STUDIES OF PROTEIN PURIFICATION ON EXPANDED BED

A

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BY

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This thesis is dedicated to my parents and teachers

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CERTIFICATE

This is to certify that the work incorporated in the thesis entitled "STUDIES OF PROTEIN PURIFICATION ON EXPANDED BED" submitted by Mr. Deba Prasad Nayak was carried out by the candidate under my supervision/guidance at National Chemical Laboratory. Such material as has been obtained from other sources, has been duly acknowledged in the thesis.

Date: September 24, 2001

(Dr. Bhaskar D. Kulkarni)
Research Guide

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(Deba Prasad Nayak)

Abbreviations

ASTM	American standard measurement
Bo	Bodestein number
C	Enzyme activity of α-CGTase in the effluent, U mL^{-1}
C*	Equilibrium enzyme activity of α-CGTase in the liquid phase, U mL^{-1}
C_0	Enzyme activity in the feed, U mL^{-1}
CD	Cyclodextrin
CGTase	Cyclodextrin glycosyltransferase
C_i	Outlet concentration of BSA from the column, mg mL^{-1}
$C_{i\,m}$	Maximum outlet concentration of BSA from the column, mg mL^{-1}
CIP	Clean-in-place
CLD	Crosslink density
C_{pi}	Mass concentration of component i
D	Distribution coefficient of solutes
d_{pore}	Pore diameter, Å
Da	Dalton
DAC	Dynamic adsorption capacity, U mL^{-1} of the adsorbent
DAR	Dynamic adsorption rate, U mL^{-1} of adsorbent min^{-1}
D_{ax}	Axial liquid dispersion coefficient of the liquid, $cm^2\ s^{-1}$
d_c	Column diameter, cm
D_e	Effective diffusion coefficient, $cm^2\ s^{-1}$
DEAE	Diethylamino ethane.
Deldrin	Poly(acetal)
D_m	Molecular diffusion coefficient, $cm^2\ s^{-1}$
DMSO	Dimethyl sulphoxide
D_{or}	Diameter of the hole of the distributor plate, mm

d_p	Particle diameter, μm
D_{pi}	Particle dispersion of component i
E(t)	Residence time distribution function
EGDM	Ethylene glycol dimethacrylate
g	Acceleration due to gravity, cm s^{-2}
Ga	Gallileo number
G (d_{pore})	Calculated pore size distribution
H_0	Settled bed height, cm
H_a	Height of the theoretical plate due to axial dispersion, cm
H	Expanded bed height, cm
HEG	Copolymeric macroporous HEMA and EGDM
HEMA	2-Hydroxyethyl methacrylate
HETP	Height equivalent to theoretical plate, cm
HMDI	Hexamethylene diisocyanate
ID	Internal diameter, mm
IPDI	Isophorone diisocyanate
K	Consistence index from viscosity measurement
K(d_{pore})	Experimental pore size distribution
K_d	Dissociation constant, U mL^{-1}
kDa	Kilodalton
k_f	Film mass transfer coefficient, cm s^{-1}
L	Mean projected solute size, Å
L_c	Ligand concentration, mg g^{-1} dry wt of matrix
M	Flow index
m_u	Units of enzyme applied to the column
m	$\varepsilon_0/(1-\varepsilon_0)$

MF	Microfiltration
MW	Molecular weight, kDa
n	Expansion index (Richardson-Zaki coefficient)
N	Number of theoretical plates
NBR	Nitrile butyl rubber
Pe	Peclet number
Pe_p	Particle Peclet number
POM	Polyoxymethylene
PMDI	Phenylene methylene diisocyanate
PP	Polypropylene
Q	Volumetric flow rate, mL min^{-1}
q*	Equilibrium adsorption capacity, U g^{1} dry weight of the matrix
q_b	Maximum bed adsorption capacity, U g^{1} dry weight of the matrix
q_m	Maximum adsorption capacity in batch adsorption, U g^{1} dry weight of the matrix
Re	Reynolds number
Re_m	Reynolds number for minimum fluidisation
Re_p	Particle Reynolds number
Re_t	Terminal Reynolds number
RTD	Residence time distribution
Sc	Schmidt number
Sh	Sherwood number
SS	Stainless Steel
t_b	Breakthrough time, min
TDI	2,4-Tolylyl diisocyanate
t_m	Mean residence time of the solute, s
u	Superficial velocity, cm s^{-1}

UF	Ultrafiltration
u_m	Minimum fluidisation velocity, cm s^{-1}
u_t	Terminal settling velocity, cm s^{-1}
V	Volume of the feed applied to the column, mL
V_e	Peak elution volume of the solute, mL
V_0	External void volume, mL
V_b	Breakthrough volume, mL
V_f	Volume of the fluidized or expanded bed, mL
V_t	Total column volume, mL
V_{tf}	Total volume passed through the bed, mL
ΔF_v	Portion of the total volume of the particles having the particle diameter within the interval $d_p-\Delta d_p/2$ to $d_p+\Delta d_p/2$.
ΔH	Difference in bed height, cm
Δp	Pressure drop across the bed, Kg cm^{-2}
δ_e	Solubility parameter of the solvent
δ_{EGDM}	Solubility parameter of EGDM
δ_g	Solubility parameter of the polymer
δ_{HEMA}	Solubility parameter of HEMA
ε	Void volume of the packed bed
ε_o	Void volume under expansion
ε_p	Particle accessible void fractions
ϕ_{EGDM}	Volume fraction of EGDM
ϕ_{HEMA}	Volume fraction of HEMA
μ	Viscosity of the liquid phase, mPa s
ρ_l	Density of the liquid, g cm^{-3}
ρ_p	Density of the particle, g cm^{-3}

σ	Standard deviation
σ_r	Dimensionless variance
θ	Dimensionless time.
τ	Fluid shear stess (m Pa)
γ	Shear rate (s^{-1})

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Chapter 1

General Introduction

1.1. Summary

Purification of proteins by expanded bed has gained considerable interest in recent years. Chromatographic purification of proteins on expanded bed is usually carried out in a single step from unclarified crude fermentation broth on stable expanded bed, thereby economising the process. The traditional multi-step methods, along with the other single step methods of protein purification, are first discussed to bring forth their merits and demerits. The principle of protein purification on expanded bed, operating conditions, its advantages and disadvantages are also discussed. Special emphasis is given to the matrix development, their characterisation methods, determination of key parameters relating to chromatographic adsorption, design and practical considerations. Dependence of various operating variables on the physico-chemical properties of the feed, adsorption performance of protein and the feasibility as well as the applicability for protein purification from a wide range of feed stocks are also discussed.

1.2. Background

Most of the proteins produced in an industrial scale are either from cells (microbial, plant and animal) or are from fluids in which such cells have been active. The latter category includes blood (plasma or serum). However, most products of interest arise from the activity of cells or of cell constituents under controlled conditions as in reactors. These proteins have end uses like enzymatic activities, specific recognition and other therapeutic uses (e.g.: proteins, enzymes, peptides, antibodies, antigens, nucleic acids, hormones etc.). The common impurities in protein are extracts, cell debris, lipids, polyphenols, starch, pectins, mucins, nucleic acids and proteases. A sequence of steps are required to purify proteins up to the desired level for an industrial operation. Each purification step has its capital and process cost, which dictates the overall economy of the process. The combination of the steps dictates the cost of protein. Therefore, the unit operations involved in these steps need to be selected very judiciously.

A majority of proteins produced by cultivation of microorganisms, animal and plant cells involve suspended particles and a combination of many biomolecules having different physico-chemical properties. The suspended particles include whole cell in fermented broths and fragments of broken cells (when the desired substance is present within the cells, a cell breakage step is necessary to release the intracellular product).

Isolation of the product of choice usually involves the following steps:

1. Separation of cells from the surrounding fluid i.e. clarification
2. Isolation of a rather impure product from the fluid i.e. concentration
3. Purification of the impure product

Depending on the extent of the separation required (determined by the nature of step 2), the first step may take the form of screening, dead-end filtration, cross flow filtration, settling and decantation or centrifugation, or a combination of two or more of these operations. Sometimes coagulation and/or filter aids may be used to assist the processing. If such additives are to be avoided, dead-end filtration is restricted essentially to cells which do not form compressible cakes under the conditions of filtration. The standard techniques used for the removal of cells and cell debris are centrifugation and microfiltration. The centrifuges in current use are well suited to industrial processes and handle volumetric flows exceeding 1,000 L h^{-1} in a completely contained environment, which is crucial to handling genetically modified organisms. However, the cost and hence effectiveness of centrifugation is highly dependent on particle size, density difference between the particle and surrounding liquid and viscosity of the feed stock. Small cells (*E. coli*, or cell homogenates), small particle size and high viscosity reduce the feed capacity during centrifugation and sometimes makes it difficult to obtain a completely particle-free liquid in which the clearance value of the particles should be in excess of 99 to 99.9% (Datar and Rosen, 1987). It has also been shown that centrifugation is not efficient enough to clarify cell culture broth for direct application onto chromatographic column (Berthold and Kempkan, 1994). Continuous centrifugation may also result in shear damage. Centrifugal force as such is generally not harmful, but significant shearing forces may

arise during entry, in passage through the channels in disc centrifuges and in the nozzles of centrifuges, which discharge the solids continuously as slurry. To obtain particle-free broth that can be further purified by traditional packed bed chromatography (PBC), centrifugation is usually combined with microfiltration.

Microfiltration is usually carried out in cross flow filtration mode (Baily et al., 1990; Fane and Radovich, 1990) of the suspended material. This subjects to shear stresses during the passage through the filter module and recirculation improves the process. Fluxes in the range of 50 to 1000 L m^{-2} h^{-1} have been reported (Fane and Radovich, 1990). However, these fluxes are dependent on the composition of the feed stock. Flux in the range of 25-85 L m^{-2} h^{-1} are used to harvest cells of *E. coli* at different cell densities (Baily et al., 1990). This is below the suggested economic limit for the cross flow filtration process, which is set to around 100 L m^{-2} h^{-1} (Kroner, 1984). A major advantage of cross flow filtration is the relative ease with which the process can be operated as a contained system. This is facilitated by the absence of moving parts as compared with a centrifuge. However, microfiltration has its drawbacks. Though microfiltration yields cell free broth, the flux of liquid per unit membrane area often dramatically decreases during the filtration (when the solid content approaches 30%) (Tutnjian, 1982; Schutte, 1982). Problems arise from fouling of the microfiltration membrane and form concentration polarisation, both of which may have adverse effect on the rate of separation and may limit the feasible degree of concentration that is achievable. Some of the problems associated with fouling can be minimised by careful design of the operating parameters (Fane and Radovich, 1990).

The combined use of centrifugation and filtration often results in long process time or the use of comparatively large units causing significant capital expenditure, with added recurrent costs towards equipment maintenance. It also results in significant product loss due to degradation.

Neither centrifugation nor microfiltration have any significant ability to reduce the volume of the feedstock and thereby increase product concentration.

In steps 2 and 3, the variety of potential processes available includes solvent extraction, precipitation, concentration, ion exchange, adsorption, gel and affinity chromatography, crystallisation, spray drying, freeze-drying and electrophoresis. A specific process is chosen taking into account the physical and chemical properties of the required substance and of the impurities present, paying due attention to the cost and safety. Particular attention is given to any adverse effects that may arise from processing conditions. In most cases these effects will take the form of decomposition or changes in molecular structure as a result of chemical action or changes in temperature. In the case of proteins in particular, account also needs to be taken of the effects of shear on flocs.

Disruption of cells also gives rise to problems other than those associated with the separation of whole cells from the surrounding fluid. In particular, the disruption of the cell wall generates many small particles including those released from within the cells. The matter is complicated further by the release from the cells of nucleic acids which may cause a significant increase in viscosity.

There is scope for exploiting the properties of cells and flocs, which might complement and improve the efficiency of existing processes. These include the response to exposure of particulate matter to steady or fluctuating electrical, ultrasonic and magnetic fields. These processes might be used to control the growth of crystals or flocs, to effect the separation by streaming in continuous-flow systems, or to reduce fouling and polarisation in membrane separations. Magnetic separation could be more widely exploited by adsorbing particles or specific molecules onto the surfaces of magnetic particles. The production of very small (50-100 nm) particles of magnetite by some bacteria is of special interest.

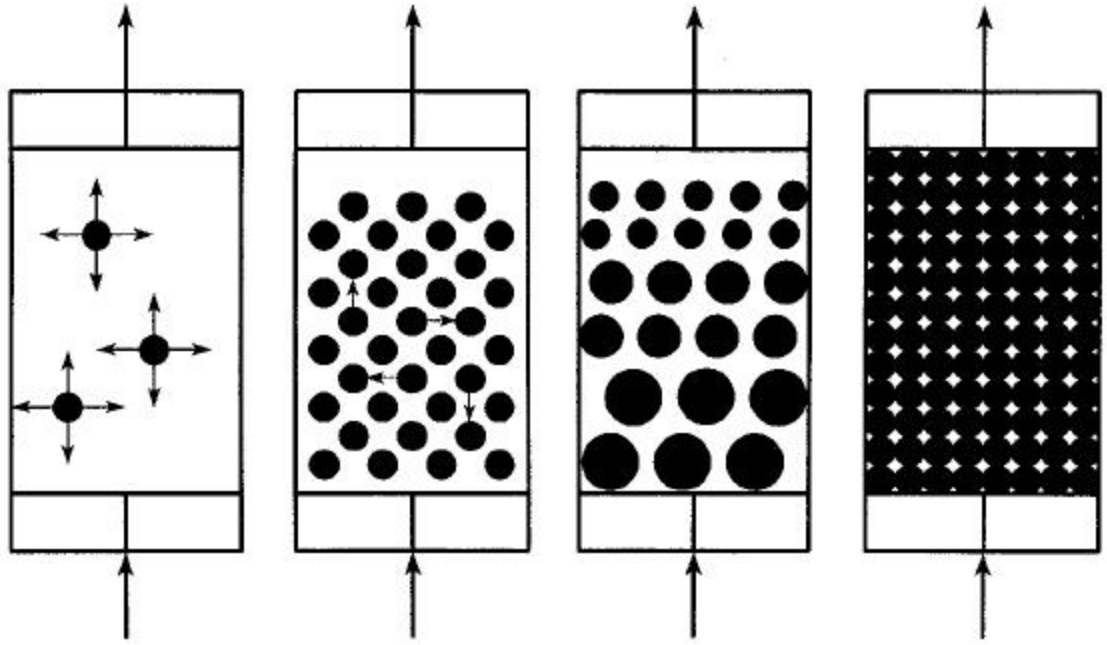
Foam floatation might offer a less-damaging, convenient and cost-effective alternative to filtration and centrifugation. Shear damage in continuous centrifugation might be reduced, especially by improved design of feed and collection arrangements for suspensions. Since it is virtually impossible to directly investigate the flow regimes in such systems, computational fluid dynamics have an important role to play. A similar approach could be applied to the use of cross flow systems and

perhaps to flow in packed beds. These are examples of processes where knowledge of the local, as opposed to overall conditions in processing equipment might lead to significant improvements in process efficiency. They also highlight the need for laboratory studies to be conducted under conditions that provide a sound basis for scaling-up. Therefore, one of the keys to reduce the costs of a biotechnological production is the simplification of the downstream process (Spalding, 1991). This may be achieved by the reduction in the number of steps involved in the process. Integrated technologies have aimed at combining single steps of a protocol into new unit operations, thus tightening the process. Up to now, several principles of integration have been described. A quite different technique to obtain a clarified solution of the product is to use aqueous two phase system extraction. In an aqueous solution of two properly selected polymers or one polymer and a salt, two phases will be formed, both containing 80% or more of water. The most studied dextran-polyethylene glycol systems are expensive to use on a large scale mainly due to the prohibitive cost of purified dextran. While some success has been obtained with crude dextran, (Kroner et al., 1982) greater experience is needed with these systems to evaluate their true potential. Polyethylene glycol (PEG)-salt systems have been successfully used on a large scale (Kroner, 1983). However, the economy would be improved if an ultrafiltration unit is used to recover PEG. The possible volume reduction will be limited by the product solubility in the presence of PEG. Most of the proteins have been tested for purification by this methodology (Birkemueier et al., 1987; Kroner and Kula 1978; Kroner et al., 1982; Woodrow and Quirk, 1986).

The introduction of membrane chromatography has helped to accelerate downstream operations by allowing concentrating very dilute solutions together with purification by chromatography (Brandt et al., 1988 and Briefs and Kula, 1992). Standard packed bed chromatography requires that particulate material be removed from the feed, since the packed bed acts as a dead-end filter and is easily blocked by the particulates. An alternative to this is the adsorption on to a resin in a stirred tank with feed stocks. At the end of the adsorption phase, adsorbent particles are separated from the depleted feedstock using a filtration or sedimentation system that is able to distinguish between adsorbent particles in the feed. Washing and elution of the loaded

adsorbents may then be carried out either in batch or packed bed mode. This method has, for instance, been used for many years on a commercial scale for the isolation of plasma coagulation factor IX using DEAE Sephadex (Brummelhuis, 1980).

The well-mixed batch adsorption (Figure 1.1a) is a single-stage process that requires more adsorbent to achieve the same degree of adsorption as in multistage process such as packed bed chromatography. Other disadvantage of this process is the mechanical difficulty in handling the adsorbents. Another approach to increasing the efficiency of batch adsorption is the use of submicron ion-exchange particles, which are later removed from the cell homogenate by centrifugation (Vorauer-Uhl et al., 1993).



(a) (b) (c) (d)

Figure 1.1. Operational modes for protein adsorption in column of a) stirred tank b) fluidised bed c) expanded bed and d) packed bed.

A variety of fluidised bed systems have been developed to remove particulates with the recovery of desired product. In a fluidised bed (Figure 1.1b) particulate adsorbent in a column is forced to rise from its settled state by introducing an upward liquid flow into the column. In the fluidised state the bed voidage increases, thereby

creating enough space-in-between the adsorbent particles so that the cell and the cell debris can pass unhindered through the bed.

By the optimal choice of adsorbent particles that bind to product, it is possible simultaneously to recover the protein of interest and to remove cells and cell debris. This should make fluidised beds a very attractive methodology for the recovery of various biomolecules. However, few applications have been documented on the industrial use of fluidised beds, mainly due to lack of suitable adsorbents. Early applications on fluidised beds describe the direct recovery of antibiotics from cell suspension. The process used a cation exchanger, which was scaled to handle up to 15 m^3 h^{-1} in a 1.2-m diameter column. A more recent example is the recovery of immunomycin from a *Streptomycin hygroscopicus* culture (Gailliot et al., 1990). The immunomycin was adsorbed on a polystyrene-divinyl benzene matrix in a methanol/water mixture. Although the initial product concentration was low, the product was recovered in very high yield from feedstocks of 14,000 L.

While fluidised beds have been successfully employed for direct recovery of low molecular weight compounds, it has been found unsuitable for the recovery of protein for a number of reasons:

1. The adsorbents used for purification of low molecular weight compounds are not suitable for protein purification because ionic and hydrophobic surfaces of the matrices result in nonspecific interaction and loss of protein during adsorption.
2. The traditional chromatographic adsorbents designed for packed bed adsorption are compatible with proteins but lack in physical and chemical properties, necessary for fluidised bed operation.
3. The relative high degree of mixing and channeling lowers the efficacy of adsorption process. The protein effluent from the column has to be recycled to minimise loss.

Several attempts have been made to minimise the mixing in the bed. One approach is to introduce segmented beds (Buijs and Wesseling, 1980 and Van der

Wiel and Wesselingh, 1989). Sectioning of a fluidised bed column into smaller compartments by inclusion of suitable sized baffles around the walls of the column reduces back mixing and increases efficiency in adsorption. This method is not suitable for batch protocol, commonly used in packed bed adsorption procedures, but is best suited for continuous countercurrent operation in which pulsed flow is used to convey adsorbent particles downwards, counter to upward flow of feedstock.

Other approaches are based on maintaining the adsorbent beads in a fixed position within the bed, yielding a stable expanded bed (Figure 1.1c), which behaves like a packed bed with greater voidage. The schematic diagram for a packed bed chromatography is depicted in Figure 1.1d. Another method that involves the stabilisation of ferromagnetic particles by subjecting to an external magnetic field is termed as magnetically stabilised fluidised bed (MSFB) (Burns and Graves, 1985; Lochmuller and Wigman, 1987; Chetty and Burns, 1991; Terranova and Burns, 1991). In MSFB, back mixing is reduced through the use of a magnetically susceptible adsorbent, such as alginate/magnetite, polystyrene-divinyl benzene/magnetite and agarose/magnetite, polyacrylamide-magnetite. While, MSFBs have been successfully demonstrated at laboratory scale for a number of operations, it has probably not been implemented in pilot/production scale. The most obvious reason is the relatively complicated equipment needed for MSFB. Furthermore, the large magnetic fields necessary to keep MSFBs in efficient operation generate considerable heat that needs to be removed from the system.

Purification of proteins by a number of steps with the single step alternative method (expanded bed chromatography) is shown in Figure 1.2.

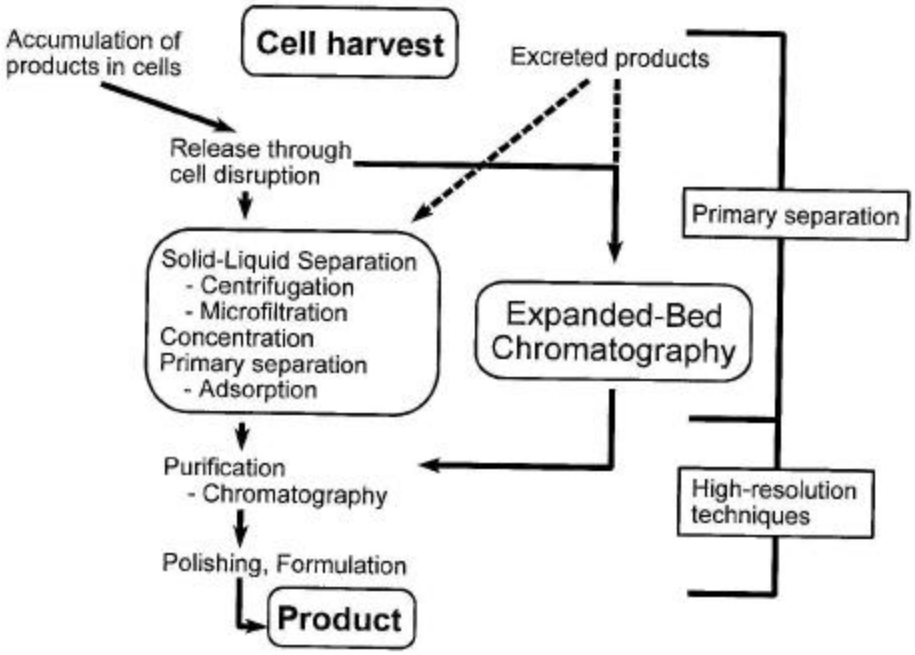


Figure 1.2. Schematic process flow sheet of conventional downstream processes.

1.3. Adsorption on expanded bed

Chase and Draeger (1992) and Draeger and Chase (1991; 1992) introduced the concept of adsorption of proteins on expanded beds. They documented that the bed voidage is larger than that in packed/sedimented beds. Expanded beds are sometimes considered as an integral part of the fluidised bed technology; some authors do not differentiate between the two expressions. A comparison of the characteristics of expanded beds versus fluidised beds is depicted in Figure 1.3.

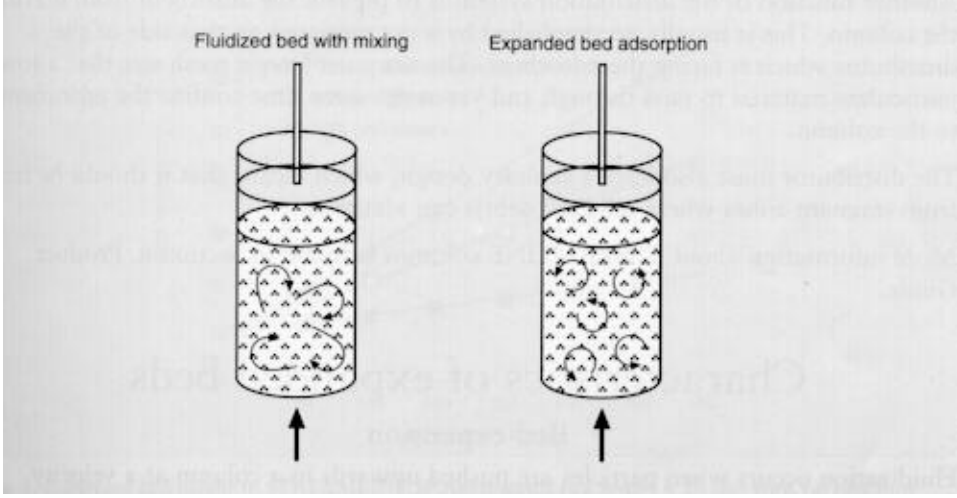


Figure 1.3. Schematic diagram of fluidised bed vs stable expanded bed.

It may be argued that all fluidised beds are expanded beds (relative to packed beds). Reviews have been published on purification of proteins on expanded beds (Chase, 1994; Hjorth, 1997 and Anspach et al., 1999).

1.3.1. Principle

Expanded beds are based on fluidisation. When a liquid is pumped at a low velocity to the bottom of a sedimented bed having a distributor plate, with free space between the adsorbent bed and the upper adapter, the liquid flows through the interstitial space of the sedimented bed without causing adsorbents to move. On increasing the superficial velocity, the pressure drop in the liquid passing through the bed increases, as shown in the Figure 1.4 by the linear segment OA. At this point A, the pressure drop equals the force of gravity on the particles and the adsorbents begin to move. First the bed expands slightly with the adsorbents still in contact. As the porosity increases, the rise in pressure drop is more gradual than earlier. When Point B is reached, the bed is in the loosest possible condition with the adsorbents still in contact. As the velocity is increased further, the adsorbents separate out and true fluidisation begins. The pressure drop sometimes diminishes marginally from point B to F. From Point F onward the particles move more and more vigorously, swirling about and travelling randomly in all directions. The contents of the column strongly resemble a boiling liquid, and the term “boiling bed” is used to describe solids fluidised in this manner.

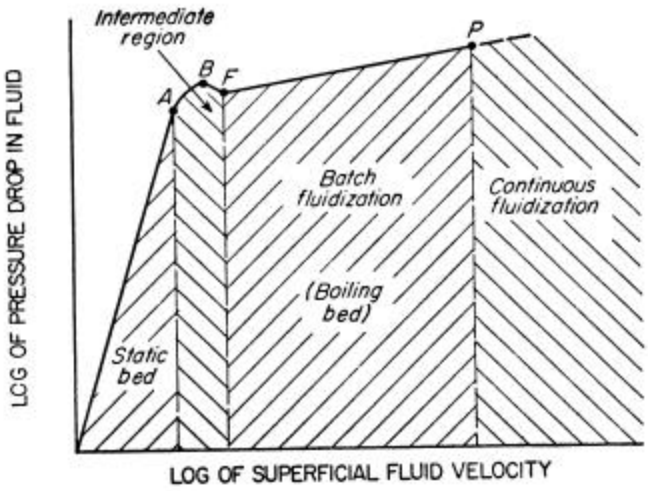


Figure 1.4. Pressure drops in fluidised solids.

Linear velocity of fluid between the particles is much higher than the velocity in the space above the bed. Consequently nearly all the particles drop out of the fluid above the bed. Even with vigorous fluidisation only the smallest adsorbent are entrained in the fluid and are carried away. On increasing fluid velocity further, the voidage (porosity) of the bed rises; the bed of solids expands, and its density falls. Entrainment becomes appreciable, and when all particles are entrained in the fluid, the porosity approaches unity, and the bed as such ceases to exist. The phenomenon then attains the state of simultaneous flow of two phases. From point F to point P and beyond the pressure drop rises with the fluid velocity but much more gradually than when the solid particles were stationary. Fluidisation without entrainment is complete, the fluidisation is said to be continuous. This point is called the relaxation point and the velocity required to cause relaxation of solid particles is called minimum fluidisation velocity, u_m, which characterises transition between the sedimented and expanded state. The adsorbent particles have high density, but with a distribution in size. When there is constant upward flow, the beads are suspended and each finds its own equilibrium position; the bed is expanded and stable. At the relaxation point, column back pressure is in equilibrium with the force due to the weight of the adsorbent particles. Further increasing the flow-rate merely causes expansion of the particle bed and a constant back pressure in the expanded bed (Figure 1.5). Only monodisperse particles show a sharp transition in the back pressure

with increasing flow rate. In practice, relaxation usually takes place over a range of flow rates owing to the particle size distribution.

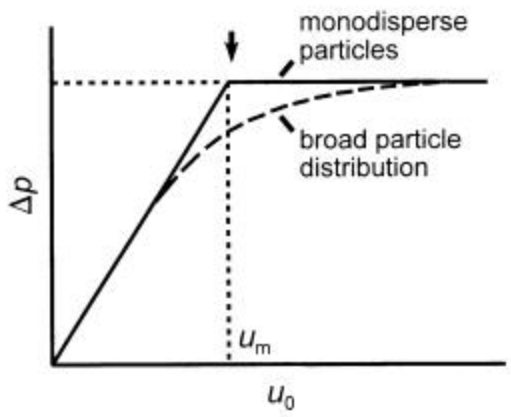


Figure 1.5. Dependence of pressure drop and minimal fluidisation on the flow rate of a sedimented particle bed.

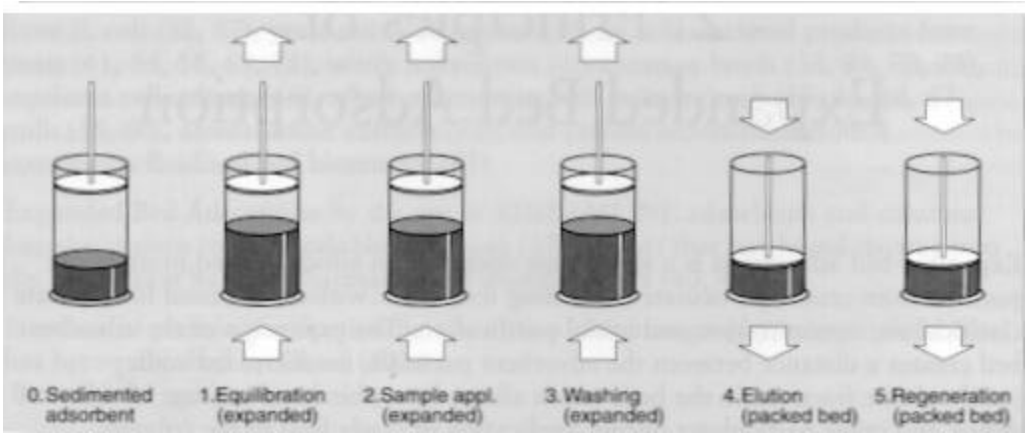


Figure 1.6. Expanded bed adsorption showing various stages of operation.

1.3.2. Operation

Loading the adsorbents in a column generates a random distribution of small and large particles in the sedimented bed. In order to achieve the particle size distribution and densities as designated by the manufacturer, it is necessary to completely mix the suspension before loading it into the column (if not, the whole content is used). Otherwise, particle size distribution will differ in subsequent column fillings due to classification.

During equilibration (Figure 1.6-1), the bed is usually expanded by a factor

of 2 to 3, thereby increasing the voids between the adsorbent particles and the bed porosity, starting from $\varepsilon_0 \approx 0.4$ in the sedimented bed. After about 30 minutes the classification within the bed is completed. This can be visually inspected, as the movement of the particles comes to an end.

The feed containing cells, cell debris or other particulate matter is applied subsequently Figure 1.6-2). As the physical properties of the feedstock are different from those of the buffer used for equilibrations-especially the viscosity being high, the bed expands further if the flow rate is not decreased. Provided that the settling velocity of the particulates in the feedstock is much lower than that of the adsorbent particles, the particulates leave the column, whereas the proteins that strongly interact with the adsorber are retained.

An intensive washing step (Figure 1.6-3) is necessary to remove particulates and weakly adsorbed proteins. It is essential to pass several column volumes (≈20 relative to the sedimented bed) of the buffer through the column in the expanded mode; otherwise, particulates may remain in the column. The flow is then stopped, the adsorbent allowed to settle and finally the upper adapter is lowered. If necessary, further washing can be done in the packed mode in either flow direction.

Elution is generally carried out in the packed configuration as smaller volume is needed to elute the product (Figure 1.6-4). As in packed-bed chromatography, reversal of flow is recommended for step elution. If gradient elution is preferred, the same flow direction, as used during adsorption, may be more effective. Due to reasons of biological safety, movable parts may be undesirable and a closed system may be preferred. In this case, the upper adapter may be fixed and washing and elution carried out in the expanded mode, the differences being an increase in elution time and volume.

Some components continue to be bound under the chosen washing and elution conditions. As the removal of undesirable components follows an exponential decline, a complete removal should not be envisaged. This aspect is particularly important for bacterial cells. A decontamination of the column requires clean-in-place

(CIP) procedures with reagents such as 1 M NaOH and 2 M NaCl, up to 70% aqueous ethanol or 6 M urea in the expanded mode. Besides the biological hazard that may develop on circumventing CIP, column operation may be severely affected. Channeling may occur due to aggregation of adsorbent particles. Regeneration of the column is carried out in a packed bed mode also (Figure 1.6-5). Also, the sedimented bed may move upwards as a whole with the next run.

1.4. Theoretical considerations

The term "expanded bed" and "fluidised bed" are used differently in literature. In expanded beds, particles are supported by frictional forces caused by fluid flow. The frictional force which supports the particles of fluidised beds are mechanically caused by other particles. The term "expanded bed" was coined to emphasise the fact that fluidised bed may behave differently (Chase and Draeger, 1992).

According to Stoke's law, the bed expansion in a column depends, among other parameters, on the viscosity of the applied solution. The viscosity of microbial homogenates is higher than water and strongly varies with the composition and concentration of the biomass. It can not be predicted but must be measured. This is usually done by plotting the data on shear stress against shear rate obtained by using a rheometer. Only if Newtonian behaviour applies or the solution is very dilute, the viscosity can be directly obtained from the slope of the graph. Furthermore, only then the simple dependance of the shear rate, γ, on the linear liquid velocity, u, and the particle diameter, d_p, according to Eq. (1.1), is valid.

$$g = \frac{u}{d_p} \qquad (1.1)$$

Higher concentrations of biopolymer solutions display non-newtonian behaviour and the viscosity is shear rate dependent. A better approximation of the shear rate in an expanded bed is provided by Eq. (1.2), considering the flow index, m, and the voidage of the expanded bed, ε (Machac et al., 1993). The flow index can be obtained from the power law (Squires et al., 1988), with τ, the fluid shear stress, while K, the consistence index, is obtained from viscosity measurements at different

shear rates. The flow index allows the estimation of the shear rate to be considered during expanded bed adsorption. As the shear rate in the expanded bed is very low (<10 s^{-1}), the viscosity is to be extrapolated from the tangential slope of the power law graph at very low shear rates.

$$\gamma = 12.u_0.\frac{(1-\varepsilon)}{\varepsilon^2 d_p}.\frac{(3m+1)}{4m} \qquad (1.2)$$

$$\tau = K\gamma^m \qquad (1.3)$$

The voidage of the expanded bed depends on the degree of bed expansion, the ratio h/h_0 of expanded bed to the settled bed. To accommodate particulate matter, such as cells or cell debris in the column, the flow rate during adsorption must be higher than u_m. On the other hand, it must be much lower than u_t in order to keep the height of the column managable and to prevent a highly diluted particle suspension. The useful range of flow-rates is provided by correlation given by Richardson and Zaki (1954). It is valid at very low particle to column diameters; at ratios d_p/d_c>0.01 wall effects are to be expected (Joshi, 1983). Eq. (1.4) correlates u_0 and the terminal settling velocity u_t of a particle with ε and the expansion index, n.

$$\frac{Re_0}{Re_t} = \frac{u_0}{u_t} = \varepsilon^n \qquad (1.4)$$

Where Re_0 and Re_t are the Reynolds number containing u_o and u_t terms, respectively. For Re_t<0.2, which applies to normal expanded bed operations, n=4.65 is constant. However, the expansion index depends on both particle and fluid properties. Furthermore, while the most fundamental investigations on fluidisation were performed with uniform, spherical, non-porous particles, particles of high densities and greater diameters were used in expanded bed chromatography. Consequently, differing values of n were described by Chang and Chase (1996) for liquids of differing glycerol content on an ion-exchange sorbent. The porosity for the sedimented bed, ε_0, can be measured by the application of a large solute that does not

penetrate into the adsorbent's pore system. The random bed theory yields ε_0=0.418 (Jakubith, 1991) and as a rule of thumb it is assumed that ε_0=0.4.

It was also shown that n depends only on the terminal Reynolds number, Re_t, of a particle. For Re_t<0.2, n=4.65 is constant. It should be mentioned that the values of n depend both on particle and fluid properties. Eventhough the Re_p for the Streamline adsorbent is in the range 0.1-0.4, no significant difference has been observed between the data simulated by the Richardson-Zaki model and experimental data. The Richardson-Zaki model is only valid for monodisperse particles. The model thus has to be corrected for the wide particle size distribution of the Streamline adsorbents. This is done by using the Perfectly Classified Bed (PCB) model presented by Al-Dibouni and Garside (1979) as follows.

$$\Delta H(\varepsilon) = h_0 \frac{(1-\varepsilon_0)\Delta F_V}{(1-\varepsilon)} \qquad (1.5)$$

where h_0 is the sedimented bed height, ε_0 is sedimented bed voidage, ΔF_v is the portion of the total volume of the particles having the diameter within the interval $d-\Delta d/2 < d < d+\Delta d/2$ and ε, is the bed voidage within that interval. The model states that the particle size group between d and $(d-\Delta d)$ is contained within the bed heights h and $(h+\Delta H)$. Using this approach, each segment of height is thought of as a bed with a specific bed voidage that can be calculated with the Richardson-Zaki equation. The total bed height is obtained by simply adding up the heights of each segment. It is important to point out that the Richardson-Zaki equation only describes expansion in the equilibrium state, i.e. when the bed has stopped expanding.

Another model that describes a segregated system was set up by Kennedy and Bretton (1966). The mechanism of the model is as follows. The particles in the superposed monocomponent beds are in constant and essentially random motion. From time to time a particle from one component will cross the interface into another component where the voidage differs from the equilibrium state experienced prior to the transfer. The effect of this is a net force on the particle that tends to return it to the bed of its own species. In this steady state, the diffusion and convective fluxes of each

component on a horizontal plane may be equated to yield the following statement of the Kennedy-Bretton model:

$$D_{p_i}\left(\frac{dC_{p_i}}{dh}\right) = C_{p_i} u_0 u_t \frac{\varepsilon^{n_i}}{\varepsilon} \qquad i = 1,2,..... \tag{1.6}$$

where D_{Pi} is the particle dispersion of component i and C_{pi} is the mass concentration of component i. The advantage of this model is the ability to predict the variance in size distribution with height, in beds with narrow size ranges with pronounced mixing. The preference for describing the segregation in Streamline using the PCB model is its simplicity. The wide particle size distribution of the Streamline adsorbent increases the stability of the bed, which makes it possible to use the simple PCB model.

Furthermore, the most fundamental investigations on fluidisation were performed with larger, denser, uniformly spherical beads as used in ion-exchange chromatography. Besides d_p, the viscosity, η, and g, the gravitational acceleration, u_t depends upon the density of the adsorbent, ρ_p, and liquid ρ_l, and can be estimated

$$u_t = \frac{(\rho_p - \rho_l) d_p^{\,2} g}{18\eta} \tag{1.7}$$

from the Stoke's law (Eq. 1.7). A more comprehensive description of the hydrodynamics in liquid fluidisation is provided by di Felici (1995).

In this equation the parameters that are seen to play a decisive role in particle fluidisation are mainly the diameter and density of the adsorber matrix that can be used to control the terminal velocity in the fluidised bed. As viscosity is an inverse function of temperature, the bed expansion increases on shifting a column from a laboratory to a cold room if the flow rate is not adjusted correspondingly. Therefore, temperature control is very important to keep a control of fluidisation of the bed.

Assuming that the volume of the solid-phase does not change with bed expansion, h/h_0 can be calculated from Eq. 1.8 with a porosity of the settled bed, ε_0 as obtained from Eqs. (1.4) and (1.7).

$$\frac{h}{h_0} = \frac{(1-\varepsilon_0)}{(1-\varepsilon)} \tag{1.8}$$

The expansion behaviour can also be estimated from empirical considerations. Hartmann et al. (1992) had the best fit to their experimental data for n by using Eq. (1.9) from Garside and Al-Dibouni (1977); this equation is valid for $10^{-3} < Re_t < 3.10^4$.

$$n = \frac{5.09 + 0.2309\,\mathrm{Re}_t^{\,0.877}}{1 + 0.104\,\mathrm{Re}_t^{\,0.877}} \tag{1.9}$$

A more general theoretical view of fluid behaviour is based on the Gallileo number (Martin et al., 1981).

$$Ga = \frac{\rho_p g(\rho_p - \rho_l) d_p^{\,3}}{\eta^2} \tag{1.10}$$

From Ga, the terminal Reynolds number can be calculated for different column and particle diameters:

$$\mathrm{Re}_t = \left[\frac{23}{Ga} + \frac{0.6}{Ga^{0.5}}\right]^{-1} \left[1 + 2.35.\frac{d_p}{d_c}\right]^{-1} \tag{1.11}$$

From which the terminal flow-rate works out to $u_t = Re_t \eta d_p$. Also, the expansion index can be estimated from Ga.

$$\frac{5.1-n}{n-2.4} = 0.016.Ga^{0.67} \tag{1.12}$$

The Reynolds number for minimal fluidisation can be estimated according to Riba et al. (1978).

$$\mathrm{Re}_m = 1.54.10^{-2} Ga^{0.66} M\upsilon^{0.67} \tag{1.13}$$

with Mυ expressed as

$$M\boldsymbol{u} = \frac{\left(\boldsymbol{r}_p - \boldsymbol{r}_l\right)}{\boldsymbol{r}_l} \tag{1.14}$$

Tailor-made expanded-bed adsorbents display a broad particle size distribution, leading to a gradual transition of bed expansion. Consequently, as long as theoretical models are not available to account for this distribution, the bed expansion characteristic is best obtained by experimental evaluation using solvents of differing viscosity.

The simplest way to describe the overall mixing in a liquid fluidised bed is via the residence time distribution (RTD) of fluid elements. RTD describes the probability distribution of fluid element spending a certain time t in the column. A wide RTD represents a situation where gross mixing of liquid element leads to a broad range of possible time t that a fluid element can spend in the column. If we consider the application to a column of infinitely narrow pulse of a suitable tracer that does not interact with the adsorbent, ideal plug flow would demand that the pulse travels unchanged through the bed, so that each tracer molecule has precisely the same residence time t'. The residential time is calculated from the ratio of the bed length to the flow velocity (h/u_0). If one or more of the factors discussed above causes axial mixing, then some elements of the pulse will be retarded and result in increased t'. Other parts will travel faster and show reduced t'. Thus a variety of t' are obtained, which are characterised by the residence time distribution function E (t).

There are two simple models that mathematically describe RTD as a function of distinct parameters and quantify the amount of axial mixing within a column; the dispersion model and the tanks-in-series model.

The dispersion model starts with a mass balance over a thin segment of an adsorbent column. A Consideration of convective and dispersive transport results in the following equation.

$$\frac{\partial C}{\partial t} = D_{ax,l} \cdot \frac{\partial^2 C}{\partial Z^2} - u_0 \cdot \frac{\partial C}{\partial Z} \tag{1.15}$$

Under the boundary conditions of an open system, an analytical solution may be obtained, which describes E(t) as a function of the dimensionless time θ (-t/τ) and of a single variable, the column Peclet number Pe, which is defined according to the equation

$$E(\theta) = \frac{Pe}{\sqrt{4.\pi.\theta}}.e^{\frac{Pe.(1-\theta)^2}{4\theta}} \qquad (1.16)$$

The axial mixing within the particle bed is determined by the Peclet number, Pe. This dimensionless number relates dispersed flow, $D_{ax,\ l}$, to convective flow ($u_0 h \varepsilon^{-1}$) (Eq.1.17).

$$Pe = \frac{u_0 h}{D_{ax,l}} \qquad (1.17)$$

The Peclet number is replaced by Bodestein (Bo) number by some of the investigators.

$$Bo = \frac{u_0 h}{\varepsilon.D_{ax,l}} \qquad (1.18)$$

At values for B_0>40, axial mixing has little effect on the adsorption performance, i.e. the expanded bed behaves similar to a packed bed (Chang and Chase, 1996a). Bodestein number increases on increasing the height of the sedimented bed. A decrease in Bodestein number during the operation indicates that the expansion is disturbed by particle aggregation or by local turbulences in the column. The second dimensionless number representing axial mixing is the particle Peclet number (Pe_p), that contains the particle diameter d_p as the characteristic length. It characterises a specific adsorbent particle rather than the specific column set-up.

$$Pe_p = \frac{u_0 .d_p}{D_{ax,l}.\varepsilon} \qquad (1.19)$$

1.5. Equipment/accessories requirement

The set up for expanded bed chromatography consists of two vessels connected through a 3-way valve to a pump, so as to create less pulsation. Higher pulsation creates channelling in the lower part of the expanded bed. Pulsation can be avoided by installing a pulse dampener between the pump and the column. Connecting narrow diameter tubing will allow the pump to run at higher speed so that pulsation can be reduced. The pump is connected to the bottom adapter of the column. The upper adapter of the column is connected to the flow cell of a spectrophotometer to monitor the tracer signal for bed stability measurement or can be used for protein measurement. The 3-way valve allows for a change in the flow direction for step input of tracer and changing the various operations during a chromatographic process e.g. loading, washing, elution and clean-in-place. A stirrer is provided in the sample vessel to stir the feed during application to avoid aggregation of the particulates. The operational view of the expanded bed chromatography is shown in Figure 1.7.

This technology owes its successes to proper design of distributor plate of the column, the adsorbent media and ligand chemistry.

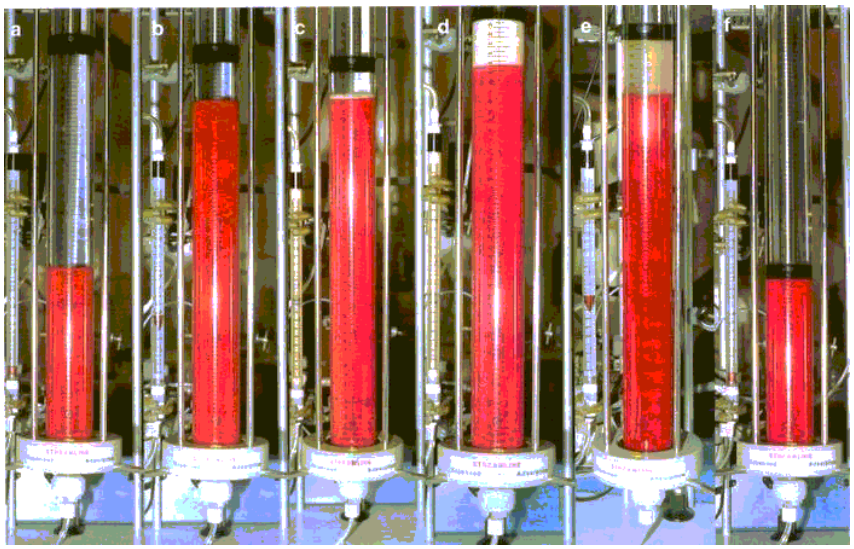
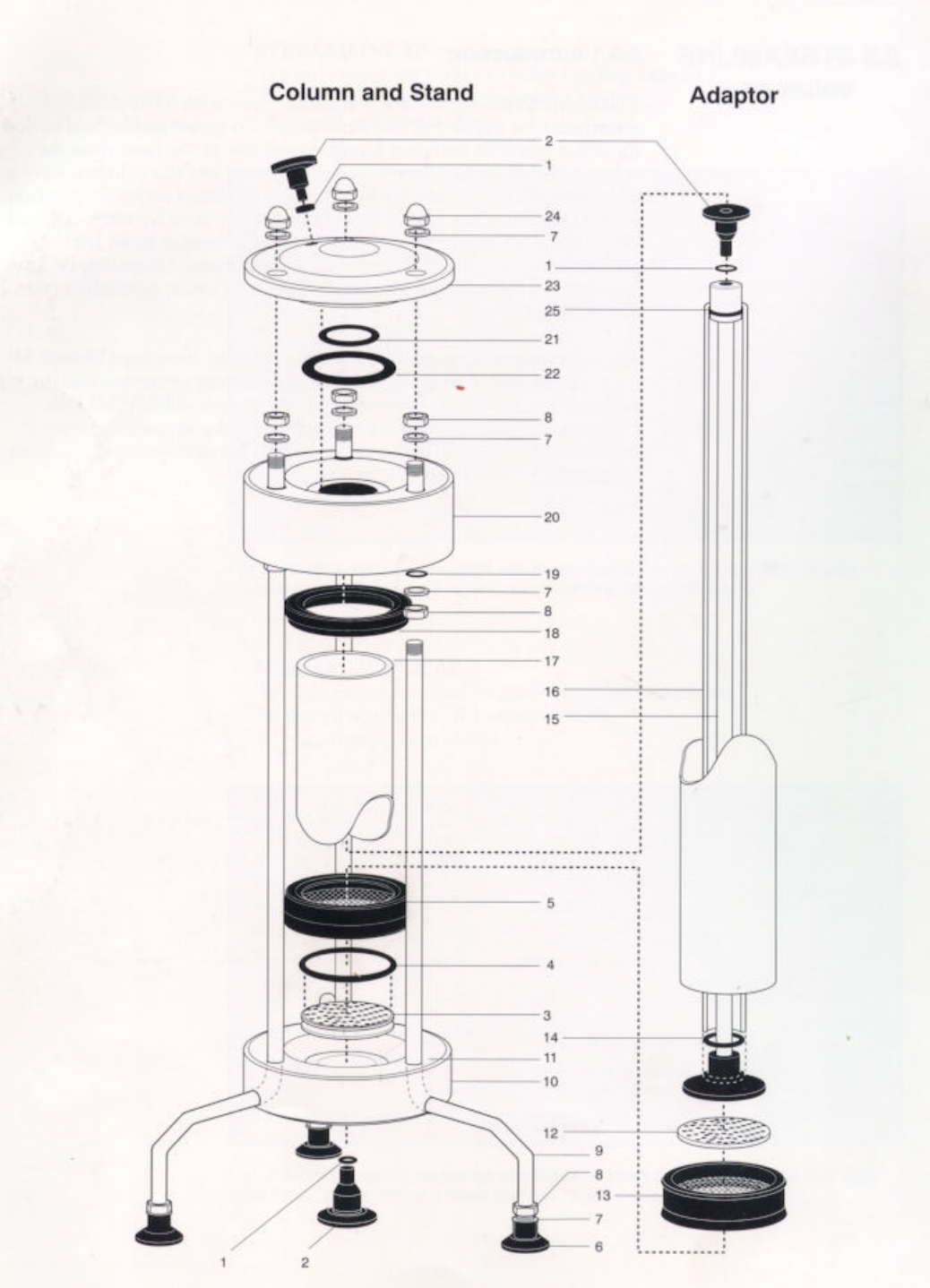


Figure 1.7. An expanded bed chromatography in operation

1.5.1. Column

The column has a significant impact on the formation of stable expanded beds. UpFront Chromatography A/S and Amersham Pharmacia Biotech market columns of various dimensions. These columns are shown in Figure 1.8 and 1.9. Streamline (Amersham Pharmacia Biotech, Uppasala, Sweden) columns are equipped with a specially designed liquid distribution system to allow the formation of a stable expanded bed. A specially designed liquid distribution system for expanded beds is required since it derives from the low pressure drop over the expanded bed. This can assist the distributor in producing plug flow through the column. Since the pressure drop over an expanded bed is much smaller, the distributor plate in an expanded bed column must produce a plug flow by itself. Consequently, it is necessary to build in an additional pressure drop into the distribution system. Besides generating a pressure drop, the distributor has to direct the flow in the vertical direction alone. Any radial flow inside the bed will propagate turbulence through the column. Shear stress associated with flow constrictions also requires a consideration while designing the liquid distributor. Shear stress should be kept to a minimum to reduce the risk of molecular degradation. Another function of the distributor system is to prevent the adsorbent from leaving the column. This is usually accomplished by a net mounted on that side of the distributor facing the adsorbent. The net must have a mesh size that allows particulate materials to pass through and at the same time confine the adsorbent to the column. The distributor must have sanitary design, which means that it should be free from stagnant zones where cells/cell debris could accumulate.

Streamline columns are also equipped with a movable adapter that is operable through a hydraulic drive. This allows the height of the expanded bed to be altered during the different stages of an expanded bed adsorption cycle. The adapter is lowered by pumping liquid into the hydraulic compartment placed above the adapter plate. It is raised by pumping liquid upwards through the column while allowing the hydraulic liquid to flush out of the hydraulic compartment.



1. O-ring, 2. Connector, 3. Distributor plate, 4. Oring, 5. Bottom net, 6. Stand feet, 7. Washer, 8. Nut, 9. Stand, 10. Bottom Flange, 11. Adapter plate, 12. Adapter distributor plate, 13. Adaptetr net, 14. Oring, 15. Adapter, 16. Rod piston, 17. Column tube, 18. Gaasket, 19. O-ring, 20. Upper flange, 21. Rod sealing, 22. O-ring, 23. Lid, 24. Domed nut, 25. O-ring.

Figure 1.8. Principal components of Streamline column

The adapter is set at its upper position for bed expansion and feed application and is lowered to the surface of the sedimented bed for desorption in the packed bed mode. It can also be lowered to the surface of the expanded bed for the wash cycle after feed application. This speeds up the wash cycle and considerably reduces the consumption of wash buffer.

The material used in Streamline columns have been chosen for their compatibility with the solvents used during the operation and the cleaning-in-place (CIP) procedures. Furthermore, Streamline columns have been designed to comply with the varying hygienic requirements at the different stages of process development, i.e. from laboratory to manufacturing scales. The component materials are listed in Table 1.1.

Table 1.1. Material of construction of Streamline column

Column tube – borosilicate glass
Stand – stainless steel (SS 2343)
Rod piston- stainless steel, electropolished (SS 2353, ASTM 316L)
Flange, top and bottom- PP (polypropylene)
Net, top and bottom- Stainless steel (DIN 1.4404)
Net gasket, vulcanised-EPDM (ethylene propylene rubber)
Distributor plate-stainless steel, el. Polished (SS 2343)
Adapter- PP (polypropylene)
O-rings (in contact with the throughput)- EPDM
O-ring (in contact with the hydraulic fluid)- NBR (nitrile rubber)
Feet- POM (polyoxymethylene)

The main feature of UpFront column is that no inlet screen is provided to block the flow of the crude feed. It is provided with a bottom stirrer for good distribution of flow over the cross-section of the column (Figure 1.9) while maintaining plug flow. Due to the very gentle stirring and special design of the

stirrer, no vertex is formed (i.e. no back-mixing is observed despite the mixing!). A provision is also made to reverse the flow for fast settling and elution. A special floating elution distributor (Figure 1.9) has been provided to minimise the volume of elution buffer. Due to this distributor, the height of the elution buffer above the fixed bed is only 1-2 cm during the elution. A concentrated eluate is obtained as a result.

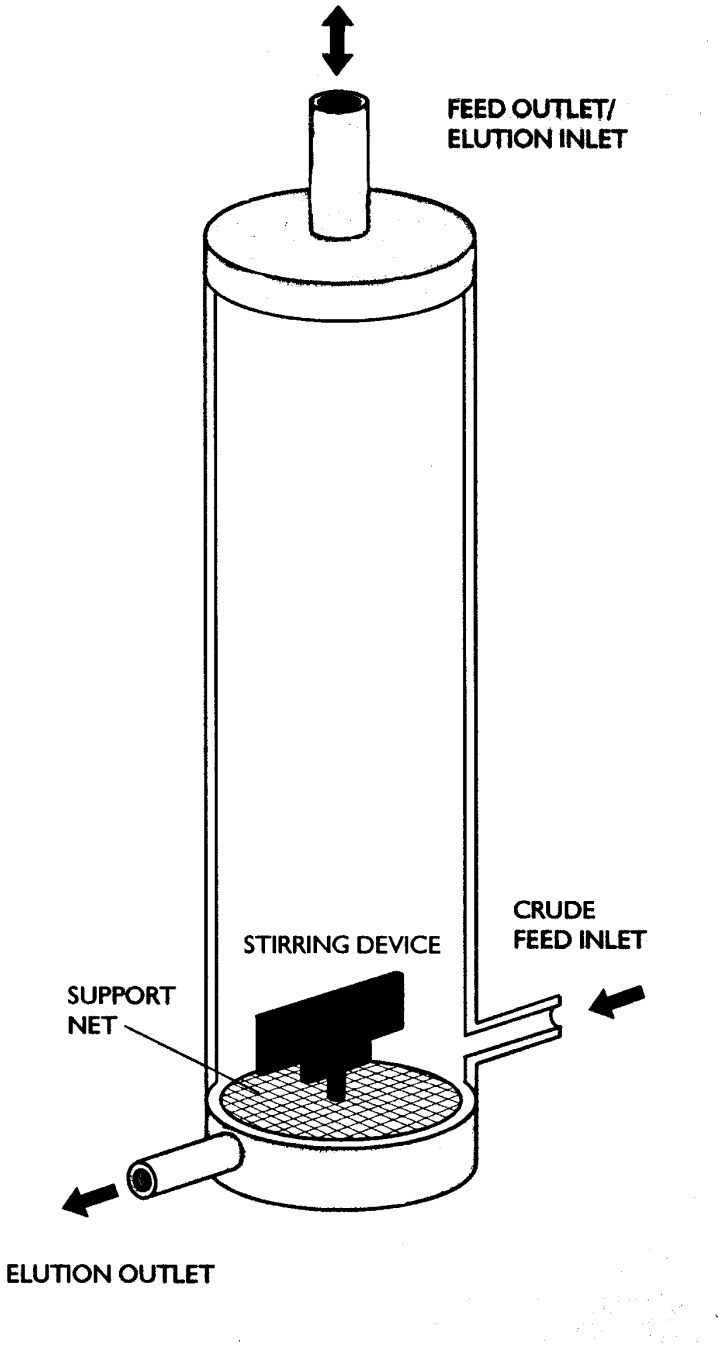


Figure 1.9. The expanded bed flow path of UpFront column.

The Streamline columns are equipped with appropriately mesh sized nets for even distribution whereas the column marketed by UpFront Chromatography realises an open inlet where the distribution is performed by stirring. Both technologies exhibit significant shortcomings: nets are prone to clog by attachment of cells or cell aggregates, while stirring suspended might have a severe negative impact on the chromatographic performance. Boehringer Ingelheim Pharma Kg and Amersham Pharmacia Biotech AB jointly accelerated the progress of a respective hardware with the dedicated emphasis to processing of mammalian cells. The advanced designed features eliminate cell attachment by continuous gentle movement in the applicator plate. The cell culture fluid is evenly fed from the applicator plate through cone-shaped distribution channels with no net required.

The effect of column verticality on liquid dispersion and separation efficiency in expanded bed adsorption was investigated by Bruce et al., 1999 using 1 and 5 cm diameter columns. For a 1 cm diameter column a 15°- misalignment resulted in a reduction of Bodestein number from 140 to 50 and from 75 to 45 for the 5 cm diameter column. Pure protein breakthrough profiles, from the application of bovine serum albumin onto Streamline Q XL, demonstrated that at 10% breakthrough, 7.8% more protein could be applied to a vertical 1 cm diameter column compared to the same column misalignment by 15°. A 55% increase in amount of glucose-6-phosphate dehydrogenase (G6PDH) was adsorbed at 10% breakthrough as compared to the same column aligned 0.185° to vertical.

1.5.2. Matrices

1.5.2.1. Properties of a matrix

An ideal expanded bed matrix should have high density, good mechanical stability, optimal stratification characteristics, fast equilibration and all the other positive features of standard packed bed matrices. Trailoring the chromatographic characteristics of an adsorbent for use in expanded bed includes careful control of the sedimentation velocity of the adsorbent beads. The sedimentation velocity is proportional to the density difference between the adsorbent and the surrounding fluid multiplied by the square of the adsorbent particle diameter. To achieve the high

throughput required in industrial applications of adsorption chromatography, flow velocity must be high through the complete purification cycle. The first result reported from expanded bed adsorption using conventional chromatographic adsorbents based on agarose (Draeger and Chase, 1990) revealed an obvious need for particles with higher sedimentation velocity to allow the operation of expanded beds at high flow velocities without the beads being carried out of the column by the lifting liquid flow. The shear force in the bed may result in breaking of the beads and the fine particles so generated may block the distributor plate and result in a fuzzy surface in the bed.

1.5.2.2. Commercial matrices

There are two types of adsorbents sold in the market by Amersham Pharmacia Biotech (Uppsala, Sweden) and UpFront Chromatography A/S (Denmark). Streamline adsorbents (Amersham Pharmacia Biotech.) are based on 6% crosslinked agarose with quartz particle inside the core, a material proven to work well for industrial scale chromatography, as claimed by the company (Carlsson, et al., 1995).

The macroporous structure of the highly cross-linked agarose matrices combines good binding capacities for large molecules, such as proteins, with high chemical and mechanical stability (Figure 1.10). High mechanical stability is essential for matrix used to reduce the attrition when particles move about freely in the expanded bed. It is readily chemically modifiable via primary and secondary hydroxyl groups. It is resistant to high salt concentrations, stable at room temperature to 0.1 M sodium hydroxide solution and 1 M hydrochloric acid for 2-3 hours. It is stable in the pH range 4-9. The chemical and mechanical stability of agarose matrix gel can be increased by crosslinking with epichlorohydrin (Porath et al., 1971; Kristiansen, 1974), 2,3-dibromo propanol (Kristiansen, 1974) or divinyl sulphone (Kristiansen, 1974; Porath et al., 1975), 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 and thermal

stability up to 121 ºC. The modified agarose matrix in Streamline adsorbents is more elastic (less brittle) compared to inorganic materials such as glass or ceramic. The mechanical stability of Streamline has been verified by repeated expansions and sedimentations and by subjecting the adsorbent to different shear forces (Hansson, 1995).

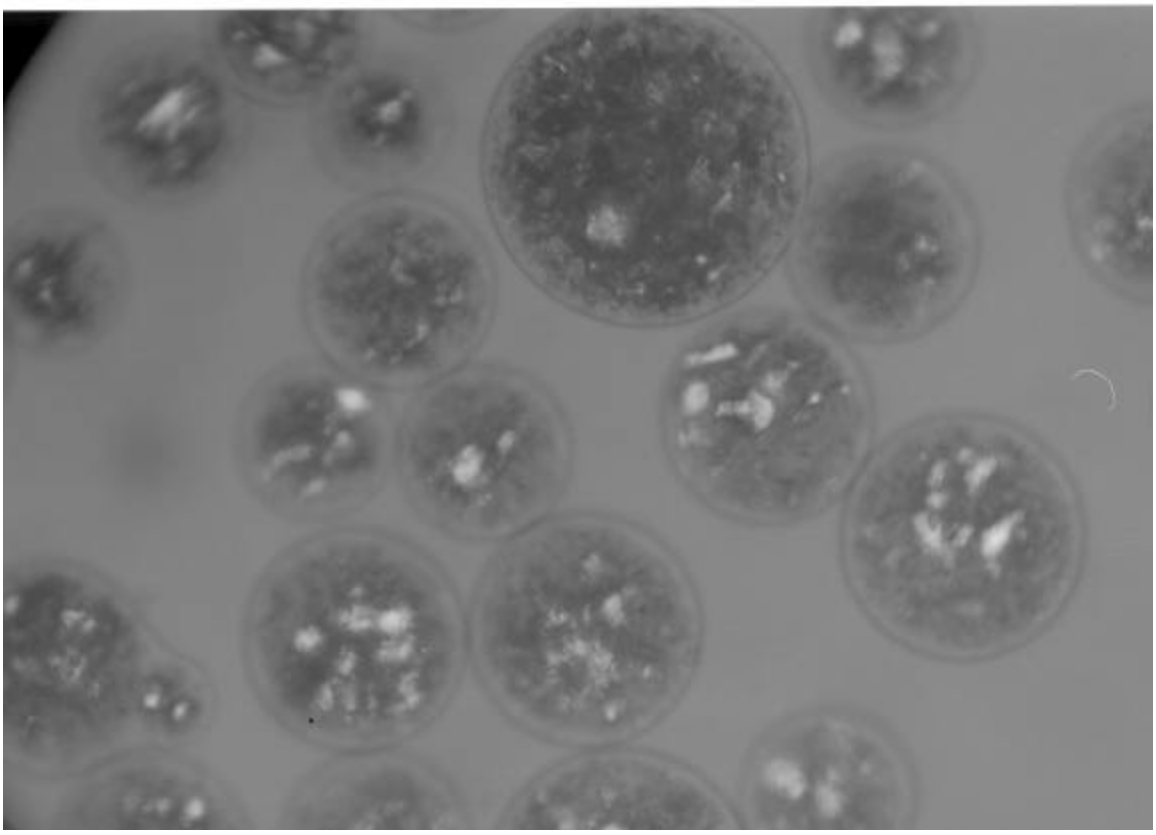
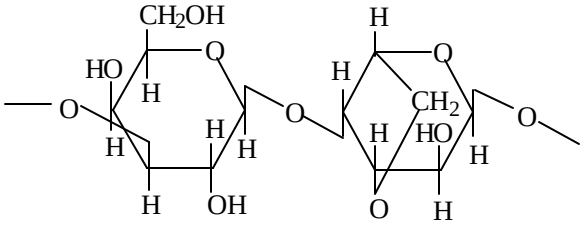


Figure 1.10. Pictorial view of Streamline matrices

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 (Porath and Axen, 1976). 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 (Guiseley and Renn, 1977). Agarose is also used in combination with synthetic polymers as a supporting material for packed bed chromatography. 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. All these matrices are used for packed bed chromatography.

Particles made only of organic material have limited density and would need to have large diameters for the high sedimentation velocity required. Such large particle diameters result in long diffusional path lengths, which cause considerable mass transfer resistance, counteracting productivity. Streamline adsorbents are therefore based on composite particle containing an inert core material that is denser than organic materials. Such particles can be designed so that their sedimentation velocity is high even for a reasonable small particle size. The polydispersity in particle size is an important design factor contributing to the stability of the expanded bed. The size and density gradients position the beads at individual adsorbent particles. The smaller, lighter particles move to a position at the top of the expanded bed, the larger, heavier particles remain at the bottom, resulting in a stable, uniform expansion. In other words, the beads find their ideal position in the column, which is the reason for low axial dispersion in expanded bed. Amesham Pharmacia Biotech (Uppsala, Sweden) markets a variety of adsorbents.. Ion-exchange chromatography separations take advantage of net charge and charge distribution at the surface of the biopolymer (Velayudhan and Horvath, 1988; Drager and Regnier, 1987). Streamline SP, a strong cation exchanger, was explored by Thömmes et al., 1995; Garke et al., 2000; Fujiwara et al., 1997 for the purification of various proteins on expanded bed. The use of Streamline DEAE, a weak anion exchanger, was reported by Johansson et al., 1996; Chang and Chase, 1996b; Pessoa et al., 1996; Chang et al., 1998. Streamline SP XL and Streamline Q XL are high capacity strong anion exchangers.

High molar masses of dextran are coupled to the agarose matrix and the strong Q and SP ion exchange groups are attached to these dextran chains through chemically stable ether bonds. The quaternary amine and the sulphonate groups maintain full protein capacity over the entire long-term pH stability ranges of 2-12 and 4-13, respectively. The Streamline Q SP (sulphopropyl) was explored by Ameskamp et al., 1999 for purification of monoclonal antibodies on a pilot scale. Streamline rProtein A, a composite material from 4% crosslinked agarose and steel beads with a particle size in the range 80-165 μ, is an affinity adsorbent. The ligand is engineered by fusing a cysteine residue to the C-terminus to favour an oriented coupling to the matrix by epoxy chemistry (Kennedy, 1996). It was explored for purification of monoclonal and polyclonal antibodies (Thömmes et al., 1996). Hydrophobic interaction chromatography in comparison exploits the accessibility and surface distribution of lipophilic or nonpolar residues. The term "hydrophobic interaction chromatography" is frequently attributed to separations affected by a decreasing salt concentration. Streamline™ Phenyl is a hydrophobic media and was explored by Noppe et al., 1996 and Farenmark et al., 1999. Streamline Chelating is obtained by substitution with iminodiacetic acid groups on spacer arms coupled to the Streamline matrix for immobilised metal affinity chromatography, Noronha, et al., 1999; Clemmitt and Chase, 2000a and 2000b had explored these matrices on EBA mode. Streamline heparin is prepared by coupling heparin, for purification of plasma proteins, e.g. coagulation factors and other types of proteins. Heparin is immobilised on the Streamline matrix by reductive amination, giving a stable coupling even at very high pH. Streamline Red H-E78, a dye affinity matrix, was used by Chang et al., 1994a.

The UpFront matrices are the composites of 6% crosslinked agarose with one solid glass bead inside, with particle size in the range 100-300 μ, having an average density of 1.3 g/mL (1.4 to 1.6 g/mL) (Oehlenschlaeger et al., 1999). These have short diffusion distance and therefore have fast equilibration. This company has come up with mixed mode media and an affinity matrix (FastMabs A). The mixed mode media is used for high efficiency, and first capture adsorption of proteins, which is claimed as an alternative to ion exchangers and hydrophobic media, whereas

FastMabs A is an alternative to Protein A adsorption and ion exchange chromatography. These mixed mode media comprise of low molecular weight ligands, which have both hydrophobic core e.g. aromatic or heteroaromatic ring structure, onto which hydrophilic or ionic substituents e.g. amino acid or carboxylic acid groups are attached. The mixed mode media are Mimo AS and AD, Mimo BS and BD, Mimo CS and CD, Mimo ES and DD, Mimo ES and ED, Mimo ES and ExF (Straetkvern et al., 1999). The Mimo standard resins are spherical crosslinked agarose beads, with a particle diameter of 40-100 μm and a density 1.0-1.1 g/mL. High-density particles are produced by the encapsulation of a glass particle, giving a 25-30% composite with a mean density of 1.4-1.5 g mL^{-1} and size range 100-300 μm. Ligand concentration of the high density media is in the range 40-60 mM. The protein-binding capacities are 50-150 mg mL^{-1}, 10 μg of Mab (Lihme and Hansen, 1997) from dilute hybridoma cell culture supernatant; 12 mg Fab fragments (Hansen et al., 1999) per mL of the matrix, respectively. Since the protein binding mechanism is rather complex and optimum binding of protein occurs under conditions where the substituents are on a non-charged form, the binding strength is therefore strongly pH dependent. In contrast to ion-exchangers, it is largely independent of ionic strength. Proteins are typically bound to the mixed mode matrices at an optimal pH –value and eluted by a change in pH, ionic strength or detergent in the binding and/or washing buffer. The number and specific nature of the substituents influences the overall binding specificity towards different proteins. The different Mimo matrices differ significantly in their ligand structure and may show significant differences in selectivity and capacity towards protein and/or the impurities in raw material. All types are stable towards regeneration with 1 M sodium hydroxide.

The hydrophilic or ionic substituents and their specific nature in terms of charge and pKa values influences the overall binding specificity towards proteins as well as the binding strength in a strongly pH-dependent matter. This means that binding will take place at pH values characteristic for the particular mixed mode ligand and the specific protein, while efficient elution of the bound protein often can be performed by a simple change in pH.

Compared to traditional ion exchangers or hydrophobic interaction chromatography, the mixed mode media offers the following advantages when applied in large-scale protein fractionation.

1. Adsorption efficiency is largely independent of ionic strength. This eliminates the need for desalting or dilution steps.

2. High concentrations of hydrophobic salts are not required for adsorption. This eliminates waste problems and extra cost.

3. Organic solvents are not required for adsorption. This increases safety, decreases waste problems and saves cost.

4. Mixed mode media have a low surface charge. This minimises irreversible fouling when crude raw materials are used.

5. Mixed mode media can be efficiently regenerated in 1M NaOH.

The FastMabs A is an agarose matrix, derivatised with a low molecular weight ligand, stable in 1 M NaOH. It has a broad specificity for mouse, rat, and human immunoglobulins, including IgM. The binding of antibodies to the affinity matrix is performed at pH 5, is largely independent of ionic strength of the raw material, while elution is accomplished at neutral pH.

1.5.2.3. Existing matrices

There are several reports on matrices used for expanded bed adsorption for protein purification. These are listed in Table 1.2.

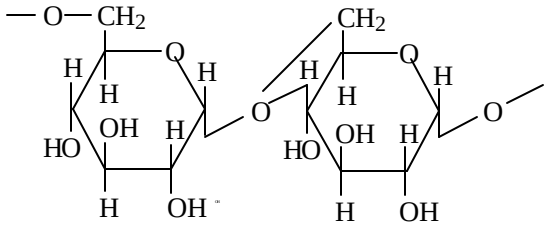
Table 1.2. Matrices and their properties used for expanded bed adsorption

Sl. No.	Materials	Particle Density ($g\ mL^{-1}$)	Diameter (μm)	Reference
1.	Cellulose/titanium dioxide	1.20	125-600	Gilchrist et al., 1994
2.	Agarose/metal alloy	1.15	100-300	Hjorth et al., 1995
3.	Triacrylate/zirconium dioxide	1.50	100-300	Cmabs
4.	Zirconium dioxide	3.20	50	Griffith et al., 1997
5.	Titanium dioxide	2.59	400 and 80	
6.	Cellulose	1.60	150-250	Pai et al., 1999
7.	Chitin			Roy et al., 2000
8.	Controlled pore glass (bioran)	2.20	130-250	Thömmes et al., 1995

1.5.2.3.1. Natural polymers

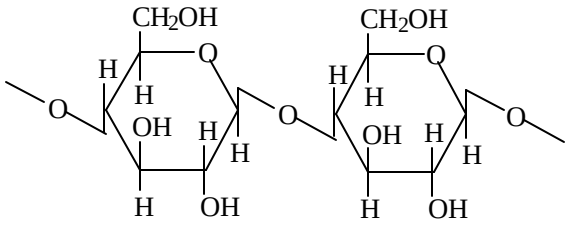
The supports (matrices) used for expanded bed can be sub-classified as organic, inorganic and composite. Organic supports include natural and synthetic polymers. The natural organics include agarose, cellulose, dextran, starch and its derivatives and chitin. The most commonly used is a composite of agarose and quartz particles and agarose with glass bead. Agarose gels offer greater bio, mechanical stability and wider pore size than other gels (Porath and Axen, 1976). Dextran and agarose gel composites were used for expanded application (Thömmes, 1999). Cross-linked supermacroporous cellulosic beads using conjugated Cibacron blue were explored for protein purification (lactate dehydrogenase from porcine muscle homogenate) on expanded bed (Pai et al., 1999).

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 (Turkova, 1978). Two types of gels, Sephadex and Sephacryl, are mainly used in the packed bed chromatography (PBC) 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 Biotech. (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 (Lowe and Dean, 1974). 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 bisacrylamide (Johansson and Lindberg, 1979). It is porous and yet mechanically stable. The main advantage of this matrix is the excellent flow characteristics, 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.

Cellulose has been widely used as a matrix in industrial processes because of its easy availability and low cost. 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 (Jameson and Elmore, 1971) and acrosin (Schleuni et al., 1973) as well as chitin used to purify lysozyme (Imoto and Yagishita, 1973) on PBC. 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 (Madden and Thom, 1982). 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.

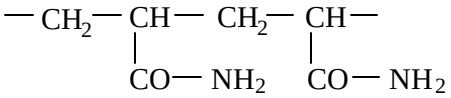
There are two other basic problems that diminish the usefulness of cellulose as a matrix (Scouten, 1981). First, because of its fibrous nature, it is easily clogged by particulate material and easily compressed by the application of even moderate pressure. Thus, it is of limited use in rapid, high-pressure applications.

1.5.2.3.2. 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.

1.5.2.3.2.1. Polyacrylamide

Polyacrylamide was first used for protein chromatography in 1962 (Hjerten and Mosbach, 1962; Lead and Sehon, 1962). 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, hey are not attacked by microorganisms. They have excellent chemical stability due to a polyethylene backbone and show low nonspecific binding characteristics.

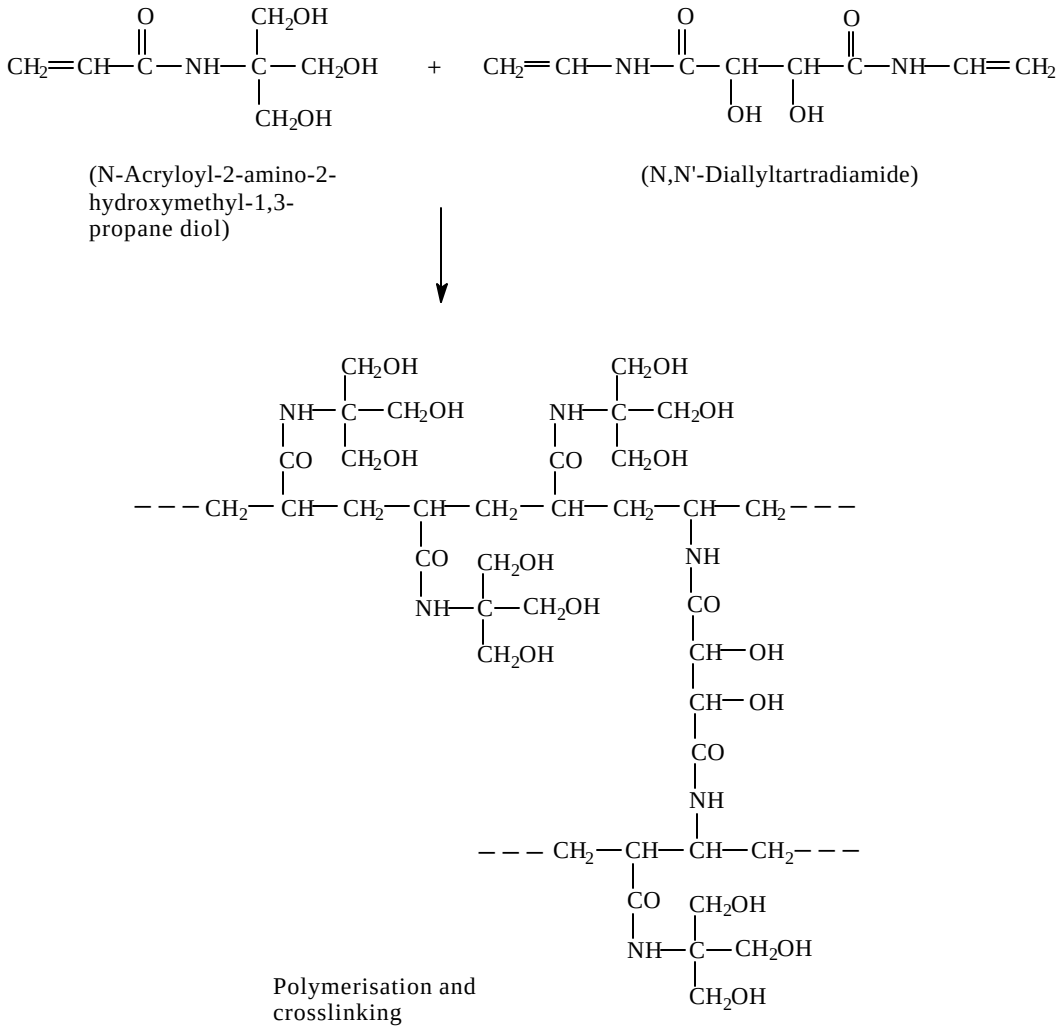
Polyacrylamide gels, however, suffer from some disadvantages, which have prevented them from becoming popular support in affinity chromatography. These gel particles strongly adhere to clean glass surfaces, so use of siliconised glass or polyethylene vessels are recommended. They have poor mechanical 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 (Epton, et al.1975; 1976).

1.5.2.3.2.2. 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 (Hermanson et al., 1992) (Scheme 1.1). 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 (Gorman and Wilchek, 1987). 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 $-20^{o}C$ to $121^{o}C$ and are also stable in organic solvents and in detergents.

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 (Mohr and Pommerening, 1985).



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

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 as 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 (Villems and Toomik, 1993).

1.5.2.3.3. 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 (Narayanan and Crane, 1990). Their stability can also be enhanced by coating them with zirconium (Szabo et al., 1988; Stout and Destefano, 1985) or with an inert polymer (Ivanov et al., 1991).

Glass and silica show non-specific adsorption of protein due to charged surface silanol groups (Weetall and Filbert, 1974; Muzutani, 1985). This effect can be reduced by surface treatment with hydrophilic silanes such as ϒ-aminopropyl triethoxy silane (Weetall and Filbert, 1974; Weetall, 1969) and glycidoxy propyl trimethoxy silane (Chang et al., 1976).

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 (Dean et al., 1985). Such a procedure cannot be applied to silica-based materials because of their solubility at alkaline pH (Jervis, 1988).

A special type of inorganic support is the magneto-gel. Iron oxide particles could be directly loaded with protein, enzyme or polysaccharide (Horisberger, 1976).

It is difficult to obtain magnetite particles that are chemically stable and are free from ferric ion leakage.

1.5.2.4. Methods of matrix preparation

Most of the matrices used for expanded bed applications are composites of inorganic/metallic core with organic coating. The composite supports are used to increase the physical and chemical rigidity and density, which is a requisite for expanded bed adsorption. The three known types are: 1) Pore-matrix composites, 2) Interpenetrating networks and 3) Core-shell grafts. The pore-matrix composites comprise of a inorganic support (e.g. glass or zirconia), soaked in a solution of the organic monomer mixture, usually containing a cross-linker and an initiator. Subsequent polymerisation under carefully controlled conditions leads to the formation of the desired organic polymer within the pores of the beaded inorganic support (Alpert and Regnier, 1979; Heinemann et al., 1987; Kolla et al., 1987). It should be emphasised, however, that the organic polymer must usually be sufficiently crosslinked to be permanently "entrapped" within the pores of the inorganic matrix. Alternatively, a less highly crosslinked organic polymer may be covalently "anchored" to the pore surface. Covalent attachment can be accomplished either by copolymerisation with vinyl residues attached to the pore surface (Wulf et al., 1985) or by reaction between appropriately chosen reactive groups on the organic polymer and the inorganic matrix (Schutyser et al., 1982). These matrices are prepared by emulsion polymerisation. To give the matrices an added mechanical stability the natural polymers like agarose are crosslinked.

Hot agarose solution mixed with vegetable oil heavy inorganic/metallic particles and cooled with continuous stirring generates spherical composite beads (Gilchrist et al, 1994). Cocker et al. (1997) synthesised polyacrylamide/magnetite beads for protein purification in magnetically stabilised fluidised beds. Hjertén (1964) first introduced agarose spheres for chromatographic use. An aqueous solution of 1-5% agarose is stirred with mixture of toluene and carbon tetrachloride at 50 ^{o}C, in presence of droplet stabiliser. The resulting droplet suspension is cooled to room temperature under continuous stirring to effect gelation, and the formation of non-

crosslinked agarose beads (*cf*. Sepharose B) (Hjerten, 1964). For better physical stability, the matrices are crosslinked. The non-crosslinked matrices are treated with epichlorohydrin, diepoxides, 2,3-dibromopropanol or divinyl sulphone at high pH and 60 ºC for 2 hours. The resulting crosslinked beads are then desulphurised at a higher temperature (120 ºC). These reactions are usually carried out in the presence of small amounts of a reducing agent (sodium borohydride) to avoid oxidative degradation of the polymer (Porath et al., 1971; Porath and Janson, 1971).

More recently, agarose gels containing two types of cross-linking have been described (Anderson et al., 1984). The aqueous agarose droplets are prepared as described above. The water in oil droplet suspension is treated with diepoxide, which is soluble in the organic phase, followed by reaction with epichlorohydrin. This crosslinking procedure probably results in the formation of microcapsular particles in which the outer shell are more hydrophobic and more highly cross-linked than the inner cores. A synthetic support with heavy zirconium core, marketed by BioSepra, has a distinct advantage of inertness to microbial attack. Although the technical know-how has not been reported, it is apparent that this matrix is prepared by suspension polymerisation.

Synthetic polymers frequently used for packed bed chromatography and immobilisation are polystyrene, polyacrylates (copolymer of acrylic acid and acrolein-Leemputten, 1977; radical copolymerisation of hydroxyalkyl methacrylate and a crosslinking agent mainly divinyl comonomer like ethylene dimethacrylate-Cuatrecassas, 1970 and Valentova et al., 1975; copolymers of glycidyl methacrylate and ethylene dimethacrylate- Svec et al., 1975), polyvinyls and copolymers based on maleic anhydride and ethylene/styrene, polyamides, polyaldehydes etc. However, these polymers should be evaluated for protein purification on expanded bed.

Controlled pore glass (CPG), prepared from borosilicate glass (SiO_2, B_2O_3, Na_2O) by acid treatment, has been extensively used (Thommes et al., 1995). The process is based on the observation that, on heat treatment, certain borosilicate compositions form two-phase systems in which tiny borate-rich particles exists within

a continuous silicate-rich matrix (Norderg, 1944). The pores formed by acid leaching are relatively small (30-60 Å). For the preparation of large pores, the acid leaching is followed by a mild caustic treatment (Lynn and Filbert, 1974). This results in controlled dissolution of silicate from the interior of the pores, and hence the formation of the larger pores. The initial phase separation process of borosilicate glass, as well as acid and base treatments, can be carefully "controlled" to produce relatively narrow pore sizes of about 3000 (300 nm) or larger. CPG produced in this way typically contains about 95-96% silica glass, 3-5% B_2O_3 and traces of related metal oxides. These supports have wide range of pore sizes. However, CPG is unstable in alkaline pH. The brittleness, which leads to attrition, can be circumvented by zirconia coating.

Naturally occurring porous minerals such as Kieselgurh, attapulgite clays, pumice stone, sand and bentonite may be investigated for use on expanded bed. These have a large pore size distribution. The other supports are magnetic oxides, nickel and stainless steel, which are nonporous. These have low surface area, which limits the available surface for protein adsorption. However, diffusional constraints are eliminated. Surface area can be enhanced by decreasing particle size. This will result in blocking the distributor plate of the expanded bed column. The protein adsorption can be enhanced by coating a thin layer of polymer onto porous or nonporous inorganic carrier, followed by derivatisation to introduce the ligands or ion-exchangers. Hence, these two methods are commonly used for matrix preparation. It is imperative to discuss these methods in detail.

1.5.2.4. Suspension polymerisation

Monomer is the single ingredient present at the start of a bulk addition polymerisation except for an inconsequential volume of initiator. As polymerisation proceeds, however, a second component, polymer, is formed at the expense of the monomer. Since a macromolecule is intrinsically much less mobile than its monomer, the viscosity of a system polymerised in bulk will increase rapidly as the monomer is converted to polymer, and the transfer of heat from the exothermic polymerisation will become increasingly more difficult. Once the viscosity of a homogenous

chemical reaction becomes sufficiently high to cause the reaction rate to be diffusion-controlled, the single-phase reaction assumes the characteristics of a heterogeneous system. The reaction subsequently must be agitated to achieve an uniform product. Bulk polymerisation of vinyl monomers become so viscous at high conversion that stirring is extremely difficult, if not impossible. In addition, bulk polymerisation reactions of vinyl monomers will develop irregular "hot spots" where heat transfer is impeded. This causes autoaccelerated reaction rates leading to a product with a broad molecular weight distribution. Since monomer will be volatilised in the area of the local hot spots with an undesirable appearance results from the bubbles trapped in the viscous matrix.

Polycondensations (step-growth polymerisation) are not generally as exothermic as are addition (chain-growth) polymerisations. A polycondensation is conducted at such a high temperature that the temperature difference with the surroundings provides reasonably efficient heat transfer. Finally, polycondensation takes place by a stepwise mechanism; therefore, the viscosity does not increase significantly until end of the polymerisation. Bulk preparation of condensation polymers is often the preferred procedure.

The undesirable consequences, which result from the increasing viscosity accompanying addition polymerisation in bulk may be reduced by the addition of an inert, miscible, low-molecular weight diluent; this technique is referred to as solution polymerisation. Although solution polymerisation provides better heat control and retards autoacceleration, the solvent is rarely inert, and a product is lower in molecualr weight than that obtained by the bulk technique will result because of chain transfer with the solvent. In addition, the solvent must be removed at the conclusion of the polymerisation; solvent removal can be difficult, dangerous, and expensive.

The problem of heat transfer created by addition polymerisation in bulk can be circumvented by decreasing the quantity of material to be polymerised. Although this is an effective solution, it is sometimes impractical and there are alternative approaches to overcoming this problem. Two immiscible liquids, vigorously agitated,

produce this exact condition because one of the phases becomes discontinuous and it agglomerates into small, spherical particles or beads, with each bead constituting a microreaction vessel. The size of the beads will be inversely proportional to the stirring rate and to the quantity of protective colloid employed. The high surface tension (which will cause immiscible liquids of lower surface tension to become the discontinuous phase), the incompatiability with most monomers, the high heat capacity, and because it is economical, nontoxic, and inert to the polymerisation reaction, water is an excellent choice as the dispersion medium. In special cases any liquid which is immiscible with the monomer may be substituted for water.

Systems in suspension are, regrettably, metastable. Although no completely satisfactory solution to this problem has yet been advanced, protective colloids formed by hydrophilic polymers like gelatin, hydroxyethyl cellulose, polyacrylamide, and poly(vinylalcohol); surface active agents and finely divided inorganic compounds like the clays or calcium, magnesium phosphate have been helpful in stabilising suspended systems. The latter systems are particularly effective during the difficult stage where the droplets become tacky and the system tends to agglomerate. The mechanism of polymerisation is altered substantially when the dispersed particles approach colloidal dimensions. Hence, the surface-active agent must be selected carefully to avoid the formation of an emulsion.

1.5.2.5. Emulsion polymerisation

Addition polymerisation can be accomplished by a number of different techniques such as bulk, solution, suspension, and emulsion. The last method offers the distinct advantage of providing a polymer of exceptionally high molecular weight, narrow molecular-weight distribution, and high rates of conversion at low temperatures, while at the same time permitting efficient control over the exothermic polymerisation reaction.

The word emulsion is used here to describe a dispersion (usually colloidal in size) of one liquid phase in another (Vold and Vold, 1964). Dispersions other than solutions will tend to settle out under the force of gravity as the forces, which caused

the dispersion, are removed. Fortunately, the rate at which an emulsion is generated is slow and is determined by two experimentally variable parameters-the particle size and the interfacial tension. In those systems where the dispersion is sufficiently great and where the interfacial tension has been minimised, settling will not be observed at all. A stable emulsion made up of a polymer as dispersed phase with an aqueous continuous phase is a latex.

Two liquids are immiscible when one is highly polar, and the other is non-polar. Water and a hydrocarbon provide a typical example. The water molecule is polar and is associated through hydrogen-bond formation. When water is mixed with another polar substance, the sphere of association of the water is increased by intermingling with or solvating the hydrophilic moiety. The hydrocarbon too will intermingle with another non-polar substance but in a vastly different sense. Molecules of a non-polar substance are associated through the relatively weak dispersion forces. Since the dispersion forces are not selective, non-polar substances can mix and there will be a very small energy change accompanying the process; in fact, the mixture is only marginally different from either of the components existing separately. When a polar and non-polar substance are brought together, they will not mix because the nonpolar substance possesses insufficient energy to separate strongly cohesive dipoles or hydrogen bonds.

Certain molecules, called surface-active agents or surfactants, are amphipathic-they present a union of the two unlike solvent types into a single molecule. Surfactants are composed of an ionic, hydrophilic end and a long hydrophobic hydrocarbon chain. The sodium salts of fatty acids (soaps), the anionic detergents like the sodium aryl sulphonates (I), and the cationic detergents like cetyltrimethylammonium chloride (II) are typical examples.

Since a surfactant has a polar end, surfactant is soluble in water. However, the nonpolar end of each surfactant molecule is present in a hostile environment. Once a minimum critical concentration of the surfactant is exceeded, the hydrophobic ends of the surfactant collect into an aggregate known as micelles. Although the exact

structure of micelles remains still a mystery, they are generally believed to consist of 50 to 100 molecules arranged with the hydrocarbon chains irregularly tangled but concentrated in the centre of the micelle. Micelles are protected and stabilised in suspension by the ionic hydrophiles distributed about the surface (Hartly, 1936). The diameter of the micelle is roughly twice that of the elongated surfactant of which it is composed (Flory, 1953).

Kinetic data coupled with the observation of high degrees of polymerisation and narrow-molecular weight distributions realised in the product make it apparent that the mechanism of the emulsion polymerisation has been considered by many authors (Flory, 1953; Bovey et al., 1955; Harkins, 1952).

1.5.2.6. Porous structure and its importance

Important requisites for protein purification are the binding of proteins on porous matrices which is governed by pore size, pore size distribution, pore volume and pore surface area. The pore size distribution needs to be precise to bind the specific protein, the pore volume should be large enough to bind and elute a large amount of specific protein. In addition, the support should comprise of ligands/ion-exchangers at the surface efficiently with the protein under the mild conditions.

The ideal supports for protein purification are tailor made synthetic porous polymers with requisite pore size, pore size distribution, pore volume, pore surface area and optimum concentration of the ligand/ion-exchanger. Therefore, the requirement of beaded, porous polymers is met by synthesising them by suspension polymerisation technique (Farber, 1977). As discussed earlier, the polymerisation takes place in the dispersed phase. Continuous agitation and a stabilising agent are normally required to maintain the dispersion. In most cases the continuous phase is aqueous and the polymerisation proceeds through free radical mediated chain growth mechanism.

Extensive study of macroporous morphology and formation of porous texture have been conducted for beaded, crosslinked styrene-divinyl benzene resins (Hodge and Sherrington, 1980; Jacobelli et al., 1979). The internal structure of the resin beads

can be controlled by different parameters in the polymerisation process, such as the amount of crosslinking monomer used, type and volume of diluent/porogen/pore generating solvent (an inert organic solvent) added to the monomer phase. Three main class of porogens (Millar et al., 1963) known are (i) solvents for the polymer (ii) non-solvents and (iii) polymers soluble in the monomer(s). The last produces only large pores. The molecular weight is then an important parameter. The pore volume is large (up to 1 mL g^1) when the molecular weight of the porogen is high (Sederel and DeJong, 1973).

When solvating diluent is used, large amounts of both diluent and crosslinking agents (divinyl benzene; DVB) are necessary to get permanent porosity. Under such conditions high surface areas (500 m^2 g^{-1}) with narrow distribution of small pores (10 nm) are obtained, together with a limited volume (0.3 mL g^{-1}) of large size pores.

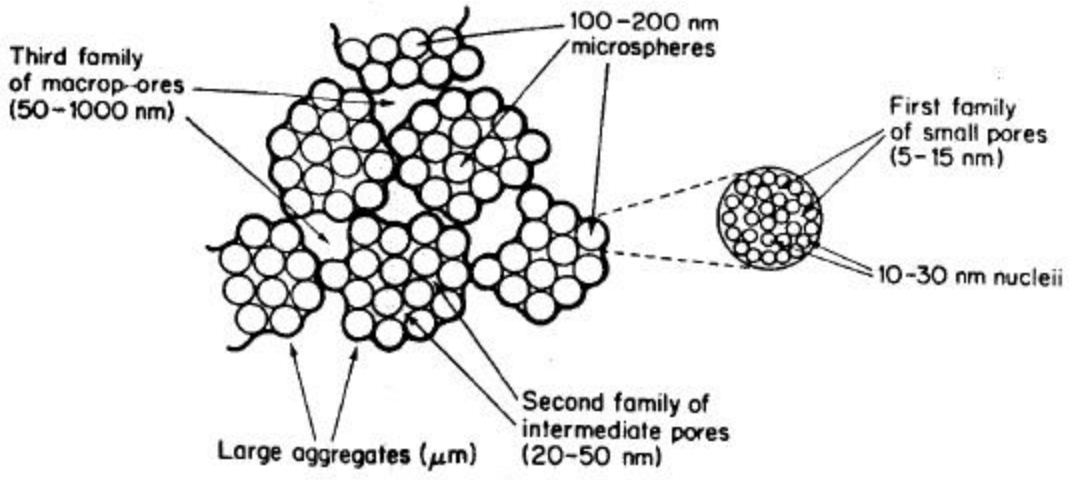


Figure 1.11. Schematic representation of the structure of macroporous domain.

The most complex and frequently investigated systems are those incorporating a non-solvating diluent (Hilgen et al., 1975). Here, observed experimentally by Jacobelli et al. (1979), (Figure 1.11) the bead contains large agglomerates of microspheres (100-200 nm). Each microsphere consists of smaller nuclei (10-20 nm), which are more or less fused together. In between the nuclei, there is a first family of very small pores (5-15 nm), which is mainly responsible for the high surface areas of these materials. In between the microspheres a second family of intermediate pores (mesopores) is

observed (20-50 nm) which account for moderate surface areas. (up to 100 m^2 g^{-1}). A third family of large pores (50-1000 nm) is located between agglomerates. This family of pores is responsible for high pore volumes (up to 3 mL g^{-1}), which can be achieved when very high relative volume of diluent is used.

1.5.2.7. Resin swellability

Resin swellability (or bulk expanded volume) in a given solvent is a multifaceted property reflecting the chemical structure of the polymer backbone, degree of croslinking and the architecture of the polymer matrix. The three dimensional structure of the polymer matrix (or network) takes shape according to the conditions prevailing during the formation of the polymer microbeads. For beaded polymer supports obtained by suspension polymerisation, the type and the percentage of the monomer diluent strongly influences the shape of the three dimensional polymer network. For polysaccharide gels produced by suspension cross-linking, the nature and the proportion of the polymer solvent has a similar role. In either case, polymer molecular weights (degree of polymerisation, DP) also contributes to matrix architecture. In general terms, these criteria also apply to inorganic gels although the process is more complicated. The extent of polymer swelling (bulk expanded volume) decreases as the nominal degree of crosslinking increases. It is also evident that, for a given degree of crosslinking, the bulk expanded volume is strongly dependent on the nature and proportion of the monomer diluent. In general, the higher the percentage of the monomer diluent, the larger the bulk expanded volume of the resin, but different diluents affect polymer swelling to different extents.

An interesting implication of these observations is that the bulk expanded volume of the gel can be maintained at a relatively constant level by simultaneously increasing both the degree of polymer crosslinking and the percentage of monomer diluent. However, this process has a far reaching effect on the gelation and precipitation of the polymer "grains" within the micro beads. The pattern of gelation and precipitation, in turn, affects the porosity and surface area of the beads.

Another practically important aspect of resin swellability is the pattern of polymer-solvent compatibility, i.e. the relative measure of the polymer swelling in different solvents. Polymer-solvent compatibility is determined by the chemical structure of the polymer backbone. An interesting illustration of this structure-property relationship is provided by the swellability data (Unger, 1979).

It should be emphasised that the extent of apparent polymer swelling (bulk expanded volume) and the solvation of the polymer chain do not necessarily coincide. The distinction between "polymer swelling' and "solvation of the polymer chains" is particularly relevant in any discussion of the reactivity (or site accessibility) of the polymer bound reactive sites. When a low-crosslinked polymer (whether porous or not) swells in a "good solvent", individual polymer chain segments become solvated. Under these conditions, the polymer bound reactive sites are rendered potentially accessible to the soluble reagent. On the other hand, highly cross-linked porous gels generally "suck up" certain volumes of various liquid, whether good or poor solvents (Negre et al., 1982; Barer et al., 1983). Here the liquids are stored in the pores (*cf*. the channels between the precipitated grains), without necessarily contributing to polymer solvation and site acessibility. For non-porous polymer beads, the extent of swelling is closely related to solubility parameter (Grulke, 1989).

1.5.3. Affinity ligand

Affinity chromatography is based on the exceptional ability of biologically active substance to bind specifically and reversibly to other substances, generally called ligands, affinity ligands or affinants. Ligands used in affinity purifications are 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.

1.5.3.1. 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 (Narayanan and Crane, 1990). 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 (Lowe et al., 1973).

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 (Ostrove, 1990). 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 (Mohr and Pommerening, 1985). 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.

1.5.3.2. Activation and coupling

Activation is the process of chemically modifying a matrix such that the products of the process will react/couple with a ligand via covalent linkages. A good ligand that is badly coupled to a good matrix will fail to purify by affinity chromatography. 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, without side reactions that introduce 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 amenable to easy scale-up.
6. The activation and coupling chemistry should be reproducible.

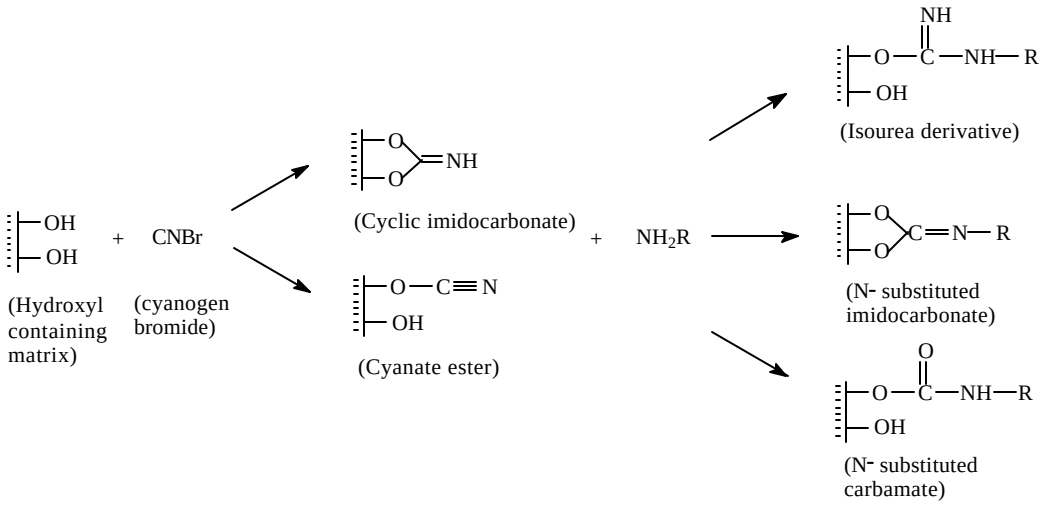
1.5.3.3. General reactions

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

1.5.3.3.1. Cyanogen bromide (CNBr)

Cyanogen bromide is one of the first methods used to activate solid supports (Axen et al., 1967). 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 be activated by this procedure (Turkova, 1976). Cyanogen bromide activated supports can be used to couple smaller ligands as well as high molecular weight biopolymers with quaternary structure such as haemoglobin (Chua, and Bushuk, 1969). The activation is relatively simple and reproducible.

Reaction is carried out either in aqueous media or in an acetone-water mixture using trimethylamine as catalyst (Kohn and Wilchek, 1982) (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 (Parikh et al., 1974; Hseu et al., 1981). This is primarily due to instability of isourea bridging group between polymer and ligand.

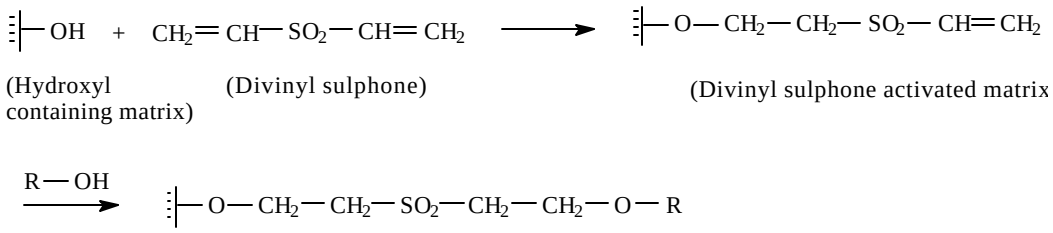


Scheme 1.2. Matrix activation with cyanogen bromide and subsequent ligand immobilisation

1.5.3.3.2. Divinyl sulphone

Divinyl sulphone (DVS) is used with alkali resistant non-shrinking hydroxyl containing polymers (Sundberg and Porath, 1975). DVS introduces vinyl groups into

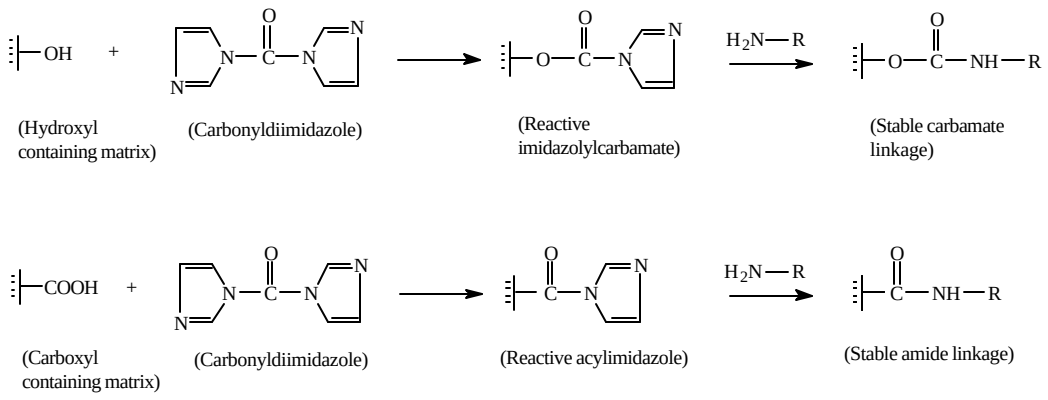
the matrix (Scheme 1.3) that couple to alcohols, amines, phenols and sulphydryls. The vinyl group introduced into the matrix is more reactive than the oxirane group. Therefore, the coupling takes place rapidly and completely at lower temperatures and at lower pH than in the oxirane activated matrices. 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 above pH 9. DVS is toxic and expensive.



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

1.5.3.3.3. Carbonyldiimidazole

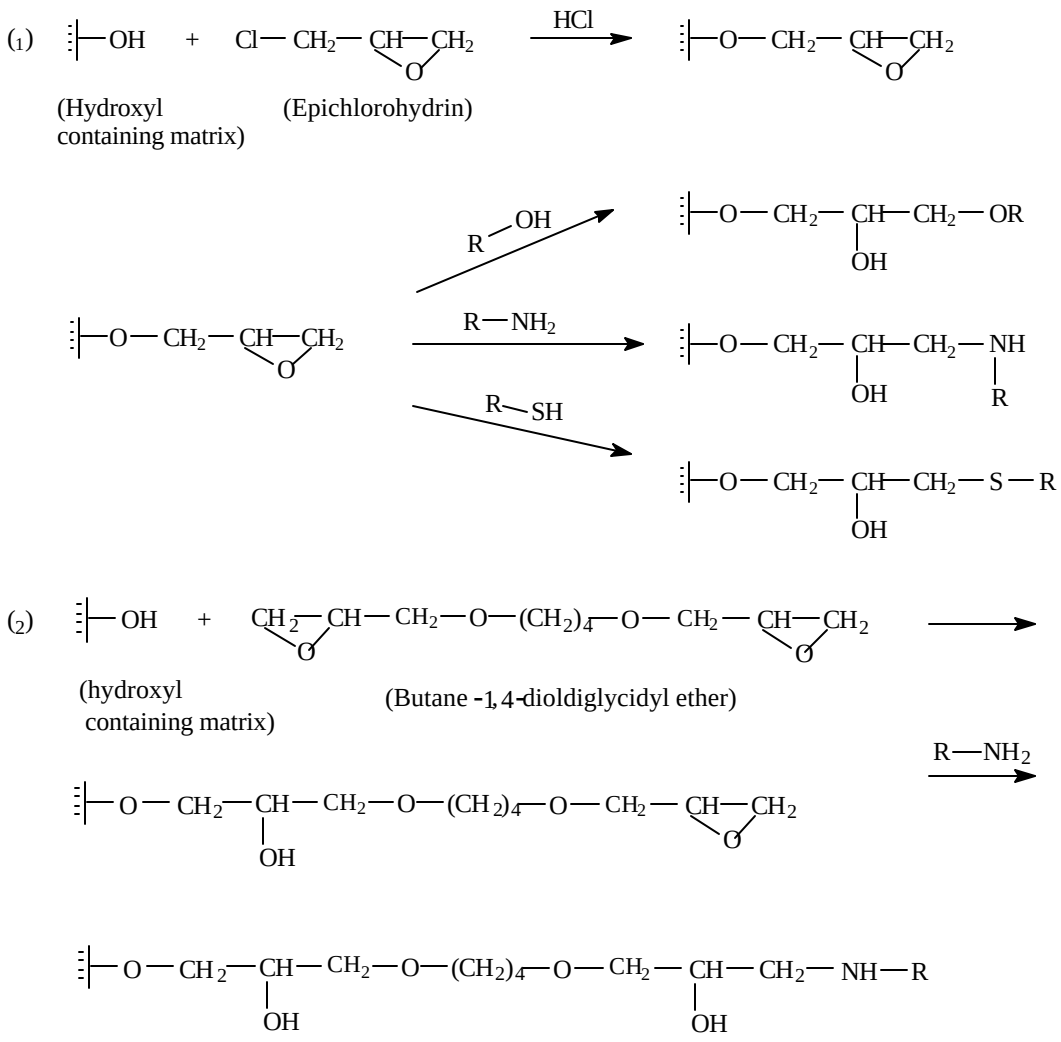
N,N'-Carbonyldiimidazole (CDI) activation can be used with both carboxyl and hydroxyl matrices that can withstand non-aqueous conditions (Hearn, 1987). The activation of hydroxyl groups with CDI forms an intermediate imidazolyl carbamate that can react with amines to give stable carbamate linkage. Hydroxyl 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 ligands 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 matrices by carbonyldiimidazole and subsequent coupling to amine ligand is presented in Scheme 1.4.



Scheme 1.4 Matrix activation with carbonyldiimidazole and subsequent ligand immobilisation

1.5.3.3.4. Epoxy (bisoxirane) activation

Reaction of –OH groups of hydroxylic polymer with epichlorohydrin leads to activated support with oxirane groups. Bisoxiranes such as 1,4-bis(2,3-epoxypropoxy) butane are also used to introduce oxirane group on the support material. During activation with bisoxirane, one of the oxirane groups reacts 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 chain. All amino-, hydroxyl-, and thiol- 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 (Sundberg and Porath, 1974), polyacrylamide (Dunnill and Lilly, 1974), polymethacrylates (Turkova et al., 1981; Kato et al., 1979), cellulose (Ayers et al., 1984), porous glass (Chang et al., 1976) and silica (Glad et al., 1980). 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

1.5.3.4. Variants of affinity chromatography

Several variations within affinity chromatography have evolved. These arise from the introduction of ligands to induce a specific type of interaction to bring forth affinity towards the biomolecule to be separated. The subclasses are presented below:

1.5.3.4.1. 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 (Cuatrecasas et al., 1968). Biologically active substances, viruses, cells, nucleic acids and antibodies 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 (Turkova et al., 1973). 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 (Turkova et al., 1973). In both cases elution was performed 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, (Sternbach, 1975; Jaehning, 1977) lipoprotein lipase (Olivercrons et al., 1971), collagenase (Sakamoto et al., 1975) 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 (Fronstedt and Porath, 1975). Immobilised lectins are used in 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 (Mohr and Pommerening, 1985). 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 have been used to purify bovine viral diarrhea virus and for the separation of influenza A virus glycoconjugates after solubilisation (Kristiansen et al., 1979).

Immobilised nucleic acids are used both as general ligands for all DNA-binding proteins and for sequence-recognising molecules. DNA-cellulose is widely

used. Isolation of protein kinase (Greth and Chevallier, 1975), DNA polymerase (Nolan, 1992), RNA polymerase (Wandzilak and Benson, 1977), RNA ligase (Cranston et al., 1974) were achieved using immobilised DNA adsorbents.

Cells can also be separated on the basis of their interaction with bioligands. Eshhar et al. isolated thymocytes on anti-thymocyte globulin bound Sepharose 4B matrix (Eshhar et al., 1974). Adipose cells were isolated using insulin incorporated Sepharose 4B column (Soderman et al., 1973). Matrix bound antigens and antibodies can be used for the separation of complementary immuno-substances. For example, Cuatrecasas prepared a column of insulin-Sepharose and adsorb insulin antibody at pH 8.8 and to elute with hydrochloric acid (Cuatrecasas, 1969). Vice versa, the antialbumin antibodies hooked to Sepharose may serve as an affinant for the isolation of albumin (Separation News, 1972).

1.5.3.4.2. 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 in protein purification are the reactive triazine dyes. The interaction of proteins with the triazine dyes was first observed in 1968. C*ibacron blue F3G-A* is the most important representative. It was shown that the dye c*ibacron blue F3G-A* could bind to some enzymes (Kopperschlager et al., 1968; Haeckel, 1968). Until then a dextran conjugate of the triazine dye, c*ibacron blue F3G-A* had been used as a void volume marker in gel filtration (Andrews, 1965). 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) (Hey and Dean, 1981).

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) (Lowe and Pearson, 1984). 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) (Lowe and Pearson, 1984). 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 done 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 (Clonis et al., 1987). 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 (Janson, 1984). 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 (Lowe, 1984; Clonis et al., 1986; Lakhin and King, 1976). In addition, dye columns are readily reusable (up to 40 times for a single column) (Hey and Dean, 1981) 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 (Hey and Dean, 1981). 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.

1.5.3.4.3. Metal chelate affinity chromatography

Porath et al. (1975) 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 (Lonnerdal and Keen, 1983). This method is based on the ability of various metal ions to form co-ordinate compounds with high- or low- molecular weight ligands (Basolo and Johnson, 1964). 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 (Chaberek and Martell, 1959). 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 to 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 (Sulkowski, 1985). 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. In addition, immobilised tris(carboxymethyl) ethylene-diamine (TED) (Porath and Olin, 1983), EDTA (Haner et al., 1984) and hydroxamic acids (Ramadan and Porath, 1985) 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 (Porath, 1978). Two enzymes, glycogen phosphatase and lactate dehydrogenase, were purified on Fe^{3+}-Sephadex column (Chaga et al., 1989). Human fibroblast interferon has been purified on Zn^{2+}-biscarboxymethyl amino-Sepharose 6B matrix (Heine et al., 1981).

1.5.3.4.4. Hydrophobic interaction chromatography

The formation of contacts between apolar groups in aqueous solution forms basis of this mode of separation and purification of proteins and nucleic acids (Yan, 1972). Hydrophobic interaction chromatography takes advantage of the hydrophobic domains or binding sites on proteins and other biomolecules. Proteins are folded in native biologically functional conformations 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 (Shaltiel, 1978). 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 (Van Hippel and Schleich, 1969; Jencks, 1969), whereas structure breaking electrolytes have a reverse effect on the water structure (Hamaguchi and Geiduschek, 1962). Various hydrogels, substituted by apolar side chains of differing 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 (Jennissen, 1976). 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 (Hofmeister, 1988). 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.

← Increasing salting out effect

Anions PO_4^{3-}, SO_4^{2-}, CH_3COO^-, Cl^-, Br^-, NO_3^-, ClO_4^-, I^-, SCN^-

Cations NH_4^+, Rb^+, K^+, Na^+, Cs^+, Li^+, Mg^{2+}, Ca^{2+}, Ba^{2+}

Increasing chaotropic effect →

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.

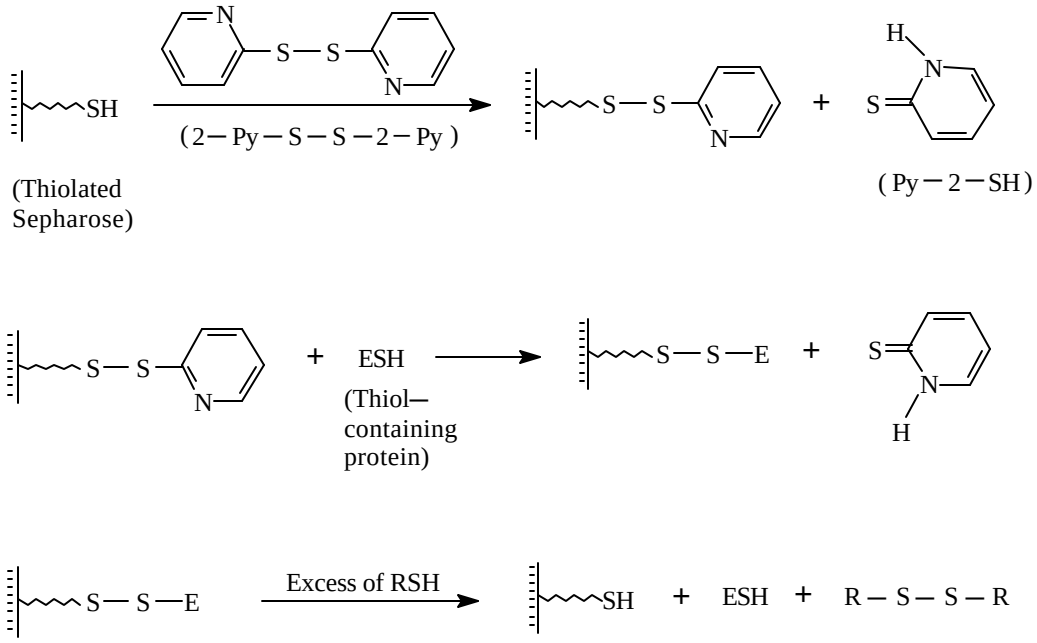
1.5.3.4.5. Charge transfer adsorption chromatography

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 (Porath, 1978; Porath and Dahlgren-Caldwell, 1977). 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 (Mohr, and Pommerening, 1985). 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 (Villems and Toomik, 1993). Egly et al. have studied the chromatographic behaviour of adenosine and adenosin monophosphate (AMP) on Sephadex G-25 epichlorohydrin support coupled to acriflavin.

1.5.3.4.6. 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 (Brocklehurst et al., 1973).

Brocklehurst et al. employed polymer with 2,2-dipyridyl sulphide for the isolation of papain, chymopapin, ficin, propapain, creatine, phosphokinase and phosphofructokinase (Brocklehurst et al., 1974). The isolation of protein with a thiol group (ESH) is presented in Scheme 1.6:



Scheme 1.6 General run of covalent chromatography

In the adsorption step, the biomolecule to be separated is covalently bound 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 (1972, 1974) 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. Ashani and Wilson (1972) 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).

1.6. Controlling elements of adsorption on expanded bed

An expanded bed is characterised by: 1) liquid dispersion, 2) film mass transfer, 3) pore diffusion and 4) adsorption. Since the protein breakthrough curve of expanded bed is similar to packed bed, their performances are similar. However, the breakthrough curve of protein, which is a measure of expanded bed performance, is based on some of the parameters including the operating variables. A minimum height is required for less protein dispersion and more dynamic binding of proteins for slow binding kinetics of protein. The minimal height of the sedimented bed needed for efficient protein adsorption is not only a function of adsorption rate but also is dependent on liquid dispersion. With the tailor-made adsorbents described in Table 1.2, a minimal sedimented bed height of 100 mm is required for the adsorption of small proteins, such as lysozyme on cation-exchanger (Hjorth et al., 1995). For larger proteins the bed height may need to be increased. This is due to the fact that slow diffusion of the larger proteins may have little contribution towards the slow exchange of proteins on the matrix and an early breakthrough emerges. Therefore, it is recommended to start with a sedimented bed height of about 150 mm during method development to avoid the risk of an early breakthrough. This required minimum sedimented bed height might be decreased for the adsorbents of higher pore size, in which the diffusion of larger proteins is not a limitation. This will provide an extra advantage of using lesser amount of adsorbents.

Unlike with the packed bed operations, the dynamic binding capacity decreases with increase in flow rate. This is probably not due to increased axial mixing as described by Chang and Chase (1996), but might be due to other factors. One possibility is due to the increased viscosity of the feed in comparison to clarified feed. The process time of protein purification by expanded bed may increase due to lower diffusion and mass transfer. The process stream frequently encountered in biological applications of expanded bed adsorption often have viscosity of the order of 1-2 cp for mammalian cell culture broths and 10 cp or higher for *E. coli* and

P. pastoris broths (Johansson et al., 1996). The current process time are long and two or more days can be required to process 250 L of mammalian cell culture broth or 14 000 L of antibiotic fermentation (Batt et al., 1995; Galliot et al., 1990). Since the terminal velocity is inversely proportional to fluid viscosity, increase in viscosity decreases the range of liquid velocities achievable in the expanded bed. Feed stock dilution may be required to decrease the viscosity in order to attain a reasonable linear velocity for fluidisation without excessive axial dispersion and product loss (Draeger and Chase, 1991; Chang and Chase, 1996). Other applications may require feedstock dilution to achieve a conductivity level necessary for resin adsorption on ion-exchange resins (Frej et al., 1994). Dilution in either case can double or triple the feedstock volume, resulting in longer process time. As a result, fluidised bed adsorption times are long, and in some cases, this technology offers only a marginal advantage over conventional primary recovery methods (Batt et al., 1995; Johansson et al., 1996).

Bodestein number was found to increase with increase in flow rate in a range from 100 to 400 cm h^{-1} (Hartmann et al., 1997; Thömmes et al., 1996) with tailor-made expanded bed matrices. A minimum flow velocity is required to obtain a stable bed (Thömmes et al., 1996). Influence of film diffusion is higher than that in the packed bed owing to the increased voids between the particles, though it is difficult to measure the film diffusion in a fluidised bed (Joshi, 1983). However, a decrease of the dynamic capacity observed at increased flow rates is attributed to pore diffusion restrictions (Chang and Chase, 1996). There are also reports on lowering of dynamic capacities when the concentration of proteins in the feed is lower than the K_d value (Draeger and Chase, 1991) on packed bed. Therefore, the most limiting in expanded bed chromatography is hindered pore diffusion of proteins, which is also found in packed bed adsorption.

It has been hypothesised that the long process times may be due to intraparticle mass transfer resistance in the resin particles and/or flow hydrodynamics in the fluidised bed. Hanratty et al. (1956) observed that turbulent diffusion caused by the random motion of particles governed the macro-mixing and dispersion

characteristics in a expanded bed. Mehta and Shemilt (1976) observed that the scale of turbulences for solid depends on the viscosity of the liquid in an expanded bed. To offset hydrodynamic instabilities, magnetised particles have been fluidised in presence of a magnetic field (Seibert and Burns, 1998). Studies indicate that magnetic stabilisation improves adsorption efficiency but intra-particle mass transfer effects have not been investigated. The effect of resin characteristics as a function of hydrodynamic parameters was investigated by Wright et al., 1999. To quantify the impact of resin characteristics in expanded bed adsorption, mass transfer effects noted while using a macroporous resin (Streamline SP) was compared with that observed for hyper-diffusive resin (S-HyperD LS). Adsorption capacity was found to be poorer under all conditions for the macroporous resin than for the hyper-diffusive resins due to intraparticle mass-transfer limitations.

1.7. Composition of feedstocks and their effects

Various crude feedstocks have been evaluated with the different chromatographic systems. Channeling and movement of the bed front have been observed on applying the feed. It has been a challenge for the researchers to apply the feedstocks to a chromatographic column on expanded bed mode. The feed containing the polysaccharides and nucleic acids interact with the ionic resin resulting in a compact bed. Therefore, the flow of the feed finds a path to come out of the bed in the shape of channel(s) or the bed is moved towards the upper adapter. The remedies to this problem are: 1) dilution of the feedstock and 2) addition of nucleases, to decrease the interaction. Therefore, there is a need to discuss the composition of the feed, which controls the binding characteristics on expanded bed. Table 1.3 describes the composition of microbial and animal feeds. Both are complex mixtures of dissolved components and particulates with varying concentration. Particulates are mainly whole cells with some cell debris. Cell debris are produced from the cell-breaking step required, if the product is intracellular. Besides the media components, the feed stock contains variety of proteins including the target protein with some other substances such as salts, vitamins, nucleic acids, polysaccharides, peptides, amino acids, lipoproteins, polynucleotides, endotoxins etc. Especially, the cell-disrupted

broth contains high concentrations of proteins, DNA, polysaccharides and endotoxins. Coloured byproducts (pigments) are also excreted which adsorb very strongly to the adsorbents and decreases the binding capacity in ion-exchangers. Contaminants are also mixed with the target products during elution. This irreversibly bound pigment necessiates the use of CIP procedure of adsorbents after each run in expanded beds.

Table 1.3. Composition of a broth after processing by microorganisms or cell culture.

Particulates
Cells (> 1μm)
Cell fragments (<1μm)
Protein precipitates (<1 μm, colloids)
Dissolved constituents
Low-molar-mass molecules (MW < 1000)
Amino acids, vitamins, nucleic acids
Buffer salts, additives etc.
Colour and aroma substances
High-molar-mass molecules (MW >> 1000)
Proteins, including lipoproteins
Polynucleotides (mainly DNA)
Endotoxins

Higher viscosity of the feed enables the bed to expand higher at lower velocity of the feed. This restricts the bed to operate at lower flow velocity and decreases the throughput and binding capacity. The higher viscosity of the feed also leads to increased restricted pore diffusion and external mass transfer. This means that the higher viscosity increases liquid dispersion, decreases external mass transfer rate and pore diffusion, which in turn decreases the adsorption characteristics. The most practical difficulty is the channeling of the bed and sometimes the whole bed front

moves and clings to the upper adapter. This leads to uneven distribution of the feed and most of the adsorbents are underutilised, which decreases the efficiency of the process. The higher viscosity of the broth in comparison to the buffer is due to the release of DNA due to accidental cell lysis in the growth cycle (one phase is the cell decline phase, where cell lysis occurs) or intentional cell breakage (for intracellular products). This effect is severe with long-chain genomic DNA. The viscosity of high cell density cultures, used in industrial scale for higher volumetric productivity, is high. However, microorganisms secreting nucleases (DNase or RNase) can be used to control the viscosity in fermentation at high cell density (Huisman et al., 1999). The viscosity and the cell concentration of the broth are summarised in Table 1.4.

Table 1.4. Characteristics of some cell suspensions

Feedstock	Viscosity (mPa s)	Cell concentration	Reference
Candida kefyr	2.2	7 g/L, dry mass	Pessoa et al, 1996
Alkaligenes eutrophus	1.08-1.11	5-10 g/L, dry mass	Draeger and Chase, 1990a and 1990b
Bacillus cereus	1.5-3.0	5-10 g/L, wet mass	Kroner, 1986
Streptomyces fradiae	50-190		Choi et al., 1998
BHK cell culture	1.36	10^7 cells/cm^3	Vogel and Kroner, 1999
Mammalian cells	1.3		Kohara et al., 1995

Both dilution and treatment with the enzyme DNase (Bezonase, degrades nucleic acids into smaller fragments; Asplund et al., 1996) were previously necessary before application onto expanded bed. However, Boynton et al. In 1999 integrated a nuclease-encoding gene from *Staphylococcus aureus* into the genomes of several poly(3-hydroxyalkanoates) (PHA) producers to reduce the viscosity of the lysate to a level similar to that observed for the wild-type strain after treatment with commercial nuclease.

Another problem is the determination of the exact viscosity at low shear rates operating during application on the expanded bed. Since determination by capillary viscometry is not possible owing to the presence of particulates, low-shear rheometers should be employed. Rheometers measure shear stress against the applied shear rate. With Newtonian fluids the viscosity can be taken from the slope of the resulting graph or even at one shear rate, since there is a linear relation between the shear stress and shear rate. However, as biomass fluids show non-Newtonian behaviour, it would be advisable to take the detailed data of shear stress vs shear rate. Since it would be difficult to measure the shear rate of the feed on the column at different feed velocity, the slope of the curve resulting from the viscosity measurement experiment can not be determined at the actual shear rate (in the column). Therefore, the viscosity should be taken at constant shear stress. The other problem may rise in exact measurement of viscosity from animal cell culture broth, which are very shear sensitive and can easily lyase during application to the column due to either change of pH or ionic strength.

1.8. Influence of cells on column performances

Infection of the adsorbents or clinging of cells to the adsorbents results in turbulent flow pattern in the expanded bed and decreases the number of theoretical plates. Therefore, studies were carried out with the influence of cells on binding capacity of BSA onto anion exchangers. The binding capacity dropped by about 75% after addition of 2% of *Alkaligenes eutrophus* cells (Draeger and Chase, 1991). The breakthrough volume also decreased substantially in presence of only 0.5% cells to less than 25% of the original value obtained in a cell-free solution. In comparison to this, a protein A affinity adsorbent yielded only about a 10% decrease in capacity. The same author found a slight difference between different types of cells, although the general observation did not change (Chase and Draeger, 1992). Ameskamp et al., 1999 observed retention of biomass of hybridoma cells on Streramline SP and Streamline SP XL. Since the cell wall of bacterial and animal cells is negatively charged, there is attractive interaction with anion exchangers at the low ionic strengths commonly chosen for protein adsorption. Therefore, competitive interactions at the adsorbents' surface are probably the reason for the observed

decrease in protein capacity. The fluidisation characteristics were observed to change (Amsekamp, 1999). This causes a decrease in the binding capacity and an increase in elution volume. The eluate carries biomass and even viable cells, and necessiates an additional clarification step. To minimise this problem, mixed mode matrices which have low surface charge compared to ion-exchangers were developed by UpFront chromatography A/S (Denmark). These matrices are claimed to be less prone to fouling when tested with crude raw materials.

Scanning electron micrographs of the DEAE anion-exchanger showed adsorbed cells of bakers' yeast after extensive washing with an elution buffer (Anspach et al., 1999). The remaining cell debris of a homogenate and from bakers' yeast is evident on the surface and on the pore entrance of the anion-exchanger even after extensive washing. The animal cells also adsorb at anion-exchangers as reported by Feuser et al. (1999). On the other hand, adsorption of protein A on affinity adsorbent was not established, in the study by Draeger and Chase (1990a). However, on long-term application, an apparent loss of cells was noted, which was related to cell adhesion at the flow distributor and the lower inlet filter of the expanded bed column. Feuser et al., 1999 carried out detailed screening of suitable biomass/adsorbent combinations, that yield a robust and reliable initial capture step by expanded bed adsorption from unclarified feedstocks.

The above examples demonstrate that the packed-bed adsorption data can not be translated to the expanded bed mode. There is a dramatic change in binding characteristics in presence of particulates, and thus process protocols should allow for some flexibility.

Cell aggregates can block the screen or the flow distributor at the lower column adapter, causing turbulence at the lower part of the column. Since the hydrodynamic behaviour of a single cell or cell debris differ, when cell aggregates increase in size in the column due to incorporation of further cells, these may not be able to exite the column as the required terminal velocity would increase. In general, the aggregation of dead cells are more pronounced. Therefore, constant stirring of the

feed and use of in-line crude mesh filter is always recommended in order to minimise the aggregation during the application of feed.

The problem is magnified for the adhesive cells. Animal cells, which are anchorage dependent and stick to any surface / each other, tend to be problematic. Hybridoma cells are more problematic where as Chinese hamster ovary (CHO) are the least.

Cell and cell aggregation also causes aggregation of adsorbents, and consequently leads to change in hydrodynamic properties. This creates channeling and increased axial dispersion and even to a complete breakdown of the expanded bed. In case of cation exchangers aggregation of the biomass takes place at low pH. This can be solved by switching to anion exchange chromatography.

Aggregation of cells and cell debris at the lower adapter can be generally controlled by the clean-in-place (CIP) procedure. However, cleaning must follow immediately after each run. It is important to remove as many cells as possible during the washing step, to avoid entrapment of the cells in the interstitial volume of the particle bed. Problems of bed or the distributor clogging due to aggregation or adhesion of cells are most likely to take place after the collapse of the bed. If aggregation of the bed is severe, normal CIP will not reach all parts of the column and the problem will persist. As the strength of the chemicals used for cleaning is dictated by the chemical resistance of the equipment, dismentling the whole system may become necessary.

Lyse of mammalian cells prior to application can be circumvented by on-line adjustment of pH and ionic strength as well as by keeping the cell suspension in the cold (Batt et al., 1995). The addition of glucose is recommended to adjust the osmolarity, but here local concentration must be avoided.

1.9. Application of real sytems

1.9.1. Separation of cells

The amount of both microorganisms and animal cells can be reduced up to five orders of magnitude (Hansson et al., 1994; Hjorth et al., 1995). This is more efficient than centrifugation and may also exceed microfiltration, if large pore sizes are used. However, extensive washings with at least 20 sedimented bed volumes is necessary in the expanded-bed mode. It was also shown that some particulates always stick to the lower adapter and are released with flow inversion (Hansson et al., 1994). Hjorth et al., 1995 showed that *E. coli* cells were released on increasing salt concentration in the washing buffer to 0.5 M NaCl, which confirms the sticking of cells to anion-exchange surfaces. Lowering the adapter and inverting the flow generally causes the liberation of a relatively low amount of cells or cell debris that ends with sedimentation of the bed. If particulates still stick at the adsorber particles they are trapped in the voids of the bed and are not found in measurable amounts in the elution fraction. The experiment conducted by Ujam et al., (2000) to separate of *E. coli* and *S. cerevisiae* in an ion-exchanger (Streamline DEAE) indicated the potential for carrying out cell separations in expanded beds as an alternative to immunomagnetic cell separations.

1.9.2. Product recovery

Probably the most crucial elements affecting large-scale process design are the physical, chemical, and biochemical properties of the contaminating materials in the original broth and those of the protein that constitutes the final product. The properties of the starting material will be partly determined by its cultivation source, viz., bacterial, yeast, or mammalian cell, the type of the cultivation medium used (e.g., presence of albumin, calf serum, proteases, solid bodies like whole cell or cell debris), the length of the cultivation, and whether the product is intracellular or extracellular. To these factors we must add the actual physico-chemical properties of the product (surface charge/titration curve, biospecificity towards certain ligands, surface hydrophobicity, MW, pI, stability) as compared to those of the contamitant components in the crude broth. The stability of the final product is also of utmost importance as this will affect the types of operations that can be used as well as the conditions and process times that can be afforded. Purification of proteins from

various sources have been cited in the literature by a number of investigators. The sources from which the proteins are captured are microbial cells e.g., bacterial (Table 1.5) and yeast cells (Table 1.6), animal cell culture (Table 1.7) and other sources e.g., milk and industrial effluents (Table 1.8). The separation methods include a variety of techniques such as ion-exchange, affinity, and hydrophobic interaction chromatography. Because of the cell interaction with the anion exchangers, most of the product purification involves cation-exchange chromatography. Besides many host-derived proteins, DNA can be reduced more than 5 orders of magnitudes on cation-exchange adsorbents. High endotoxin concentration can be reduced by at least 3 order of magnitude. DNA and endotoxins are negatively charged and are therefore repelled from a cation-exchager. In case of strongly positively charged human basic fibroblast growth factors (p*I*=9.6), purification and concentration factors of 17 and 5 were obtained using Streamline SP (Garke et al., 2000).

The tendency of association of various proteins to the membranous structure (phospholipids), was negated by the addition of Triton X-100, of 1% (v/v) concentration, to the homogenate prior to purification (Barnfield et al., 1994). The IL-8 was solubilised, denatured and finally renatured (Barnfield, 1996). One of the problems associated with the intracellular yeast cells is the disruption of the cells, which are not easily disintegrated. Generally cell rupturing is through bead milling. High pressure homogeniser is an alternative method for this purpose.

Despite the problem discussed above anion-exchange adsorbents were employed to capture net-negatively charged proteins from unclarified homogenates, such as annexin V (Barnfield et al., 1994) and endotoxin A from recombinant *E. coli* (Johansson et al., 1996), extracellular inulinase from C*andida kefyr* (Pessoa et al., 1996), or BSA from recombinant *S. cerevisiae* (Barnfield et al., 1997). Even plasmid DNA was purified by a DEAE anion-exchanger from a *E. coli* lysate (Hitchcock et al., 1996). Hansson et al. (1994) indicated that anion exchange adsorption of *E. coli* cell is prevented at pH 5.5. However, no particular note about destabilisation of the expanded bed due to adsorbent aggregation is reported in other studies, although higher pHs were used. It seems that the concentration of solid biomass and the

viscosity is limiting (Barnfield et al., 1994). In order to avoid deterioration of the anion exchange adsorber due to cell adsorption, an extensive CIP procedure is recommended, involving 0.5 M NaOH with 1 M NaCl for contact time of at least 4 hours, and subsequently washing with distilled water, 30% isopropanol and 25% acetic acid (Johansson, 1996).

Despite adhesion of cells or clogging of cell aggregates at the lower column adapter, expanded bed chromatography has been successfully applied to animal cell cultures, such as antibodies from hybridoma cell lines using protein A sorbents (Thömmes et al., 1996) or from CHO cells using cation exchangers (Batt et al., 1995).

A further application is seen in the separation of soluble proteins from precipitated proteins. This was shown by the purification of recombinant human protein C from milk of transgenic pigs (Degener et al., 1998). Here, milk proteins were selectively precipitated by the addition of Zn^{2+} and the suspension was applied to an DEAE expanded-bed adsorber. Actinorhodin (antibiotics) was purified on variety of ion exchangers such as P-11 (cation-exchanger) resulting in 26% adsorption and 2% recovery whereas the DE-52 (anion-exchanger) yielded in 99% adsorption and 56% recovery. Streamline DEAE anion-exchanger, which is especially designed for EBA applications, yields 82% adsorption and 50% elution of actinorhodin fed into the chromatography column directly from the fermentation broth.

Table 1.5. Purification of products produced from bacterial cells

Cells	Product	Adsorbent	Fold conc.	%Yield	Reference
E. coli homogenate	Recombinant Annexin V	Streamline DEAE		>95	Barnfield et al., 1994
E. coli homogenate	Recombinant anti HIV Fab-fragment	Streamline SP	9.2	>95	Jägersten et al., 1994
E. coli lysate	Recombinant Endotoxin A	Streamline DEAE	13	79	Johansson, et al., 1996
E. coli inclusion bodies (disrupted and solubilised)	Recombinant human interleukin 8	Streamline SP	>10	97	Barnfield, et al., 1996
E. coli	Recombinant secreted fusion protein of two synthetic IgG-binding domains (ZZ)	Streamline DEAE	16	>93	Hansson et al., 1994
Recombinant *E. Coli*	Human Fab fragment	FastMabs AD	8.7	90	Hansen et al., 1999
E. coli	Recombinant Interleukin 1 receptor antagonist	Streamline SP	90-92% purity	85	Maurizi et al., 1997
E. coli homogenate	Fusion protein	Streamline SP	100 fold in total protein reduction	70	Brobjer, 1999
Lactobacillus amylovorus	amylovorin	Streamline SP		30	Callewaert et al., 1999
E.coli lysate	*Lactobacillus* phage LL-H muramidage	Streamline SP	74 fold purification and 5.9 concn.	60	Vasala, 1998
E. coli homogenate	modified dephtheria toxin	Streamline Chelating			Noronha et al., 1999
E. coli homogenate	Glutathione S-transferase [GST -$(His)_6$]	Streamline Chelating	3.34 fold purification	80	Clemmitt and Chase, 2000b
E. coli homogenate	β-galactosidase	Streamline Chelating	5.95 fold purification	86.4	Clemmitt and Chase, 2000a
High cell density recombinant *E. coli*	Human basic fibroblast growth factor (rh-bFGF)	Streamline SP	17 fold purification	87	Garke et al., 2000

Table 1. 6. Purification of products from yeast cells

Cells	Product	Adsorbent	Fold conc.	%Yield	Ref.
S. cerevisiae homogenate	Glucose-6-phosphate dehydrogenase	Streamline DEAE	PF-12	98.1	Chang and Chase, 1996
S. cerevisiae homogenate	Glucose-6-phosphate dehydrogenase	Streamline Procion Red H-E7B	103	98.8	Chang et al., 1995
S. cerevisiae homogenate	Alcohol dehydrogenase (ADH)	Streamline Phenyl		>95	Smith et al., 1996
H. polymorpha	Recombinant aprotinin	Streamline SP	3.8	76	Zurek et al., 1996
P. pastoris	Recombinant HAS	Streamline SP		87.1	Noda et al., 1996
P. pastoris	Therapeutic protein	Streamline SP		84	Purvis et al., 1996; Binieda et al., 1996
Pichia pastoris	recombinant human angiostatin (rhAngiostatin)	Cation exchange			Shepard et al., 2000
S. cerevisiae	Malaria transmission-blocking vaccine	Streamline Chelating			Noronha et al., 1999
Candida kefyr	Extracellular inulinase	Streamline DEAE	2.8	93	Pessoa et al, 1996

Table 1.7. Purification of products from animal cell culture broth

Cells	Product	Adsorbent	Purification Factor	Fold conc.	%Yield	Reference
CHO	Nerve growth factor	Streamline SP	11	30	100	Beck et al., 1996
CHO	Recombinant mooclonal antibody	Streramline SP		5	99	Zapata et al., 1996
CHO	antibody	Streamline SP	6	19	75	Batt et al., 1995
Myeloma Cell	Humanised IgG_4 antibody	Streamline rProtein A		15	111	Jägersten et al., 1996
Hybridoma cell	Murine IgG_1	Streamline rProtein A	90% purity	15-21	95-100	Lütkemeyer et al., 1996
Hybridoma cell culture	IgG_{2a} antibody	Streamline rProtein A		30-50	78-95	Thömmes et al., 1996
Hybridoma Cell cuoture	Monoclonal antibody	Streamline SP	3.2-8 (pH from 5.5 and 6, dilution 1:1 to 1:3)			Thömmes et al., 1995
Hybridoma Cell culture	Monoclonal antibodies	Streamline SP XL		Concn. Factor 6.9-7.3	65.9-58.1	Ameskamp et al., 1999
Chicken intestine	Alkaline phosphatase	Cibacron Blue-linked cellulose beads	48 fold purification		70%	Roy and Gupta, 2000

Table 1.8. Purification of proteins from other sources

Source	Product	Adsorbent	Purification Factor	Fold conc.	%Yield	Reference
Milk	Lysozyme	Streamline SP	8.3	-	88.9	Noppe et al., 1996
Defatted milk	α-lactoalbumins	Streamline phenyl				Noppe et al., 1999
Milk of transgenic livestock	Recombinant human protein C (rhPC)	Streamline DEAE	200		89-94	Degener et al., 1996
Crude potato juice	patatin	Mimo ES and Mimo ExF	1.2 (ES) and 2.4 (ExF) fold purification, respectively	0.65 (ES) and 1.9 (ExF)	37% (ES) and 50% (ExF) recovery	Straetkvern et al., 1999

1.9.3. Analytical procedures on expanded beds

Flow injection analysis were established for on line detection and quantification of intracellular lactate dehydrogenase (LDH) (Nandakumar et al., 2000a) and nisin (Nandakumar et al., 2000) during fermentation. A constant online monitoring from within the expanded bed using stopped-flow analysis (SFA) (Willoughby et al., 2000) was carried out to provide data for the control of expanded bed operation.

1.9.4. Refolding of inclusion body proteins inside expanded bed

In-vitro refolding of overexpressed proteins in inclusion bodies has been a bottleneck for large-scale production of recombinant biopharmaceuticals. Aggregation and large volume due to high dilution have been addressed as major problems. To circumvent these drawbacks, Lee (2001) developed a novel refolding process that combined the solid phase, or matrix assisted refolding technique inside expanded bed adsorption (EBA) chromatography. He tested a fusion protein (hGH-GST) and a single-chain polypeptide (rlFN-α-2a) as model proteins in a recombinant *E. coli*. The released inclusion bodies were first dissolved by urea and then fed to an expanded bed column to allow the unfolded proteins to bind to the resin surface via anionic interaction. As the urea is slowly washed off, the bound proteins are refolded as attached to the resin. After the denaturant was completely removed and thus the bound proteins were fully folded, they were eluted by salt gradient. The inclusion body proteins were refolded with much improved yield and purity directly from cell homogenate. This simplified process could reduce the number of renaturation steps.

1.10. Scope of the thesis

Biotechnology companies incur approximately the same costs as traditional drug makers during discovery and testing, but manufacturing-especially downstream processing-adds significantly to the cost of finished drugs. Downstream processing is typically a multi-stage process requiring such unit operations as filtration, centrifugation or precipitation, concentration, and often chromatography. The incentive to reduce the number of these operations and improve their efficiency is

tremendous. For example, the overall yield from a seven-stage process, each with a 75% yield, is 13%. Improving the yield to 85% and reducing the number of operations to three increases the overall yield to 61%, a savings that goes directly to the bottom line.

Bioprocesses typically live by three chromatography steps: capture, intermediate, and polishing. Existing techniques for the last two steps are highly effective and are unlikely to be fertile areas for cost reduction. But the capture step has caught the imagination of bioprocess innovators for its potential to combine or eliminate several pre-chromatography steps. One could envisage, for example, replacing cell removal, concentration, and capture into a single unit operation. This could be achieved through expanded-bed technology, which has the advantage over fixed-bed chromatography in its particle-independent operation. In other words, process fluids could conceivably be fed directly from the fermenter to the expanded bed.

1.10.1. Why new chromatography media?

To be suitable for use in expanded bed, the chromatography matrix must be highly dense to permit the high linear flow velocities necessarily encountered in this process. In addition, the process must be selective enough to achieve a high degree of purification. The matrices available in the market (Amersham Pharmacia, UpFront and BioSepra) are exorbitantly expensive. No existing material fits the bill. We observed loss of the quartz particles from the Streamline matrices, which blocks the column distributor plate and subsequently destabilises the system.

1.10.2. The model system

A starch degrading enzyme cyclodextrin glycosyltransferase (CGTase), which produces predominantly α-CD, is chosen as model protein in the fermentation broth, to study on expanded bed chromatography, due to following reasons.

1. Removal of cell mass from the crude fermentation broth by centrifugation did not result in clarified broth (clarification 99%) even when operated at high speed (≈9,600 g).

2. Flocculation of the fermented broth with flocculating agent (Magnafloc, 5 mL/L of fermented broth) and the subsequent clarification by centrifugation at 2,300 g for 20 min at 10 °C resulted in more than 99.9 % clarification without any loss in enzyme activity. The ionic flocculating agents may block the pores of UF membranes and the resins used for concentration and clarification.

3. Microfiltration (channel diameter 2 mm with 19 channels, surface area 0.19 m^2; Techsep, France), alternative method for the clarification of fermented broth, decreased the flux with time (reduced from 70 to 26 mL/min) and thereby resulting in a longer filtration time (1 hour 38 minutes for a volume of 3. 38 L). A small fraction of the enzyme and protein were lost. By washing the retentate side of the membrane module with 50 mM Tris-HCl buffer (pH 7.0), 3.7 % were recovered. The rest of the enzyme and proteins were found to be concentrated on the retentate side. Total recovery of the enzyme was 72 % after washing. This results in a dilution of the clarified fermented broth to nearly double of its initial volume.

4. Protease activity (2.8 U/mL) in the micro-filtered fermented broth was observed to digest the enzyme, resulting in loss in enzyme activity with time even at low temperature (6 °C).

5. The unclarified, centrifuged fermented broth blocked the packed bed chromatographic column and resulted in high pressure drop and subsequent blocking of the column. Sometimes flow channeling occured during loading of the unclarified centrifuged fermented broth for its purification.

6. Ultrafiltration of the clarified fermentation broth took additional time and could not separate protease from the broth.

Furthermore, Gawande (2000) observed the following problems during purification of α-CGTase.

1. Recovery of the enzyme from packed starch column was time consuming, since the brown pigments present in the fermentation broth adsorbs to the highly porous

starch and washing of these pigments in the washing step took long time. The recovered protein has several bands, which was visible in PAGE.

2. Additional gel filtration by Sephacryl S-200 was required to obtain a single band protein.

3. The overall recovery of the enzyme from fermented broth was 49% with 736 fold purification.

4. The amounts of buffer required for the purification of 10 L fermented broth was 3.5 L and the processing time was 61 hours.

In contrast, due to loss of enzyme activity in multistep purification, process problems in centrifugation and subsequently in packed bed chromatography, lower flux in microfiltration and the protease activity, which degraded the enzyme, the above enzyme system was chosen as candidate for purification on one step purification methodology i. e. on expanded bed.

This thesis describes the investigation of the above topic in an interdisciplinary (material science, chemical engineering and biology) way for the purification of α-CGTase. The objectives of the present study are outlined below.

1. Total synthesis and testing of the affinity matrix for purification of α-CGTase.

2. Determination of pore size distribution of the matrix by inverse size exclusion chromatography (ISEC).

3. Determination of key parameters such as adsorption, dissociation constant, diffusion coefficients and elution conditions on packed bed.

4. Design and testing of the distributor plate and testing the affinity matrix for purification of α-CGTase on expanded bed.

Chapter 2 deals with synthesis of a number of polymeric matrices and selecting the best matrix for use in expanded bed for protein purification. Coupling of

α-cyclodextrin to the matrix by a various diisocyanates (bifunctional spacers) for preparation of affinity matrix to purify α-Cyclodextrin glycosyltransferase (CGTase) are also reported. Based on the study the optimal affinity matrix was selected for α-CGTase purification.

Chapter 3 describes determination of pore size distribution of the best matrix by inverse size exclusion chromatography. It compares the pore size distribution in aqueous solution, with that in 1,4-dioxane. The observed increased pore size in 1,4-dioxane is also explained.

Chapter 4 entails determination of optimal ligand density on the best matrix, diffusion coefficient of various proteins and development of an empirical equation for calculation of diffusion constants for various proteins. Dissociation constant of enzyme ligand complex was established from the isotherm. The enzyme adsorption and elution on packed bed were also standardised.

Chapter 5 describes the design of distributor plates and testing for use on expanded bed. The selected affinity matrix was evaluated on expanded bed for its mechanical stability and microbial degradation. Optimisation of the feed dilution for easy column operation and sharp breakthrough of the enzyme through the bed is also presented.

Chapter 2

Novel affinity matrices:
Total synthesis and its application for adsorption of protein

2.1. Summary

Chapter 2 describes selection of matrix on the basis of density and nonspecific interaction, coupling of ligand (α/β-CD) by various diisocyanates, evaluation and selection of the right spacer arm for coupling reaction, their evaluation for α/β-CGTase purification and assessment for clean-in place procedure.

Four crosslinked macroporous beaded copolymer matrices consisting of 2-hydroxyethyl methacrylate (HEMA)-co-ethylene dimethacrylate (EGDM), glycidyl methacrylate (GMA)-co-pentaerythritol triacrylate (PETA), allyl glycidyl ether (AGE)-co-ethylene dimethacrylate (EGDM) and polyacrylamide were synthesised. The suitability of these polymers for expanded bed adsorption was evaluated. Beaded HEMA-EGDM copolymer with 25 mole% crosslink density (CLD)-HEG beads was selected for Expanded Bed Adsorption (EBA) application based on its higher density and low non-specific interaction. Novel affinity chromatography matrices were synthesised using various diisocyanates (hexamethylene diisocyanate, HMDI; isophorone diisocyanate, IPDI; phenylene diisocyanate, PMDI and tolylene diisocyanate (TDI) as bifunctional reagents to couple the macroporous beaded HEMA-EGDM copolymeric supports, of controlled particle size distribution, with α and β-cyclodextrins (CDs). The optimal conditions to couple the hydroxyl groups present in cyclodextrin (ligand) and the polymeric supports through urethane linkage were established iteratively using various diisocyanates. Efficacy of ligand binding on the matrix and non-specific interactions of the synthesised affinity matrices were evaluated. The characteristics of the synthesised affinity matrices towards the adsorption of α and β-Cyclodextrin glycosyltransferase (CGTase) were investigated. The binding of β-CGTase was the highest on affinity matrices with PMDI spacer. TDI wass established as the best spacer arm on the basis of high ligand binding and low non-specific interactions. The optimal conditions to regenerate the matrices were also established.

2.2. Introduction

Beaded macroporous support materials for use in EBA operation require

following characteristics: i) high density (1.2 to 1.3 g mL^{-1}), ii) low non-specific interactions, iii) good mechanical integrity, iv) stability towards various clean-in-place reagents and v) ease of derivatisation chemistry. EBA is an increasingly popular unit operation for single step recovery of proteins from the feed stocks (Chase, 1994). The matrices that have been evaluated for EBA are based on crosslinked agarose/quartz composites (Hjorth et al., 1995), denser glass coated with crosslinked agarose (Zakirakos et al., 1996), agarose/metal alloy composites (Hjorth et al., 1995), agarose/kieselgurh composites as solid phases (Desai et al., 1990), cellulose/titanium dioxide (Gilchrist et al., 1994), trisacrylate/zirconium dioxide (Biosepra S. A., France) and fluoride modified zirconia supports (Griffith, 1997). These matrices are tailor-made for protein adsorptions in expanded beds and comprise of hydrophilic natural or synthetic polymers. The densities of the matrices are enhanced by incorporation of heavier particles, such as quartz, glass, titania, zirconia or metal alloys. The composites of crosslinked cellulose and silica may have limitations due to chemical instability in acidic solutions due to the acid-catalysed hydrolysis of the siloxane bonds (Si-O-Si) anchoring the bonded phase to the surface (Glajch et al., 1987). The smaller size zirconia supports may block the distributor plate and support screen during fluidisation. Pai et al. (1999) explored the suitability of non-composite supermacroporous crosslinked cellulose in expanded adsorption. Cellulosic matrices are unstable towards stringent clean-in-place reagents frequently used in EBA operation. The investigators, therefore, used mild regenerating reagents for clean-in-place procedures. Moreover, the higher swelling of these materials caused problems during elution in packed bed operation.

The total synthesis of new affinity matrices entails the sequential optimisation of procedures aimed at: (i) synthesis of suitable support material with functional group, (ii) modification of the functional group and iii) attachment of the ligand to the modified support material. With an established matrix, for the above steps to be successful, the derivatisation and coupling reactions should be optimised. The derivatisation and coupling chemistry depend on the functional groups present on the support matrix as well as on the ligand. The matrices and the ligands with hydroxyl group are conventionally activated by epoxides, cyanogen bromide and divinyl

sulphone. The ether bond generated by the transformation of the epoxy group, formed by reaction of matrices with epoxides, is very stable. This epoxidation is carried out in the pH range 9.0 to 13.0 (alkaline conditions). Thus, this procedure is suitable for ligands stable under alkaline conditions (Porath and Fornstedt, 1970). The hydroxyl can be transformed into the oxirane group, by reaction with bisoxirane, such as 1,4-bis(2,3-epoxypropoxy) butane (Sundberg et al., 1974). The desired long hydrophilic spacer arm, to delink the ligand from the bulk of the support, is also introduced. The simultaneously occurring cross-linking, as the side reaction, increases the stability but at the same time decreases flexibility and permeability. Cyanogen bromide offers facile activation of the matrices. However, it is hazardous and the process is a multi-step one (Axen et al., 1967 and Porath et al., 1973). The matrix shrinks very noticeably under extremely high activation as a result of cross-linking (Axen et al., 1967). The other method of activation of the hydroxyl matrices is with divinyl sulphone (DVS). DVS is useful with alkali-resistant nonshrinking polymers. This activation increases the rigidity of the matrices (Porath and Sundberg, 1972).

This chapter describes the selection of the appropriate support material (matrix) for use on EBA from the four synthesised macroporous copolymeric beads (HEMA-co-EGDM; GMA-co-PETA; AGE-co-EGDM and polyacrylamide) on the basis of higher density and low non-specific interactions towards proteins. There have been no literature reports on using diisocyanates as binding reagent in the development of affinity matrices. This work reports the use of reactive diisocyanates in the preparation of affinity chromatography matrices. Macroporous, beaded, crosslinked copolymers of 2-hydroxyethyl methacrylate (HEMA) and ethylene dimethacrylate (EGDM) were synthesised as model matrices, α and / β-CDs as ligands and α and / β-CGTase as protein. The hydroxyl groups of the matrices were coupled to the hydroxyl groups of the CDs using a variety of diisocyanates to obtain affinity matrices differing in spacer arm. The non-specific interactions, extent of ligand binding and stability towards clean-in-place reagents were assessed to evaluate these affinity matrices.

2.3. Materials and Methods

2.3.1. Chemicals

Allyl glycidyl ether (AGE), 2-hydroxyethyl methacrylate (HEMA), ethylene dimethacrylate (EGDM) and glycidyl methacrylate (GMA) were procured from Fluka AG (Germany). Cyclohexanol and pentaerythritol triacrylate (PETA) were obtained from Aldrich Chemical Co., USA. Poly(vinyl pyrrolidone) was procured from Polysciences, USA. α and β-Cyclodextrins were gifted by Cerestar (USA) and SA Chemicals (Mumbai, India), respectively. 1,6-Hexamethylene diisocyanate (HMDI), isophorone diisocyanate (IPDI), 2,4-tolylene diisocyanate (TDI) and dibutyltin dilaurate were obtained from Sigma (USA). Phenylene methylene diisocyanate (PMDI) was from BASF (Germany). 1,4-Dioxane, dimethyl sulphoxide (DMSO), Tween 20, dichloroethane, toluene, acrylamide, N,N'-methylene bisacrylamide and cyclohexanol were procured from SD Fine Chemicals (Boisar, India). Azo bis(isobutyronitrile) (AIBN) was obtained from SISCO (Mumbai, India). All fermentation media components were obtained from Himedia (Mumbai, India). Streamline DEAE was kindly gifted by Amersham Pharmacia, Uppasala, Sweden. Soluble starch was from E-Merck (Mumbai, India).

2.3.2. Instrumental

Infrared (IR) spectra were recorded on a model IR 470 spectrophotometer (Shimadzu, Japan). Samples of HEMA-EGDM copolymer beads, α and β-CD as well as affinity polymer were mixed thoroughly, pelletised in KBr, and analysed.

2.3.3. Methods

2.3.3.1. Preparation of the polymeric matrices

Three beaded macroporous copolymeric matrices were synthesised by suspension copolymerisation (Kotha et al., 1991) of HEMA with EGDM, GMA with PETA and AGE with EGDM. The mole ratio of the crosslinking comonomer (EGDM and PETA) relative to monomer (HEMA, GMA and AGE) was in the range of 25 to

200%. Polyacrylamide matrices were prepared by emulsion polymerisation (Cocker et al., 1997).

The density of each size fraction of Streamline DEAE and affinity HEG beads were measured under swollen state by floatation method (McCaffery, 1981). The solvent mixture used in this experiment was methylene chloride and methyl salicylate.

2.3.3.2. Coupling of ligands

1,4-Dioxane was made anhydrous by keeping in sodium hydroxide pellets overnight and was distilled and stored over dried molecular sieves (3 Å). Similarly, DMSO was kept overnight over chromatographic grade alumina, distilled and stored over dried molecular sieves (4 Å).

Diisocyanates were efficiently coupled to macroporous HEMA-EGDM copolymer matrices in the ambient in an aprotic solvent. This step was carried out in a conical flask with suitable aprotic solvent under stirring at 200 rpm for 24 hours with air and dried nitrogen overlay. After completion of the activation step, the matrices were washed with anhydrous 1,4-dioxane. The second step was the coupling of the ligand (CD) to the activated macroporous copolymer matrices with isocyanate linkages so obtained by a sequential procedure. α/and β-CD solutions were made in anhydrous DMSO and added to the activated matrices. The reaction was carried out under stirring at 200 rpm in 250 mL conical flask for 24 hours. The affinity matrices so prepared were washed sequentially with 20, 40, 60 80% methanol and dried under vacuum at 60 °C. The detailed procedure is described in Section 2.4.2.

2.3.3.3. Swelling of HEMA-EGDM copolymer beads

10 g of HEG beads were tested for swelling in water, 50 mM Tris-HCl buffer (pH 7.0, 8.0, 9.0 and 10.0), 1 N HCl and 1 N NaOH. The packed bed volume after swelling was noted. The skeletal volume of HEG beads was calculated by multiplying the packed bed volume with 0.6, considering the external void volume as 0.4. The skeletal volume of the

dried HEG beads was calculated from the ratio of weight to density. The swelling ratio was calculated from the ratio of skeletal volumes in swelled and in the dry state.

2.3.3.4. Ligand concentration measurement

The α and β-CD affinity matrices synthesised were assayed by methyl orange (Lejuene et al., 1989) and phenolphthalein methods (Kaneko, et al., 1987), respectively. The affinity matrices were added to the specified dyes till the decrease in absorbance was nearly 5 to 6%. The respective underivatised HEMA-EGDM matrices were used as controls. The amount of CD bound was calculated from difference in test and control values.

2.3.3.5. Estimation of bound diisocyanates

The bound diisocyanates were estimated as follows. 1 g of the affinity matrices were delivered to a round bottom flask and 1 mL of 0.001N solution of di-n-butylamine in 25 mL toluene solution was added. The mixture was heated under reflux for 15 minutes. On cooling, 50 mL of methanol were added through the condenser and the content of the flask titrated against 0.001 N HCl solution using bromophenol blue as indicator. A blank experiment was run in parallel. The isocyanate content (expressed in μmols NCO g^{-1} of the matrix) was calculated from the difference of blank and sample reading.

2.3.3.6. Porosity measurement

The pore size distribution, pore volume and pore surface area were measured by mercury intrusion porosimetry instrument in the pressure range of 0-4000 kg cm^{-2} (Quantachrome, USA). The mercury contact angle was 140°. The polymers were also sieved and were in the range of 105 to 250 μm.

2.3.3.7. Non-specific interactions

In a 50 mL conical flask, the affinity matrix (1 g; 2μmols of α-CD g^{1} of dried matrix) was equilibrated with 0.05 M Tris-HCl buffer of pH 7.0 to check for non-specific ionic interactions. 10 mL of 0.1 mg mL^{-1} bovine serum albumin (BSA) (made in 50 mM Tris-HCl buffer, pH 7.0) was added to each flask and shaken well at

12 °C, 200 rpm for 8 h. The BSA solution in plain matrices served as control. BSA solution was also shaken in native form under similar conditions, to estimate change in its concentration. The matrices were decanted and washed with 0.05 M Tris-HCl buffer (pH 7.0) at 12 °C. The adsorbed protein was eluted with 5 mL of 6 M urea solution. The desorbed protein was estimated as described by Lowry et al. (1951).

Similarly, hydrophobic interaction was studied at room temperature with protein solution containing 1 M NaCl, under conditions identical to that used for non-specific ionic interactions. The matrices were washed with 1 M NaCl solution at 25 °C and eluted with 0.05M Tris-HCl buffer (pH 7) at 12 °C.

2.3.3.8. Production of α and β-CGTase

α and β-CGTase were produced by fermentation, using *Klebsiella pneumoniae pneumoniae* NCIM 5121 (Gawande and Patkar, 1998) and *Bacillus firmus* NCIM 5119 (Gawande et al., 1998) in a 1 L magnetically driven top suspended fermenter (Gallenkamp, UK). α-CGTase was produced in a fed batch mode whereas β-CGTase was produced in a batch mode operation. The batch run of α-CGTase was started with 0.5 L medium containing (in g L^{-1}) dextrin 49, urea 5, yeast extract 5, $(NH_4)H_2PO_4$ 6, $MgSO_4$ 0.2 and pH 7.0. After 6.5 hours of batch cultivation, feed containing dextrin (172.5 g L^{-1}) and other components (at the double the concentration used in the batch mode of operation) was started. The feed rate was controlled using a fed-back control based on dissolved oxygen (DO), which is maintained between 0-25% of its saturation value. The addition of the feed was completed after 15 hours.

β-CGTase was produced in 0.6 L medium containing (g L^{-1}) corn starch 24, yeast extract 26, pharmamedia 25, Na_2HPO_4 1, $MgSO_4.7H_2O$ 0.2 and $NaCO_3$ 10 (sterilised separately). The optimised fermentation media used was as described earlier (Gawande, 2000 and Gawande and Patkar, 1999). The fermented broth was centrifuged at 7,200 g for 20 min. The supernatant was assayed for adsorption of the enzyme in the synthesised affinity matrices.

2.3.3.9. Adsorption studies

Adsorption of both α and β-CGTase were studied in shake flasks. The matrices (1 g; 2 μmol of α / β-CDs) were equilibrated with 10 mL of 0.05 M Tris-HCl buffer, pH 7.0. The fermented broth containing α and β CGTase was added and the flasks were shaken at 12 °C for 16 h. The plain matrices were also shaken with the fermented broth under similar conditions, to check for non-specific binding. Similarly, fermented broth was also shaken alone to establish the stability of the enzyme under these conditions. The adsorbed α and β-CGTase were eluted with 10 mM $CaCl_2$ solution in 50 mM Tris-HCl buffer, pH 7.0 at 25 °C and 55 °C, respectively. Protein was assayed by Lowry method (Lowry et al, 1951). α and β-CGTase were assayed by methyl orange (Lejuene et al., 1989) and phenolphthalein methods (Goel and Nene, 1995), respectively.

2.3.3.10. Measurement of stability of the coupling group

To one gram of affinity matrix, 10 mL of various regenerating solutions were added and shaken for 2 hours. The regenerating solutions were decanted and the treated affinity matrices were washed 5 times with 10 mL of distilled water till free from regenerating solutions. The matrices were dried under vacuum at 60 °C and then analysed for α/β-CD content, as described in Section 2.3.3.4.

2.4. Results and Discussion

2.4.1. Synthesis of polymeric beads

The matrices prepared comprise of 2-hydroxyethyl methacrylate (HEMA), glycidyl methacrylate (GMA) and allyl glycidyl ether (AGE) as monomer and ethylene dimethacrylate (EGDM) and pentaerythritol triacrylate (PETA) as the corresponding crosslinking comonomer. Three macroporous beaded copolymers were synthesised by suspension (HEMA-co-EGDM; GMA-co-PETA; AGE-co-EGDM) and one (polyacrylamide) by emulsion polymerisation, respectively in jacketed

cylindrical polymerisation reactor (11 cm diameter and 15 cm height) provided with six blade Ruston turbine impeller with controlled variable revolution. The schematic diagram of the reactor is shown in Figure 2.1. All the suspension polymerisation reactions were carried out at 70 °C using azobis(isobutyronitrile) (AIBN) as free radical thermal initiator. The continuous phase comprised of 1% (v/v) aqueous solution of protective colloid, poly(vinyl pyrrolidone) (PVP) 2.5 g in 250 mL water.

The discontinuous organic phase consisted of monomer, the crosslinking comonomer, pore generating solvent (cyclohexanol) and initiator. Monomer to pore generating solvent ratio was kept 1:1. The polymerisation was allowed to proceed for 3 hours. The polymer beads obtained were then thoroughly washed with water and methanol. The beads were dried under vacuum at 60 °C. The feed-ratios of the monomers used for the synthesis of the beaded copolymer matrices with 25 mole% crosslink density (CLD) are presented in Table 2.1. CLD is defined as the mole percent of crosslinking comonomer relative to the moles of reactive functional monomer. Since all copolymerisations were taken to 100% conversion, the average crosslink density in all copolymers is effectively the relative mole percentage of the crosslinking comonomer in the feed.

Table 2.1. Composition of the tailor made matrices.

S. N.	Matrices	Monomer	Crosslinking monomer	Volume, mL		CLD, mole%
				monomer	crosslinking monomer	
1	HEG	HEMA	EGDM	21.6	8.4	25
2	GMPTA	GMA	PETA	25.0	7.7	25
3	AEG	AGE	EGDM	26.8	10.7	25

HEG represents HEMA-co-EGDM; GMPTA, GMA-co-PETA; AEG, AGE-co-EGDM macroporous copolymer beads respectively. CLD is defined as mole percent of the crosslink monomer relative to the moles of reactive functional comonomer. HEMA, EGDM, GMA, PETA and AGE represent 2-hydroxyethyl methacrylate, ethylene dimethacrylate, glycidyl methacrylate, pentaerythritol triacrylate and allyl glycidyl ether, respectively.

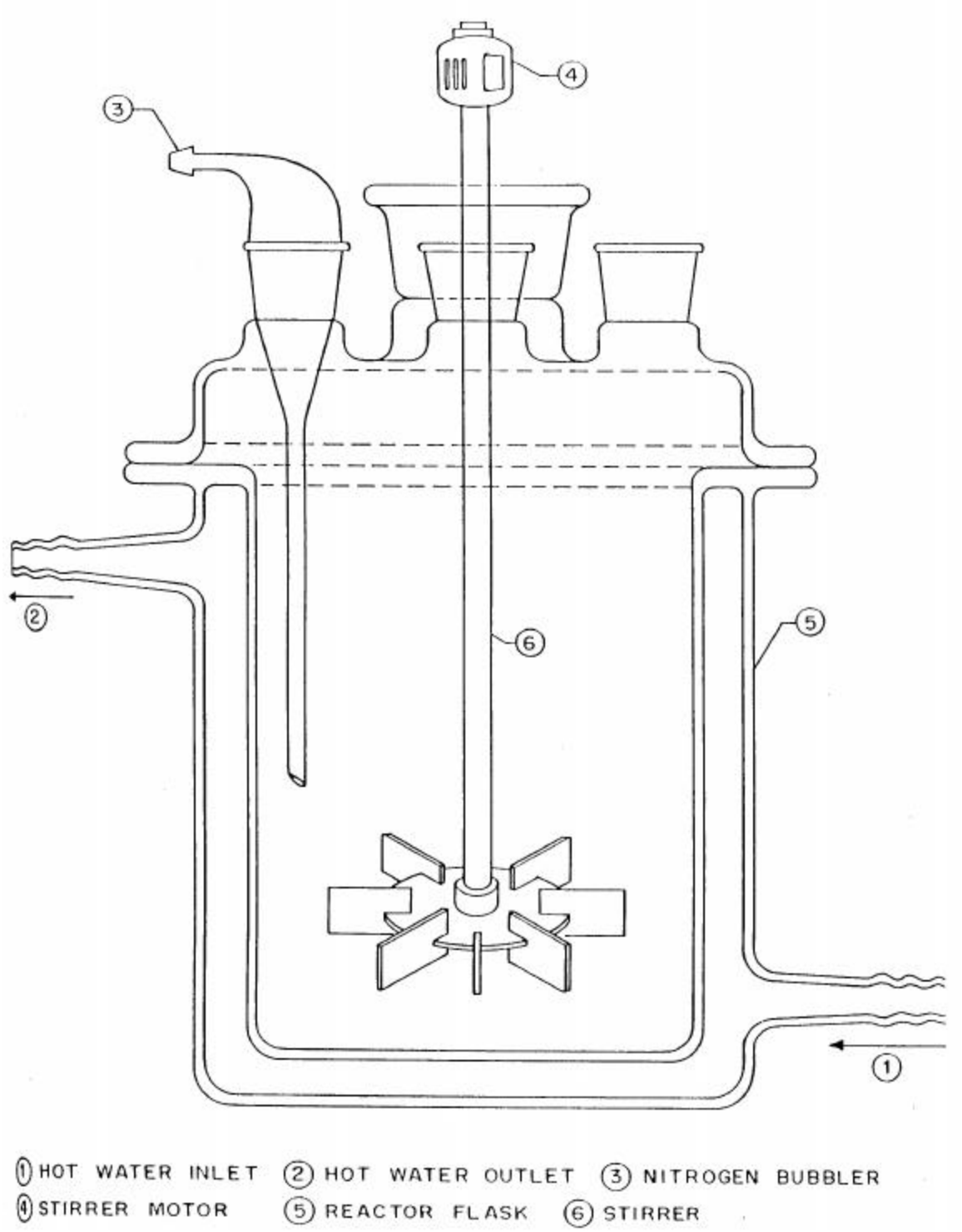


Figure 2.1. Polymerisation Reactor

Polyacrylamide (PACRY) gels were synthesised by emulsion polymerisation methodology. Acrylamide (8.88g) and N,N'-methylene bisacrylamide (5.14 g) were chosen as monomer and crosslink comonomer, respectively. The monomer and the crosslink comonomer with 50 mL of toluene and 61.9 mL of ethylene dichloride (EDC), 37.5 mL water, 3 mL Tween 20 were stirred at 400 rpm in the above described reactor. Ammonium persulphate (0.25 g) and 0.675 g of sodium sulfite with 6.7% (v/v) of DMSO were added to initiate the reaction. The reaction was continued for 8 hours at room temperature and the PACRY beads were recovered after washing with 1% (w/v) NaCl solution, and repeated washing with 20% (v/v) methanol. The beaded copolymer matrices were dried under vacuum at 60 °C. The prepared matrices, HEMA-co-EGDM, GMA-co-PETA, AGE-co-EGDM and polyacrylamide were abbreviated as HEG, GMPTA, AEG and PACRY respectively for convenience.

2.4.2. Screening of support material for use in expanded bed

The non-specific interaction of proteins on the matrices and density of the matrices under swollen conditions were evaluated to arrive at the best matrix for use in EBA, since EBA is a protein purification method which requires matrices of higher density (1.15 to 1.3 g mL^{-1}). PACRY has minute ionic and no hydrophobic interactions, whereas, HEG beads has no non-specific and little hydrophobic interactions. The lower density of PACRY beads ruled out its usefulness on EBA, whereas the rest were suitable for EBA. HEG beads were therefore, useful in EBA application due to its higher density and less nonspecific protein interactions. However, GMPTA and AEG had higher nonspecific interactions towards BSA (Table 2.2) and thus were not useful for purification of proteins. The HEG beads and Streamline DEAE (marketed by Amersham Pharmacia, Sweden for EBA operation) were observed under microscope (Lietz, Germany), which showed spherical (Figure 2.2) shape. The particle size and density distribution of the HEG beads were compared with Streamline DEAE matrices and this is discussed in Table 5.1 . The pore size distribution under swollen state is discussed in Chapter 3. The scanning electron microscopic (SEM) view of a cross section of HEG beads shows that the

matrix is macroporous (Figure 2.3). Series of copolymers, differing in crosslink density (CLD), were synthesised (Table 2.3), since it has been established that with increasing CLD, broader pores are generated (Kotha et al., 1996).

Table 2.2. Comparison of density and non-specific interaction of BSA with the tailor made matrices.

S. N.	Matrices	Ionic ($\mu g\ g^{-1}$ dry wt.)	Hydrophobic ($\mu g\ g^{-1}$ dry wt.)	Density ($g\ mL^{-1}$)
1	HEG	Nil	95.0	1.21
2	GMPTA	222.0	131.0	1.16
3	AEG	794.0	222.0	1.18
4	PACRY	20	Nil	1.0

HEG, GMPTA, AEG and PACRY represent copolymeric macroporous beads of HEMA-co-EGDM, GMA-co-PETA, AGE-co-EGDM and acrylamide-co-N,N'methylene bis-acrylamide, respectively. CLD is defined as the mole percent of crosslinking comonomer relative to the moles of reactive functional monomer. Ionic and hydrophobic interactions were measured with 10 mL of 0.1mg mL^{-1} of BSA solution in 50 mM Tris-HCl and 1N NaCl solution respectively. CLD of all the matrices were kept at 25 mole%

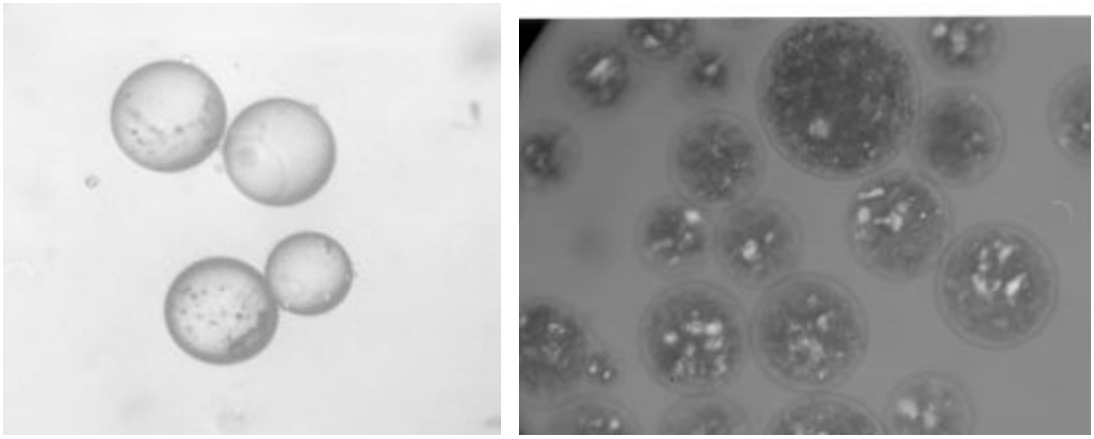


Figure 2.2. Microscopic view of HEG (CLD = 25 mol%) beads (left) and Streamline DEAE (right)

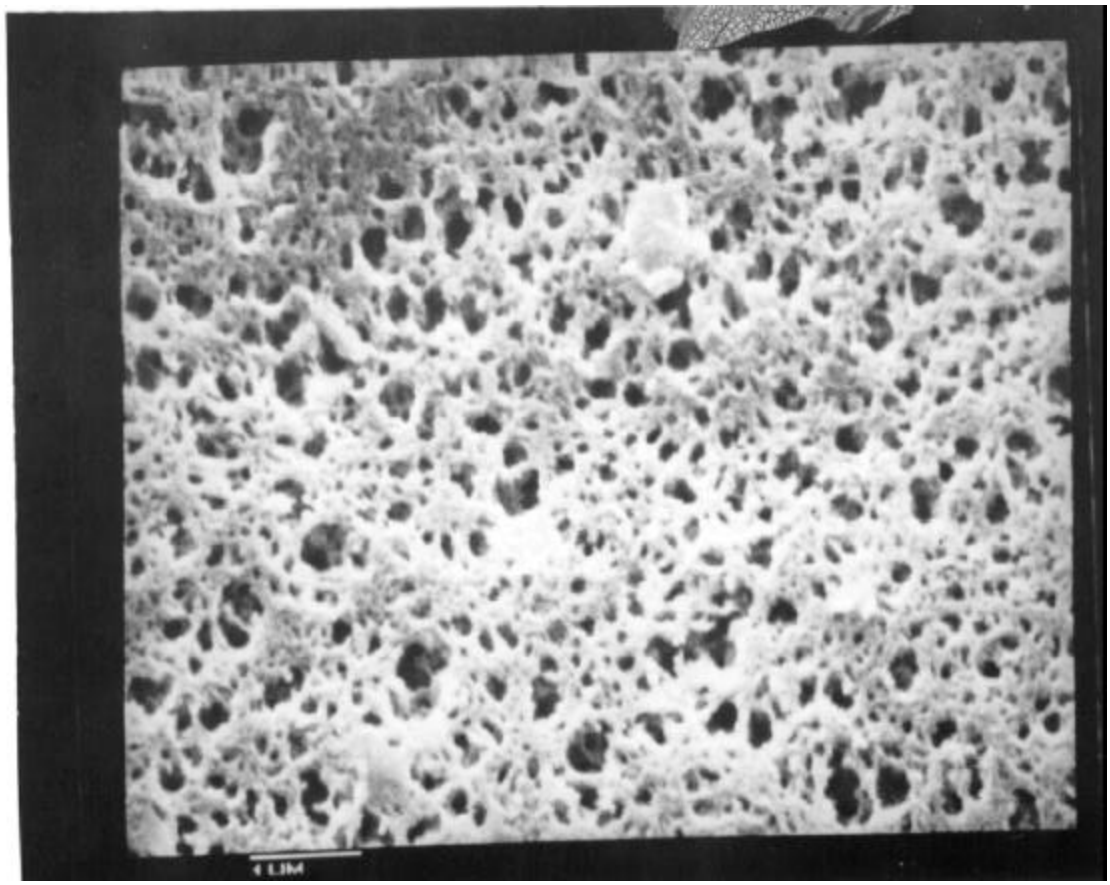


Figure 2.3. Internal porous structure of HEG beads (CLD 25 mole%) as observed with SEM

Owing to high crosslink density and low affinity for the aqueous environment, HEG beads exhibit low swelling (9.96 mL of dried HEG beads swell to 24.6 mL in 50 mM Tris-HCl buffer, pH 7.0). The swelling characteristics did not change with pH (1-12) and salt concentration from 10 mM to 1 M of NaCl.

2.4.3. Development of affinity matrices

The activation and coupling chemistry used here employs very facile bifunctional reagents such as diisocyanates. Isocyanates are carbonyl compounds with double bonds. The urethane bond formation starts with the reaction at ambient temperature between the carbonyl carbon of the isocyanates and the alcoholic oxygen. Ozawa (1967) employed hexamethylene diisocyanate (HMDI) as bridging reagent to stabilise lysine and enzyme proteins. However, this reagent has not been applied for

Table 2.3. Composition of HEMA EGDM matrices

SL. No.	HEMA		EGDM		HEG
	Volume, mL	Mol	Volume, mL	Mol	Crosslink Density (%)
1	21.60	0.178	8.40	0.044	25
2	16.88	0.139	13.12	0.070	50
3	13.85	0.114	16.15	0.086	75
4	11.74	0.097	18.26	0.097	100
5	10.19	0.084	19.81	0.105	125
6	9.00	0.074	21.00	0.111	150
7	8.06	0.066	21.94	0.116	175
8	7.30	0.060	22.70	0.120	200

HEMA=2-hydroxyethyl methacrylate, EGDM=ethylene dimethacrylate, HEG= copolymeric macroporous beads of HEMA and EGDM; The continuous phase comprised of 1% PVP in water. The discontinuous phase consists of 30 mL of HEMA and EGDM with 0.6 g of AIBN. The polymerisation was carried out at 70 °C. Other parameters are same as described in Table 2.1.

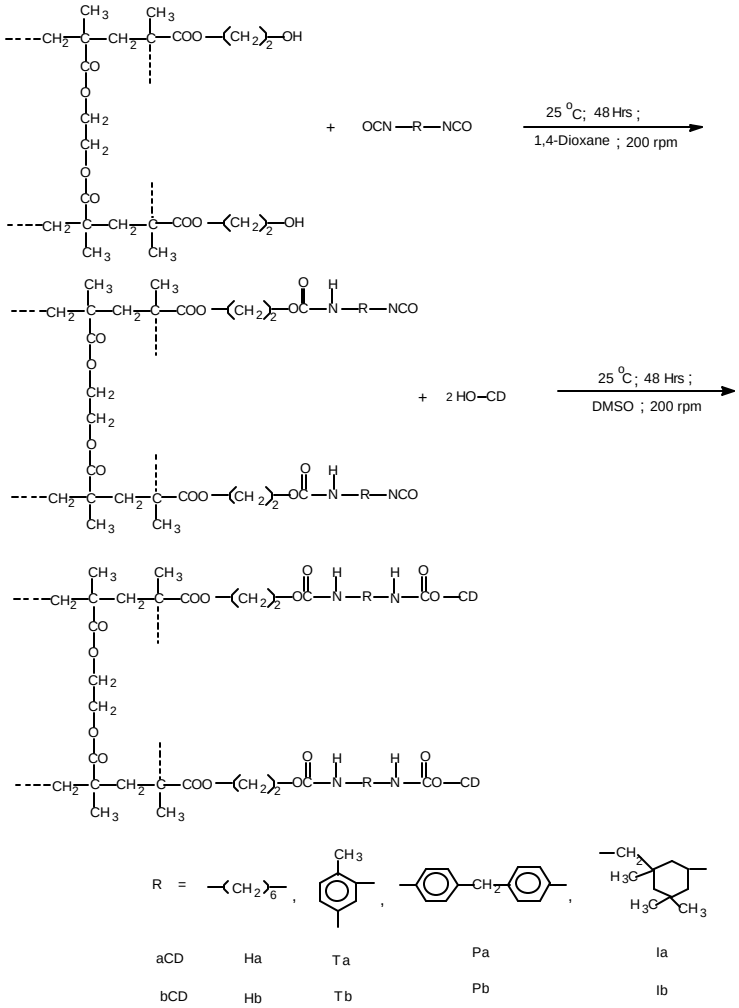
development of affinity matrices through urethane linkage.

A detailed study is undertaken to study these highly reactive reagents as linking groups in affinity matrices. A number of diisocyanates were evaluated for their ability to couple the ligands to the matrices. HEG beads (CLD-25 mole%) were coupled in two sequential steps to α and β-CDs through urethane linkages using different diisocyates. The first activation step was optimised using different molar quantities of isocyanate (-NCO) group (present in diisocyanates) with respect to a specific mole of hydroxyl (-OH) group in the matrix. This step was conducted in a stoppered 250 mL conical flask in a suitable aprotic solvent under stirring. The other parameters varied were temperature, addition of catalyst, and the nature of gaseous overlay. After completion of the activation step, the matrices were washed with

anhydrous 1,4-dioxane. The second step was the coupling of the ligand (CD) to the activated matrices. Cyclodextrin solution was prepared in anhydrous DMSO and added to the activated matrices. The coupling reaction was conducted at a constant stirring rate of 200 rpm. The other parameters established were the effects of temperature and addition of catalyst (Scheme 2.1).

The IR spectra of HEMA-EGDM copolymer, α-CD and a typical affinity matrix comprising of HEMA-EGDM copolymer coupled to α-CD are shown in Figure 2.4. The spectra show strong absorption peaks at 3500 (-OH), 2920 (-CH_3), 1715 (-C=O) and 1040 cm^{-1}, respectively for HEMA-EGDM copolymer, at 1027 cm^{-1} (-OH) for α-CD and at 3300, 2950, 1700 and 1020 cm^{-1} for HEMA-EGDM copolymer coupled with α-CD through tolylene diisocyanate (TDI). Strong peaks at 3300, 2950 and 1700 cm^{-1} confirm urethane bond formation in the derivatised copolymer. The typical spectra of α-CD are the strong peak concentrated around 1020 cm^{-1}. It can be seen that the spectrum of α-CD coupled to HEMA-EGDM copolymer by 2,4-TDI has all the features of spectra of HEMA-EGDM copolymer and α-CD. This indicates the formation of urethane linkage between HEMA-EGDM copolymer and α-CD. With 1,6-hexamethylene diisocyanate (HMDI) in anhydrous 1,4-dioxane in a sealed conical flask, a maximum of 1.45 μmol of α and β-CD could be bound to one gram of matrix (Table 2.4). The maximum binding of α-CD with reactive IPDI, TDI and PMDI spacers were 24.9, 11.4 and 7.4 μmol per g of dried matrix, respectively (Table 2.4). The optimal binding of α-CD to the IPDI matrices is not known since the binding continued to increase within the concentration range used in the study. From these observations it can be concluded that the reactivity of different diisocyanates in absence of inert environment is in the order IPDI>TDI>PMDI>HMDI.

Scheme 2.1. Activation and coupling of HEG beads (CLD 25 mol%).



Step 1: Activation of the matrix by HMDI, IPDI, PMDI and TDI. Step 2: Coupling of α (aCD) and / or β-CD (bCD) to the activated matrix. The -R group in the affinity matrix is represented as Ha/Hb (HMDI), Ta/Tb (TDI), Pa/Pb (PMDI) and Ia/Ib (IPDI) coupled with α and β-CD, respectively.

Table 2.4. Effect of various diisocyanates on coupling of a cyclodextrin to the HEMA-EGDM copolymer matrices in air.

S.N.	OH / NCO	μmols of α-CDs bound g^{-1} of matrices by the spacers			
		HMDI	IPDI	PMDI	TDI
1	1:1	0.07	12.65	4.63	6.47
2	1:2	0.09	12.65	7.36	7.82
3	1:3	1.12	12.89	4.45	6.87
4	1:4	1.25	13.13	4.56	6.85
5	1:5	1.45	13.31	4.45	6.30
6	1:6	1.42	14.59	4.36	6.27
7	1:7	1.38	16.19	4.36	11.44
8	1:8	1.41	20.27	4.28	ND
9	1:9	1.40	24.54	4.28	ND
10	1:10	1.43	24.87	4.13	11.4

ND – not detected; HMDI=1,6-hexamethylene diisocyanate, IPDI=Isophorone diisocyanate, PMDI=Phenelyne diisocyanate and TDI=2,4-Tolylene diisocyanate; OH/NCO is the molar ratio of –OH group present on HEG beads to –NCO group present on various diisocyanates; CLD, is mole percent of the crosslink monomer relative to the moles of the reactive comonomer of the polymeric matrix, was kept 25 mole%.

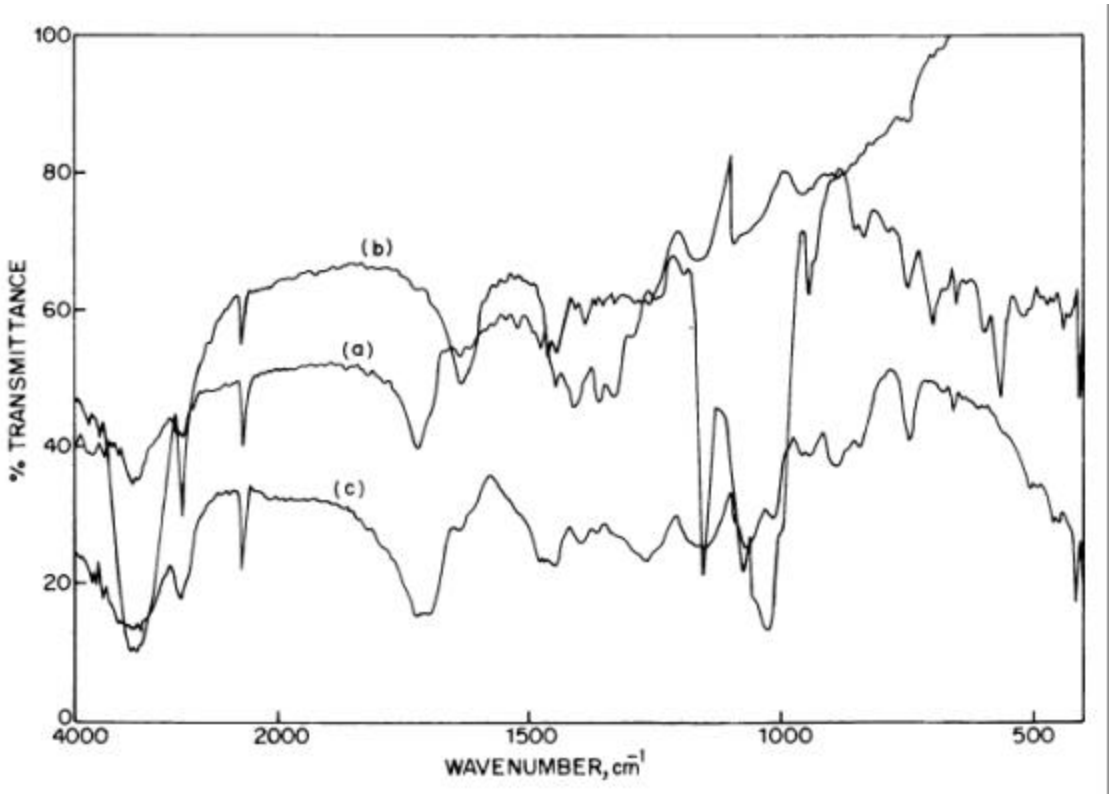


Figure 2.4. **IR spectra of a) HEMA EGDM copolymer beads, b) a-CD and c) HEMA-EGDM copolymer beads activated with 2,4-TDI and coupled to a-CD.**

2.4.3.1. Effect of nitrogen atmosphere

The reactions carried out earlier were under air (non-inert environment). The reactions were also repeated using dry nitrogen overlay. The reactions were conducted using 2 μmols each of α-CD and the various diisocyanates per g of dry matrix. Maximum cyclodextrin binding was noted on copolymer with TDI while the minimum was with copolymer coupled to IPDI (Table 2.5) as spacer arm. The differing reactivities of the isocyanates in presence and absence of dry nitrogen does not reveal a trend, especially since the relative humidity with air as overlay was not known. The differing binding of cyclodextrins to the activated matrix is due to differences in the degree of sensitivity of the various isocyanates towards moisture, which is a competing reaction to the coupling reaction with α/β-CDs. The use of dry nitrogen atmosphere enhanced the covalent binding of α-CD by 309 fold to the HMDI activated matrix, by 1.19 fold for IPDI, by 15.56 fold for PMDI and by 13.45

fold for TDI, respectively. The increase in binding of α-CD under nitrogen atmosphere is probably due to the prevention of hydrolysis of isocyanate group. Diisocyanates are extremely sensitive to moisture. It may be concluded that HMDI is most and IPDI is least sensitive to moisture. From these observations, the reactivity of different diisocyanates in inert atmosphere (nitrogen) is as follows: TDI>PMDI>HMDI>IPDI. Therefore, in subsequent studies of binding of cyclodextrins to the HEMA-EGDM polymeric support the reactions were carried out with TDI as the spacer under dry nitrogen atmosphere.

Table 2.5. Effect of nitrogen atmosphere on binding of cyclodextrin to the HEMA EGDM matrix.

S. N.	Diisocyanates	μmols of α-CDs bound g^{-1} of matrices
1	HMDI	21.6
2	IPDI	15.0
3	PMDI	72.0
4	TDI	87.0

HMDI=1,6-hexamethylene diisocyanate, IPDI=Isophorone diisocyanate, PMDI=Phenelyne diisocyanate and TDI=2,4-Tolylene diisocyanate. The molar ratio of -CNO to -OH group was kept at 1:1 and mole percent of the crosslink monomer relative to the moles of the reactive comonomer of the polymeric matrix was 25%.

2.4.3.2. Effect of crosslink density of the polymeric beads

As the maximum binding of α-CD was noted with TDI, copolymers of differing crosslink density (CLD) were next evaluated for binding of α-CD using TDI as spacer. With increase in CLD, as discussed in Section 2.4.2, the pore surface area is known to increase while pore size distribution tends to broaden (Kotha et al., 1996). In addition to this, we observed an increase in density of the polymeric beads from 1.21 to 1.26 g mL^{-1} with increase in CLD i.e., increase in mole fraction of crosslinking monomer, EGDM. The higher density of the polymeric beads is useful in expanded bed adsorption (EBA) of proteins from the fermentation broth.

CLD, the mole percent of crosslinking comonomer relative to reactive monomer, was increased to synthesise a series of HEMA-EGDM copolymer beads. A maximum of 1,495 μmols of α-CD were bound to the HEMA-EGDM polymer of 200% crosslinking density, which is an increase of 17 fold over that on HEMA-EGDM copolymer with 25 mole% CLD (Table 2.6). The higher binding of cyclodextrins on highly crosslinked matrices was due to higher surface area and pore volume. The degree of swelling within the series of matrices (differing in CLD) was evaluated in DMSO to determine whether the permeation of CD into the beads causes the higher binding of α-CD. This means increased swelling would lead to higher permeation of CD into the beads resulting in greater binding of CD. Since DMSO was the solvent used in the coupling reaction of cyclodextrins, the swelling study was conducted with this solvent. It was observed that the swelling of the matrices decreased with an increase in CLD of HEMA-EGDM copolymer matrices (Table 2.7). This indicates that the increasing binding of α-CD to higher crosslinked polymeric matrices is not due to swelling of the polymer matrix, but rather due to increased pore surface area and broader pore size distribution of the matrix.

Reactive porous synthetic copolymer beaded matrices ought to embody specific pore size, pore size distribution, pore volume and pore surface area and optimum concentration of reactive groups to be effective for the covalent binding of the ligands. Pore size distribution should be definitive to anchor the larger ligands. The pore surface should comprise of functional groups, which can react efficiently with the ligand (irrespective of the size of the ligand). It was observed that the pore surface area was not in an increasing trend with increase in CLD as described by Kotha et al., 1996. This anomaly in pore surface area with increase in CLD is probably due to the randomness in precipitation polymerisation. However, the pore surface area has the same trend (Table 2.8) as the coupled α-CD to the matrices (Table 2.6). The pore volume is increased and broader distribution of pores is observed with increase in CLD. Furthermore, since HEMA contains one –OH group, whereas EGDM does not contain -OH group, increase in CLD decreases the number of reactive functional groups (-OH) per gram of the macroporous copolymer. This

indicates that the combined effect of the irregular profile of pore surface area, which is an important parameter of accessibility for coupling of the ligand to the reactive functional group of the copolymeric matrix, and the decreasing trend of –OH group with increase in CLD are not sufficient clues to explain the abnormality in cyclodextrin coupling to the copolymeric matrices (Table 2.6). It is well known that the pores present in highly crosslinked polymers are permanent ones while those in less crosslinked (25 mole% CLD) are due to solvent induced swelling. Since both degree of swelling of the matrices in DMSO and the pore surface area data (Table 2.8) could not explain the abnormality in the trend of cyclodextrin binding with the matrices varying CLD, the bound diisocyanates were analysed. The bound diisocyanates showed the same trend as the binding of cyclodextrin to the matrices of varying CLD (Table 2.7). Therefore, it is concluded that the surface area has the dominating role than the concentration of -OH groups in coupling and degree of swelling of the HEMA-EGDM copolymeric matrices, of varying CLDs.

Table 2.6. Effect of crosslink density of HEMA-EGDM matrices on binding of a -cyclodextrin with TDI.

S. N.	Mole% CLD	μmols of α-CD bound g^{-1} dry wt. of matrices
1	25	86
2	50	598
3	75	822
4	100	608
5	125	910
6	150	506
7	175	372
8	200	1495

Crosslink density (CLD) is defined as the mole percent of crosslinking monomer relative to the moles of reactive functional comonomer.

Table 2.7. Effect of crosslink density of HEMA-EGDM matrices on swelling in DMSO and bound diisocyanates

S. N.	Mole% CLD	Swelling ($mL\ g^{-1}$)	Bound diisocyanates ($\mu mols\ g^{-1}$)
1	25	4.10	95
2	50	4.05	605
3	75	4.00	855
4	100	3.94	615
5	125	3.90	920
6	150	3.80	485
7	175	3.70	408
8	200	3.70	1502

Swelling is defined as the packed volume of in the swollen state in DMSO per gram dry weight of the matrix.

Table 2.8. Pore characteristics with varying CLD.

% CLD	Pore size distribution (vol%), radius in Å									Pore volume ($mL\ g^{-1}$)	Surface area ($m^2\ g^{-1}$)
	<50	50-100	100-150	150-200	200-300	300-500	500-1000	1000-3000	>3000		
25	58.8	32.4	1.7	1.1	1.1	1.1	1.7	1.3	0.8	0.1	1.7
50	50.6	30.6	6.9	2.8	2.6	3.5	3.0	0.0	0.0	0.13	9.1
75	68.6	14.0	4.2	0	0	8.0	2.9	1.0	1.3	0.61	93
100	48.5	21.6	12.5	5.4	4.8	3.2	2.0	2.0	0.0	0.55	85
125	14.0	14.3	12.1	10.4	18.5	18.6	8.6	3.4	0.1	0.92	162
150	15.8	8.8	15.5	29.3	21.3	5.8	2.7	0.8	0.0	0.63	145
175	17.4	8.0	10.0	19.0	36.4	5.7	2.4	1.3	0.0	0.53	127
200	10.0	8.0	6.5	4.0	7.6	11.8	17.0	27.1	7.9	1.87	283

2.4.3.3. Effect of catalyst

One wt. percent of dibutyltin dilaurate was added as catalyst to facilitate the rate of the reaction between -NCO groups of diisocyanates with -OH groups of the HEMA-EGDM copolymer (Hostettler and Cox, 1960). This reaction was carried out with HEMA-EGDM copolymer beads of 25 mole% CLD under nitrogen atmosphere. Each isocyanate showed differing reactivities, in all the cases coupling reactions (second reaction) were much slower than the activation step (first reaction). This is evident from the activation energies and rate constants for the first and second reaction cited in literature (Saunders and Frisch, 1962). Therefore, catalyst was tested at two temperatures to see their effects. The matrices were activated at room temperature. The coupling step with β-CD were studied at ambient (25 °C) and at 45 °C. In a parallel control set, without catalyst, both reactions were conducted at 25 °C. In a third set, again devoid of catalyst, the first step was at 25 °C while the second step was carried out at 45 °C.

The profile in Figure 2.5 indicates that higher reaction temperature is preferable for the coupling step with CD, while the activation step between matrix and isocyanate should be performed at lower temperature. The first (activation) step was slower in absence of catalyst and at 25 °C whereas the second (coupling) step was moderate when carried out either without catalyst at higher temperature or at 25 °C in presence of catalyst. However, the exact reason of the kinetics of binding is not clear, as the reactive group to one end of the isocyanate (first reaction) is a crosslinked polymer whereas the other end (second reaction) comprised of cyclodextrin. The groups differ in structure and thus might show different reactivity. This kinetics shows a differing trend from that observed for various diisocyanates with n-butanol in toluene (Saunders and Frisch, 1962). At higher temperature (45 °C), in presence of catalyst, the coupling step did not increase the binding of β-CD with an increase in concentration of diisocyanates. However, at lower temperature (25 °C) there was a greater increase in binding of β-CD with the diisocyanate concentration.

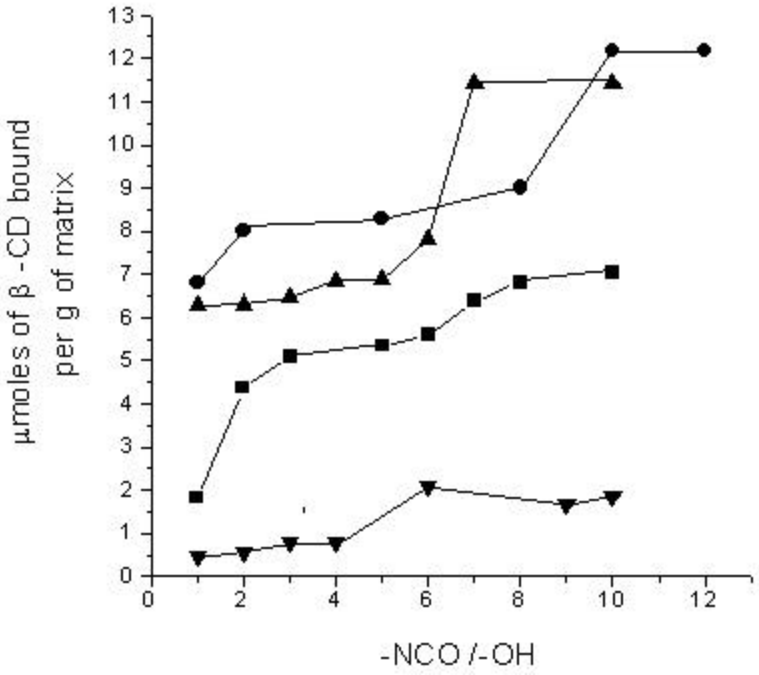


Figure 2.5. Effect of catalyst on coupling of b-CD to HEG beads.
■ Activation of the matrix and coupling of β-CD to the activated matrix were at 25 °C in absence of catalyst. ● Activation of the matrix and coupling of β-CD to the activated matrix were at 25 and 45 °C respectively in absence of catalyst. ▲ Activation of the matrix and coupling of β-CD to the activated matrix were at 25 °C in presence of catalyst. ▼ Activation of the matrix and coupling of β-CD to the activated matrix were at 25 and 45 °C respectively in presence of catalyst. -NCO/-OH is the molar ratio of -NCO (of TDI) to -OH group (polymeric matrix).

2.4.4. Nonspecific interactions

Non-specific interactions of proteins with solid supports, such as affinity matrix, arise as a result of retention of the proteins by some general factors like ion exchange, hydrophobic interactions, charge-transfer complexion of phenolic groups etc. The effectiveness and selectivity of an affinity chromatography matrix is depressed by these non-specific sorptions. Even a small percentage of non-specific adsorption on the column destroys its usefulness. Here, it is essential to have hydrophilic non-ionic matrix with little or no capacity for non-specific adsorption.

Non-specific adsorption should not occur in the derivatised matrix, though the unreacted matrix may provide considerable adsorption or hydrophobicity.

The adsorption of BSA was tested on affinity matrix at pH 7.0 and 12 °C in shake flask. BSA adsorbed to the matrices was eluted with 6 M urea at room temperature. It is presumed that the adsorption of protein to the matrices was non-specific, if the protein could be eluted with the desorbing buffer. The non-specific interaction (ionic and hydrophobic) studies were tested on the 25 mole% CLD HEMA-EGDM copolymer beads coupled to α-CD using different diisocyanates. Results presented in Table 2.9 reveal low non-specific ionic interaction for matrices with HMDI and PMDI spacers. The absence of non-specific ionic interaction of HMDI and PMDI can be explained on the basis of resonance and inductive effects, which create charged pockets and bind BSA. However, it would be difficult to explain the binding of BSA to the affinity matrix comprising of polymer, two urethane units and cyclodextrin since the system is structurally complex. However, the observed trend is that the non-specific interaction of BSA increased with increase in CLD of the polymeric matrix (Table 2.10). Therefore, polymeric matrix of lower CLD (25 mole%) was used in the adsorption and desorption studies.

Table 2.9. Nonspecific (ionic and hydrophobic) interactions of BSA with the matrices

S. N.	Affinity matrices	ionic	hydrophobic
1	Plain matrices	Nil	95.0
2	HMDI	6.0	Nil
3	IPDI	Nil	Nil
4	PMDI	5.0	30.0
5	TDI	Nil	Nil

Ionic and hydrophobic interactions are the amount of BSA adsorbed per g of dried matrix at 12 °C in 50 mM Tris-HCl buffer, pH 7.0 and in presence of 1% NaCl solution in 50 mM Tris-HCl buffer, pH 7.0 at 25 °C, respectively. HMDI, IPDI, PMDI and TDI in the Table indicate the coupling agents to couple CD with HEG beads (CLD= 25 mole%).

Hydrophobicity of the matrices denatures the protein and binds at higher salt concentration and temperature. The protein is desorbed by low concentration of buffer at low temperature. BSA was used as a model protein to study the adsorption to the matrices with 1% NaCl at 25 °C. BSA was desorbed at 4 °C and with 0.05 M Tris-HCl buffer, pH 7.0. The result indicates that the very marginal hydrophobicity of the plain HEMA-EGDM matrices disappears on activation with HMDI, IPDI and TDI (Table 2.8). With PMDI modification, the hydrophobicity of the matrix is depressed. Long chain aliphatic compounds and aromatic rings have hydrophobic pockets. However, the combined effect of the polymer coupled to neutral cyclodextrin by urethane bond on hydrophobicity is not known. The increase in hydrophobic interaction with crosslink density arises from higher hydrophobicity of the crosslinking monomer (EGDM), which is more hydrophobic relative to HEMA (Table 2.9). Though the density of the matrix and the pore surface area increase with increase in crosslinker content, the higher non-specific adsorption of protein to the matrix with increasing CLD of copolymer limits the use of copolymers with CLD in excess of 25 mole% in chromatographic operation.

Table 2.10. Non specific interaction (ionic and hydrophobic) of protein with HEG beads of varying crosslink density.

S. N.	Mole% CLD	Ionic	Hydrophobic
1	25	nil	95
2	50	59	153
3	75	65	284
4	100	71	297
5	150	77	478
6	175	123	487
7	200	183	547

Crosslink density (CLD) is defined as the mole percent of crosslinking monomer relative to the moles of reactive functional comonomer. Ionic and hydrophobic interactions are the amount of BSA adsorbed per g of dried matrix at 12 °C in 50 mM Tris-HCl buffer, pH 7.0 and in presence of 1% (w/v) NaCl solution in 50 mM Tris-HCl buffer, pH 7.0 at 25 °C, respectively.

2.4.5. Adsorption on shake flask

α and β-CGTase were produced by fermentation. α-CGTase was produced by fermentation of *Klebsiella pneumoniae pneumoniae* in a 1 L fermenter at 600 rpm, in a fed-batch mode whereas, β-CGTase was produced using *Bacillus firmus* in a batch mode using similar capacity of fermenter. The medium composition and the fermentation operating conditions are presented in Section 2.3.9. The adsorptions of CGTases on the optimally synthesised affinity matrices were evaluated next. Since the HEMA-EGDM matrix showed lowest non-specific interaction at 25 mole% CLD, affinity matrices prepared from this copolymer, with different diisocyanate spacers and α/β-CD as ligand were taken for adsorption studies in shake flask. One gram of affinity matrices prepared with different diisocyanates spacers and having 2 μmols of ligand g^{-1} of dried matrices were used. The affinity matrices were shaken with 15 mL (6.95 U mL^{-1}) of β-CGTase and 10 mL (31.45 U mL^{-1}) of α-CGTase from the clarified fermented broth at 100 rpm and 12 °C for 16 h. The matrices were washed with 10 mL of 1mM $CaCl_2$ solution in 50 mM Tris-HCl buffer (pH 7.0). The adsorbed enzyme were desorbed with 5 mL of 10 mM $CaCl_2$ solution in 50 mM Tris-HCl buffer shaken for 4 h at pH 7.0 at 25 °C and 55 °C for α-CGTase and β-CGTase, respectively. The data is presented in Table 2.10. Approximately identical amount of α-CGTase was observed to be adsorbed to the affinity HEMA-EGDM copolymer matrices coupled to α-CD through HMDI, IPDI and TDI spacers. On the contrary, affinity matrix with PMDI spacer coupled to β-CD adsorbs higher amount of β-CGTase. Elution of the enzymes from the respective affinity matrices resulted in lower recovery from PMDI modified β-CD affinity matrix. The higher amount of adsorption and lower recovery of β-CGTase from the matrix modified with PMDI is due to higher hydrophobicity of PMDI. The fermentation medium of β-CGTase from *Bacillus firmus* contained 1% Na_2CO_3 and pH was 8.9. The adsorption studies with BSA also confirm the increased adsorption at higher salt concentration due to the hydrophobic interaction (Table 2.8). Thus, the increased binding of β-CGTase relative to α-CGTase on HEMA-EGDM copolymer beads modified with PMDI is due to increased hydrophobic interactions, which is prominent

at higher salt concentration. This also results in a lower desorption of β-CGTase (Table 2.11).

Table 2.11. Adsorption and elution of a and b-CGTase on affinity HEG beads.

S. N.	Matrices	U of CGTase adsorbed g^{-1} of matrices	% eluted
1	HEG 25- HMDI-β-CD	70.0	89%
2	HEG 25-IPDI-β-CD	69.0	91%
3	HEG 25-PMDI-β-CD	76.0	79%
4	HEG 25-PMDI-α-CD	65.5	92%
5	HEG 25-TDI-β-CD	69.0	95 %

HEG 25- HMDI-β-CD=HEG beads comprised of 25 mole% CLD coupled with β-CD by HMDI; HEG 25-IPDI-β-CD=HEG beads comprised of 25 mole% CLD coupled with β-CD by IPDI; HEG 25-PMDI-β-CD=HEG beads comprised of 25 mole% CLD coupled with β-CD by PMDI; HEG 25-PMDI-α-CD=HEG beads comprised of 25 mole% CLD coupled with α-CD by PMDI; HEG 25-TDI-β-CD=HEG beads comprised of 25 mole% CLD coupled with β-CD by TDI.

2.4.6. Stability of the coupling group

The operational stability of the matrices requires stable ligands, coupling groups and matrix. The affinity matrices were tested with various regenerating reagents employed in clean-in-place procedures. The regenerating reagents used in our experiments were 1 and 2 M NaCl, 0.5 and 1 M NaOH, 30% (v/v) isopropanol/water, 6 M urea, 10% ethanol amine, sodium dodecyl sulphate (SDS) and water at 121 °C for 30 minutes. The matrices (CLD-25 mole%) coupled with α-CD (2 μmols g^{1} of dried matrices) by TDI were shaken with the regenerating reagents for 5 hours in a shaker at 400 rpm. The regenerating solution was decanted and the matrices were washed five times with 10 mL of water. The matrices were dried under vacuum at 60 °C. The dried matrices were assayed for α-CD. Drastic conditions like 0.5 and 1 N NaOH cleaved the urethane bond. Milder reagents (NaCl up to 2M concentration, 30% (v/v) isopropanol, 6M urea, 10% ethanol amine and SDS) and

higher temperatures (up to 121 °C) were found to be suitable for clean-in-place procedure.

Chapter 3

Pore size distribution of HEG beads by inverse size exclusion chromatography

3.1. Summary

The pore size distribution of macroporous hydroxyethyl methacrylate-ethylene glycol dimethacrylate (HEMA-EGDM) copolymer (100:25) beaded matrix (HEG beads) was characterised by inverse size exclusion chromatography (ISEC) to explore it for chromatographic applications. Since blue dextran (used for size exclusion chromatography measurements) was irreversibly bound to HEG beads, the size exclusion of all proteins could not be tested. Therefore, porosity measurements were conducted in 1,4-dioxane with narrow molecular weight distribution polystyrene standards as the probe solutes. The theoretically estimated pore size distribution data matched well with experimental data in 1,4-dioxane. This was extrapolated to establish the porosity of HEG beads in aqueous buffer using proteins as probe solutes. Larger pores are generated in 1,4-dioxane, as predicted by closer solubility parameter values.

3.2. Introduction

As described in chapter 2, HEG beads (macroporous copolymer beads of 2-hydroxyethyl methacrylate (HEMA) and ethylene glycol dimethacrylate (EGDM) with CLD 25 mol%) were found suitable for expanded bed application due to its higher density and low non-specific interaction towards protein. The matrix should have optimal pore size distribution, since restrictive diffusion of the proteins into the pores is undesirable, especially in EBA mode. Hence, measurement of the pore characteristics is essential. In general, porosity of the macroporous beaded polymers are estimated either with gas adsorption or mercury intrusion. However, these two methods are applicable only to dry samples of sufficient mechanical strength (Guyot and Bartholin, 1982). For swellable polymers, inverse size exclusion chromatography (ISEC) is probably a more exact methodology to characterise the pores in the wet state, which differ from the pore characteristics in the dry and unswollen states. The difference between size exclusion chromatography (SEC) and ISEC is its focus: SEC is biased towards the solute properties, whereas ISEC generates information regarding the porous solid.

Separation in size exclusion chromatography (SEC) depends on the size and shape of the probe solutes (Ryle and Porter, 1959; Hagel, 1993). Proteins and

polystyrenes are globular and random coils in aqueous buffer solutions and 1,4-dioxane, respectively. The pores of the beaded copolymer behave differently to these probe solutes during fractionation. In most porosity determinations by ISEC, the eluting solvent used is tetrahydrofuran (THF). Since the polarity of THF differs widely from that of water, the porosity data generated with polystyrene as probe solute is not suited to selecting the optimal matrix for protein purification. The purifications of proteins and other biomolecules on chromatographic matrices involve the use of aqueous solutions as the eluting solvents since biomolecules tend to denature in organic media. For this reason, the porosity is measured in aqueous buffer. HEG beads irreversibly bind blue dextran, used for the void volume (V_0) measurement in aqueous ISEC. Therefore, porosity could not be measured in buffer with proteins as probe solutes. In this study porosity was measured in 1,4-dioxane, of polarity similar to THF, and the porosity data was extrapolated to buffer. The characterisation of pores of HEG beads in aqueous buffer and in dioxane, using protein and polystyrene have been compared.

In this chapter, a reproducible methodology is presented to characterise the pores of HEG beads in terms of its utility for the preparative purification of protein by adsorption in expanded bed.

In size exclusion chromatography (SEC), a mixed bag of the solute molecules are differentially distributed between the eluting solution and the stationary phase on the basis of their size and shape. Small molecules (such as those of eluent) permeate all the available pores in the support material and are eluted at the volume, V_t. Large molecules, which cannot enter any of the pores, are totally excluded and are eluted at the external void volume, V_0. Molecules of intermediate size are eluted after volume V_e, which lies in between V_0 and V_t. The distribution coefficient is given as

$$D = \frac{V_e - V_0}{V_t - V_0} \tag{3.1}$$

V_t, the total permeable volume of the column, is obtained from the peak retention volume of the small probe solute (acetone).

We make the following assumptions:

(i) the distribution coefficient, D, of a solute depends upon the size and shape of the pores; (ii) the molecules of the solute can be considered as rigid; (iii) the band spreading arises only from kinetic effects and (iv) the solute is polydisperse. The most complex model of random size touching spheres offers only a marginal improvement in accuracy. For simplicity we have assumed the cylindrical model, taking into account that the pore sizes are randomly distributed. Thus, the marginal but definite loss in accuracy is compensated by the simplicity of the calculations connected with the SEC determination of the pore-volume distribution.

Knox and Scott (1984) showed that the pore size distribution $G(d_{pore})$ for a continuous distribution of pore diameters can be obtained from the plot of $K(d_{pore})$ against ln (d_{pore}) by a simple differentiation procedure, as presented in equation 3.2. This method, while is subjective, is less time consuming and shows stability.

G (d_{pore}) is given by the following expression:

$$G\left(d_{pore}\right) = K\left(d_{pore}\right) - 1.5.\left(\frac{dK\left(d_{pore}\right)}{d\left(d_{pore}\right)}\right) + 0.5.\left(\frac{d^2K\left(d_{pore}\right)}{d\left(d_{pore}\right)^2}\right) \qquad (3.2)$$

where, d_{pore} is the pore diameter and $K(d_{pore})$ is the pore volume distribution determined by experiment.

Elucidation of protein structure by X-ray diffraction studies clearly demonstrated that most proteins are elliptical (Perkins, 1988). The shapes of the probe solutes (polystyrene and proteins) are assumed to be spherical for simplicity. According to Giddings et al. (1968), a characteristic dimension L (mean projected solute size) governs partitioning behaviour in porous systems. For polystyrenes and proteins, the dependence of L on molecular mass M is described by the equation

$$L = a.MW^n \qquad (3.3)$$

At a constant a, various values are reported in literature for polystyrenes, according to the approach of different researchers (Halasz and Martin, 1978; Freeman and Poinescu, 1977 and van Kreveld and van den Hoed, 1973). In the present study we have used a = 0.246 Å and n = 0.588, as determined by van Kreveld and van den Hoed (1973) for polystyrene. For protein a = 0.794 and n = 0.333 were used, as described by Squire (1981).

3.3. Materials and Methods

3.3.1. Chemicals

Tris(hydroxymethyl) methylamine (Trizma base) was obtained from Sigma (USA). The source from where analytical grade 1,4-dioxane, dimethyl sulphoxide (DMSO), methylene chloride, methyl salicylate and cyclohexanol used in the study of this Chapter is listed in Section 2.3.1. Triton X-100 was from SD Fine chemicals (Boisar, India). The protein standards: Cytochrome C, horse heart (12,400 Da); Lysozyme, chicken egg white (14,300 Da); BSA (66,000 Da); alcohol dehydrogenase, yeast (1,50,000 Da); and β-amylase, sweet potato (2,00,000 Da) were obtained from Sigma-Aldrich, USA. The monodisperse polystyrene standards of molecular weights 3,600, 8,500, 35,000, 110,000, 233,000, 470,000 and 27,00,000 Da were from Waters Associates Inc., Massachusetts, USA.

3.3.2. Matrix

The spherical HEG beads (macroporous beaded copolymer of 2-hydroxy ethyl methacrylate (HEMA) and ethylene glycol dimethacrylate (EGDM) of CLD 25 mol%) was used in this experiment. The particle sizes between 105 and 355 μm were wet sieved through standard sieves (Jayant Scientific India, Mumbai, India) using 0.01% Triton X-100 as the wetting agent. The bead diameters were determined by observing under a polarised microscope (Lietz, Germany) with a measuring device. The particle size was calculated from diameter obtained from microscope by volume moment mean of 70 to 80 particles in five sets of observations. The standard deviation was less than 5% of the total diameter in all observations. This particle size distributed matrices were used in our subsequent studies. Density of the particles under swollen state was

determined as described in Section 2.3.3.1.

3.3.3. Experimental arrangement

The experimental set up consisted of a jacketed glass column (diameter 10 mm and length 27 cm), a 3-way injection valve, a six roller peristaltic pump (LKB, UK), one jacketed glass eluting solution reservoir, circulating temperature controlled water bath and a spectrophotometer (UNICAM UV/Vis UV2, UK). The eluting solution from the reservoir was pumped by the six roller peristaltic pump and then passed through the 3-way injection valve to the top of the column. The bottom part of the column was connected to the online spectrophotometer equipped with a flow through cell for measurement of outlet solute concentration.

The wet-sieved HEG beads were packed in their respective eluting solution in a column (10 cm diameter and 27 cm length). The column temperature was kept at 10 ºC. The polystyrene (5mg mL^{-1} in 1 mL 1,4-dioxane) and protein (2 mg mL^{-1} in 0.5 mL 50 mM Tris-HCl buffer pH 7.0) standards were injected in the column with the eluting solution in separate experiments. An injection time of 0.25 min was used, which helped to reduce concentration-dependent effects, such as viscous fingering. The injection port of the 3-way valve was switched towards the reservoir containing the eluting solution (buffer or 1,4-dioxane). The concentrations of polystyrene and protein standards in the effluent were monitored at 270 and 280 nm, respectively.

3.4. Results and Discussions

3.4.1. Preliminary investigations

In previous chapter, it was shown that the non-specific adsorption of protein on the matrix increased with increase in molar% of crosslinking comonomer relative to reactive monomer (%CLD) (Table 2.10). Macroporous 2-hydroxyethyl methacrylate (HEMA)-co-ethylene glycol dimethacrylate (EGDM) beaded copolymer matrix of 25 mol% CLD, abbreviated as HEG beads, was established as the optimum crosslinked polymer for lower non-specific protein interaction (minimum amounts of 95 μg of BSA was adsorbed per 1 g of HEG beads in presence of 1% (wt/v) NaCl solution) and moderate density under swollen state (1.21 g mL^{-1}), which is suitable for expanded bed

operation. The other suitable properties, which are useful for operation in expanded bed, are described in Sections 5.4.1 and 5.4.2.

The particle size distribution of HEG beads follows Gaussian distribution (Figure 3.1), which ensures less liquid dispersion in the column. Since matrix with this particle size distribution was evaluated for EBA application (Section 5.4.2.), particles with similar size distribution were used for porosity estimation. The average particle size was found to be 258 μm.

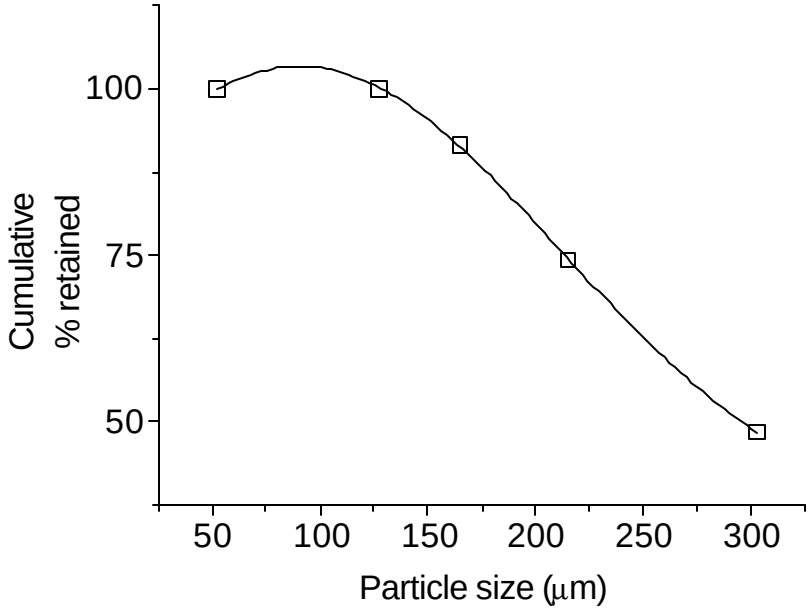


Figure 3.1. Particle size distribution of HEG beads.

There are many reports of aqueous size exclusion chromatography using dextran and pullulan as probe solutes for exclusion measurements. The exclusion data of these probe solutes can not be completely translated to the probable exclusion of proteins from the pores of matrix as desired in the evaluation of the matrix for chromatographic purification of proteins. However, Brissova et al. (1996) evaluated the permeability of microcapsules (hollow hydrogel spheres) using dextrans as probe solutes and converted this into the exclusion limit of protein on the basis on the relationship between solute size and molecular weight of dextran. The polydispersity factor of dextran used in the experiment was 1.16 to 1.94. This could generate erroneous elution volume. In the dextran elution curves, the observed broadening of the

peaks and long tails might be due to the increased polydispersity factor, which leads to difficulty in the calculation of peak elution volume. Therefore, in this experiment, despite change of solvent system, polystyrene standards with narrow polydispersity factor of 1.05 were used. The results were extrapolated to permeability of protein in aqueous buffer for pore size distribution measurement. The elution profile of polystyrene standards (Figure 3.2) in 1,4-dioxane shows sharp peaks, which is essential to chromatographic operations.

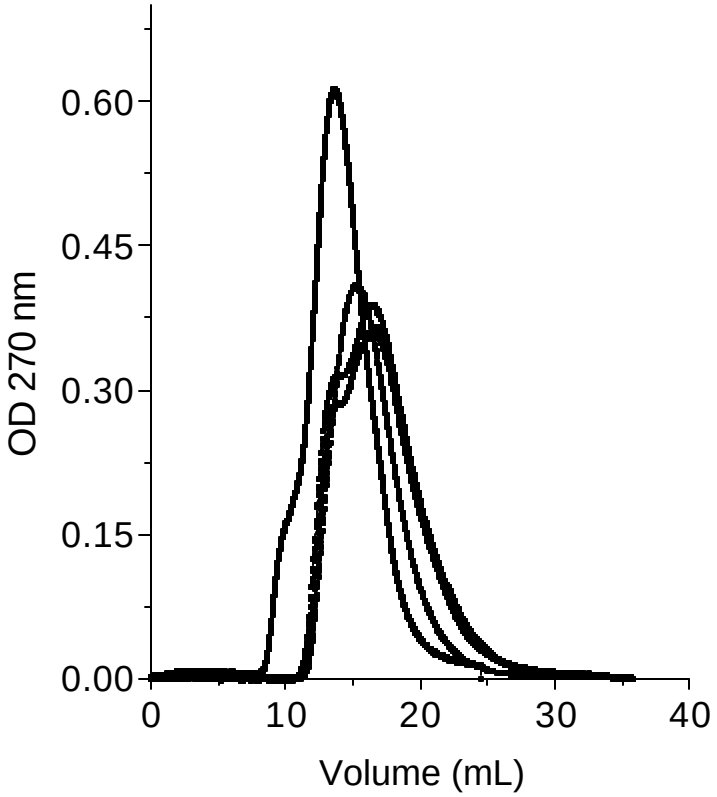


Figure 3.2. Elution of polystyrene standards on HEG beads in 1,4-dioxane.

3.4.2. Porosity of HEG beads in 50 mM Tris-HCl buffer

Proteins are the most appropriate solutes to measure pore characteristics, as applicable to chromatographic separation. The size of protein standards is tabulated in Table 3.1. Vytášek et al. (1976) showed that many proteins (trypsin, pepsin, cytochrome C, cytozyme, chymotrypsin, chymotrypsinogen and albumin (calf serum) did not interact with commercial HEMA-EGDM copolymer (Spheron) beads. The only exception is Spheron 100,000. Spheron 100,000, with the highest mol% cross-linking

agent is consequently the most hydrophobic. Mikes et al. (1976) obtained similar results with Spheron 300 gel using acidic pepsinogen, basic chymotrypsin and serum albumin, which are known to adsorb strongly. The retention of these proteins by the column is traced to the molecular sieving effect of the gel pores.

Table 3.1. Calculation of molecular size of polystyrene and protein standards.

Molecular weight of polystyrenes (Da)	Size (Å)	Proteins	Molecular weight (Da)	Size (Å)
3, 600	30.34	Cytochrome C, horse heart	12,400	18.32
8, 500	50.28	Lysozyme, chicken egg white	14,300	19.21
35, 000	115.57	BSA	66,000	31.97
1, 10, 000	226.61	Alcohol dehydrogenase, yeast	1,50,000	42.02
2, 33, 000	352.32	β amylase, sweet potato	2,00,000	46.24
4, 70, 000	532.27			
27, 00, 000	1487.91			

Diameter of polystyrene standards were calculated by using the correlation $L\ (Å) = 0.246 \times (MW)^{0.588}$ (van Kreveld and van den Hoed, 1973). Diameter of protein standards were calculated by using the correlation $L\ (Å) = 0.794 \times (MW)^{1/3}$ (Squire, 1981)

The polymer did not adsorb BSA in 50 mM Tris-HCl buffer, pH 7.0, at 12 ºC. The extent of non-specific binding was estimated from the recovery of injected protein in the effluent. The peak shape was found to be Gaussian (Figure 3.3). Percent recoveries of alcohol dehydrogenase (yeast), β-amylase (sweet potato), BSA, cytochrome C (horse heart) and lysozyme (chicken egg white) were found to be 96, 100, 99, 93 and 97, respectively.

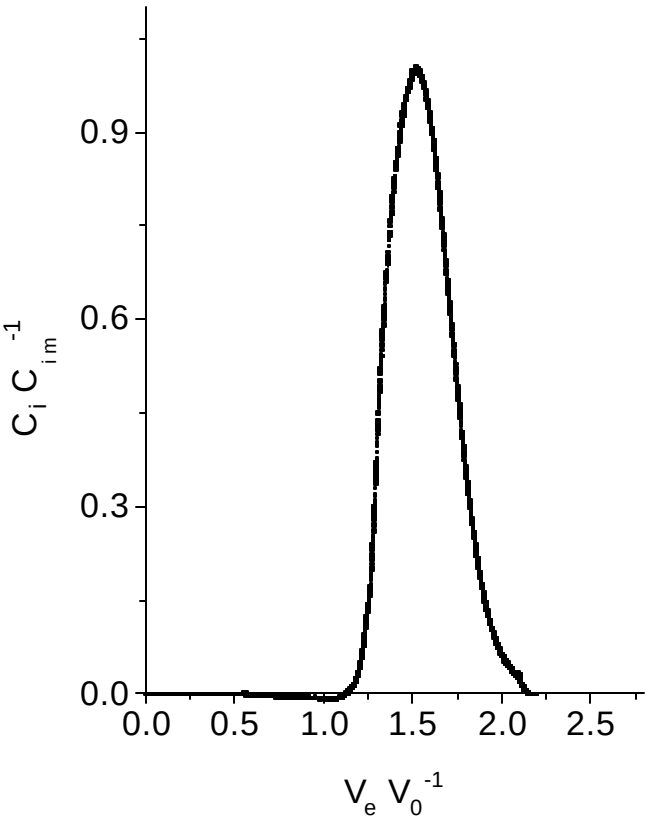


Figure 3.3. **Normalised elution profile of BSA on HEG beads in 50 mM Tris HCl buffer.**
C_i and C_{im} are the outlet and injected concentration of BSA, respectively. V_e and V_0 are the peak elution volume of BSA and void volume of the column, respectively

Addition of detergents or salts is known to increase the resolution of protein separation on the column. The (sodium dodecyl sulphate) SDS-protein complexes penetrate the Sepharose 4B gels to a much greater extent than globular proteins of the same Stokes radius (Nozaki et al., 1976). However, the binding of blue dextran (MW 20,00,000 Da) on the matrix was irreversible, which could not be eluted with 1 M NaCl solution. The cause of this binding to the matrix is not known. Therefore, the pore size distribution could not be evaluated experimentally over the entire range of pore sizes, though the protein retention curve is described by one linear region of molecular weight 14,300 to 2,00,000 Da (18.32 to 46.24 Å). The profile is shown in Figure 3.4. Therefore, the porosity of HEG beads was determined in a solvent having polarity

similar to THF, using polystyrene as probe solutes.

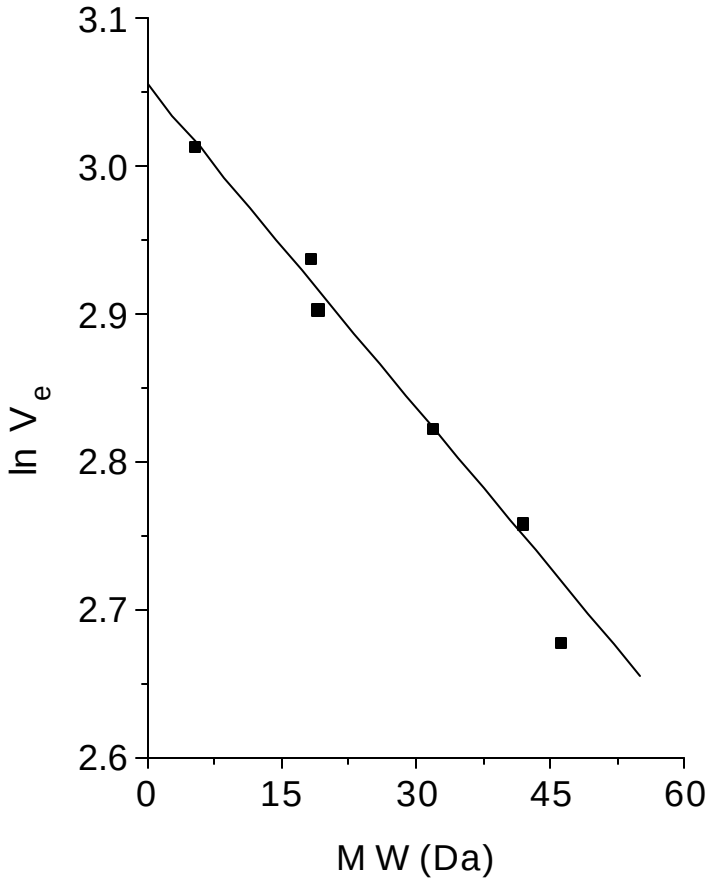


Figure 3.4. Retention of protein standards in 50 mM Tris HCl buffer
V_e is the peak elution volume of the solute.

3.4.3. Extrapolation of porosity measurement in buffer from the porosity measurement in 1,4-dioxane

Ampiphilic and hydrophobic solutes are difficult to separate on the basis of exclusion alone. Therefore, to circumvent the problems associated with charge/hydrophobic interaction, monodisperse, neutral polystyrenes are used as described in Table 3.1. HEG beads did not adsorb polystyrene (MW 35,000) in a batch adsorption experiment. The polystyrene retention curve (Figure 3.5) for HEG beads can be described by two linear regions of differing slopes in the molecular weights range 78 to 1,10,000 (5.3 to 227 Å) and 1,10,000 to 27,00,000 Da (227 to 1488 Å). This shows that the porosity of polymeric beads in 1,4-dioxane are bimodal. Most porous materials are known to have bimodal pore size distribution (le Maire et al., 1987; Harlan, 1995

and Biffis et al., 1995), including silica-based systems (Nicolov, 1986) with a distribution around 1 to 3 nm.

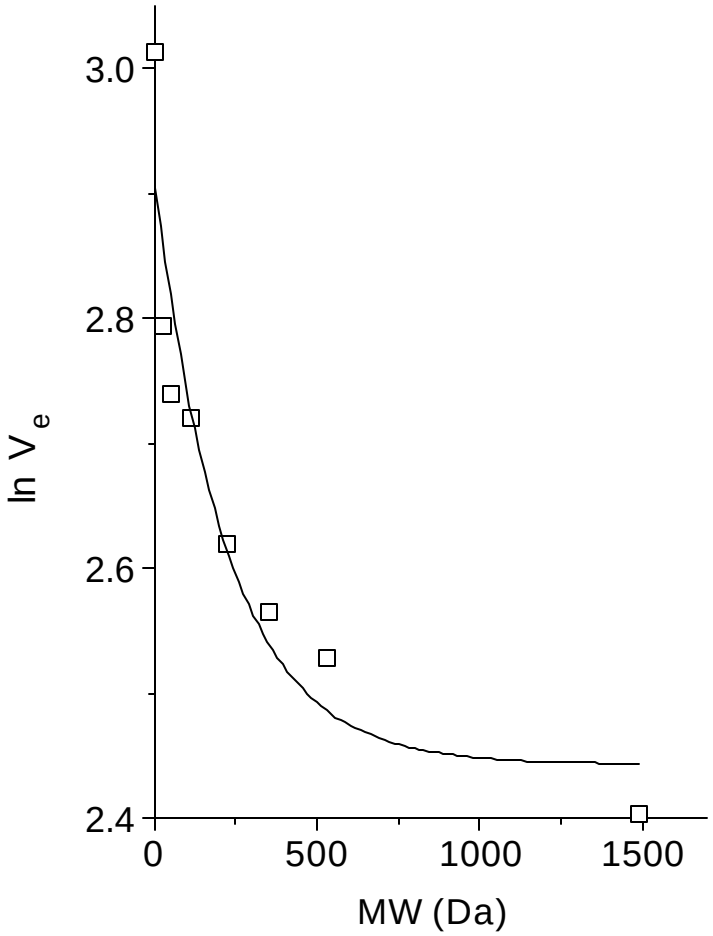


Figure 3.5. Retention of polystyrene standards in 1,4-dioxane.
V_e is the peak elution volume of the solute.

The total separating volume (V_t-V_0) was set to 100%. A correlation between the percentage cumulative pore volume and the pore size was estimated from the graph presented in Figure 3.6. A plot of (V_e-V_0) divided by the internal void volume (V_t-V_0) as a function of pore radius, generated a cumulative pore distribution of the swollen gel, shown in Figure 3.6 (the filled square symbols). The curve (solid line fitting to the hollow squares) is described by the equation $K = 72.67\ e^{dpore/11.04} + 55.83\ e^{dpore/380.3}$, where K ($d_{pore}$) is the cumulative pore volume and d_{pore} is the pore diameter.

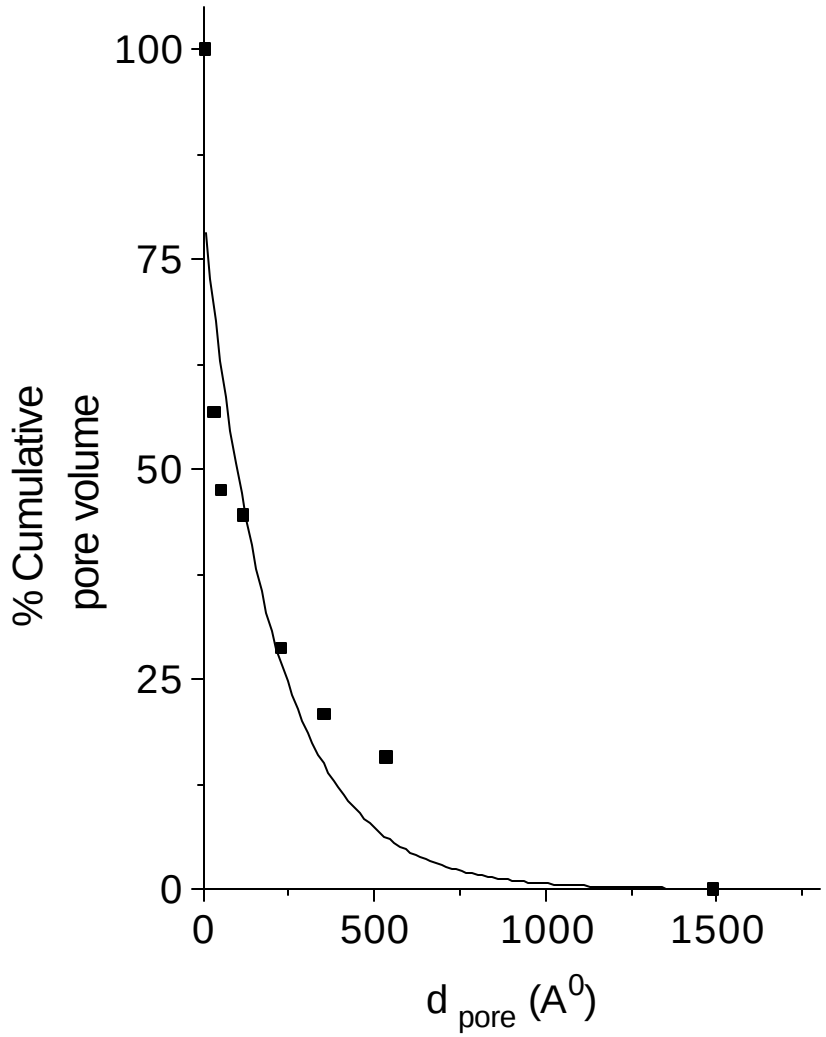


Figure 3.6. Experimental pore size distribution of HEG beads in 1,4-dioxane using polystyrene standards as probe solutes.

Assuming that hydrodynamic volume is the molecular parameter determining the retention, the radii of gyration are set to be equivalent to the radii of pores into which the standard polystyrene molecules are able to diffuse into. Since the retention volume is a function of pore radius, the normalised cumulative pore size distribution curve was differentiated. The correlation derived by Knox and Scott (1984) was used to theoretically calculate the pore size distribution, using equation 3.2 presented in Figure 3.7.

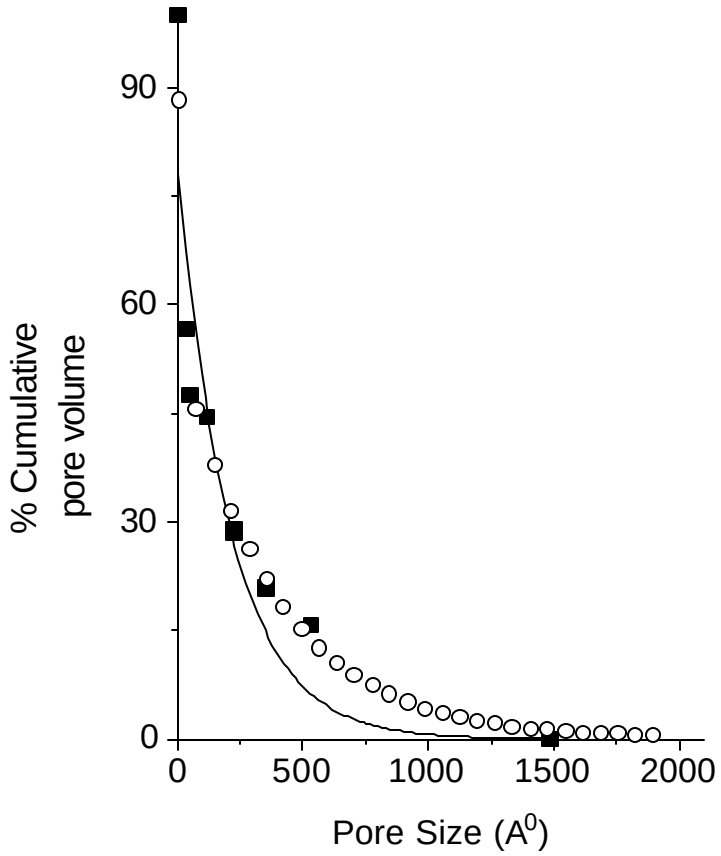


Figure 3.7. **Fitting of the calculated pore volume distribution curve (○) of HEG beads in 1,4-dioxane and polystyrenes as standard solutes, to the experimental curve (■).**
The experimental points are fitted to the equation $K(d_{pore}) = 72.67\ e^{dpore/11.04} + 55.83\ e^{dpore/380.3}$.

In 1,4-dioxane, the theoretical and experimental pore volume distribution on this matrix matched well. Therefore, the above model, which was found to be valid for narrow monodispersed polystyrene in dioxane, was extrapolated for data obtained with protein in buffer. On that basis, a cumulative pore size distribution is calculated from the differentiation of the experimental results and the equation 3.2 of buffer swollen HEG beads. The points (filled squares) in Figure 3.8 are described by the equation $K = -38.45+48.44\ e^{-(dpore\ -13.25)/31.60)}$, where K is the cumulative pore volume and R is the pore radius. The theoretical pore size distribution was calculated from equation 3.2 described by Knox and Scott (1984) (Figure 3.8).

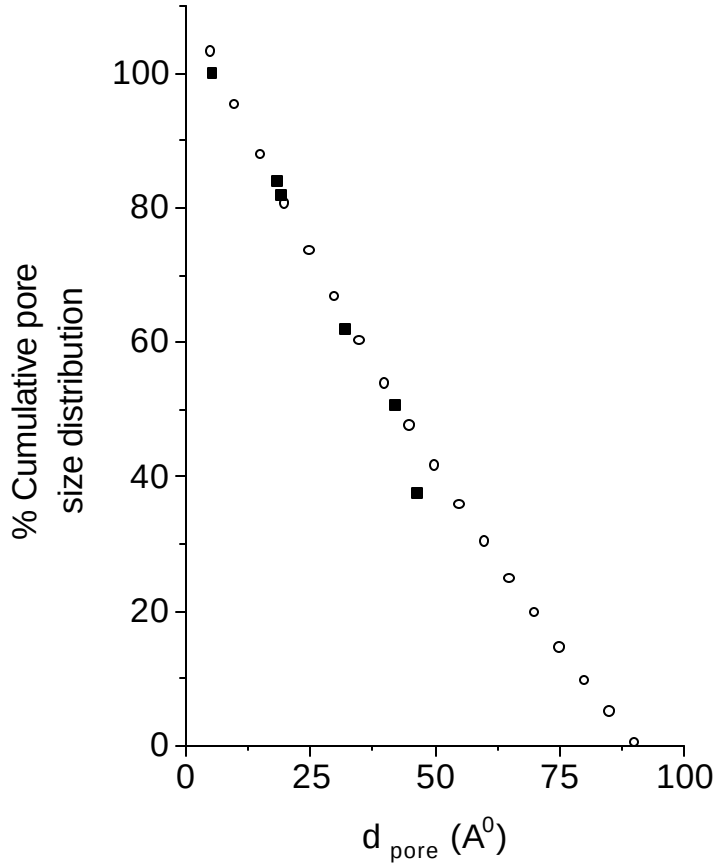


Figure 3.8. Experimental determination of % cumulative pore volume distribution in 50 mM Tris HCl buffer using protein as probe solute (■).
The line connecting the experimental points (■) is described by the equation $K = -38.45+48.44\ e^{-(dpore-13.25)/31.60)}$. The pore size distribution curve (○) of HEG beads in 50 mM Tris HCl buffer, pH 7.0 and protein as probe solute was calculated from differentiation of the curve connecting filled squares and the above equation.

The pore size data presented in Table 3.2 indicates that 50% of the pores in 1,4-dioxane are below 100 Å. The rest of the pores are between 100 and 700 Å. The polydispersity is clearly brought out. In buffer solution, on the other hand, all pores are below 100 Å. The molecular size of blue dextran bound irreversibly to the matrix is 61.68 Å, as calculated from the correlation used by Squire (1981). This size corresponds to 27% of the pore volume (Figure 3.8). The pore size distribution by aqueous (buffer) ISEC is compared to mercury intrusion porosimetry. The pores having less than 50 Å and between 50 to 100 Å by mecury porosimetry are 58.8% and 32.4%, respectively (Table 2.8) as compared to 61.09 and 38.28%, respectively in aqueous

ISEC. This shows some similarity between these two techniques, whereas the difference is much higher in organic ISEC. This is due to greater swelling of the beaded polymer in dioxane as compared to aqueous buffer. In 1,4-dioxane the HEG beads swelled and a viscoelastic macroporous gel was evolved. This elastic swelling of polymer network in 1,4-dioxane resulted in bigger pores. These bigger pores were generated at the expense of smaller pores due to network swelling of the polymer. Furthermore, the pore size up to 100 Å was half of the total number (Table 3.2) indicating that these pores were suppressed under bigger pores during network swelling.

Table 3.2. Comparison of pore volume distribution in 1,4-dioxane and buffer.

Pore diameter (Å) in 1,4-Dioxane	% pore volume	Pore diameter (Å) in buffer	% pore volume
d_{pore}< 100	50.0	d_{pore} <25	27.85
100< d_{pore} <200	30.9	25< d_{pore} <50	33.24
200< d_{pore} <300	19.1	50 < d_{pore} <75	25.68
300< d_{pore} <400	11.85	75 < d_{pore} <90	12.6
400< d_{pore} <700	14.7		
700< d_{pore} <1320	0.15		

1,4-Dioxane was used as eluting solvent and polystyrene standards as probe solute.
50 mM Tris-HCl buffer, pH 7.0 was used as mobile phase and protein standards as probe solute.

Polymer materials swell in solvents characterised by a value of solubility parameter δ_e similar to the value of the solubility parameter δ_g of the polymer. The solubility parameter describes the attractive strength between molecules of the material. This may be affected by variations in their chemical constituents, i.e., the number of crosslinks and the distribution of the chain branches/substitution groups along the polymer backbone. Techniques to determine solubility parameter of polymers are well described in literature. These include, refractive index (Koenhen and Smolders, 1975), intrinsic viscosity (Mieczkowski, 1991 and 1992; Siemann, 1992), turbidimetric

titration (Marco et al., 1986), gas chromatography (DiPaola-Baranayi, 1982; Ashworth and Price, 1986), and swelling (Yagi et al., 1992; Hamurcu and Baysal, 1994; Sen and Güven, 1998). Sen and Güven (1998) established that the measured solubility parameter of poly (N-vinyl 2-pyrrolidone/EGDM) gels by swelling measurement was identical to that estimated theoretically. The solubility parameter of HEG beads was estimated by the additivity rule using the following equation and was found to be 8.01.

$$\partial_g = f_{HEMA}\partial_{HEMA} + f_{EGDM}\partial_{EGDM} \qquad (3.4)$$

where, ϕ_{HEMA} and ϕ_{EGDM} denote the volume fractions of HEMA and EGDM in the matrix, δ_{HEMA} and δ_{EGDM} are the calculated solubility parameters of pure HEMA and EGDM, respectively.

The solubility parameters for 1,4-dioxane and water are 10 and 24.3 respectively. The closer the value of solubility parameter between the solute and solvent, greater will be the probability of interaction between the two. When $(\delta_e-\delta_g)^2 = 0$, solution is assured by entropy factor. With linear polymers this results in dissolution. In crosslinked systems such as HEG beads under study, this increases its size due to swelling. Nakazato and Suzuki (1989) reported that there is a linear relationship between $\Delta\delta$ (solvent-gel) and exclusion limit (exclusion limit/log MW) in acrylamide terpolymers. They interpreted that the exclusion limits are due to the formation of large, macropores. They also found an inverse linear relationship between solubility parameter of the gel and the exclusion limit. The exclusion limits are so high that the values of δ of the terpolymer are small. In other words, the magnitude of the hydrophobicity is high. Since the solubility parameter for the HEG beads is closer to 1,4-dioxane than water, the polymer swells in 1,4-dioxane while it is in an unswollen state in water.

Chapter 4

Determination of key parameters for adsorption of protein onto the matrix

4.1. Summary

Effective diffusion coefficients of the HEG beads (HEMA:EGDM=100:25) were evaluated with four standard proteins. The effective diffusivity of α-Cyclodextrin glycosyl transferase (CGTase) was calculated from the empirical correlation developed between molecular weight of proteins and the ratio of effective to molecular diffusion coefficients. The value of effective diffusivity shows transportation of α-CGTase in the pores was restricted to 76%. The adsorption of α-CGTase to the pores of the matrix was favourable in low concentration of the enzyme in the fermented broth. α-CGTase was adsorbed on the affinity-HEG beads. A number of elution procedures were investigated and 10 mM $CaCl_2$ in 50 mM Tris-HCl buffer, pH 7.0 at 25 °C was established as the best eluent. One hundred and fifteen fold purification of the enzyme was achieved with 48% recovery of the protein.

4.2. Introduction

The principal parameters, which govern adsorption/desorption of proteins on to a matrix, are: diffusion of protein on to the pores and rate of adsorption / desorption of the protein on to the ligand achored to the functional groups inside the pores. Although Chapter 3 deals with the pore size distribution, these data are not sufficient to describe the protein movement inside the pores. Therefore, some of the key parameters e.g., effective diffusion coefficient of the proteins and protein-ligand dissociation constant were investigated to study diffusional and kinetic aspects of the model protein α-CGTase on to the affinity-HEG beads.

Macroporous copolymer matrix of 2-hydroxyethyl methacrylate (HEMA) and ethyleneglycol dimethacrylate (EGDM) have been employed as adsorbents for purification of proteins in chromatography (Vondruska et al., 1976; Kleinmann et al., 1989; Vytasek et al., 1976; Mikes et al., 1976 and Turkova et al., 1973). Due to higher mechanical (Vondruska et al., 1976), chemical, thermal stability (Coupek, et al., 1973), lower non-specific interaction (Mikes et al., 1976), easy derivatisation of the functional groups (Turkova et al., 1973; Valentova, 1975) and better size

exclusion limits (Coupek et al., 1973; Vytasek et al., 1976), the matrix is popularly used in HPLC and packed bed liquid chromatography. The pore characterisations and exclusion limits were studied by Coupek et al., 1973 and Vytasek et al., 1976 but a detailed study of pore and surface diffusion of proteins is required for its use in chromatographic purification of proteins.

Cyclodextrin glycosyl transferase (CGTase) is an enzyme that cleaves starch molecules, cyclises and transglycosylates to a valuable cyclic compound called as cyclodextrin (CD). These cyclic compounds are termed as α, β and γ depending upon the number of glucose units and have various industrial applications in food, pharmaceutical, cosmetics. The purification of enzyme involves many steps depending on the desired purity. Among all the purification methods, affinity purification is gaining popularity due to the selectivity to the proteins to be purified. There are a number of reports on purification of CGTase by using α-CD coupled to Sepharose supports (Laszlo et al., 1981; Wind et al., 1995). Systematic adsorption and mass transfer studies of purification of this enzyme on this matrix has not yet been reported.

Diffusion and adsorption/desorption are the main mechanisms of chromatographic separation that occur inside the pores of the adsorbent. Pore characterisation by various methods determines the exclusion of proteins, pore size, surface area and volume of the macroporous polymeric beads. These values are not sufficient to study transportation of the macromolecules into and out of the pores during adsorption and desorption processes. Furthermore, understanding the behaviour of protein transportation inside the pores, their interaction with the ligands at the surface are the key to develop more accurate multicomponent isotherms and models of chromatographic separation of the biological molecules.

Several techniques and models are used for the estimation of the mass transfer parameters, such as shock layer theory (Sajonz et al., 1997 and Miyabe et al., 1999) and homogeneous Fickian diffusion model (Yoshida et al., 1994). Staining method (Carleysmith et al., 1980 and Firouztale et al., 1992) and light scattering technique (Phillies et al., 1989 and Warren et al., 1989) were also explored for the determination

of effective diffusivity of the chromatographic matrices. Regardless of the accuracy of the given model, all models require accurate values of key parameters, to quantify a given mechanism.

The object of this study is to evaluate effectiveness of the affinity material for the purification of α-CGTase. Height equivalent theoretical plate (HETP) method has been used for the determination of film mass transfer coefficient and pore diffusion coefficient of four standard proteins, whereas batch adsorption experiment has been performed for measurement of dissociation constant and maximum adsorption capacity. These adsorption and mass transfer data would be helpful in process design. Although HETP method of determining effective diffusivity has some limitations, it has been successfully applied to the Gaussian distribution of solutes in a packed column (Boyer and Hsu, 1992).

4.3. Materials and Methods

4.3.1. Chemicals

Calcium chloride was purchased from Sarabhai Chemicals (Baroda, India). The sources of four standard proteins (Lysozyme, chicken egg white; albumin, bovine serum; alcohol dehydrogenase, yeast; and β-amylase, sweet potato) and Tris(hydroxymethyl) methylamine (Trizma base) were summarised in Section 3.3.1.

4.3.2. Methods

Synthesis of HEG beads was described in Section 2.4.1. The matrices having similar particle size distribution as described in Section 3.3.2, were used in this study for HETP, adsorption and elution experiments in a packed column. The affinity-HEG beads prepared by coupling of α-CD with HEG beads through urethane linkage (Section 2.4.3) were tested for all the above experiments. The ligand concentration was assayed by methyl orange method (Section 2.3.3.4).

4.3.2.1. Adsorbate

α-CGTase (mol. wt. 75 kDa) was used as a model protein for adsorption studies. The enzyme α-CGTase was produced by fermentation (Gawande, 2000). The

fermented broth was clarified by using 5 mL L^{-1} of flocculating agent (Magnafloc), allowed to settle overnight at 10 ºC and centrifuged at 2,300 g for 20 minutes (Eltech, India, Model No. SC 7500).

4.3.2.2. Batch adsorption for ligand concentration optimisation

Equilibrated adsorption of α-CGTase was determined at different ligand (α-CD) concentration on HEG beads. A range of affinity-HEG beads, of varying ligand concentrations on beads, were prepared by mixing the affinity-HEG beads, with high loading of ligands, with underivatised HEG beads. Thirty millilitre of clarified fermented broth was added to 1 g of affinity-HEG beads and shaken for 5 hours at 12 ºC on a constant temperature shaker incubator (PsycroTherm™, New Brunswick Scientific Inc., USA). The amount of enzyme adsorbed was estimated from the decrease in activity in the liquid phase. The enzyme was desorbed from the matrices by shaking for 4 hours at 25 ºC with 10 mM $CaCl_2$ in 50 mM Tris-HCl buffer of pH 7.0.

4.3.2.3. Adsorption isotherm studies

To each of series of conical flasks, 30 mL of the clarified fermented broth was added in the concentration range 0%, 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90% and 100% (v/v), relative to the undiluted fermented broth containing 34.2 U mL^{-1} of α-CGTase. All dilutions were carried out using 50 mM Tris-HCl buffer (pH 7.0). 1 g of affinity-HEG beads was soaked in water and vacuum was applied to eliminate air bubbles from the pores. The adsorbents were then equilibrated with the same buffer used for fermented broth dilution. The diluted adsorbates were shaken with the adsorbents for 10 hours at 12 ºC on a constant temperature shaker incubator (PsycroTherm™, New Brunswick Scientific Inc., USA). The enzyme activities in the liquid phase were calculated from the residual enzyme activities. The enzyme activity in the solid phase was estimated from the difference of initial and the residual enzyme activities.

4.3.2.4. Determination of effective diffusion coefficient

A column of diameter 10 mm and length 27 cm was packed with

underivatised HEG beads for size exclusion experiments. HETP method was used to determine effective diffusion coefficient. The standard proteins used in this study were Lysozyme (chicken egg white, MW 14,300 Da), albumin (bovine serum, MW 66,000 Da), alcohol dehydrogenase (yeast, MW 1,50,000 Da) and β amylase (Sweet potato, MW 2,00,000). Column temperature was maintained at 10 ºC. The protein standards made up to 1 mg mL^{-1} in 0.25 mL volume were introduced. The injection time was kept at 0.25 min. This allowed to negate concentration dependent effects and to neglect the time with respect to the mean residence time of the protein samples. The mobile phase, 50 mM Tris-HCl buffer, pH 7.0 was run at various flow rates.

4.3.2.5. Packed bed studies

The column used had a diameter of 10 mm and length 100 mm. It was packed with 6.8 mL of the affinity-HEG beads having a ligand concentration 6.65 mg of α-CD g^{-1} (dry weight). The column temperature was maintained at 10 ºC by circulating cold water through the jacket of the column. The column was equilibrated with Tris-HCl buffer (0.05 M, pH 7.0) by passing twenty times bed volume of the buffer. The fermented broth was applied from the top at a flow rate of 0.3 mL min^{-1} till the effluent α-CGTase activity matched the α-CGTase activity in the feed. 10 mL fractions of the effluent were collected and assayed for enzyme activity and protein content. The column was washed at 10 °C with Tris-HCl buffer, pH 7.0 at a flow rate of 0.3 mL min^{-1} till the optical density at 280 nm of the effluent reached zero. The enzyme elution was tested with various reagents at a flow rate of 0.3 mL min^{-1}. Protein content and α-CGTase activity of 2 mL fractions were estimated using Lowry method (Section 2.3.3.7) and the methyl orange method described by Lejuene et al. (Section 2.3.3.4) respectively.

4.4. Results and Discussions

4.4.1. Optimisation of ligand concentration for binding and elution of α-CGTase

Prior to adsorption of α-CGTase to the affinity-HEG beads from the clarified fermented broth, the effect of ligand concentration on binding and elution was

investigated. The ligand (CD) concentration attached to the HEG beads was varied from 4.5 to 67.5 mg g^{-1} of matrix (dry weight) by blending affinity matrices with underivatised matrices in varying proportions. Cyclodextrin has been investigated as a ligand for purification of CGTase (Ahn et al., 1990, 1991; Larsen et al., 1998; Mori et al., 1994; Wind et al., 1995). The effect of CD concentration as ligand on the matrix for adsorption and elution has not been studied so far. Therefore, the batch adsorption study was performed using affinity-HEG beads to probe the extent of binding and the recovery during elution. No binding of α-CGTase was observed in an independent experiment with underivatised HEG beads. The enzyme was eluted with 10 mM $CaCl_2$ solution (in 50 mM Tris-HCl buffer, pH 7.0) at 25 ºC by shaking the flasks for 4 hours in an orbital shaker. The results showed increased binding and decreasing trend of recovery during elution of the enzyme as the ligand concentration was increased (Table 4.1).

Table 4.1. Optimisation of ligand concentration for adsorption and recovery of a -CGTase on affinity HEG beads.

L_c (mg g^{-1}) (dry wt.)	Adsorbed (U g^{-1})	Eluted (U g^{-1})	% Recovery	Sp. activity (U mg^{-1})
4.5	157	150	95.5	493
9.0	189	166	87.8	443
13.5	220	193	87.7	405
18.0	283	215	76.0	310
22.5	378	280	74.0	296
27.0	409	304	74.3	297
31.5	473	331	70.0	266
36.0	504	339	67.3	206
40.5	599	406	67.8	173
45.0	662	418	63.1	nd

nd- not detected; L_c=ligand conc.

The decreasing trend in the specific activity of the eluted enzyme indicates non-specific elution of other proteins at higher temperature. The basic reaction in affinity chromatography is the formation of a specific complex between the substance to be isolated and the ligand bound to the matrix. A measure of the strength of this complex is the dissociation constant or equilibrium constant. At higher ligand concentration, the dissociation constant is larger. An increase in dissociation constant enhances the interaction and drastic conditions are needed to elute the enzyme. On the other hand, at lower ligand concentration, the enzyme does not separate from the impurities (Cuatrecasas, 1972).

The concentration of the immobilised ligand has a distinct influence on the effectiveness of an affinity support. Holroyde and co-workers (1976) observed in the affinity chromatography of glucokinase with 2-amino-2-deoxy-D-glucopyranose-N-(6-aminohexyl)- Sepharose, an optimal concentration of the ligand of 3.75 μmol g^{-1}. It has also been observed by Turková et al. (1982) that the affinity chromatographic efficiency of (ε−aminocaproyl-L-Phe-D-Phe-OCH_3)-Separon, a specific adsorbent for porcine pepsin, depends strongly on the inhibitor concentration (0.85 μmol inhibitor g^{-1} dry gel). Pepsin was eluted from the column by increased ionic strength of the elution buffer in a single sharp peak, where as at high inhibitor concentration (155 μmol g^{-1} dry carrier) several peaks of pepsin, exhibiting the same proteolytic activity were observed. Therefore, in this study on adsorption of α-CGTase on affinity-HEG beads, the ligand concentration was restricted to 6. 65 mg g^{-1} of dry matrix.

4.4.2. Equilibrium adsorption isotherm

The batch adsorption study was performed to evaluate the binding strength of α-CGTase for immobilised α-cyclodextrin. Figure 4.1 shows that the isotherm obeys Langmuir isotherm, which is of the form

$$q^* = \frac{q_m C^*}{(K_d + C^*)} \qquad (4.1)$$

where, q* is the equilibrium binding capacity (U g^{-1}, dry weight of matrix), C* is the

solution phase enzyme activity at equilibrium (U mL^{-1}), q_m is the equilibrium binding capacity (U g^{-1}, dry weight of matrix) and K_d is the dissociation constant of the enzyme ligand interaction (U mL^{-1}).

The maximum binding capacity of 666 U g^{-1} (195 U mL^{-1} of adsorbent) dry weight of affinity-HEG beads was obtained. This corresponds to 0.27 μg of CGTase adsorbed per mL of affinity-HEG beads (the specific activity of pure α-CGTase is 736 U mL^{-1}, Gawande, 2000). The dissociation constant is low, as calculated from Figure 4.2, (6.21 U mL^{-1}or 8.43 μg L^{-1}, 1.12×10^{-7} M), indicating that α-CGTase can be recovered from low concentration of fermented broth.

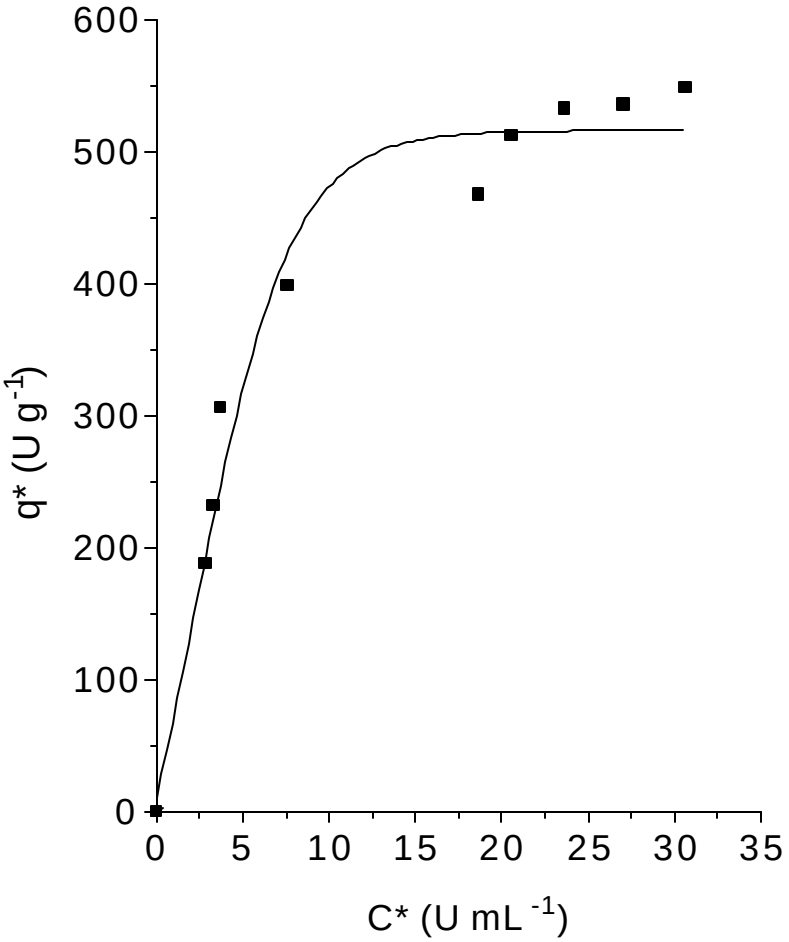


Figure 4.1. **Equilibrium adsorption curve of a -CGTase from clarified fermented broth on to affinity-HEG beads.** q^* is the solid phase equilibrium capacity and C^* is the liquid phase equilibrium enzyme activity.

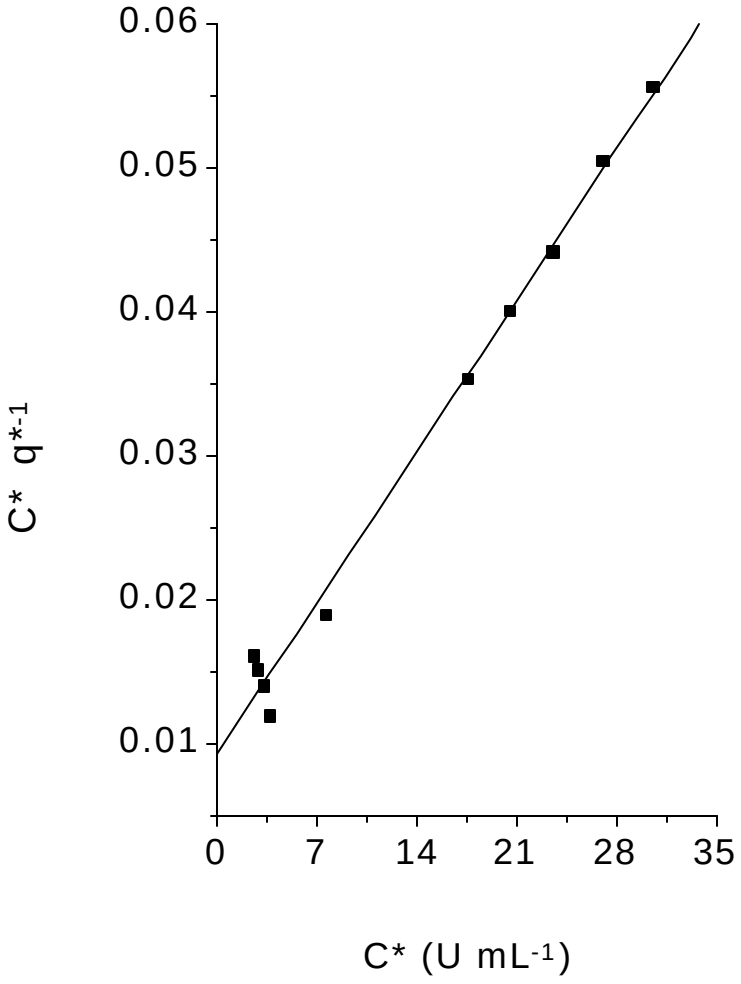


Figure 4.2. **Calculation of a -CGTase ligand dissociation constant.**
q^* is the solid phase equilibrium capacity and C^* is the liquid phase equilibrium enzyme activity.

4.4.3. Determination of effective diffusivity

Knowledge of the hindered diffusion of proteins is of fundamental importance to the mechanism of transportation into the pores of the beads. Since the packing pores are of size comparable to the protein solutes, the diffusion of proteins in the porous packing is highly hindered. Therefore, the determination of the effective diffusion coefficient of proteins in chromatographic column is of critical importance in mass transfer kinetics.

Film and the intraparticle-hindered diffusion are assumed to dominate the HETP model equation. The axial dispersion and adsorption of the proteins in the porous underivatised packing is negligible. However, our previous studies on non-specific interaction of proteins to HEG beads had indicated adsorption up to 95 μg of BSA in presence of 1% NaCl (wt/v) (Table 2.9). The interaction of proteins to HEG beads was not observed in 50 mM Tris-HCl (pH 7.0). Since there was no difference in measured surface area of HEG and affinity-HEG beads having α-CD concentration within 10 mg g^{-1} of the dry matrix, we assumed that there was change in pore size distribution of HEG beads after coupling with α-CD. However, the pore surface area of the affinity-HEG beads decreased to 90% of the HEG beads when α-CD concentration reached 900 mg g^{-1} of dry matrices.

Arnold et al. (1985) developed the HETP method to estimate kinetic parameters in a SEC column, where the height of an equivalent transfer plate, HETP, was defined by

$$HETP = \frac{2D_{ax}}{u} + \frac{2\varepsilon_p^2 r_p u}{3mk_f(1+\varepsilon_p/m)^2} + \frac{2\varepsilon_p r_p^2 u}{15mD_e(1+\varepsilon_p/m)^2} \qquad (4.2)$$

Where, D_{ax} is the axial liquid dispersion coefficient, u is the superficial velocity of the liquid, ε_p is the accessible void fraction, r_p is the radius of the particle (matrix), k_f is the film mass transfer coefficient, D_e is the effective diffusivity and m is the ratio of packed bed void volume to the volume of the matrix ($\varepsilon_0/(1-\varepsilon_0)$).

There are two major contributions to HETP: film diffusion (H_f) and hindered diffusion in the porous packing in extreme right hand of the equation (4.2). The exit peak of BSA is Gaussian in shape (Figure 4.3).

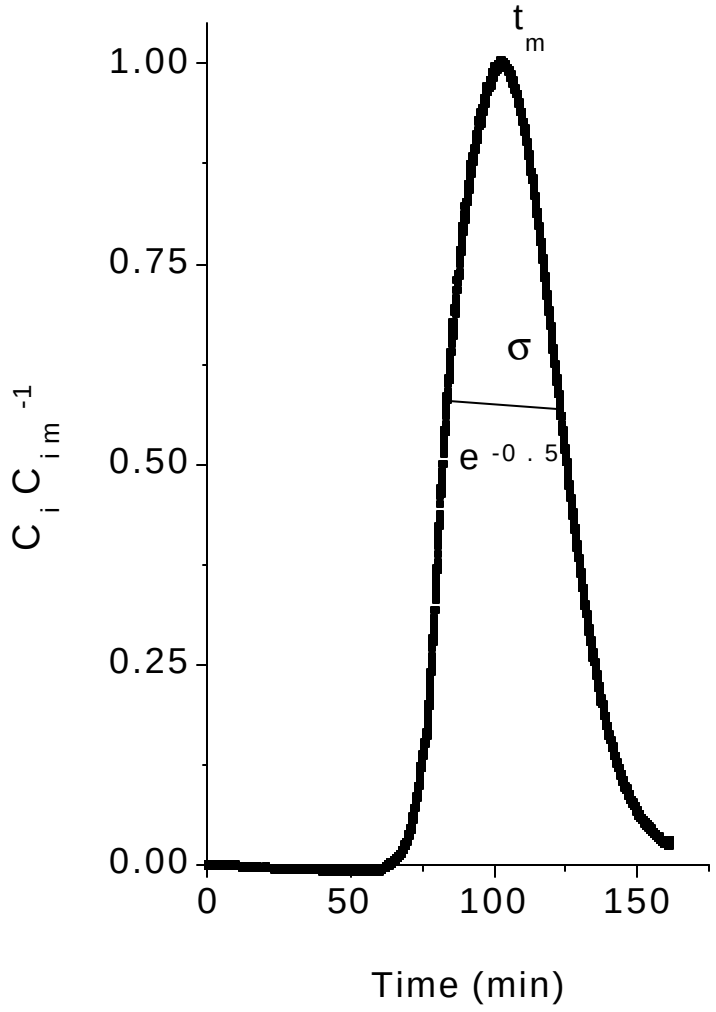


Figure 4.3. Normalised elution profile of BSA on HEG beads in 50 mM Tris-HCl buffer.

σ (standrd deviation) is calculated from the difference in elution time which corresponds to $e^{-0.5}$ times the normalised outlet concentration of the protein. t_m is the peak mean residence time of the curve. C_i and C_{im} are the outlet and maximum outlet concentration of BSA, respectively.

Hence, HETP can be calculated (Arnold et al., 1985**)** as

$$HETP = \frac{\sigma^2}{t_m} H_0 \qquad (4.3)$$

where the standard deviation σ and mean residence time t_m are calculated

experimentally by the chromatogram of a SEC process (Figure 4.3) (Arnold et al., 1985). H_0 is the paked bed height of the column. The standard proteins used in this study are summarised with their physical characteristics in Table 4.2. The diffusion coefficient of the proteins in bulk solution is calculated using correlation given by Young et al. (1980) (Table 4.2). Particle accessible void fraction (ε_p), which is defined as the ratio of pore volume permeated by a solute to the packing volume, is tabulated for different proteins (Table 4.3). ε_p is the slope of the plot of t_m versus $H_0\ u^{-1}$, as shown in the Figure 4.4.

Table 4.2. Physical characteristics of four standard proteins used in this experiment.

Proteins	Source	MW (Da)	D_m ($cm^2\ s^{-1}$) × 10^{7}
Lysozyme	Chicken egg white	14,300	8.95
Albumin	Bovine serum	66,000	5.38
Alcohol dehydrogenase	Yeast	1,50,000	4.09
β -amylase	Sweet Potato	2,00,000	3.72

Table 4.3. Determination of intraparticle void fraction of proteins on HEG beads.

Proteins	Intraparticle void fraction (ε_p)
Lysozyme	0.87
BSA	0.80
Alcohol dehydrogenase	0.70
β amylase	0.69

A correlation obtained between logarithm of molecular weight and ε_p is as follows

$$\boldsymbol{e}_p = 0.0715.\ln(MW) + 1.56 \qquad (4.4)$$

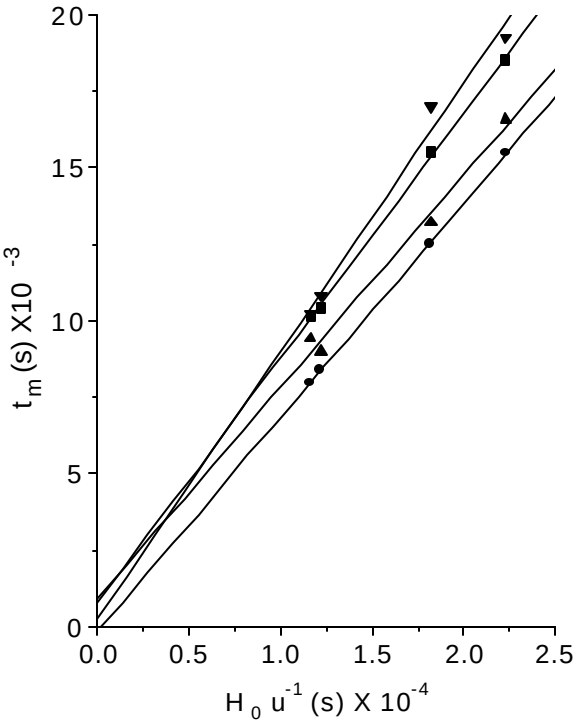


Figure 4.4. Mean residence time of the outlet peaks of four standard protein samples in HEG beads packed in a column.

The slope of the lines is the ratio of the pore volume permeated by a solute. (▼) Lysozyme, (■) BSA, (▲) alcohol dehydrogenase, (●) β-amylase. t_m, H_0 and u are the peak elution time, packed bed height and flow velocity of the eluting solution, respectively.

According to Funk and Houghton (1961), the diffusion resistance of solutes from the mobile phase to the stationary phase is a result of the film mass transfer resistance between two phases. Geankopolis (1983) proposed a typical correlation of the film mass transfer coefficient (k_f) as follows

$$k_f = \frac{2D_m}{d_p} + 0.31\left(\frac{m}{D_m \rho_i}\right)^{-2/3}\left(\frac{\Delta\rho_l \mu g}{\rho_l^{\,2}}\right)^{1/3} \qquad (4.5)$$

Where k_f is the film mass transfer coefficient, D_m is the molecular diffusivity of the protein, ρ_l is the liquid density, μ is the liquid viscocity, $\Delta\rho_l$ is the difference in viscosity, d_p is the particle (matrix) diameter and g is the acceleration due to gravity.

To estimate the bulk mass transfer coefficient in a packed bed, the following correlation of Foo and Rice (1975) was used:

$$Sh = 2 + 1.45\,\mathrm{Re}^{1/2}\,Sc^{1/3} \qquad (4.6)$$

where, Sh = $k_f\, d_p/D_m$, Re = $u\rho_l d_p/\mu$, Sc = $\mu/(\rho_l D_m)$ and u is the superficial velocity of the liquid flow through the column.

The film mass transfer data for four protein standards at varying flow velocities are plotted, as shown in Figure 4.5. The film mass transfer coefficient of four standard protein samples increases only marginally with increase in interstitial velocity or with a decrease in the relative molecular mass of proteins. The contribution of H_f to HETP in HEG column is found to be within 15%.

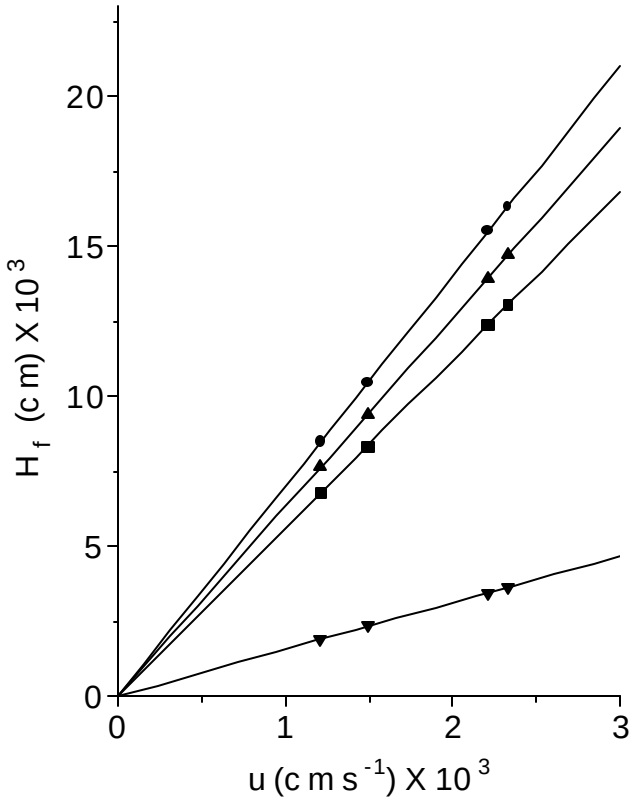


Figure 4.5. Film mass transfer data for four standard protein samples on HEG beads.

(▼) Lysozyme, (■) BSA, (▲) alcohol dehydrogenase, (●) β-amylase

The axial dispersion coefficient for each protein is tabulated in Table 4.4.

Table 4.4. Axial dispersion of four proteins in HEG beads.

Proteins	$H_a \times 10^3$
Lysozyme	9.22
BSA	4.15
Alcohol dehydrogenase	0.99
β-amylase	0.95

The effective diffusion coefficient is calculated from equation (4. 2)

$$HETP - H_f = H_a + \frac{2\varepsilon_p R^2}{15 m D_e (1+\varepsilon_p / m)^2} u \qquad (4.7)$$

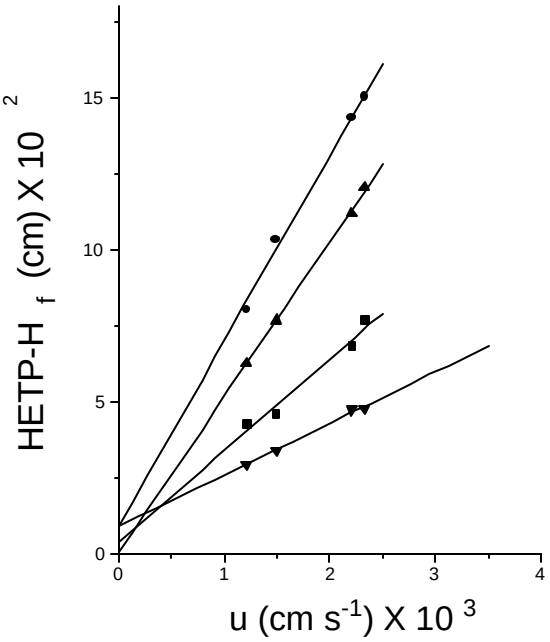


Figure 4.6. HETP analysis of four protein samples on HEG beads.

(▼) Lysozyme, (■) BSA, (▲) alcohol dehydrogenase, (●) β-amylase**.**

The plot of $HETP\text{-}H_f$ versus u is linear (as shown in Figure 4.6). Therefore, the effective diffusion coefficient can be calculated from the slope of each line. The D_e of four standard proteins on HEG beads is summarised in Table 4.5.

Table 4.5. Intraparticle diffusion and diffusion restriction of the proteins.

Proteins	D_e (cm^2 s^{-1}) $\times 10^7$	D_e/D_m
Lysozyme	2.89	0.32
BSA	1.46	0.27
Alcohol dehydrogenase	0.76	0.19
β-amylase	0.63	0.17

It can be found from Table 4.5 that the effective diffusion coefficients of four proteins are lower than the values in the bulk solution, by 17.0 to 31.9%. This means that the intrapore diffusion in the porous packing is highly hindered for molecular weight in excess of 1,50,000. The mass transfer of the proteins in HEG column is dominated by intraparticle diffusion. Hence, the mechanism of the mass transfer can be better explained by the intraparticle-hindered diffusion.

The result of intraparticle diffusion in Sepharose CL-6B (Boyer, 1992) is compared with HEG beads. As the molecular weight of the proteins increases, the intraparticle diffusion coefficients in HEG beads have higher value than that in Sepharose CL-6B. The effective diffusion coefficient of proteins in certain gel matrices was observed experimentally by Laurent and Killander (1964). They claimed that the extent of diffusion hindrance varied exponentially with the size of the protein. In order to correlate the effective diffusion coefficient in macroporous HEG beads with relative molecular mass, ln (D_e/D_m) is plotted against $(MW)^{1/3}$ (Figure 4.7), using the correlation proposed by Young et al. (1980). The correlation developed is as follows:

$$\ln\left(\frac{D_e}{D_m}\right) = -0.0267\left(MW^{1/3} + 30.87\right)(1-\varepsilon_0)^{1/2} \qquad (4.8)$$

From this correlation the effective diffusivity of any protein of known molecular weight can be calculated. The bulk diffusivity of CGTase (MW 75, 000,

Gawande, 2000) was calculated from equation 4.8. The ratio of D_e to D_m of α-CGTase in HEG beads was found to be 0.237.

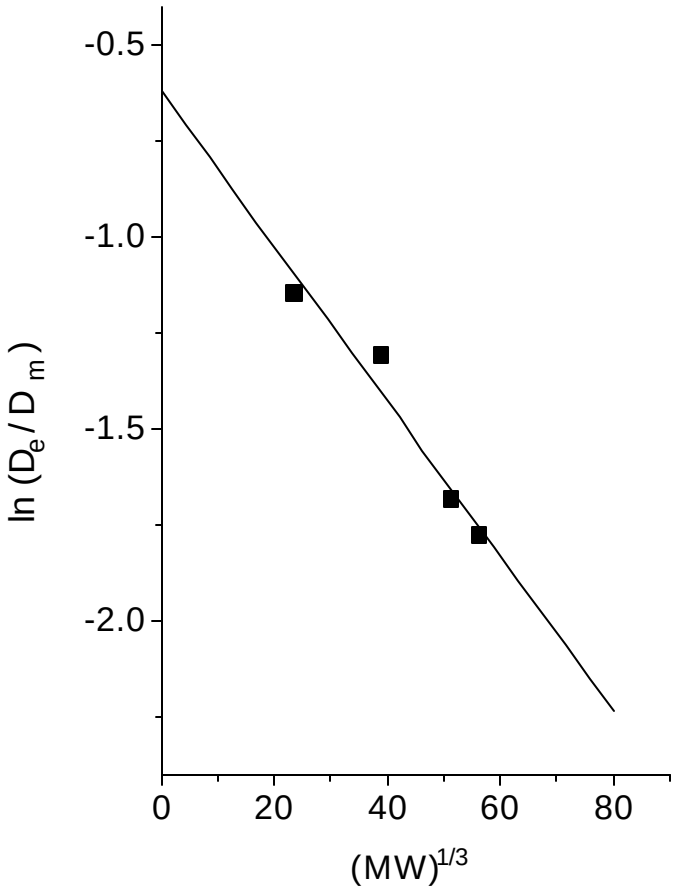


Figure 4.7. **Correlation of effective diffusion coefficient based on relative molecular mass.**

D_e is the effective diffusivity coefficient and D_m is the molecular diffusivity coefficient, respectively.

4.4.4. Adsorption and elution studies

Breakthrough curve is a measure of the adsorbate passed through the bed per unit amount of adsorbent with a target permissible loss of 5% enzymes / proteins (of the feed concentration) in the effluent. This gives an estimate of amount of adsorbent to be used for specified amount of protein to be purified. The breakthrough curve of α-CGTase adsorbed on to the affinity matrix described above is shown in Figure 4.8. Maximum bed adsorption capacity (q_b) was found to be 50.5 U of α-CGTase per mL of the bed. Taking effluent concentration target as 5% of the feed concentration,

dynamic adsorption capacity was found to be 38.5 U mL^{-1} of the adsorbent.

Several eluents were tested for possible elution of CGTase from the packed bed (Table 4.6). Elution with 10% $CaCl_2$ yielded 48% recovery with 115-fold purification. However, keeping the eluent buffer the same and increasing the temperature decreased the recovery of protein, probably due to deactivation of the enzyme at higher temperature (Table 4.7).

Table 4.6. Elution of a -CGTase with various eluents at 25 °C.

Eluent	% Recovery	Fold purification
50 mM Tris-HCl buffer, pH 7.0	19.0	4.5
2% soluble starch in 50 mM Tris-HCl buffer, pH 7.0	58.4	25.0
10 mM $CaCl_2$ in 50 mM Tris-HCl, pH 7.0	48.0	115.0

Table 4.7. Elution with 10 mM $CaCl_2$ in 50 mM Tris-HCl buffer, pH 7.0 at different temperatures.

Temp (° C)	Protein Conc. (mg mL^{-1})	Enzyme activity (U mL^{-1})	Sp. activity (U mg^{-1})	Fold purification	% Recovery
25	0.065	25.6	393.3	115.0	48.0
38	0.072	29.4	407.0	119.0	18.8
55	0.070	22.2	318.0	93.0	10.5
70	0.022	1.0	46.2	13.5	4.5

Enzyme adsorption and washing profile (both protein concentration and enzyme activity) is shown in Figure 4.8 and elution with 10 mM $CaCl_2$ in 50 mM Tris-HCl, pH 7.0 is shown in Figure 4.9. Purification results of α-CGTase by various reported methods in a packed bed column is compared with the present method is discussed in Section 5.4.5.

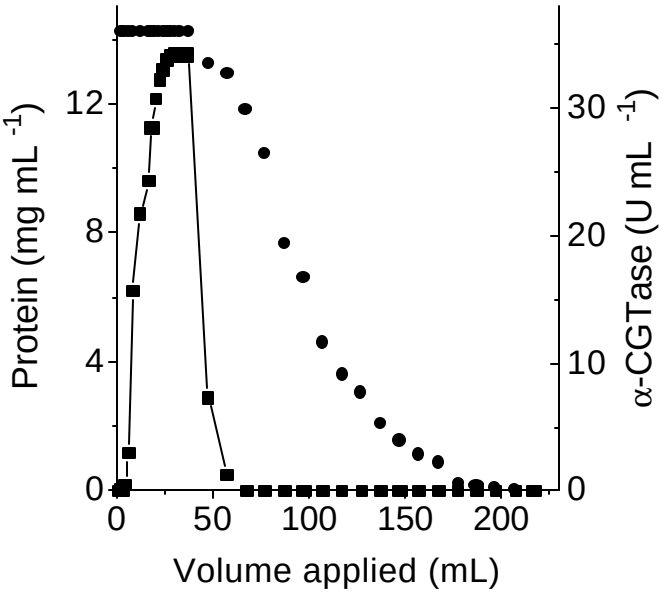


Figure 4.8. Loading and washing profiles for protein and a -CGTase of the effluent clarified fermented broth.

(•) Protein and (■) α-CGTase.

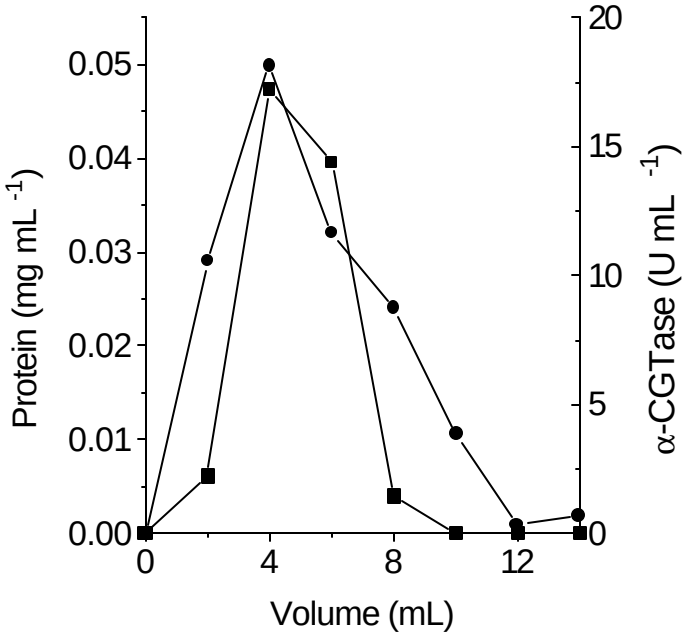


Figure 4.9. Elution profile of a -CGTase and protein.

(•) Protein and (■) α-CGTase

Chapter 5

Application of EBA to a real system: Purification of a-cyclodextrin glycosyltransferase

5.1. Summary

The affinity-HEG beads were evaluated for their suitability in expanded bed chromatography. The optimum thickness of the distributor plate for stable expanded bed for use in expanded bed adsorption (EBA) was established. The affinity-HEG beads were comparable in density to Streamline DEAE and exhibited better mechanical stability at higher superficial velocity under fluidisation. The affinity-HEG beads were used as affinity chromatography matrices for the purification of α-Cyclodextrin glycosyltransferase (CGTase). Feeding of 5 fold diluted fermented broth to the column containing affinity-HEG beads of settled bed height 7.5 cm (ID 26 mm and length 42 cm) at double bed expansion resulted in sharp breakthrough curve of α-CGTase. The adsorbed enzyme was eluted from the bed in 50 mM Tris-HCl buffer containing 10 mM $CaCl_2$ at 25 ºC in packed bed configuration.

5.2. Introduction

The success of purification of protein on expanded bed is due to appropriate design of the distributor plate and the denser adsorbent (Chase, 1994). The distributor plate distributes the feed liquid uniformly by imparting sufficient pressure drop by itself, whereas the particle size and density distributed adsorbents expand and segregate at different parts of the bed at higher flow velocity to stabilise the bed and to increase the throughput.

The objective of this chapter is to evaluate the hydrodynamic characteristics of HEG beads (macroporous crosslinked copolymer beads of HEMA and EGDM, 100:25) synthesised in this study. These beads were modified by coupling with α-cyclodextrin (CD) and are abreviated as affinity-HEG beads. These affinity-HEG beads were used as affinity chromatography matrices for purification of α-CGTase as a real system on EBA mode. The effect of adsorption of α-CGTase at various dilution rates has been evaluated.

5.3. Materials and Methods

5.3.1. Chemicals

The sources from where the media components, soluble starch and Streamline DEAE were purchased are presented in Section 2.3.1. Magnafloc was a kind gift from Hindustan Antibiotics Ltd., Pune, India.

5.3.2. Methods

5.3.2.1. Adsorbents

The affinity-HEG (HEMA:EGDM=100:25 and α-CD concentration 6.65 mg g^{-1} dry wt.) and Streamline DEAE beads were wet sieved between diameter 105 to 355 μm and 105 to 250 μm, respectively as described in Section 3.3.2. The density of each size fraction of Streamline DEAE and affinity-HEG beads were measured under swollen state as described in Section 2.3.3.1. α-CD bound on the matrices was assayed by the methyl orange method (Section 2.3.3.4).

5.3.2.2. Column

The column used in this study was XK 26 (inner diameter 26 mm and height 42 cm, Amersham Pharmacia Biotech AB, Sweden) (Figure 5.5.). The distributor plates were fabricated (SS plates 1 and 2mm thickness and tapered deldrin plates of 2 mm thickness, 15° angle). The distributor plate was supported with a support screen facing upword to retain the adsorbents in the column.

5.3.2.3. CGTase production

CGTase (E. C. 2.4.1.19) was produced by fermentation using *Klebsiella pneumoniae* pneumoniae, NCIM 5121, in a 1L magnetically driven top suspended fermenter (Gallenkamp, UK) (Gawand and Patkar, 1998). The optimised fermentation media and conditions described earlier was used (Gawande, 2000).

5.3.2.4. Feed characteristics

The viscosity of the feedstock was measured by Bohlin CVO 50 cone and plate Rheometer (Bohlin Instrument, UK) in double gap geometry with ID 40 mm

and OD 50 mm at steady shear stress mode. The temperature of the rheometer was controlled at 10 °C (± 0.1 °C) by means of computer controlled constant water bath (Julabo, Sweden).

The density of the feedstock was calculated from the ratio of the weight to the volume of 250 mL of the diluted feedstock. Ten mL of the fermented broth of various dilutions were centrifuged at 9,600 g for 20 to 30 minutes, supernatant was discarded and the residue from the centrifuged mass was dried at 60 ºC in a vacuum oven till constant weight was obtained. Dry cell weight of the samples was calculated from the dry weight of the residue per mL of the sample. Protein concentration and enzyme activity were analysed by the method of Lowry (1951) and Lejuene et al. (1989).

5.3.2.5. Bed expansion characteristics

Bed expansion was evaluated by the Richardson-Zaki equation $u=u_t\varepsilon^n$ (Richardson and Zaki, 1954), where u is the liquid superficial velocity, u_t is the particle terminal velocity, ε is the bed voidage and n is the Richardson-Zaki coefficient. The value of ε was calculated from $H/H_0=(1-\varepsilon_0)/(1-\varepsilon)$ where H_0 is the sedimented bed height, H the expanded bed height and ε_0 is the voidage in the sedimented bed. In this study a value of 0.4 was used for ε_0, as determined earlier (Draeger and Chase, 1991; Thommes et al., 1996). Number of theoretical plates (N) was calculated as $N=t^2/\sigma^2$, where t is the residence time and σ^2 is the variance. The parameters t and σ were determined by residence time distribution (RTD) analysis for expanded bed mode from the F-curve using the procedure described by Levenspiel (1972).

Mixing in the liquid phase was evaluated by determination of axial dispersion. The axial dispersion coefficient, D_{ax}, was calculated using $D_{ax}=uH/2N\varepsilon$ (Barnfield Frej et al., 1997), where u is the liquid superficial velocity, H is the bed height under expansion and ε is the bed voidage.

5.3.2.6. Measurement of Uptake rate of enzyme

Adsorption kinetics of α-CGTase (34.2 U mL^{-1}) on affinity-HEG beads (α-CD concentration 6.65 mg g^{-1} of the matrix) were carried out at 12 °C with a variety of dilutions of unclarified fermented broth. The fermented broth was diluted to 2, 5, 10 and 20 times in 50 mM Tris-HCl buffer, pH 7.0 in 250 mL conical flask. In each of the experiments, 50 mL of diluted unclarified fermented broth with 1 mL of adsorbents were taken. These flasks were stirred at 100 rpm in controlled environment incubator shaker at 12 ºC (PsychroTherm™, New Brunswick Scientific, USA). 0.5 mL of liquid was withdrawn from the flasks at 60 minutes interval, centrifuged at 9,600 g and assayed for α-CGTase. The decrease in volume of the liquid during experiment was less than 8 %.

5.3.2.7. Expanded bed adsorption experiments

5.3.2.7.1. Stable bed characteristics with various distributor plates

The experimental set up consisted of a jacketed column (ID 26 mm and length 42 cm having movable adopters) connected through a double jacketed glass vessel (600 mL volume), 3-way valve and a six roller peristaltic pump (LKB, UK) to the bottom of the chromatographic column. The upper and lower adapter of the column of 26-mm diameter and 42-cm length (XK26, Pharmacia Biotech, Sweden) were modified to suit the higher flow rate operated in the column.

Circulating cold water using constant temperature water bath (Julabo, Sweden) controlled the temperature of the whole system (at 10 °C). The degree of expansion of HEG beads were measured at various flow velocities and using various distributor plates. The testing of distributor plate experiments comprised of SS plate of 1 and 2 mm thickness, tapered deldrin plate of 2 mm thickness (15 º angle) and the support screen devoid of distributor plate. In each of the experiments with varying distributor plate, a support screen was incorporated which faced towards the adsorbents. The expanded bed height was measured when the motion of the particles appeared visually to be of stable configuration. The initial bed height in all

experiments was kept at 7.5 cm (bed volume-39.8 mL). The bed was equilibrated with 50 mM Tris-HCl buffer, pH 7.0 from one vessel and acetone (0.25%, v/v in same buffer) was used as tracer (step input) from other vessel by switching the 3-way valve. The positive step input UV signal recorded till measurement of the maximum absorbance (100%) is called as F-curve. The output signal was measured at 266 nm through a flow cell connected to a spectrophotometer (ATI Unicam UV/Vis spectrophotometer UV2, UK). When the signal at 266 nm stabilised, the buffer feeding was started by switching the 3-way valve to the flask-containing buffer. Data were compiled in a personal computer.

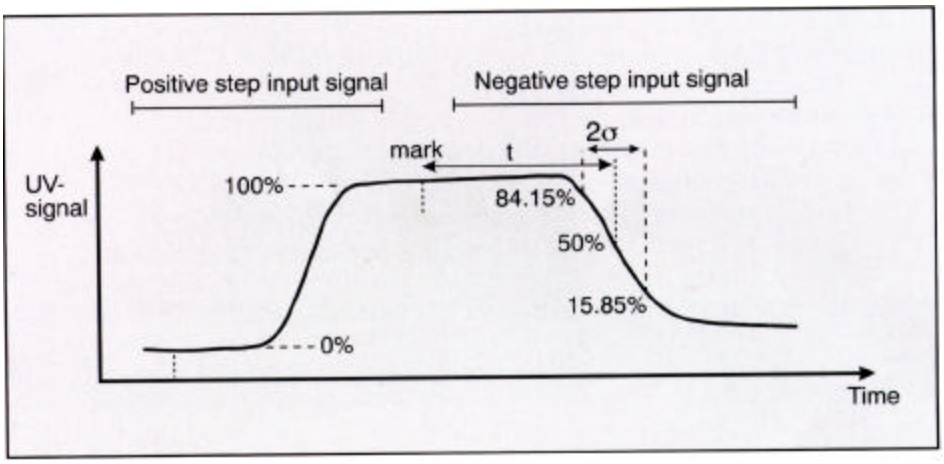


Figure 5.1. Calculation of s and t from F-curve.

5.3.2.7.2. Frontal bed analysis of Streamline DEAE and affinity-HEG beads in expanded bed

Frontal bed analysis was carried out to compare the binding of α-CGTase on affinity-HEG beads. Various distributor plates (with 1mm hole) were designed to compare the stability and adsorption characteristics in expanded bed. Affinity-HEG beads were added to the column in separate experiments, varying the distributor plates. The settled bed height was noted as 7.5 cm and the calculated bed volume was 39.8 mL. The bed was expanded to double the settled bed height and the flow of buff (50 mM Tris-HCl buffer, pH 7.0) was continued for 45 minutes to 1 hour to get a stable expanded bed at a flow rate (19.8 mL min^{-1}) that corresponds to 224 cm h^{1}. Unclarified fermented broths of varying of dilution (1, 2, 5, 10 and 20) were pumped into the bed at appropriate flow rates (depending on the feed characteristics) to

expand the bed to double of its settled bed height. Fractions collected at regular time intervals were assayed for α-CGTase, protein and cell mass. The affinity-HEG beds were washed with 50 mM Tris-HCl, pH 7.0 and 10 mM $CaCl_2$ (prepared in 50 mM Tris-HCl, pH 7.0) consecutively at 10 ºC. The flow rate was increased slowly to 19.8 mL min^{-1} till the absorbance reached zero to keep the bed expansion constant. The bed was allowed to settle by stopping the pumping of the washing buffer and the upper adapter was lowered down to pack the bed. The enzyme was eluted with 10 mM $CaCl_2$ in 50 mM Tris-HCl buffer, pH 7.0 at 25 ºC at flow rate of 4.4 mL min^{-1} (50 cm h^{-1}). The bed was regenerated at 70 ºC with 2 M NaCl solution and washed with water in packed bed mode at flow rate of 4.4 mL min^{-1}.

5.4. Results and Discussions

The matrices used in this study were affinity-HEG beads (of same particle size and density distribution as described in chapters 3 and 4; average particle size: 258 µm and average particle density: 1.21 g mL^{-1}) and Streamline DEAE (average particle size: 198 µm and average particle density: 1.18 g mL^{-1}). The particle size and density distribution of affinity-HEG beads and Streamline DEAE are summarised in Tables 5.1. Both beads have Gaussian type particle size distribution (Figure 5.2).

Table 5.1. Comparison of particle size and density distribution of affinity-HEG beads and Streamline DEAE.

Particle size (µm)	Affinity-HEG beads		Streamline DEAE	
	wt % of matrix	Density (g mL^{-1})	wt % of matrix	Density (g mL^{-1})
250- 355	48.5	1.21	Nil	nil
180-250	25.8	1.208	41	1.19
150–180	17.3	1.202	28	1.17
105–150	8.4	1.18	19	1.16
0-105	nil	nil	12	1.15

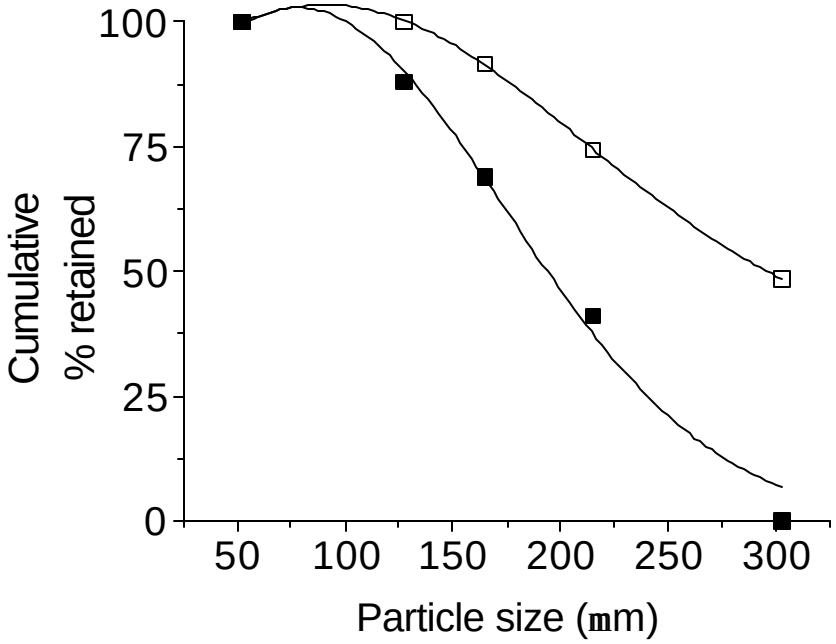


Figure 5.2. Particle size distribution of Streamline DEAE and affinity-HEG beads

■ Streamline DEAE , □ Affinity-HEG beads

5.4.1. Mechanical, microbial, chemical stability and non specific interaction of affinity-HEG beads

Affinity-HEG beads are also resistant to microbial attack. The water-swollen beads were exposed to air for a month and the size and density distribution of these beads were evaluated and compared. There was no change in size and density distribution of these water-swollen beads after exposure to air. Exposure of the affinity-HEG beads to 1M NaOH, 1M HCl and at elevated temperature 121 °C for 30 minutes did not change the size and density distribution of the particles. There was no change of fluidising characteristics of the affinity-HEG beads after exposure to higher flow rate 450 cm h^{-1} for 7 days, which was also confirmed from the size and density distribution balance of these beads before and after fluidisation (Table 5.2). These findings indicate that the affinity-HEG beads are resistant to microbial, chemical and physical environments and are suitable for expanded chromatographic operation.

Table 5.2. Comparison of mechanical stability of affinity-HEG beads with Streamline DEAE.

Particle size (μm)	Before fluidisation				After fluidisation			
	Affinity-HEG beads		Streamline DEAE		Affinity-HEG beads		Streamline DEAE	
	wt % of matrix	Density ($g\ mL^{-1}$)	wt % of matrix	Density ($g\ mL^{-1}$)	wt % of matrix	Density ($g\ mL^{-1}$)	wt % of matrix	Density ($g\ mL^{-1}$)
250- 355	48.5	1.210	nil	nil	48.4	1.210	nil	nil
180-250	25.8	1.208	41	1.190	25.7	1.208	40.5	1.190
150–180	17.3	1.202	28	1.170	17.2	1.202	25.5	1.170
105–150	8.4	1.180	19	1.160	8.3	1.180	17.6	1.160
0-105	nil	nil	12	1.150	nil	nil	8.5	1.150

The beds were fluidised continuously for a week at superficial velocity 428 cm h^{1}. The difference in particle size distribution before and after fluidisation is the loss of particles from the bed.

5.4.2. Effectiveness of flow distribution

To check the bed stability, various distributor plates were fabricated and evaluated. The residence time distribution (RTD) test is a tracer stimulus method used to assess the degree of longitudinal axial mixing (dispersion) in the expanded bed by defining the number of theoretical plates (N). The hydrodynamic characteristics of Streamline DEAE and affinity-HEG beads are compared. The effectiveness of distributor plates of various thickness with the metallic (SS) screen mounted on the side of the distributor plate facing the adsorbent, is investigated using affinity-HEG beads. Use of thin metallic support screen (12 mesh cm^{-2}) devoid of distributor plate had less distribution of the liquid on the bed, which is evident from lesser number of theoretical plates (N). Therefore, distributor plates of 1 mm and 2 mm thickness were fabricated. At lower superficial velocity, the effect of liquid distribution depends upon the density of the holes (Asif et al., 1991). It is suggested that maximum hole density of distributor plate be used for fluidised immobilisation column. The density of holes was calculated on the basis of packed bed porosity. It was assumed that the flow of the liquid passing through the distributor plate was comparable to the flow through the void spaces of a packed bed. Therefore, the ratio of hole cross section

area to distributor plate cross-section area was similar to the packed bed void space through which the liquid passes. The combined effect of perforated SS plate of 1 mm thickness (D_{or} = 0.1 cm, hole density 20.73 holes cm^{-2}) and the support screen imparted more pressure drop and distributed the feed liquid throughout the bed. The number of theoretical plates of the bed containing 1 mm perforated SS plate and the support screen increased with superficial velocity with respect to the support screen. Thickness of the distributor plate had positive effect on the stability of the bed, which was evident from the increase in N. However, the additional effect due to the tapered configuration of the distributor plate (15 ° angle) was not observed from the number of distributor plate measured (Figure 5.3), which is a measure of liquid dispersion in the bed.

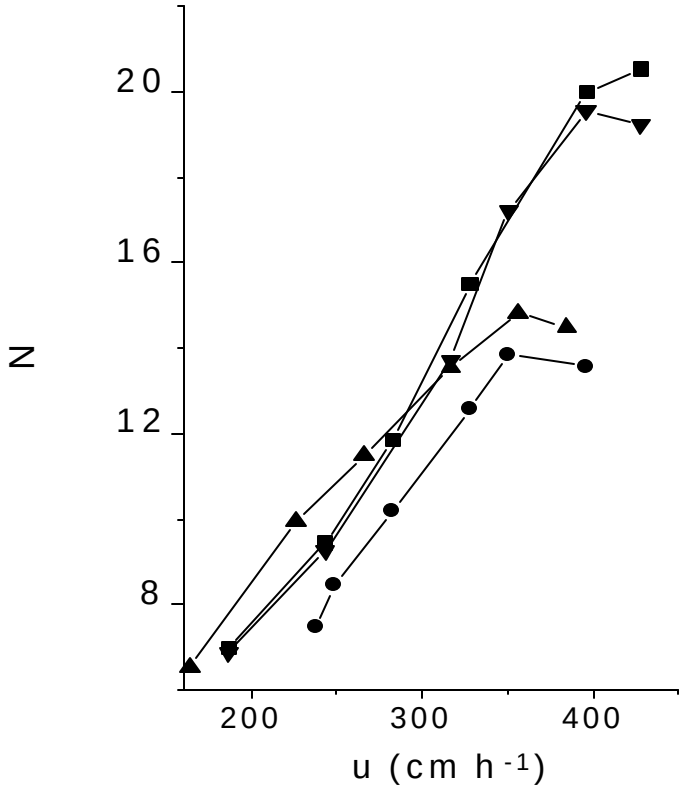


Figure 5.3. Number of theoretical plates (N) with varying superficial velocity (u) of affinity-HEG beads.

● No distributor plate, ▲ perforated SS plate (1mm), ▼ perforated SS plate (2mm), ■ perforated tapered plate (2mm)

The pressure drop was also increased by increasing the thickness of the plate and keeping the diameter and the density of the holes constant. This helps in uniformly distributing the liquid in the bed. The degree of expansion of both the matrices with increase in superficial velocity (u) are shown in Figure 5.4. The N versus u profile was found to increase for Streamline DEAE whereas with affinity-HEG beads N increased initially and then leveled off with increasing superficial velocity (Figure 5.4). As is evident from Table 5.1, the density of affinity-HEG beads varies with bead size. Therefore, the gradation of affinity-HEG beads in the expanded bed was due to density and as well as the size of the beads.

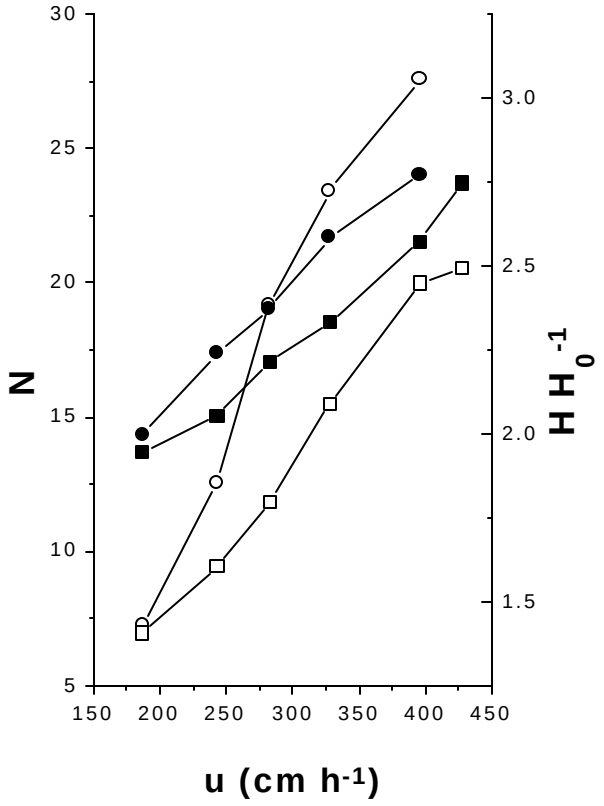


Figure 5.4. Comparison of degree of expansion (H/H_0) and number of theoretical plates (N) of Streamline DEAE and affinity-HEG beads.

● H/H0 Streamline DEAE, ■ H/H0 affinity-HEG beads, ○ N Streamline DEAE, □ N affinity-HEG beads

The axial dispersion coefficient (D_{ax}), which is a measure of liquid mixing in the bed, was measured for both the matrices using the tapered distributor plate under various flow rates. At lower flow rates, the dispersion in both beds were similar. The dispersion in affinity-HEG beads increased and exceeded that of Streamline DEAE, with increase in degree of expansion (Table 5.3). The number of stages per settled bed height (N/H_o) is a measure of liquid dispersion. The value of N/H_o was in the range of 93 to 273 and 97 to 367 for affinity-HEG beads and Streamline DEAE, respectively.

Table 5.3. Bed expansion characteristics of affinity-HEG beads and Streamline DEAE at various flow velocities.

u ($cm\ h^{-1}$)	HEG beads					Streamline DEAE				
	H (cm)	N	σ^2	$(D_{ax})_{X10}{}^6$ ($m^2\ s^{-1}$)	N/H_0 (m^{-1})	H (cm)	N	σ^2	$(D_{ax})X10^6$ ($m^2\ s^{-1}$)	N/H_0 (m^{-1})
187	14.6	7.0	0.143	7.83	93	15.0	7.25	0.138	7.68	97
243	15.4	9.5	0.106	7.73	127	16.8	12.56	0.08	6.16	167
283	16.6	11.9	0.084	7.52	159	17.8	19.20	0.052	4.88	256
328	17.5	15.5	0.064	6.92	207	19.4	23.45	0.043	4.90	313
396	19.3	20.0	0.050	6.92	267	20.8	27.56	0.036	5.30	367
428	20.6	20.5	0.049	7.64	273	nd	nd	nd	nd	nd

N= number of theoretical plates, u= liquid superficial velocity, H= bed height under expansion, H_0= packed bed height and D_{ax}=axial dispersion coefficient

This value is supported by Lindgren et al. (1993), who showed hydrodymnamic properties of commercial material (Streamline) i.e. ~200 stages/m (N/H_o). The Richardson-Zaki coefficients for affinity-HEG beads were higher (6.62) than that for the Streamline DEAE beads (6.53). This indicates that at the same degree of expansion higher superficial velocity is observed for affinity-HEG beads than for the Streamline DEAE. The calculated terminal velocity was also higher for affinity- HEG (37.95 cm min^{-1}) beads than the Streamline DEAE matrice (31.55 cm min^{-1}). Karau et al. (1997) investigated the expanded bed characteristics of particles of varying

diameter. The authors observed experimental terminal settling velocities (u_t) in the range of 22.1 to 40.7 cm min^{-1} and Richardson-Zaki coefficients (n) between 4.9 to 5.5, respectively. Similarly, the values of n and u_t reported in buffer for particles of size 247 μm were 4.79 and 25.44 cm min^{-1}, respectively (Chang and Chase, 1996a). A comparison of bed expansion characteristics of affinity-HEG beads with Streamline DEAE is summarised in Table 5.3.

5.4.3. Feed characteristics

α-CGTase was produced by fermentation of *Klebsiella pneumoniae pneumoniae* in a 1 L fermenter at 600 rpm, in a fed-batch mode as discussed in Section 2.3.3.8. The dry cell mass concentration, protein concentration, enzyme activity, density and viscosity of the fermented broth are listed in Table 5.4. The findings that prompted the selection of the fermented broth of α-CGTase as a model system for the purification using expanded bed mode was described in Section 1.10.2.

The feed characteristics comprises of the properties i.e. dry cell weight, protein concentration, enzyme activity and density. DNase is reported to lower the viscosity drastically of the crude feedstocks (Garke et al., 2000). However, the feed characteristics are varied with dilution, without the help of DNase addition. The values of these parameters decreased on dilution of the fermented broth. Viscosity of the diluted fermentation broth was found to decrease with dilution up to 5 fold and then to level off (Table 5.4). The exact reason for this anomalous behaviour of viscosity upon dilution is not known. However, *Klebsiella pneumoniae* produces extracellular polysaccharide bio-flocculent, which might participate *in vivo* bacterial aggregation (Kuniho and Ryuichio, 1999). The cell aggregation of *Klebsiella pneumoniae pneumoniae* may not decrease beyond 5 fold dilution of the fermented broth with the buffer, which is responsible for higher viscosity.

Table 5.4. Physical Characteristics of the feed.

Dilution factor	Dry cell wt. ($g\ L^{-1}$)	Protein ($mg\ L^{-1}$)	Enzyme activity ($U\ mL^{-1}$)	Density ($g\ L^{-1}$)	Viscosity (mPa s)	Shear Rate (s^{-1})
1	65.20	13.42	34.20	1.023	98.20	0.075
2	32.40	6.74	17.12	1.015	42.04	0.476
5	13.80	2.65	6.81	1.013	21.67	0.923
10	6.42	1.32	3.40	1.010	21.59	0.926
20	3.20	0.65	1.70	1.005	21.05	0.950

5.4.4. Measurement of uptake rate of enzyme

The effect of adsorption rate of the various diluted fermentation broths on the affinity-HEG beads was measured from the uptake rate of α-CGTase in a batch adsorption system. The viscosity of the broth decreased with increasing dilution till 5 fold dilution and then remained invariant till 20 fold dilution (Table 5.4). The reason may be due to the fact that the cell aggregation of *Klebsiella pneumoniae pneumoniae* does not decrease further with addition of the buffer beyond 5 fold dilution of the fermented broth. Therefore, the intraparticle diffusion in case of 5, 10 and 20-fold dilution are similar. The other parameter, which affects the rate, is adsorption dissociation constant. Our previous study on batch adsorption of the clarified fermented broth had indicated that the adsorption isotherm followed Langmuir isotherm. The maximum adsorption capacity was 666 $U\ g^{-1}$ of the adsorbent (containing 6.65 mg of ligand g^{1} of adsorbent) and the dissociation constant was 6.21 $U\ mL^{-1}$ respectively (Section 4.4.1.).

From Figure 5.5, it can be understood that up to double the dilution, intraparticle diffusion was dominant and that the rate of adsorption was slow. On the other hand, on comparing 5, 10 and 20-fold diluted feed samples, it bears out that the adsorption kinetics was dominating. Uptake rate of α-CGTase present in 5 fold-diluted feed was faster than 10 and 20 fold diluted feed owing to the fact that the

slower adsorption occurs when the enzyme concentration in the feedstock is lower than the K_d value.

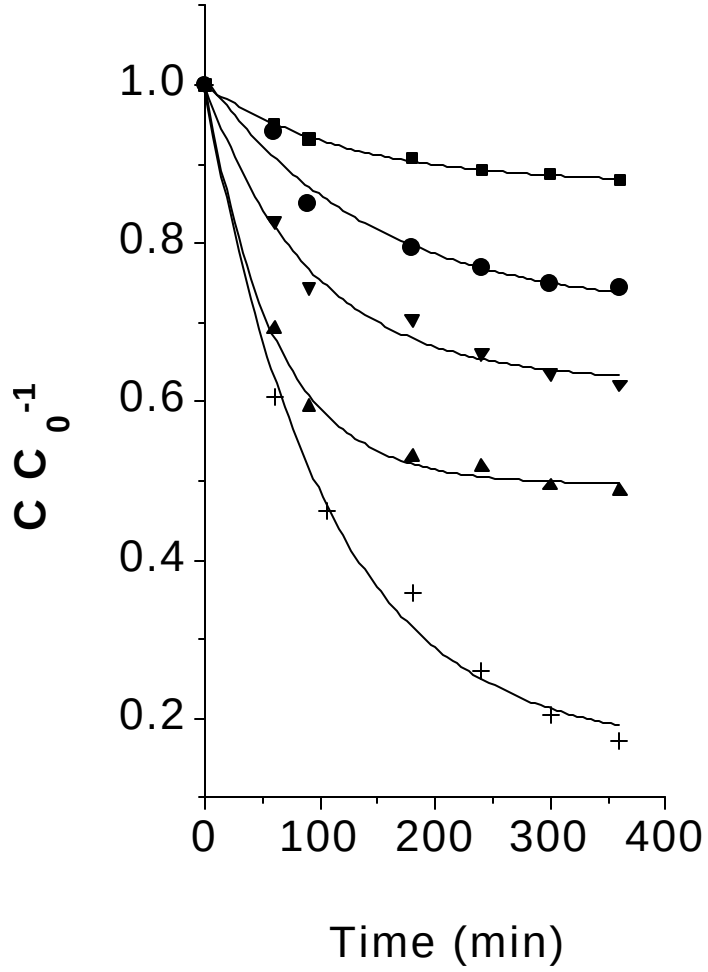


Figure 5.5. **Uptake curve for adsorption of α-CGTase to the affinity-HEG beads at different dilution of the fermented broth.**

C is the residual enzyme activity in the liquid phase at time t (U mL^{-1}) and C_0 is the initial enzyme activity of the diluted fermented broth (U mL^{-1}). ■ undiluted, • 2 fold diluted, + 5 fold diluted, ▲ 10 fold diluted, ▼ 20 fold diluted fermented broth

5.4.5. Frontal analysis in expanded bed

Similar values of axial dispersion and breakthrough profile at double expansion of the bed irrespective of the viscosity, density and superficial velocity of the feed has been reported (Chang and Chase, 1996a). Therefore, the bed was

expanded to double of its settled bed height in all experiments (settled bed volume 39.8 mL, settled bed height 7.5 cm). In this expanded bed operation, the sample was fed continuously into the column until the available capacity of the column was exhausted and adsorbate began to appear on the column outlet (Figure 5.6) The variation of the concentration of the adsorbate in the column outlet as a function of time is known as breakthrough curve, and the measurement of breakthrough curves for such system is referred to as frontal analysis. The Frontal analysis was employed to determine the dynamic adsorption capacity (DAC), dynamic adsorption rate (DAR), breakthrough volume (V_b), breakthrough time (t_b) as well as the shape of the breakthrough curve. The influence of dilution factor of the fermented broth at double expansion of the bed was investigated by varying the flow velocities. The application of the feedstock was carried out at pH 9.0 for affinity-HEG beads till the enzyme activity of the effluent α-CGTase reached that of the feed CGTase. The bed dynamic adsorption capacity (DAC) was calculated by integrating the curve (volume applied versus effluent CGTase concentration) when the effluent enzyme activity reached 5% of the feed enzyme activity. The efficiency of separation of CGTase was evaluated by calculating dynamic adsorption rate (DAR) defined as the ratio of DAC and breakthrough time.

$$q^* = \int_{v=0}^{v_{rf}} \frac{(C_0 - C)\partial V}{V_f} \qquad (5.1)$$

where, C_0 = initial concentration of adsorbate (U mL^{-1}); C = enzyme concentration in the effluent at time t (U mL^{-1}); V = volume of the feed applied to the column (mL); V_f = volume of the fluidised or the expanded bed (mL); v_{tf} = total volume passed through the bed (mL); and q* = units of adsorbate bound to the adsorbent at equilibrium (U mL^{-1}). The amount of enzyme applied to the column is given by

$$m = C_0.t.Q = C_0.V_{rf} \qquad (5.2)$$

where, Q is the volumetric flow rate (mL min^{-1}).

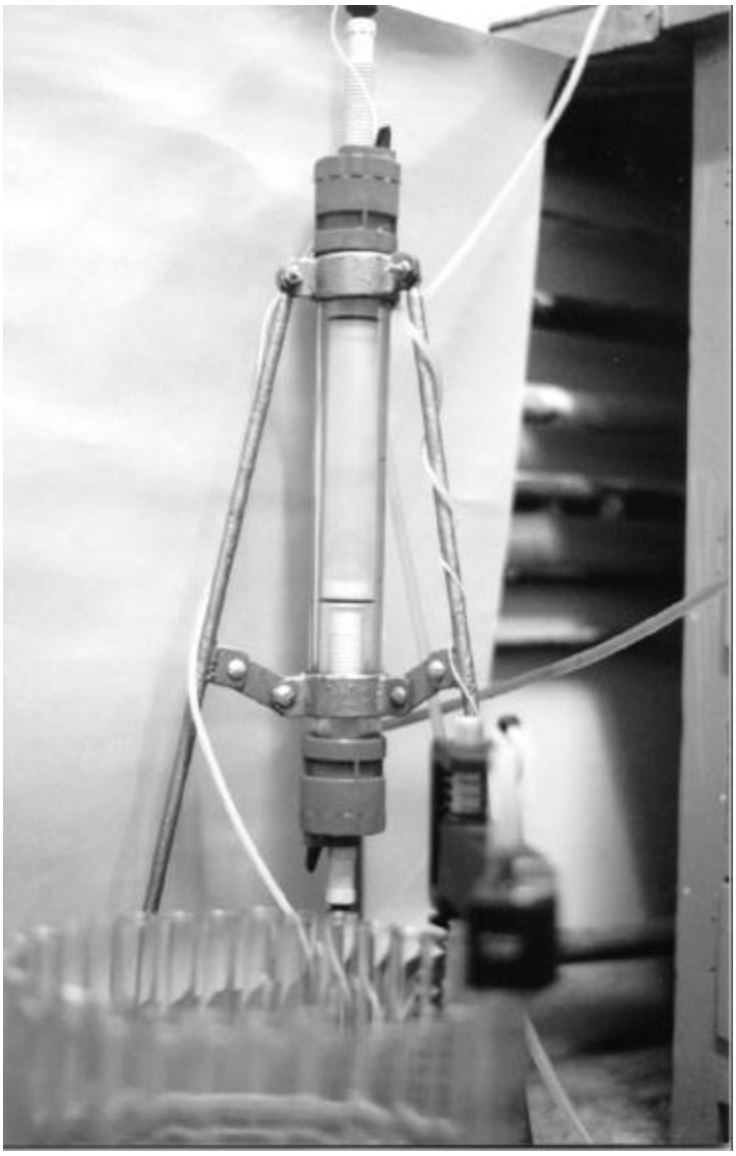


Figure 5.6. Pictorial view of experimental set up of Expanded Bed Adsorption.

In the graphical integration method, the area behind the curve also includes a contribution from the dead volume as well as the bed voidage. The bed voidage was considered as 0.4 of the total column volume and was same as determined in our previous work of inverse gel filtration packed bed porosity (different column with same tubing). Following integration of the area behind the breakthrough curve, the amount of the enzyme bound then was determined by subtraction. Loading of undiluted fermented broth (98.2 mPa s, shear rate 0.075 s^{-1}) at flow rate 5.8 mL min^{-1} (65.6 cm h^{-1}) resulted in multichanelling of the bed and bed collapsed to close to the

settled bed height. Introduction of double diluted feed (42.04 mPa s, shear rate 0.476 s^{-1}) resulted in channelling of the feedstock which could not be overcome either by sudden change in pulse of flow of feed stock or by reversing the direction of the flow. Similar observation has been noted in the recovery of Annexin V using unclarified *E. coli* homogenate (Barnfield Frej et al., 1994) and purification of glucose-6-phosphate dehydrogenase from unclarified yeast homogenates (Chang and Chase, 1996. However, trouble free expansion of the bed occurred when the viscosity was close to 21 mPa s.

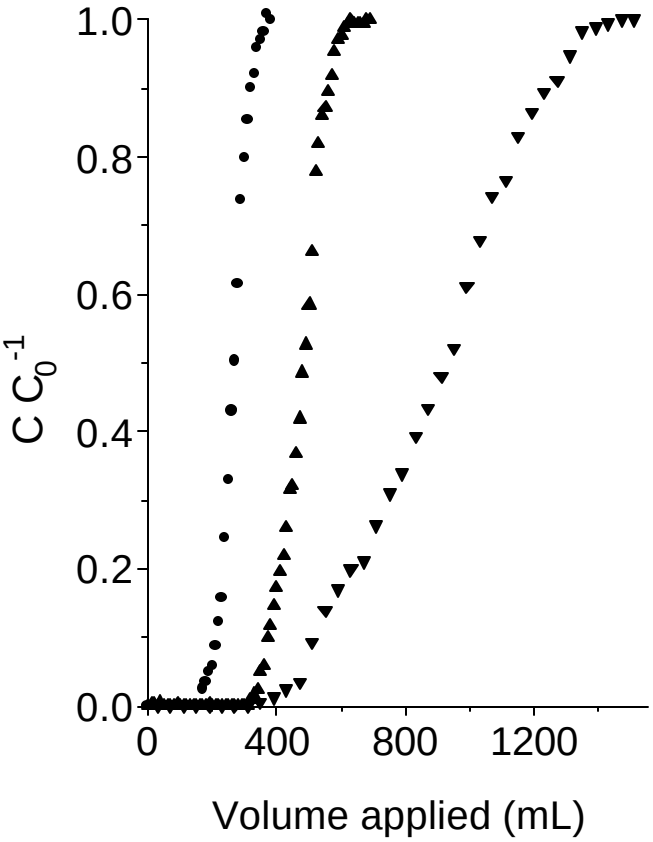


Figure 5.7. Breakthrough curve of a -CGTase on affinity-HEG at different dilutions of the fermented broth.

C is the enzyme activity in the effluent at time t (U mL^{-1}) and C_0 is the initial activity of enzyme (U mL^{-1}). • 5 fold diluted-105 cm h^{-1}, ▲ 10 fold diluted-130 cm h^{-1}, ▼ 20 fold diluted-152 cm h^{-1}

Sharp breakthrough curve was observed at 5 and 10-fold dilution of the fermented broth (Figure 5.7). This may be due to lower viscosity of the diluted feed. Comparing 5, 10 and 20 fold diluted feed samples, the feed density, cell, protein and

enzyme concentration decreased with increase in dilution, whereas there was no appreciable change of viscosity with dilution (Table 5.4).

The required superficial velocity of 5, 10 and 20 fold diluted feed sample for double expansion of the bed increased with increase in dilution due to decrease in feed density with increased dilution (Table 5.5). The V_b, t_b, q_b, DAC and DAR of various diluted samples were calculated and are tabulated in Table 5.6. The breakthrough volume and breakthrough time increased with dilution. The DAC and DAR value for 5-fold diluted feed sample gave the maximum figure due to the dominance of adsorption kinetics as evident from the uptake curve (Figure 5.4). The bed equilibrium capacities of the feed on expanded bed were less than the maximum batch equilibrium capacity. This is perhaps due to intraparticle hindrance (at higher viscosity of the feed) on expanded bed, which slowed the adsorption. On the other hand in batch adsorption, the capacity of the adsorbents were calculated till equilibrium, so has higher value. The q_b value in expanded bed of 5,10 and 20 times feed dilutions are 92.8%, 79.1% and 54% of the q_b value in packed bed, respectively. The DAC values of 5, 10 and 20 times diluted feed on expanded bed are 87.9%, 77.7% and 72.0% of DAC value on the packed bed. Dilution of the fermentation broth decreased the viscosity, decreased the enzyme activity below the K_d value, and thereby slowed the adsorption rate. The decreased slope of the breakthrough curve at 20-fold dilution is due to the slower adsorption kinetics on the bed.

Table 5.5. Comparison of breakthrough points (volume/time) on affinity-HEG beads at various dilution of fermented broth.

H_0 (cm)	Dilution Factor	u (cm h^{-1})	V_b (mL)	t_b (min)	DAC (U mL^{-1})	DAR (U $mL^{-1}min^{-1}$)	q_b (U mL^{-1})
7.5	5	105.0	194.0	20.90	33.86	1.62	46.85
7.5	10	130.0	355.0	30.90	30.46	0.99	39.24
7.5	20	152.0	447.2	33.27	20.78	0.62	36.37

H_0 is the settled bed height and u is the superficial velocity of the feed. V_b and t_b are the breakthrough volume and time of the bed considering effluent enzyme concentration 5 % of the feed enzyme activity. DAC and DAR are the dynamic adsorption capacity and rate of the bed respectively. q_b is defined as maximum bed adsorption capacity.

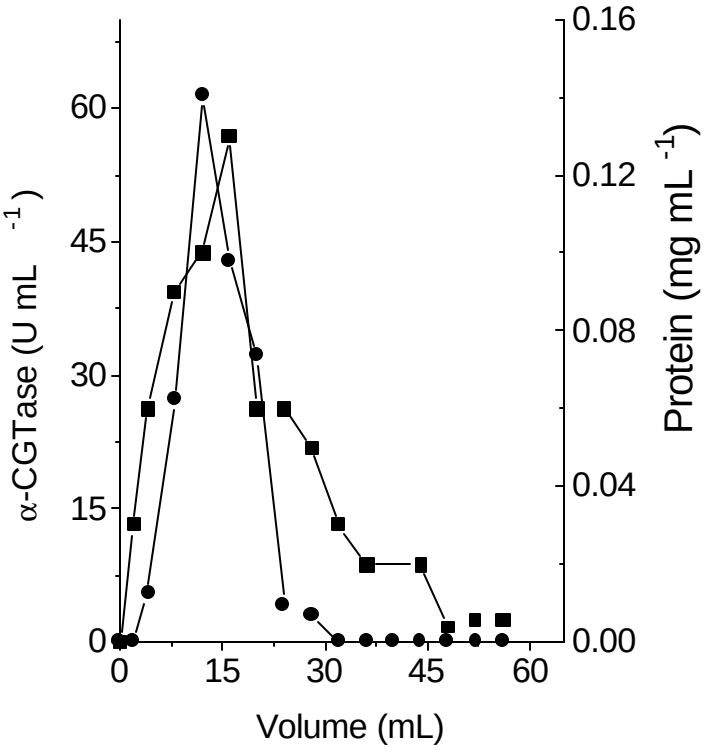


Figure 5.8. Elution profiles of a -CGTase and protein.

• CGTase activity (U mL $^{-1}$), ■ Protein concentration (mg L^{-1})

The adsorbed enzyme from 5-fold diluted fermented broth eluted with 10 mM $CaCl_2$ in 50 mM Tris-HCl at 25 °C, at a flow rate of 4.4 mL min^{-1} (Figure 5.8) yielded 94.7 fold increase in purification as compared to the feed with 37.92% recovery of the enzyme (Table 5.6).

Table 5. 6. Summary of purification of CGTase on expanded bed from 5 fold diluted sample of fermented broth

Steps	Total Volume (mL)	% recovery	CGTase (U mL^{-1})	Protein (mg mL^{-1})	Specific Activity (U mg^{-1})	Fold purification
FB (5X)	380	-	6.81	2.65	2.57	1
EBC	28	37.92	25.25	0.10	243.47	94.7

5x represents 5 fold dilution

This purification of α-CGTase on expanded bed was compared to the reported literature of purification of this enzyme by various methods. An UF concentrated CGTase was applied to a modified starch column and the enzyme was eluted from the

column with 10 mM $CaCl_2$ (in 50 mM Tris-HCl, pH 7.5) at 25 °C with 82% recovery (Gawande, 2000). This increased recovery of the enzyme resulted as the modified starch (as adsorbent) liquefied during elution and came out easily from the pores. Starch, cyclodextrin produced from liquefied starch and $CaCl_2$ provided the extra stability to the enzyme. The purification of the enzyme from the starch column was 29 fold. There are a number of references on CGTase purification using α-CD as ligand on packed bed. In one report, α-CD was coupled to Sepharose 6 B via dioxirane to purify CGTase (Laszlo et al. 1981). This one step purification of α-CGTase yielded 113-fold purification, with 90-92% recovery. In another work, Sepharose 6FF-α-CD matrix was used to purify of α-CGTase directly from cell free culture broth (Wind et al., 1995). 35-fold enzyme purification, with 79% enzyme recovery, was noted. Crude extract was adsorbed on starch, the desorbed enzyme was concentrated by $(NH_4)_2SO_4$ precipitation and finally purified on Sepharose 4B-α-CD (Ferrarotti et al., 1996). A 59.7 fold purification with 54 % recovery of the enzyme was reported by this method. In all these CD affinity based separation of CGTase, the enzyme was eluted with the corresponding cyclodextrin solution, which resulted in increased recovery.

Purification of α-CGTase on affinity-HEG beads on expanded bed was compared with packed bed chromatography described in chapter 4. The specific activity and % recovery of the purified enzyme obtained from packed bed (393.3 U mg^{-1} and 48%) was higher than that from expanded bed (243.5 U mg^{-1} and 38%). The exact reason of the greater recovery and specific activity of the protein purified using packed bed rather than expanded bed is not known.

Chapter 6

General discussions and concluding remarks

Expanded bed operation for protein purification is becoming popular in an industrial scale. There are number of industries which have tested a number of proteins to purify on pilot and large scale and many of them are successful (EBA '96). A number of academic institutes are also upgrading the system and involved in many of other novel applications. Three academic institutes of India (NCL, Pune; UDCT, Mumbai and IIT, New Delhi) are involved in working with expanded bed. The biopharmaceutical industries of India are also taking interest to purify their products in a large scale (EBA & cGMP Biopharmaceutical Conference, 2001, Hotel Orchid, Mumbai, India). Apart from these therapeutic proteins, in this gene therapy age, there will be a huge demand of vectors for functional expression of genetic constructs to target cells. These vectors will eventually be synthesized and readily assembled in customised producer or packaging cell lines, recovered and purified to an appropriate specification and formulated into stable states suitable for administration to patients at some later date. These vectors should be ultra pure and cheap. The price of these products can be minimised by application of expanded bed purification of proteins. Expanded bed chromatography has been successfully used in the recovery of plasmid DNA and adenovirus also. In most of the large molecular weight protein separation using Streamline matrices suffers diffusion problem (the breakthrough curve is more inclined to x-axis, Thommes et al., 1995). For example purification of IgG molecules on Streamline SP shows hindered diffusion. The Streamline matrices will have diffusional problems to the other large molecules e.g. IgM, α_2-macroglobulin, IgA. The viral vaccines produced on mammalian cell are ought to be purified and concentrated in an industrial scale. EBA is a suitable method for this purpose. These large sized molecules (300 to 400 nm) should be purified on supermacroporous beaded matrices.

The present thesis presents extensive studies of protein purification on expanded bed. This thesis covers many topics: 1) Synthesis of matrix, preparation of affinity matrix and their evaluation for expanded bed chromatography; 2) characterisation of the affinity matrix by inverse size exclusion chromatography; 3) determination of some key parameters (pore diffusion coefficient, optimum ligand

concentration and dissociation constant for ligand-protein complex etc.) involved in protein purification. 4) Design and fabrication of distributor plates, their evaluation in an expanded bed. Hydrodynamic, chemical and physical comparisons of in-house-made matrices with Streamline matrices were also studied. Application of feedstock containing α-CGTase to the in-house-made affinity matrices and its purification was the final assessment for completion of this thesis.

Synthesis of matrices (Chapter two)

The price of the commercially available matrices is exorbitantly high. The composite Streamline matrices tend to lose their quartz particles and thereby the density of the matrices decreases. These quartz particles were mixed up with the product and most of the time blocks the upper adapter. Our present investigation deals with synthesis of some of the dense matrices and evaluating for chromatographic operation. Out of four matrices synthesised, Copolymer beaded matrices of HEMA and EGDM (100:25, mole ratio) (HEG beads) was found suitable for expanded bed chromatographic operation due to its higher density and low non-specific interactions with proteins. The matrix was found to be resistant to a number of chemicals and no difference in swelling was observed by varying salt concentration and at a number of pHs. This matrix was coupled to α-cyclodextrins (CDs) by various diisocynates.

Preparation of affinity matrices and their evaluation (Chapter two)

Diisocyanates are the bifunctional reagents, which can be effectively coupled to the molecules having -OH and $-NH_2$ groups. The affinity matrices were prepared by derivatisation of HEG beads with a number of diisocyantes. Tolylene diisocyanate was found to be most reactive diisocyanate in terms of higher coupling of HEG beads with α-CDs and low non-specific interactions. The molar ratio of reactive monomer to the crosslinking comonomer (4:1) was selected based on its low non-specific interaction. The affinity matrix so prepared was evaluated for the clean-in-place procedures by various reagents.

Pore characterisation of the affinity matrices by inverse size exclusion chromatography (Chapter three)

The matrices are ought to be characterised for permeation of various proteins. The other important aspect of it is to what percentage of the pore the target protein is transported through. We observed irreversible binding of blue dextran to the HEG beads in aqueous size exclusions. Therefore, the complete profile of the protein exclusion could not be evaluated by this method. For this reason, polystyrene and 1,4-dioxane as the probe solute and eluting solvent, respectively to permeate through the pores of the matrices. A relation was built between the solute size and the % cummulative pore volume. The theoretical model fits the experimental result and was extrapolated for the protein data. Thus, a complete exclusion data of proteins was evaluated on HEG beads. The higher pore sizes of the HEG beads in 1,4-dioxane were predicted due to its higher swelling in dioxane. The pore size of the HEG beads shows its macroporosity.

Determination of key parameters used in chromatographic separation of proteins (Chapter four)

Although the pores of the matrix were characterised, the significance of the physical observation by hindrance of protein through the pores was not evaluated. This is called as pore diffusion, which dictates % of protein hindrance through the pores of the matrix. Therefore, effective diffusion coefficients were evaluated with four standard proteins and the effective diffusivity of α-CGTase was calculated from the empirical correlation developed between the molecular weight of the proteins and the ratio of effective to molecular diffusion coefficients. The value of the effective diffusivity showed transportation of α-CGTase in the pores was restricted to 76.3%.

It has been reported that increasing ligand (α-CD) concentration on the matrix decreased recovery and specificity of the enzyme in shake flask experiments. Therefore, α-CD concentration on the matrix was optimised and kept 6.65 mg g^{-1} of the adsorbent in all subsequent adsorption of batch as well as on chromatographic studies.

The measurement of ligand-protein dissociation constant is an important parameter in deciding the minimum concentration of the target protein in the broth to be captured by the affinity matrix. The batch adsorption studies of α-CGTase on the affinity matrices synthesized in this study showed maximum binding 666 U g^{-1} of the dried adsorbent. The measured dissociation constant from this study also indicates that the pores of the matrix were favourable in the dilute concentration of the enzyme in the fermented broth.

A number of elution procedures of α-CGTase were investigated on the adsorbents. One hundred and fifteen fold purification of the enzyme was achieved with 48% recovery by 10 mM $CaCl_2$ in 50 mM Tris-HCl buffer (pH 7.0) as eluent.

Design, fabrication and evaluation of the distributor plate of the expanded bed column (Chapter five)

The price of the commercial columns is high. Distributor plate of the column is the heart of the process hardware. Distributor plate of the expanded plate exerts additional pressure drop and thereby distributes the feed liquid inside the bed. Therefore, the thickness of the distributor plate was optimised for decreased liquid dispersion. Distributor plate of 2-mm thicknees in a 26-mm diameter column was found optimum. The material chosen for the fabrication of the distributor plate was deldrin (polyacetal) since its price is low, easily machinable and chemically resistant to all the liquids involved in the process.

Hydrodynamic, chemical and physical properties of the in-house-made matrices (Chapter five)

The number of theoretical plate is a measure of liquid dispersion and bed stability of expanded bed. The variation of number of theoretical plates with superficial velocity of affinity-HEG beads was comparable with the Streamline DEAE matrix. The mechanical stability, which is a measure of physical characteristics, of the affinity-HEG matrices was found better in fluidised conditions than the Streamline matrices. The matix is also found microbially more inactive.

Application of the real system (chapter five)

The purification of the model protein in the fermentation broth had several problems in dealing with centrifugation and microfiltration. Feeding undiluted fermented broth resulted channelling and colapse of the bed. This is due to increased viscosity of the feed. Though treatment of nuclease is one of the methods to reduce the viscosity, we opted for diluition of the feed. Feeding of 5-fold diluted fermented broth to the column containing affinity matrices of settled bed height 7.5 cm (ID 26 mm and length 42 cm) at double expansion of the bed resulted in sharp breakthrough curve of the enzyme. The enzyme was eluted from the bed in 50 mM Tris-HCl buffer containing 10 mM $CaCl_2$ at 25 °C in packed bed configuration. The enzyme α-CGTase was purified on EBA with 95 fold purification and 38% of the enzyme was recovered

Further work

The present study of protein purification containing α-CGTase was selected since all the biochemical characteristics have been well studied in our laboratory and the Biochemical Engineering group is engaged in commercialisation of the enzyme and the product cyclodextrin. The in-house-made matrix, we studied was found suitable for expanded bed application and some of the Indian companies are interested to purchase this product. The companies are interested in purification of biotherapeutics. The future work will involve optimisation of process variable for these therapeutics by this technology. Since, the matrix is more robust than the Streamline matrices, it can be explored in an industrial scale for purification of various proteins. The decrease of non-specific interaction and increase of pore size (may be heplful for purification of large biomolecules, e. g. vectors and viruses) can be manipulated according to the need by decreasing the amount of crosslinking comonomer.

Chapter 7

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