AE-1-BB matrix. It also comprised the evaluation of the effect of cross-link density (CLD) of the copolymer on degree of purification of penicillin G acylase. The *Basilen blue E-3G* matrix with higher CLD was also tested for regeneration.

3.2.2.3.1 Binding capacity of AE-1-BB matrix

The binding capacity of AE-1-BB matrix was estimated as described in Section 3.2.1.2.4. The elution profile of penicillin G acylase on the dye matrix is presented in Figure 3.21.

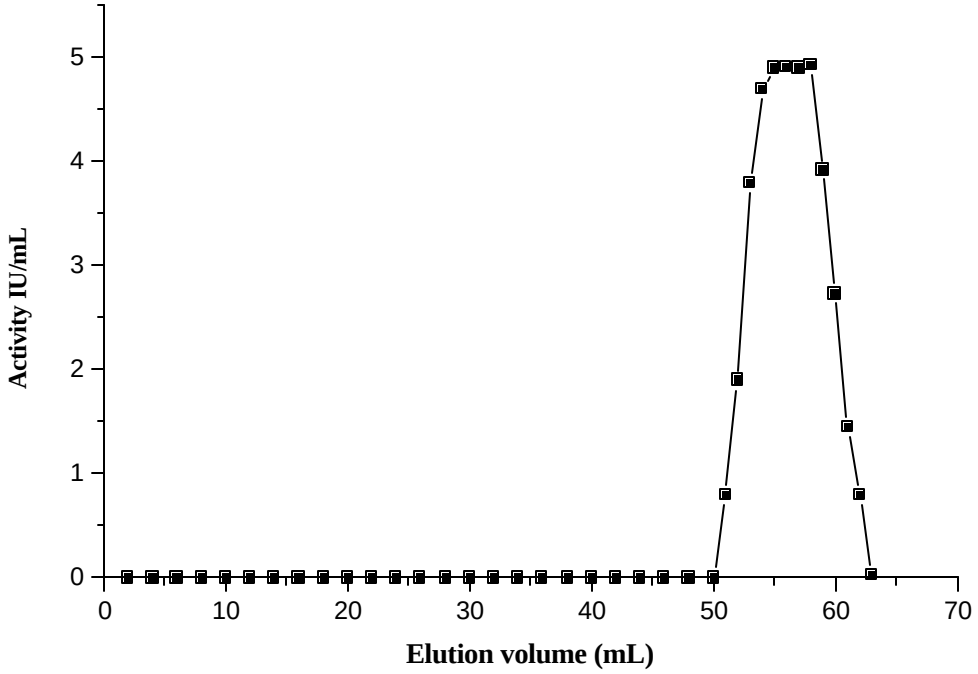


Figure 3.21 Elution profile of penicillin G acylase from *E. coli* on AE-1-BB column for estimating its binding capacity

Graphical presentation clearly indicates that till a loading of 50 mL of penicillin G acylase, enzyme was not eluted out from the column, which attributes to 100% adsorption of the enzyme. Further addition of enzyme indicated only a partial adsorption. After 55 mL of enzyme loading, the activity of enzyme in the eluant

equaled that in the original enzyme solution. Thus, the binding capacity estimated for 1 g of matrix was 452.7 IU of penicillin G acylase. The adsorbed enzyme was eluted from the column with 92% recovery. The binding capacity achieved with basilen blue E-3G for penicillin G acylase was higher than some of the earlier reports in literature.[13,14] It is even higher than the corresponding amine matrices studied in present work.

3.2.2.3.2 Regeneration of AE-4-BB matrix

Regeneration of support material is a prerequisite to repeated use. The conditions of regeneration are dependent on the nature of affine gel and of the sample and must not change the chromatographic behaviour. Sufficient washing with elution buffer and subsequent equilibration with the starting buffer frequently suffice.[41]

Basilen blue E-3G was attached to the AGE-EGDM copolymer beads with 200% cross-link density by procedure presented in Section 2.3.1.3. The resulting dye incorporated beads were termed as AE-4-BB copolymer. Regeneration study of *Basilen blue E-3G* matrix (AE-4-BB) was performed in a plastic column. Slurry of the matrix prepared in 0.05 M phosphate buffer, pH 7.6 containing 30% ammonium sulphate was packed in a column. After equilibration of column with the same buffer, crude dialysed extract of penicillin G acylase saturated with 30% ammonium sulphate and having activity 6.4 IU/mL was loaded. Packed matrix was washed with binding buffer and adsorbed enzyme was eluted with 0.05 M phosphate buffer, pH 7.6, without addition of salt. Penicillin G acylase was quantitatively bound to *Basilen blue E-3G* adsorbent in presence of ammonium sulphate and got eluted to 91% with same buffer devoid of ammonium sulphate. After elution of enzyme molecule, the column was successively washed with elution buffer, distilled water and again equilibrated with a binding buffer. The column was then ready for the second cycle. Adsorption-elution studies were carried out under the same conditions used in the first cycle. The enzyme activity was estimated in eluted fractions. The column was then regenerated by washing with elution buffer and distilled water followed by equilibration in binding buffer. Nine cycles of penicillin G acylase adsorption-elution were carried out using the procedure. Till 9th cycle there was absolutely no loss in the binding of

enzyme. Binding of penicillin G acylase onto the blue column was 100%. Up to 8th cycle, the elution and recovery of enzyme were almost 90%. In the 9th cycle percent recovery was reduced to 84%. So the resin was regenerated with 0.1 M sodium hydroxide containing 1 M sodium chloride for 1 hour. The resin was further washed with distilled water till neutral. After washings the matrix was loaded in the column and equilibrated with a 0.05 M phosphate buffer, pH 7.6, saturated with 30% ammonium sulphate. Crude penicillin G acylase extract was passed through the column in the same fashion as mentioned above. For next two cycles (i.e. 10th and 11th) 100% binding with 86% enzyme recovery was achieved. For 12th cycle adsorption of enzyme was reduced by 8% with subsequent reduction in recovery of enzyme. Percentage recovery of enzyme after 12th cycle was 78%. When the 13th cycle was run, there was further loss in binding of penicillin G acylase. Only 85% of the enzyme was loaded onto the column with 68% recovery. Thus it was established that the AE-4-BB column could be successfully used for 12 continuous cycles without much loss in its performance. The fold purification achieved was 3.5 with about 91% recovery. Thus, this column could be useful for large-scale purification. In addition, the dye ligands are cheap and are easy to immobilise. These factors are very important when economy of the purification is considered on industrial scale.

3.2.3 Metal chelate affinity chromatography

Metal chelate affinity chromatography (MCAC) is a well-known technique for protein purification. The principle of this chromatographic method is that, some amino acid residues such as histidine, cysteine, lysine, tryptophan, aspartic acid or glutamic acid, which are accessible on the protein surface, can specifically interact with metal ions e.g. Cu (II), Ni (II), Co (II), Zn (II), Cr (II), Fe (II), Al (III) etc. via electron donor groups on the amino acid side chain.[42]

Present study aims at purification of penicillin G acylase on metal chelate matrices. AGE-EGDM copolymer beads of 50 mole % cross-link density (AE-1) were selected as the base material for the synthesis of affinity matrices. The chelating copolymers were prepared by modifying AGE-EGDM copolymer with ligands like 2-picolylamine, 3-picolylamine and iminodiacetic acid. The respective chelating

polymers are termed as AE-1-2PA, AE-1-3PA and AE-1-IDA copolymers. The detailed procedure of polymer modification and data for extent of modification is given in Section 2.3.1.4. The metal ion complexation with the chelating copolymers was performed at pH 7 by using copper sulphate pentahydrate as source for copper ions. The metal ion capacities of chelating copolymer are presented in Table 3.25.

Table 3.25 The copper ion coordination to chelating matrices

Polymer matrix code	Chelating copolymer used	Cu^{2+} bound to matrix mmole/g
AE-1-2PA-Cu	AE-1-2PA	4.36
AE-1-3PA-Cu	AE-1-3PA	1.62
AE-1-IDA-Cu	AE-1-IDA	2.98

Slurry of matrix in a binding buffer, 0.025 M phosphate buffer pH 7.0 was used to prepare a column. Enzyme, penicillin G acylase having activity 0.92 IU/mL, protein 3.62 mg/mL and specific activity 0.25 IU/mg was applied to the column. After washing away unbound proteins with 0.025 phosphate buffer, pH 7.0, the enzyme was eluted with 0.025 M phosphate buffer containing 2 M sodium chloride. The chromatographic behaviour of metal chelate matrices with penicillin G acylase was estimated from the enzyme and protein concentrations in the pooled fractions. Data is presented in Table 3.26.

Table 3.26 The purification profile of penicillin G acylase on metal chelate affinity matrices

Polymer matrix code	Adsorption %	Elution %	Recovery %	Specific* activity IU/mg	Fold purification
AE-1-2PA-Cu	49.00	91.90	24.40	1.65	6.6
AE-1-3PA-Cu	85.00	62.00	53.00	5.00	20.00
AE-1-IDA-Cu	58.00	27.70	16.08	10.00	40.00

* Crude penicillin G acylase with 0.92 IU/mL activity, 3.62 mg/mL protein and 0.25 IU/mg specific activity was used.

Maximum adsorption (85%) and recovery (53%) of penicillin G acylase was achieved with 3-picolylamine matrix. After passing the enzyme through this column specific activity increased from 0.25 IU/mg to 5.0 IU/mg thus, resulting in 20-fold enzyme purification. When penicillin G acylase was contacted with iminodiacetic acid matrix, out of 58% of adsorbed enzyme only 16% was recovered with 40-fold enzyme purification. Therefore, even though fold purification achieved by using IDA-matrix is greater than that of 3-picolylamine matrix, over all performance of 3-picolylamine matrix was better than iminodiacetic acid matrix based on percent adsorption, percent recovery and fold purification. The observations may be due to the following reason: When chelated by tridentate IDA-resin, Cu^{2+} has one co-ordination site free for interaction with enzyme whereas bidentate 3-picolylamine-resin has two such sites. Thus, more sites are available for 3-picolylamine matrix-enzyme interactions, which is reflected in higher adsorption of penicillin G acylase. But it is interesting to note that even though 2-picolylamine is a bidentate ligand, its performance is poorer than IDA-matrix. In essence molecular modeling of ligand-polymer matrix structure is essential to describe above observations in detail, which is beyond the scope of present investigation, since the binding phenomenon of chelating polymer and metal is more complex.

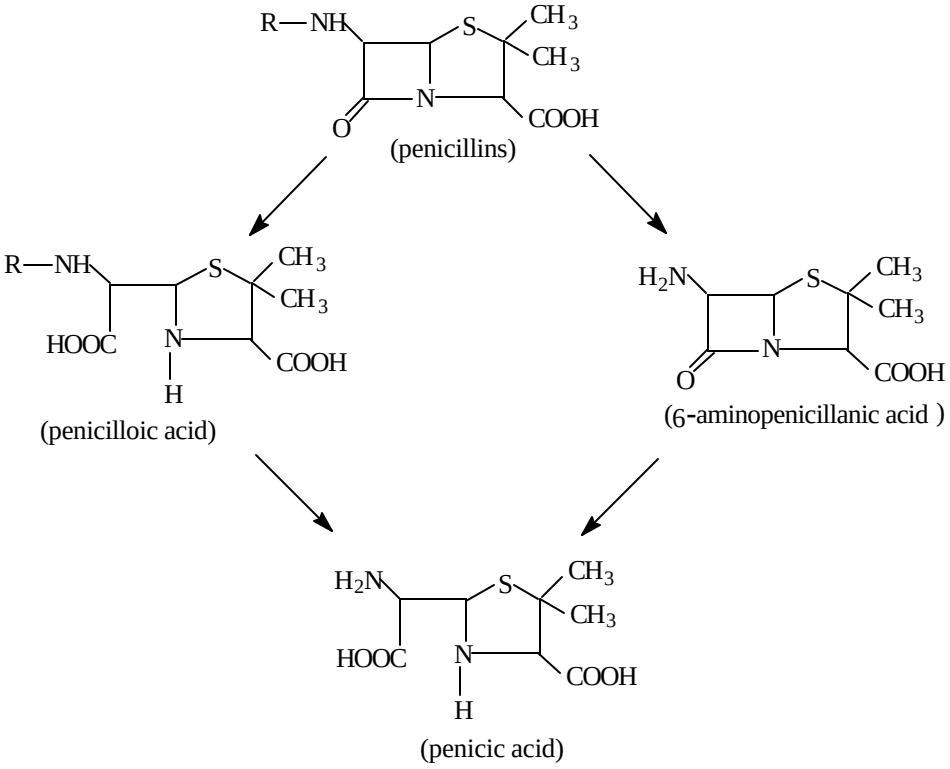
The chelating matrices without the copper ion were also tested for the purification of penicillin G acylase. No binding of the enzyme was achieved, indicating the role of copper ion in enzyme purification process.

3.3 Penicillinase (β-lactamase)

β-lactamase activity was first described by Abraham and Chain.[43] Term β-lactamase includes both penicillinase and cephalosporinase. β-lactamase catalyses hydrolysis of the amide bond in β-lactam ring of 6-APA or 7-ACA and their N-acyl derivatives.[44] Penicillin and cephalosporins are thereby transformed into penicilloic and cephalosporoic acids respectively, compounds without any antibacterial activity.

According to several Authors, β-lactamase activity occurs together with penicillin acylase activity in the same microbial strain.[45] The presence of penicilloic acid, 6-APA and eventually penicic acid in the reaction mixture is a qualitative

confirmation of the co-existence of penicillin acylase and β-lactamases. Combined action of acylase and β-lactamase on penicillin is shown in Scheme 3.1.



Scheme 3.1 Combined action of acylase and b -lactamase on penicillin

3.3.1 Purification of penicillinase

Purification of penicillinase using affinity matrices is a simple, rapid and efficient procedure, which can be adapted for the production of purified enzyme on a preparative scale. Affinity chromatography essentially involves attaching to an insoluble support, ligand molecules with which the enzyme will interact reversibly. Under appropriate conditions the enzyme is adsorbed specifically, while other proteins pass straight through. The enzyme is subsequently eluted, either non-specifically by an increase in ionic strength, or specifically by washing the column with a solution of a substrate or inhibitor which is capable of releasing the enzyme.[46] Various affinity gels have been developed for purification of β-lactamase type enzymes.[46-51]

Penicillinase, a member of β-lactamase group of enzymes, plays a significant role in nature and in chemotherapy since it inactivates some potent penicillin antibiotics. Various diagnostic kits have been developed using penicillinase as an enzyme marker, e.g. early pregnancy detection kit, fila test, dipstick ELISA kit, toxokit-G, toxokit-M etc.[52] One of the important criteria for ideal enzyme marker is high specific activity of enzyme.

Although many successful applications of affinity chromatography have been reported, these have usually employed activation reactions, there by coupling the ligand to the matrix.[49,53,54] Reagents commonly used for the activation include cyanogen bromide, divinyl sulphone, glutaraldehyde etc. which are highly toxic and corrosive. Present study utilises oxirane copolymers as support material. These copolymers have built-in reactive epoxy groups for coupling of ligands to the matrix. Thus, matrix activation is not required. Attachment of ligands to the support is a single step process. All reactions are eco-friendly, as they do not involve any hazardous chemicals.

Allyl glycidyl ether-ethylene glycol dimethacrylate copolymer with 50% CLD (AE-1) were synthesised as described earlier (Section 3.2.1.1). The affinity matrices were prepared by covalent binding of different β-lactam compounds as ligands (Table 2.11) to the crosslinked macroporous AE-1 copolymer beads. Binding reactions were conducted in 0.1 M phosphate buffer, pH 8.5 at room temperature for 48 hours. After the completion of reaction, the thoroughly washed polymer beads were suspended for 2 hours in water, pH of which was adjusted to 10.5 with sodium hydroxide. This step is essential, as the cyclic amide bond present in the β-lactam compound is labile and hence is hydrolysed at pH 10.5 to form corresponding acids, which are either product or product analogues. This step of hydrolysis of β-lactam bond further ensures the chemical homogeneity of the ligand.[55]

The slurry of affinity matrices were prepared in a binding buffer, 0.1 M phosphate buffer, pH 7.5, packed in a plastic column (0.7 cm x 5.5 cm) and equilibrated. 6800 U of crude penicillinase extracted from *Bacillus cereus* 5/B NCTC 9946 was loaded in the column. The column was washed with the equilibration buffer

and eluted with 0.1 M phosphate buffer, pH 7.5 containing 10% (wt/v) sodium chloride. Elution of penicillinase with sodium chloride is a common procedure used in other laboratories.[46,47,56] Fractions were collected and assayed for enzyme and protein. Protein concentration was measured by method of Lowry et al. with bovine serum albumin as a standard.[57] Enzyme activity was estimated by a modified method of Ghosh et al. (Section 2.6.3). Preliminary data is presented in Table 3.27.

Penicillinase from *Bacillus cereus* 5/B NCTC 9946 accepts only penicillin molecules as substrate and cephalosporin molecules are not hydrolysed.[58] However from the results presented in Table 3.27, it is evident that the penicillinase exhibits affinity interactions with various penicilloic acid and cephalosporoic acid, especially with 7-phenylglycinamido cephalosporoic acid. Ampicilloic acid- and 7-phenylglycinamido cephalosporoic acid-copolymers behave in the same fashion with the penicillinase with respect to percent adsorption, elution and recovery. This may be attributed to their identical side chain structures. Ampicillin and cephalexin have the same side chain structures and vary in β-lactam nucleus. Ampicillin is composed of five membered thiaozolidine ring where as cephalexin has six membered dihydrothiazine ring. Among the matrices studied, good results were obtained with ampicilloic acid- and 7-phenylglycinamido cephalosporoic acid-copolymers. Quantitative adsorptions (93%) with good recoveries were achieved with both the matrices. Recovery of penicillinase obtained with 7-phenylglycinamido cephalosporoic acid matrix (70.7%) is slightly greater than that with ampicilloic acid matrix (67.5%).

The difference between ampicillin and amoxycillin as well as between cephalexin and cephadroxyl is the presence of hydroxy (-OH) group on the phenyl moiety of the latter molecules. The introduction of -OH group on the phenyl ring i. e. amoxycilloic acid reduced the adsorption and hence recovery of penicillinase by about 18%. Similarly, copolymer with 7-(p-hydroxy)phenylglycinamido cephalosporoic acid ligand showed 30% loss in the recovery of enzyme as compared to the matrix modified with 7-phenylglycinamido cephalosporoic acid.

Table 3.27 Affinity interactions of penicillinase with AGE-EGDM (AE-1) matrices

Polymer matrix code	Ligand used	Adsorption %	Elution %	Recovery %
AE-1-ampi	Ampicilloic acid (Ampicillin)[a]	93.70	72.00	67.50
AE-1-amoxy	Amoxycilloic acid (Amoxycillin)[a]	71.00	69.80	49.60
AE-1-apa	6-Aminopenicilloic acid (6-Aminopenicillanic acid)[a]	26.00	38.00	9.90
AE-1-adca	7-Aminodesacetoxy cephalosporoic acid (7-Aminodesacetoxy cephalosporanic acid)[a]	7.5	100.00	7.50
AE-1-aca	7-Aminocephalosporoic acid (7-Aminocephalosporanic acid)[a]	10.6	65.00	6.90
AE-1-cephc	Cephalosporoic acid C (Cephalosporin C)[a]	19.90	32.00	6.40
AE-1-cephl	7-Phenylglycinamido cephalosporoic acid (Cephalexin)[a]	93.00	76.00	70.70
AE-1-cephr	7-p-hydroxy Phenylglycinamido cephalosporoic acid (Cephadroxyl)[a]	85.8	47.00	40.30
AE-1-val	DL-Valine	23.70	2.20	5.20

[a] Paranthesis gives the parent compound used for binding.

From the study it is clear that 6-aminopenicillanic acid-, 7-aminodesacetoxy cephalosporanic acid- and 7-aminocephalosporanic acid- matrices were enabled to adsorb the enzyme quantitatively under the experimental conditions. These molecules are comprised of β-lactam nucleus without a side chain, emphasising the role of side chain moiety in enzyme-matrix interactions. It is interesting to note that the enzyme,

penicillinase, was not adsorbed quantitatively on cephalosporoic acid C-matrix. Structure of side chain of cephalosporoic acid moiety is different from the other ligands studied. The result suggests that the structure of side chain is also an important factor in determining the interaction of enzyme with the matrix. Based on the above results, cephalexin was identified as the ligand of choice for the purification of penicillinase. Reproducible results are obtained for the repeated set of experiments.

The copolymers differing in hydrophilicity, AE-1, AP-1, AD-1 and AT-1 (Table 2.3) were converted into the cephalexin matrices under optimised conditions and the binding of penicillinase was evaluated. The data is summarised in Table 3.28.

Table 3.28 The purification profile of penicillinase on cephalexin matrices

Polymer matrix code	Adsorption %	Elution %	Recovery %	Specific* activity IU/mg	Fold purification
AE-1-cephl	96.00	84.00	81.00	3529.40	4.69
AP-1-cephl	83.70	58.90	49.30	2543.40	3.38
AD-1-cephl	56.50	44.00	25.00	1875.00	2.49
AT-1-cephl	74.00	18.00	13.30	1954.73	2.60

* Crude penicillin G acylase with 1233.00 U/mL activity, 1.64 mg/mL protein and 751.82 U/mg specific activity was used.

AE-1-cephl, AP-1-cephl, AD-1-cephl and AT-1-cephl matrices were prepared by derivatising AGE-EGDM (AE-1), AGE-PETA (AP-1), AGE-DVB (AD-1) and AGE-TMPTMA (AT-1) copolymers respectively with 7-phenylglycinamido cephalosporoic acid. Allyl glycidyl ether is the common reactive monomer in all four matrices. The difference in these matrices is the variation in the hydrophilic/hydrophobic characteristic of the crosslinking monomer. It is clear from Table 3.28 that among the matrices studied best results are obtained with AE-1-cephl matrix (96% adsorption of penicillinase with 81% recovery). The specific activity of the enzyme eluted increased from 751.82 U/mg to 3529.4 U/mg, thereby causing 4.7-fold enzyme purification. The results can be explained on the basis of steric parameters offered by crosslinking monomers. Ethylene glycol dimethacrylate is the

smallest among the cross-linkers. Therefore the enzyme has easier access to the matrix bound ligand. The monomer, pentaerythritol triacrylate (PETA) is bulkier than EGDM but less than TMPTMA and DVB, which is ultimately reflected in the % adsorption (83.7%) and fold purification (3.38) of penicillinase. However, the recovery of enzyme was only 49% indicating stronger and irreversible binding of enzyme with AP-1-cephl matrix. Similarly, 74% adsorption with 13.3% recovery of penicillinase was achieved with AT-1-cephl matix. Poor adsorption, 56.5% of penicillinase, was obtained with AD-1-cephl matrix. This may be due the steric hindrance offered by phenyl ring of divinyl benzene. The elution profile of penicillinase on AE-1-cephl matrix is graphically presented in Figure 3.22.

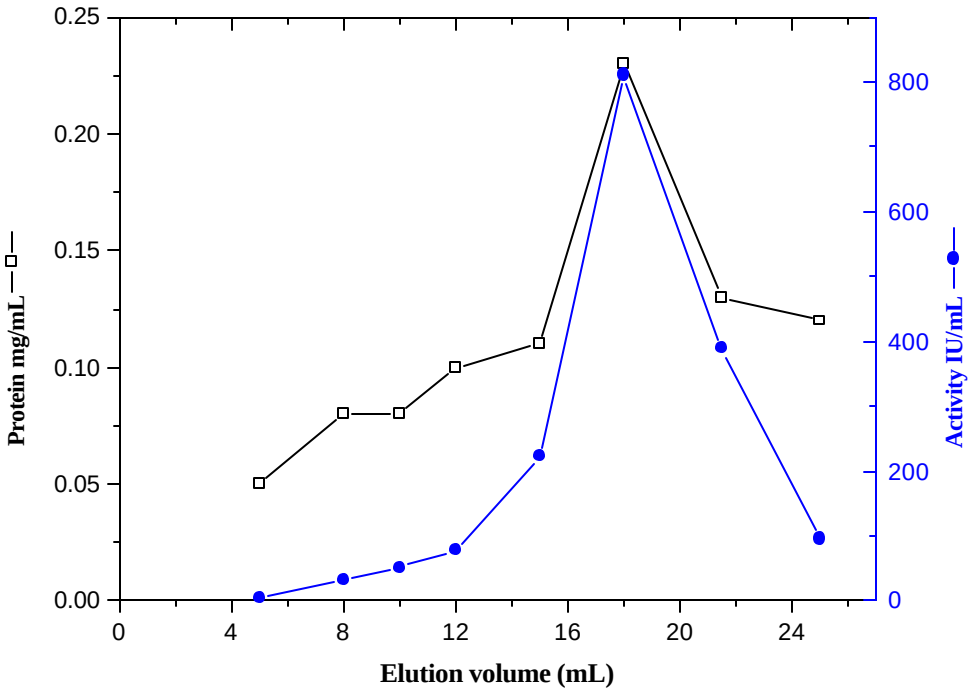


Figure 3.22 **Elution profile of penicillinase from** *Bacillus cereus* 5/B NCTC 9946 **on AE-1-cephl column**

3.4 Conclusion

The methodology to prepare base copolymer matrices of desired particle size and distribution having functional epoxy groups have been established. The spherical

beaded copolymers were synthesised by suspension polymerisation. The copolymer of allyl glycidyl ether (AGE)-ethylene glycol dimethacrylate (EGDM) with 50 mole % cross-link density (AE-1) was selected for optimisation of parameters controlling the particle size and its distribution. 71% of the copolymer particles were obtained in the range of 53-105 μm (140-270 mesh) by conducting polymerisation under constant stirring speed of 600 rpm using 8-bladed Ruston turbine stirrer with 2% wt/v protective colloid. After the standardisation of suspension methodology, a series of copolymers were synthesised by varying the relative mole rations of AGE and EGDM. Similarly, copolymers of allyl glycidyl ether with varying degrees of hydrophilicity were prepared by replacing EGDM with crosslinkers of differing hydrophilic/ hydrophobic character such as divinyl benzene (DVB), pentaerythritol triacrylate (PETA), trimethylol propane trimethacrylate (TMPTMA) and trimethylol propane triacrylate (TMPTA).

A number of probable affinity matrices were synthesised. Matrices for penicillin G acylase were synthesised by derivatising base copolymers with different aliphatic and aromatic amines. Amounts of each ligand coupled with copolymer were estimated by wet and elemental analysis. Adsorption of penicillin G acylase was investigated on amine matrices in presence and absence of 30% ammonium sulphate. Penicillin G acylase has the best hydrophobic interaction with butyl amine matrices and has the best affinity interaction with benzyl amine matrices. Copolymers differing in their hydrophilicity (AE-1, AP-1, AD-1, AT-1 and ATa-1) were converted into the corresponding butyl amine matrices. Good results are obtained with marginally hydrophobic AE-1 copolymer. Effect of cross-link density of copolymers and ligand concentration on purification of penicillin G acylase was also investigated. There is a marginal influence of ligand concentration on the purification of penicillin G acylase. Highest ligand loading was achieved when the molar ratio of butyl amine:epoxy group was set at 5:1. This is also reflected in the maximum fold purification. Cross-link density of copolymers, which controls pore size and its distribution, did not dramatically change the purification of penicillin G acylase. In fact, this enhanced the applicability of matrices on industrial scale, as higher CLD offers better rigidity and thereby easier handling.

The hydrophilic/hydrophobic character of the base copolymer influenced the adsorption-elution profile of penicillin G acylase on benzyl amine matrices. With partially pure enzyme, good results were achieved with moderately hydrophobic AGE-EGDM and AGE-TMPTMA copolymers. However, with the crude enzyme, percent adsorption drastically decreased on AGE-EGDM-benzyl amine matrix. A 5.06 fold enzyme purification with increase in specific activity from 2.38 IU/mg to 12.05 IU/mg was achieved with AGE-TMPTMA-benzyl amine matrix (AE-1-BzA). The loading concentration of benzyl amine on to the base copolymer has a substantial influence on the purification of penicillin G acylase. Promising results (91.50% adsorption, 72.61% elution and 5.06 fold purification) were obtained with matrix prepared at a benzyl amine:epoxy molar ratio of 5:1.

Hydrophobic interaction of penicillin G acylase with triazine dye matrices was responsible for the purification of the enzyme. Quantitative binding of penicillin G acylase was obtained with all dye matrices. A 100% adsorption with recovery of 93% and purification of 3.43 was achieved using *basilen blue E-3G* adsorbent (AE-1-BB). B*asilen blue E-3G* matrix with 200% CLD (AE-4-BB) permitted repeated use up to 12 cycles for the purification of penicillin G acylase with out much compromise in its performance.

Binding capacity determined per gram dry weight of AE-1-BuA, AT-1-BzA and AE-1-BB matrices were 240.6 IU, 248.2 IU and 452.7 IU of penicillin G acylase respectively.

Among the chelating copolymers studied maximum adsorption (85%) and recovery (53%) with 20-fold penicillin G acylase purification, was achieved with 3-picolylamine matrix (AE-1-3PA-Cu). No interaction of enzyme was observed with the chelating matrices in the absence of copper ion.

Matrices for the separation of penicillinase were prepared by the incorporation of different β-lactam compounds on AGE-EGDM copolymer with 50 mole % CLD. β-lactam nucleus with out a side chain showed poor interaction with penicillinase. Structure of side chain also plays a vital role in enzyme purification. Cephalexin was selected as the best ligand for the enzyme interaction. The influence of variance in

hydrophilic/hydrophobic character of copolymers coupled with cephalexin on purification of penicillinase was investigated. The hydrophilicity/hydrophobicity of the base copolymer distinctly influenced purification profile of penicillinase. Adsorption of the enzyme was highest on AE-1-cephl matrix with 4.69 fold purification.

In the present study, synthetic polymer matrices used for the purification of penicillin G acylase and penicillinase offer following advantages:

(1) Base copolymer has built-in reactive epoxy group for the coupling of ligands. Hence, no activation of the copolymer is required which eliminates the use of toxic chemicals generally involved in the activation of support material.

(2) Ligand coupling reactions are carried out at ambient temperature.

(3) As the support materials are the synthetic polymers, these are susceptible to microbial attack.

(4) Purification of enzymes is achieved in a single step process at room temperature.

(5) The matrix used is a rigid cross-linked copolymer, hence no bed compression occurs during column operation.

(6) Process is advantageous for industrial application because various cross-link density materials can be generated as per the requirements of the industrial process without much compromise on its performance.

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(a)

(b)

Figure 3.6 SEM of AGE-EGDM copolymer (AE-1). (a) Sieved sample (53 μm to 105 μm); (b) Unsieved sample.