OPTIMISATION AND APPLICATIONS OF EXPANDED BED ADSORPTION CHROMATOGRAPHY.

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EXPANDED BED ADSORPTION CHROMATOGRAPHY.

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Summary

Expanded bed adsorption (EBA) is a separation technique in which biomolecules are purified from crude, particulate-containing feedstocks without prior clarification or concentration. Operation of the bed in an expanded mode increases the void fraction and this allows particles (cells, cell debris and other particulate material) to pass through the column whilst the target product is being adsorbed. Thus historically expanded bed chromatography was developed to avoid the need for filtration prior to adsorptive separations. In addition to applying EBA to capture molecules, this thesis also shows that EBA can be applied to the purification of nano-particles. The hydrodynamic bed stability and product capture were first optimised followed by the purification of glutathione-s-transferase (GST) and Bacteriophage T7, both of which were propagated in E.coli.

The hydrodynamic bed stability was investigated by examining the effect of bed height, flow velocity, column diameter and bed aspect ratio on axial dispersion. It was observed that axial dispersion decreased with increasing bed height, flow velocity and bed aspect ratio. The effect of column diameter was not conclusive. However small diameter columns showed far better stability than larger columns if the same volume of beads were used. The optimisation of product capture was investigated by examining the effect of loading modes, cell density, feed concentration and solid content on the dynamic binding capacity, purification factor, product concentration and feed clarification of glucose-6-phosphate dehydrogenase (G6PDH), using Streamline DEAE as adsorbent. The two loading modes showed minor differences with loading at constant flow showing higher purification factor and binding capacity in high cell density suspensions. The binding capacity increased slightly with feed concentration and cell density. Product concentration was similar irrespective of experimental conditions and the solid content had negligible effect on G6PDH purification.

GST purification involved use of in-house made affinity matrices and a commercially available Streamline™ DEAE. Using these affinity matrices, GST was purified to homogeneity in a single step. These matrices were also better than commercially available GSH-Sepharose 4B. The success of EBA in the purification of particles was investigated with Bacteriophage T7. The effect of temperature, loading pH, elution pH and divalent cations were investigated. Temperature and the loading pH had significant impact on phage adsorption and recovery. There was selective elution of infectious phage from cells, cell debris and non-infectious phage.

Key words: Expanded bed adsorption, Glutahione-s-transferase, Bacteriophage T7 purification, Protein purification and hydrodynamic bed stability.

DEDICATION

To

my mother,

without whose sacrifice, persistence

and patience I would not

have come this far,

this early.

RESEARCH COMMUNICATIONS AND PUBLICATIONS

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CHAPTER ONE

INTRODUCTION AND LITERATURE REVIEW

1.0 INTRODUCTION

All companies require profits to invest in new technology, provide employment, pay taxes and reward shareholders. To make profits in highly competitive markets they must optimise their operations to reduce the cost of manufactured goods. This is particularly true for biotechnology industries that manufacture labile, easily degradable products that are usually found in only small quantities in nature. That the price of enzymes (e.g. alcohol dehydrogenase) is two to three times higher than that of precious metals (e.g. gold) is due to the high cost of producing the enzymes. Hence, optimisation of protein purification processes will reduce the production cost, so increasing the return to the company.

To optimise product capture in protein purification processes, a clear understanding of the biochemistry of the proteins is essential (Osterlund 1997). Proteins are polymers comprised of amino acids (Lehninger 1981). The side chain of these amino acids can be hydrophobic (e.g. phenyalanine, tyrosine, trptophan, isoleucine, methionine, valine) and seven of the amino acids are readily ionizable (i.e. histidine, aspartic and glutamic acids, cysteine, tyrosine, lysine and arginine). A protein can also be a dimer or tetramer and has a three dimensional structure (Lehninger 1981). Hence the chemistry of proteins results from the amino acids and the three dimensional nature of the protein molecule.

To purify a single protein, one has to understand its biochemistry. Unfortunately accurate biochemical data for any protein can only be produced by working with pure samples (Scopes 1996). The problem that was faced by the pioneers of protein purification was how to come up with a homogeneous sample of a protein when nothing about that protein was known. It is no surprise, then, that crude methods were employed. From such humble

beginnings protein purification grew into an important area and with more experiments much information about proteins was accumulated.

Batch as well as column purification processes can be used in the purification of proteins. Mass transfer in batch modes is low compared to column separations (Gailliot 1990, Chase 1992, Chang 1993, Osterlund 1997). This is because most mass transfer processes are more efficiently executed under plug flow conditions which give high contact efficiencies (high number of theoretical plates). In batch modes, it is aimed to achieve perfect mixing. It has been shown that for a given amount of mass transfer, an extraction column with plug flow would be shorter than the one behaving as a perfectly mixed tank (Micklenburgh 1975). This means for any process volumes and using the same volume of adsorbent material, plug flow systems would require less process times than perfectly mixed systems. As such most purification processes are conducted in columns.

Today protein purification is based on molecular parameters such as

a) hydrophobicity (Smith 1996, Shaltiel 1974, Porath 1973, Staby 1996, Feng 1996)
b) molecular size (Porath 1959, Minelli 1991, Siegel 1966, Abe 1991)
c) solubility (Hofland 2000, Yarmush 1992, Inouye 1993, Morimoto 1992, Sue 2000)
d) affinity for other bio-molecules or metals (Arnold 1991, Scouten 1985, Clemitt 1998)
e) net electric charge on their surface (Chang 1996, Hansson 1994, Frej 1994, Finette 1998, Chang 1996b, Raymond 1998)

Consequently a variety of adsorbents that could effectively interact with the proteins (depending on their molecular properties) were developed. This led to the development of unique methods for protein purification. Section 1.1 is a general summary of these methods and how they are applied in the purification of proteins.

1.1 A SUMMARY OF METHODS USED IN THE PURIFICATION OF PROTEINS

1.1.1 Purification of proteins based on their hydrophobicity

Hydrophobic Interaction Chromatography (HIC) was developed in the early seventies independently by Shaltiel (1974) and Porath (1973). It was discovered unintentionally during the development of affinity adsorbents. During the testing of affinity adsorbents, some matrices with non-polar spacer arms (used to couple the ligand to the matrix) were used as controls. To the surprise of the researchers some controls adsorbed as much protein as the affinity ligands (Shaltiel 1974). As there were no charges on the matrix, it was clear that there was some form of interaction between the protein and the non-polar spacer arm. This is what is now called hydrophobic interactions. Since then HIC has become a popular technique (Queiroz 1996) for the large scale separations of proteins and biopolymers (Staby 1996, Feng 1996). Queiroz investigated the effect of polyethylene glycol (RMM 10 000) as a hydrophobic ligand for the purification of a lipase. Although yield was good (79%), the purification factor was 1.1. Perhaps this is due to the long chain of hydrocarbons used as a hydrophobic ligand. HIC has been applied to the purification of a wide variety of enzymes such as alcohol dehydrogenase (Smith 1996), lysozyme and cytochrome *c* (Feng 1996). Feng investigated the effect of sample overloading on HIC matrices. His work showed that as more sample was loaded, retention time decreased. His work also demonstrated the importance of preparing the sample in the loading buffer. Smith (1996) conducted a comparative study on the performance of HIC against ion exchange for the purification of alcohol dehydrogenase. The HIC matrix showed a better purification factor (7.6) and a better yield (93%) than the ion exchange matrix (0.19 and 3 respectively). This work highlights the fact that for some proteins, especially those with strong hydrophobicity, HIC matrices may be the best choice.

HIC makes use of the hydrophobic side chains on proteins. The interaction between hydrophobic groups can be explained thermodynamically. A chemical process is energetically favourable if the change in free energy (ΔG) of the process is negative. ΔG is defined as

$$\Delta G = \Delta H - T\Delta S \qquad (1.1.1)$$

where H is the enthalpy of the process, T is absolute temperature and S is entropy.

When a protein P, with non-polar side chains, is put in water, the water molecules become ordered around the hydrophobic patches on the protein. The process can be represented as

$$P + nH_2O \longrightarrow P.nH_2O \quad (1.1.2)$$

ΔS for this process is negative because of the smaller number of moles on the right of this process. This implies ΔG has increased but it is still negative since this process is known to occur (Scopes 1994) which means ΔH is negative.

When two solubilised hydrophobic proteins come together, they tend to interact and the process can be represented as

$$P.nH_2O + P'mH_2O \longrightarrow P\text{-}P' + (n+m)H_2O \quad (1.1.3)$$

The number of moles on the right of this equation is higher than the number of moles on the left, hence ΔS for this process is positive and large. Therefore ΔG has decreased (is more negative) and hence the process is energetically favourable. However ΔH for 1.1.3 is the summation (with negative sign) of ΔH for the solubilisation of the two proteins. From equation 1.1.2, ΔH is negative, thus ΔH for equation 1.1.3 is positive. Therefore the actual sign of ΔG depends on the magnitude of ΔS and ΔH.

It is this behaviour of proteins (i.e. the interaction of hydrophobic patches) that is exploited in HIC. A non-polar chain, usually C_4-C_{10} (Scopes 1994), is immobilised onto a matrix. When a protein suspension is applied to a column packed with a hydrophobic matrix, proteins that have strong hydrophobic interactions are adsorbed. In this purification process, binding capacity is high and recoveries are very good as long as the interactions are not too

strong so as to lead to irreversible binding. The process is conducted in the presence of salt ions (Huddleston 1994, Smith 1996) and this increases hydrophobic interaction. The water molecules around the two proteins shield the hydrophobic patches of the two proteins from interacting with each other. Salt ions interact strongly with water (hydration) and thus removes the shielding effect thereby increasing hydrophobic interactions. It could also be that when the process is conducted in the presence of salt ions, Le Chateliers principle is in operation. The interaction of hydrophobic patches releases water. When salt ions are present, the water molecules are removed and hence driving the process in the forward direction.

Hydrophobic interactions are known to be affected by the type of salt used, pH, temperature and additives (Queiroz 1996). In this work, adsorption was better at high pH, and high salt. The main disadvantage of HIC is that it is difficult to improve selectivity by changing experimental conditions. This is made worse if salting out was used early in the downstream processing as both of these processes operate on the same principle. However HIC can be linked to other chromatographic processes such as ion exchange, affinity and gel filtration without prior sample preparation (Feng 1996). An ion exchange eluate can also be applied directly to an HIC column without pre-treatment.

1.1.2 Purification of proteins based on their size.

The purification of proteins can be achieved by using chromatographic methods (gel filtration) as well as non chromatographic methods (such as dialysis). In Gel Filtration Chromatography (GFC), also called molecular exclusion or molecular sieve chromatography, proteins are separated according to their differences in size as they pass through a bed of porous beads. Small proteins can penetrate the pores of the beads while larger proteins are excluded (hence the term molecular exclusion) from the pores. These larger molecules will consequently elute earlier than the small molecules.

Buffers play an insignificant role in improving the resolution in gel filtration (as long as there are no interactions between the protein and the matrix). Assuming that column packing is properly done, resolution can only be improved by using beads of different pore sizes or increasing the retention time by either increasing column length or decreasing flow rate. If everything is in place, it has been argued that GFC is easy to run even for beginners (Amersham Pharmacia Biotech Manual). Due to this ease of operation, GFC has found use in separation processes, molecular weight determinations (Minelli 1991, Siegel 1966, Locascio 1969, Ansari 1977) and desalting of chromatographic separations.

Gel filtration as a tool for molecular weight determinations

The retention time of a molecule in a gel filtration column depends on the molecular weight and the column parameters. It therefore follows that molecules with the same molecular weight will elute at the same time on the same column. If the column has been calibrated with proteins of known molecular weight, the molecular weight of an unknown protein sample can thus be estimated. This has been applied with success by Minelli (1991) in the determination of the molecular weight of a nucleotidase from human seminal plasma, Siegel (1966) on molecular weight determinations of crude sulfite and hydroxylamine, Locascio (1969) by using agarose beads and by Ansari (1977). For accurate estimations of molecular weights, it is important that the proteins are of the same shape, as the rate at which a spherical molecule moves down the column is different to that of a linear molecule. This is made possible by the addition of urea or guanidine chloride, which transform

proteins into a random coil thus reducing structural differences. Use of guanidine chloride introduced another problem in gel filtration of proteins. The matrices in use then were not stable in this solvent (Ansari 1977). In this paper Ansari used Sepharose CL-6B in molecular weight determinations of proteins by using gel filtration. Not only was the matrix stable over a long period (10 months) but also poorly resolved proteins were well resolved on this matrix. However this matrix could only be applied in the molecular weight determinations of low to medium size proteins (3000 to 80 000 Daltons). Thus agarose beads which were found to be ideal for large proteins by Locascio (1969) would still be of great use. One of the points that stand out in Locascio's paper is that there was an error in the molecular weight determinations due to structural differences of the proteins. At this stage of gel filtration development, guanidine chloride was not in use.

Gel filtration as a separation tool.

Gel filtration is widely applied in separation processes. However its main limitation is its low capacity and a relatively broad distribution of pore sizes. Thus only small sample sizes of up to 5% column volume (Amersham Pharmacia Biotech Manual) can be applied. The large distribution of pore sizes means that gel filtration is only a useful technique if the proteins to be separated have large differences in size. As a result, gel filtration has been found to be useful as a final polishing stage during downstream processing when the product is present in small volumes (Abe 1991, Melnikova 2000).

Gel filtration is particularly important in desalting or buffer exchange of chromatographic eluates. Eluates from ion exchange columns may have to be further purified on another ion exchange column. Thus the ionic strength of the eluates have to be reduced. Since ionic salts have a significantly smaller size than proteins, gel filtration is a convenient way of separating the salt ions from the rest of the proteins. Similarly in buffer exchanges, gel filtration has been found to be efficient. The protein is washed with the new buffer until it elutes. The process can take minutes whilst by dialysis it may take hours or even days. The major problem is that only small volumes of sample can be processed by the size exclusion columns.

1.1.3 Purification of proteins based on their affinity for other molecules or metals.

In theory it should be possible to purify any protein to homogeneity using ion exchange chromatography and other conventional methods such as salting out. However, because the selectivity is not very high, it takes a cascade of process steps to purify some proteins to homogeneity (Chang 1996). This requires much time and the yield is usually low, as some of the product is lost during transfer from one purification unit to the other. The development of affinity chromatography in the late 1960s gave new hope in protein purification (Porath 1992).

Affinity adsorbents can generally be grouped into two classes: those with immobilised metals and those with biospecific ligands. The principle of affinity chromatography is based on specific interaction between the protein and the ligand or metal. Each protein has an affinity for a bioligand and others can chelate a metal. Affinity for a bioligand is usually due to specific functional groups on the protein. The bioligand may be a small molecule, e.g. glutathione for glutathione-s-transferase, or conjugated as is the case for fusion proteins or it may be an antibody (Scopes 1996). The support matrix is first activated using commonly CNBr or epoxides (Porath 1974, Scouten 1985) to which the biospecific ligand is attached. When protein samples are contacted with the ligands, only those proteins with affinity for the bioligands would be adsorbed.

Affinity for a metal ion or Immobilised Metal Affinity Chromatography (IMAC) was developed by Porath (1975). The affinity is mainly due to histidine, cystein and tryptophan residues which can co-ordinate to a metal ion (Porath 1975, Arnold 1991, Wong 1991). The metal ions, which are first row transition metals (Arnold 1991, Wong 1991) are immobilised on the matrix through a spacer arm which is usually Imino Diacetic Acid (IDA) and Tris (Carboxymethyl) Ethlene Diamine (TED). When proteins with histidine, cystein and tryptophan moieties are contacted with the adsorbent, they get bound to it through their electron donating centres (imidazole group of histidine, thiol group of cystein and indoyl group of tryptophan). Binding occurs at pH 6-8 where the groups that co-ordinate to the metal ion are deprotonated. Consequently elution can be achieved by lowering the pH

(Wong 1991, Porath 1992) or by using a competitive ligand such as glycine (Wong 1991, Porath 1992). It is obvious therefore that if the ligand is to be used again, strong chelating agents which can strip the metal ion off the matrix should be avoided during elution.

Compared to biospecific ligands, IMAC was validated to have higher ligand stability, loading capacity, ligand recovery and lower costs than biospecific affinity chromatography (Arnold 1991). However they have a lower selectivity than bioselective ligands.

Because of the high specificity of affinity interactions, affinity adsorbents can be used in the purification of target molecules from complex biological mixtures with high purification. In column separation processes, the feed must be clarified first as the cell debris accumulates at the distributor thereby blocking the column (Chang 1996; Chang 1993). Although batch mode can be used, contact efficiency is low because the system acts as though it has one theoretical plate (Gailliot 1990, Chase 1992, Chang 1993, Osterlund 1997). This leads to poor binding and hence more adsorbent has to be used.

Despite the high purification achieved on affinity adsorbents, they suffer from leakage of the ligand, are costly and the capacities are lower than ion exchange matrices for example. Thus the less specific ion exchange matrices still hold number one position in protein purification (Scopes 1996).

1.1.4 Protein purification based on net surface charge.

The net charge of a protein depends on its constituent amino acids and on the pH of the buffer in which it has been solubilised. At its isoelectric point (IEP), the net charge of the protein is zero. At pH values above the isoelectric point, the protein is negatively charged hence an anion. Therefore if a matrix has positively charged groups (an anion exchanger), it can be used to adsorb a protein which has been solubilised in a buffer whose pH is above the isoelectric point of the protein. Similarly for a protein solubilised in a buffer whose pH is below the isoelectric point of the protein, a cation exchange matrix can be used to adsorb it.

Ion exchange chromatography is by far the most common and well-developed technique for the separation of proteins (Scopes 1996). Over 75% of all preparation schemes (Chang 1996) employ ion exchange chromatography. This popularity is because it is cheap, it has high resolving power, and has high binding capacity (Chang 1996). This facilitated the application of ion exchange in the purification process of various proteins from various sources such as E.coli (Hansson 1994, Frej 1994), humans (Finette 1998), yeast (Chang 1996, Chang 1996b, Raymond 1998) and milk (Manji 1985).

Purification achieved on ion exchange adsorbents is usually low because of their low specificity. However use of step elution or gradient elution improves the purity of the product as some form of selective elution is introduced. Indeed it has been pointed out that the selectivity of ion exchange adsorbents is in the elution stage whilst that of affinity adsorbents is in the adsorption stage (Scopes 1996). A detailed study on the efficiency of the elution stage to optimise glucose-6-phosphate dehydrogenase was undertaken by Chang (1996). In this study they found that using a two step elution with 0.15M and 1M NaCl gave a better purification factor than the other combinations they tested.

The development of chromatofocusing in the late seventies (Sluyterman 1978, Sluyterman 1978, Sluyterman 1981) tremendously improved the purity that could be achieved on ion exchange columns. Chromatofocusing is a chromatographic technique in which proteins are eluted from an ion exchange column by a pH gradient. The proteins are separated according to their isoelectric points. The protein with the highest IEP elutes first.

In principle, proteins can be desorbed from an ion exchange column by changing the pH to below the isoelectric point (for anion exchange) or above the isoelectric point for cation exchange. However when ordinary buffers are used, sudden pH changes occur in the column (Scopes 1994). As a result proteins with different isoelectric points elute at the same time, hence poor resolution is observed. To prevent this sudden change in chromatofocusing, amphoteric buffers (which are polymeric) and matrices with a strong

even buffering capacity over a wide pH range are used. It is claimed that proteins whose isoelectric points differ by 0.05 units could be resolved by chromatofocusing (Scopes 1994).

1.1.5 Purification of proteins based on their solubility.

Precipitation methods are employed in this field. They have been used for decades both at laboratory scale and at industrial scale to reduce processing volumes, fractionate protein mixtures or to solidify product in the finishing stage (Iyer 1995).

1.1.5.1 Salting-out.

Divalent ions such as $(NH_4)_2SO_4$ affect the solubility of proteins in two ways. At low ionic strength, they increase the solubility of proteins. This phenomenon is called salting in. As the ionic strength is increased, the solubility of the protein decreases. This is called salting out (Scopes 1994). With further increase in ionic strength, the protein may eventually precipitate out of solution. Different proteins precipitate at different ionic strengths depending on their hydrophobicity, as such purification is achieved to some extent. The precipitated proteins retain their native conformation and can be re-dissolved usually with negligible loss of activity. The purification of Immunoglobulins from whole serum is commonly achieved by this method. This is done by using either ammonium sulfate (Yarmush 1992, Inouye 1993, Morimoto 1992) or caprilic acid (Polten 1991). Milk proteins have also been purified by ammonium sulphate precipitation (Hachem 1996). In another work (Chen 1992) β-lactoglobulin and α-lactoalbumin were separated by ammonium sulphate precipitation.

1.1.5.2 Isoelectric Precipitation.

An isoelectric point (IEP) is the pH at which the protein has no net charge. At such a pH the solubility of the protein is at its minimum. At pH values above or below this pH value, the solubility rises very sharply. There is no electrostatic repulsion between neighbouring groups at its IEP. They coalesce and precipitate. Some authors (Scopes 1994) have argued that it is hydrophobic forces that lead to the eventual precipitation of the proteins. Perhaps a

classic example of isoelectric precipitation is the separation of casein from milk (Hofland 1999, Bobe 1998, Stromqvist 1995, Visser 1991). The isoelectric point of caseins is about 4.6. By reducing the pH to around the IEP, the caseins precipitate out of solution. They can be filtered off from the rest of the proteins (whey proteins). Isoelectric precipitation has also been applied to the separation of soy bean proteins (Hofland 2000, Yarmush 1992) and plant seeds (Sue 2000).

1.1.5.3 Separation of proteins by solvents

Protein solubility at fixed pH and ionic strength is a function of the dielectric constant. Water miscible neutral organic solvents, such as acetone or ethanol, have a lower dielectric constant than water. When such solvents are added to an aqueous protein suspension, they increase the attractive forces between opposite groups on the protein. This in turn decreases the degree of ionisation of the side chain on the protein. Thus the proteins tend to aggregate and then precipitate. Solvent extraction has been included in the purification of animal as well as plant proteins (Molloy 1999, Stark 1999).

Aqueous two phase systems (ATPS) also fall in this category. The separation is based on differential fractionation between an organic phase and an aqueous phase. ATPS composed of polyethylene glycol (PEG) of low molecular weight (Mwt 1000 -1500) have been shown to be a good system for differential fractionation of milk proteins (Chen 1992, Wharton 1981, Dos Reis Coimbra 1994). A major advantage of ATPS is that enrichment and clarification are combined in a single step (Chen 1992). As a result ATPS have been recommended as a first step in the recovery of milk protein.

This is not restricted to milk proteins. ATPS has been effective in separating cells and inclusion bodies (Walker 1998), and cell debris from proteins (Zijlstra 1996, Rito-Palomares 1996) due to differential fractionation. The purification factor obtained by using ATPS is low and in most cases the solutions are heterogeneous. Consequently they are used in conjunction with other purification techniques in protein purification process.

1.2 EVENTS LEADING TO THE DEVELOPMENT OF EXPANDED BED CHROMATOGRAPHY

The majority of biotechnology processes for producing pharmaceutical products involve the purification of enzymes, other proteins and other biological molecules. The sources of these target molecules may be bacterial (usually E.coli), yeast, milk, plants and mammalian tissue. Inevitably, suspensions will contain cell debris and other particulate material that must be removed early in the downstream process. Thus traditionally, protein purification has had to be accomplished using a multi-step process to ensure complete removal of impurities and contaminants. These purification steps can be as many as eight or more (Minelli 1991, Yofiriji 1997, Nishihara 1997, Abe 1991). The problem with using such a cascade of purification steps is that each recovery process affects the overall process economy by increasing the operational costs and process time, and also by causing product loss during transfer from one purification unit to the other (Chang 1996b; Cole 1997). This leads to low recoveries. For example, Minelli and co-workers (1991) obtained a yield of 0.1% in the purification of a low K_m 5'-nucleotide from human seminal plasma. Abe (1991) could only achieve a yield of 14% in the purification of g-glutamyltransferase of human semen. Tran (1993) obtained a yield of 5% from the purification of glutathione peroxidase from *Hansenula mrakii*. Apeler (1997) achieved a 3% yield in the purification of a protease. Such yields are economically undesirable. The overall result is that the product cost is high (Chase 1992, Chang 1994, Chang 1993, Osterlund 1997).

The standard techniques for solid/liquid removal have been centrifugation and filtration. The problem is that the efficiency of a centrifugation step is a function of particle size, the density difference between the particles and the liquid and the viscosity of the liquid. Thus when working with cell suspensions and small cells such as E.coli, it is often a problem to achieve a particulate-free suspension. Hence to obtain a completely particle-free solution, centrifugation is combined with microfiltration which in turn is seriously affected by membrane fouling and this adds to the operation costs.

It is not surprising therefore that bioprocess engineers have been looking for ways to reduce operational costs without compromising the purity of the product. Obviously the first idea was to reduce the number of steps. Attempts had been made to apply unclarified homogenates to packed column. However the column blocked because the packed bed would act as a depth filter causing cells and debris to build up in the column and flow rate to decrease tremendously (Chang 1996; Chang 1993).

A number of techniques have been developed to overcome this problem including the use of well mixed stirred tank systems. The advantage of this technique over packed beds is that in addition to handling unclarified feed stock, it can be used to recover target substances from large volumes. Its disadvantage is that it is a single stage process (it resembles a system with a single theoretical plate). Thus it requires more adsorbent and longer contact times to achieve the same degree of adsorption that would be achieved in a packed bed process (Slater 1991, Chang 1993).

Fully fluidised beds which use high density beads were also investigated. Because of the high velocities employed, channelling and backmixing are extensive. Thus this system has low number of theoretical plates and at worst it can resemble a system with a single theoretical plate, just like the stirred tank (Gailliot 1990, Chase 1992, Chang 1993, Osterlund 1997). Other problems with fluidised beds are low capacity due to mixed flow through the bed which is linked to the movement of adsorbent particles within the bed, product leakage, rapid breakthrough curves and consumption of large volumes of buffers (Chang 1994).

These problems can be overcome if the adsorbent particles appear to be at the same position but allowing cells and cell debris to pass freely through the bed. The most promising discovery was the use of magnetic adsorbent particles in which a magnetic field was used to stabilise the beads (Burns 1985, Nixon 1991, Nixon 1992). Although this works well at laboratory scale processes, scaling-up requires complicated and expensive equipment.

It was not until 1991 that Draeger and Chase (Draeger 1990) proposed a promising solution. They equilibrated a column in upward flow and by carefully controlling the flow velocity, they could expand the bed to a desired height and the beads were seen to be stable. In such a system, the void fraction is higher and hence it can handle unclarified feedstock while the target substances are being adsorbed. This system is now called Expanded Bed Adsorption (EBA) chromatography.

The conventional Sepharose Fast Flow adsorbents were not suitable for EBA because of their small size and low density. The bed expanded greatly at small flow velocity and the bed was not as stable. This is undesirable as it leads to low productivity. High density beads posed another problem - high flow rates to expand the bed. This impaired protein binding as diffusion through the bead was restricted (Hjorth 1997). Streamline™ adsorbents were developed to overcome this problem. (Chang 1996b, Chang 1994, Chang 1993). These beads are larger (about 200μm) and have a density of 1.2 g/ml. It is important that the density of the particles is within the same range as that of the equilibrating buffer. It was established that this condition leads to low liquid-liquid mixing, a factor that is crucial in chromatographic separations (Finette 1994). The size of particles that can be used is also another limiting factor. Larger particles give poor mass transfer (Finette 1994) because of low diffusion in the pores of the particles.

1.3 THE PRINCIPLE OF EXPANDED BED ADSORPTION (EBA) CHROMATOGRAPHY.

The principle of Expanded Bed Adsorption (EBA) chromatography is the same as that of packed beds with the exception that in EBA, the bed can be expanded to a desired height. The column is packed with adsorbent and the adapter is positioned just above the desired expanded bed height. The bed is then fluidised with suitable buffer by applying it in the upward direction. When the bed is stable, feed is applied. If the feed is more viscous than the buffer, the bed will expand further during feed application. Depending on preference,

the flow velocity may be reduced to keep the bed height constant or the application may be conducted at constant flow. Feed application is followed by washing usually with the same equilibrating buffer in an expanded mode. Washing is stopped and the bed is allowed to settle. Direction of flow is then reversed and elution commences by passing through the column an appropriate solution. If necessary the flow velocity is reduced. This reduces dilution of the product. The column is then cleaned and regenerated for future use. The whole process is summarised in Figure 1.1

The void fraction in an expanded bed is higher than in a packed bed. This allows cells and cell particulate material to pass freely whilst the proteins are being adsorbed. Thus EBA offers the dual advantage of removing particulate material and adsorbing the proteins in a single step. Consequently product cost decreases and yield increases. In the purification of G6PDH, a yield of 98% was achieved without prior removal of cell particulates (Chang 1996b). As there is no need for feed clarification, the number of purification steps is significantly reduced. Figure 1.2 compares the expanded bed procedure with the standard procedure using packed columns.

For any process, labour elements of the fixed costs depend on the number of process steps (Heaton 1996). Thus it is obvious from Figure 1.2 that the expanded bed route is cheaper than the standard procedure. Other workers (Batt 1995) have also verified this. In fact Pharmacia Biotech reported a potential saving cost of 40% (McCormick 1993) when using expanded beds compared to packed beds.

Back mixing in an expanded bed is low, so the number of theoretical plates is high. Hence EBA combines the attractive properties of both the fluidised bed (ability to handle unclarified feedstocks) and packed beds (good contacting efficiency) (Chang 1993). In fact research has shown that there is no difference in adsorption between packed beds and EBA if the bed is expanded 3-4 times (Chang 1996). Since the expanded bed route is shorter there is a substantial time saving as well. Synergen engineers reported a time saving of 56% (McCormick 1993). It is therefore not surprising that expanded bed chromatography is

growing in popularity (Chang 1996b, Hansson 1994). On the negative side though, buffer consumption in expanded beds is higher than in packed beds.

1.4: THIS RESEARCH PROJECT.

Since the development of expanded bed chromatography, there has been research into optimising the performance of expanded beds to capture proteins. Most of the research centred on one or a few aspects of optimisation. For example Koh (1995) conducted some expanded bed experiments. They used phenylalanine, which adsorbed to the packing material and they investigated the effect of column diameter, packing height and flow velocity on breakthrough curves and mass transfer. The differences in the breakthrough curves were attributed to axial dispersion in the columns. Their work centred on low bed expansions (<2 times). By contrast a 2-3 times bed expansion is normally used in most expanded bed separation processes. This is probably due to the fact that at such bed expansions, adsorption resembles that of packed beds (Chang 1996).

Mullick (1998) conducted similar experiments. They used BSA as a tracer and a 75-103 Cim fluoride-modified zirconia (FmZr), an adsorbent whose application in downstream processing is not as widely used as Streamline™ adsorbents. Their work concentrated on low $H:D_c$ values, i.e. a maximum settled bed height of 7.5 cm in a 25 mm column. (H is bed height and D_c is column diameter)

Hjorth (1995) investigated the effect of flow velocity, temperature of feed, and settled bed volume on breakthrough capacity of BSA. High flow velocities reduced the binding capacity as expected whilst higher operation temperatures increased the amount of BSA bound. Longer bed heights were expected to give better bed stability and therefore better binding than shorter beds and this was indeed what was observed by Hjorth.

Other work was carried out using streamline 25-600 columns (Frej 1997). Axial dispersion was evaluated by calculating the number of theoretical plates at a bed expansion of about 3 times the settled bed height. The number of theoretical plates, N, ranged from 70 in the streamline 25 column to 50 in the streamline 600 column. Thus according to these results there is negligible loss in bed stability if larger diameter columns are used. Similarly, the breakthrough pattern was almost identical in all the columns tested.

An interesting piece of work was carried out by Chang and Chase (Chang 1996) who investigated the effect of bed expansion on axial dispersion. They used a settled bed height of 10 cm and buffer containing 0% to 32% w/v glycerol and observed that at the same bed expansion, axial dispersion is the same irrespective of the glycerol concentration. They also observed that at the same flow velocity, axial dispersion increased with the solution viscosity. This is to be expected as in highly viscous solutions the bed expansion is larger than in low viscous solutions at the same superficial liquid velocity.

Asif (1992) challenged the assumption that axial dispersion coefficient, D_a, is constant. Indeed from this work it was apparent that D_a depended upon the distance between sample injection and sampling, the Reynolds number, bed voidage and the type of beads used. They were also able to show that axial dispersion was not a function of sampling location (at the same bed height). By using KCl and $MgSO_4$ which have different diffusivities, they also showed that D_a is independent of molecular diffusivities.

From literature it is possible to pool together data on the effect of different parameters on axial dispersion. However it is difficult to determine optimal bed stability conditions by using data from various research groups since the experimental conditions under which such data were generated is likely to be different. It was thus important that the hydrodynamic bed stability be optimised under the same set of experimental conditions. In **Chapter Two**, a wide spectrum of parameters that can influence bed hydrodynamics have been investigated. The settled bed height has been extended from 5 cm to 20 cm and the

bed expansion has also been extended from 1.5 to 3 times the settled bed height. These experiments have been conducted in 12.5 mm, 25 mm and 50 mm columns. A wide range of bed aspect ratios have also been investigated by comparing data from the three different columns that were used. As Streamline™ adsorbents are commonly used in expanded bed chromatography, Streamline™ SP was used in investigating axial dispersion in all experiments.

Whilst Chapter Two deals with the hydrodynamics of the bed, **Chapter Three** deals with the selectivity of the column when using a stable bed configuration. In particular Chapter Three investigates the influence of experimental conditions (other than hydrodynamic) on binding capacity, purification factor and product concentration. The objective was to find if the binding capacity, the purification factor and the product concentration respond differently to the same set of experimental conditions, in which case it would be possible to selectively choose the parameters that would maximise the parameter of interest in that process. For example, if the protein is to be used in obtaining biochemical data, then the purification factor is of prime importance. During purification processes, experimental conditions that would maximise the purity of the product would be used. This work stemmed from the fact that EBA can be used to clarify, capture, concentrate and purify a product in a single step (Hjorth 1997, Karau 1997, Chang 1996b). However there is a lack of literature on how these parameters vary with different experimental conditions.

Having optimised the hydrodynamic stability of the bed and product capture, **Chapter Four** examines the application of expanded beds in the purification of a typical molecule; glutathione s-transferase (GST) propagated in E.coli. The selectivity of in-house-made affinity matrices and ion exchange adsorbents is assessed both quantitatively (by protein and enzyme assays) and qualitatively (by SDS-PAGE).

GST purification has been going on since its discovery. Perhaps the most cited references are the papers by Simons et al. (1977) and Habig (1984). The work by Simons et al. concentrated on producing a good affinity matrix for GST. In particular they recommended

coupling the ligand (glutathione) at pH 7 as the matrix produced at this pH showed better selectivity and capacity than the one produced at higher pH. The work by Habig was mainly on developing an assay protocol for GST. Amongst the protocols developed, the CDNB method is the most commonly applied.

Today an increasingly wide application of GST is its use as a fusion partner for the purification of recombinant proteins (Guan 1991, Nishida 1994, Sehgal 2000). This technology has been applied for the production of different types of proteins for various end uses including antibodies and DNA binding proteins. The fusion complex comprising the target recombinant protein is attached to GST through a protease cleavage site. Purification can be effected by using the GST portion as an affinity handle to bind specifically to glutathione. The most common protocol involves adsorption onto glutathione-Sepharose, a commercially available adsorbent manufactured by Amersham Pharmacia Biotech. There are examples of the material processing both as single contact batch systems (Guan 1991) as well as in packed columns (Simons 1977, Reade 1999).

The literature on the affinity purification of fusion proteins involving GST indicates that most are conducted as laboratory scale operations usually as a tool by molecular biologists investigating new compounds expressed and produced as recombinant products in organisms such as *E. coli* (Nishida 1994, Guan 1991, Sehgal 2000). There has been no reported development of this technology from a bioprocess point of view, especially with regards to scale up. This is perhaps a result of a lack of any immediate requirement for large scale processing of these new compounds. It is therefore likely that much of the work presented hitherto was not well optimised and may have restricted application when developing protocols for obtaining products in larger quantities.

The work reported here involved use and optimisation of the EBA to capture glutathione-s-transferase. Two matrices were used (i.e. ion exchange and affinity matrix tailor made for

use in expanded bed chromatography). The affinity adsorbent developed was an in-house synthesised glutathione coupled to the Streamline™ quartz base matrix.

Expanded bed adsorption chromatography was developed so that feed containing particulate material could be processed without prior removal of the cells and debris. Usually the interest has been in capturing the target molecules whilst the debris pass through the bed. **Chapter Five** addresses a new dimension on the use of expanded beds in which EBA is used to capture nano-particles (i.e. Phage T7 propagated in E.coli). Not only is the system ideal for phage purification but also the ion exchange matrix used shows selective elution of infectious phage from the non-infectious ones and the cells and cell debris of the E.coli.

Traditionally, phage particles have been purified using CsCl density gradient ultracentrifugation (Hirsh 1982, Sain 1981). It gives generally good purity and is easy to use (Maramorosch 1967, Lyttle 1984). On the other hand this procedure is not cost effective, is time consuming and recovery of the phage requires skill (Sain 1981). Other methods have been found to be better for some phages. Aqueous two-phase systems for example, especially those of dextran sulphate and Polyethylene Glycol of molecular weight 6000 (PEG_{6000}), have been found to be particularly good for the isolation of lytic phages (Douglas 1975). For Lambda c126, gel filtration chromatography was found to be better than the CsCl method in terms of the recovery and cross contamination. The purity, though, was comparable to that obtained using the CsCl method (Sain 1981). Gel filtration has also been applied to the purification of barley yellow dwarf virus (Hewish 1983) and bovine papilloma virus (Hjorth 1982).

Viruses are known to be negatively charged at neutral pH (Farber 1983). This meant ion exchange resins could be employed in the purification of viruses. This was first applied in 1978 (Gerba 1978) using negatively charged membranes. The viral particles had to be acidified to adsorb to the membranes. The problem is that phages are sensitive to extreme pHs and so this technique offered little hope. The development of positively charged membranes and matrices offered more advantages in the purification of phages. DEAE in

particular was found to be better with a purification factor of 350 times higher than that achieved by PEG precipitation (Carlsson 1994).

Huyghes (1995) compared the performance of ion exchange chromatography, Hydrophobic Interaction Chromatography (HIC), gel filtration and Immobilised Metal Affinity Chromatography (IMAC) for the purification of a recombinant adenovirus. It was observed that DEAE gave better recovery but purity was poor. Gel filtration gave poor yields. HIC gave pure samples but the recovery was poor. IMAC gave better recovery than both gel filtration and HIC and it also gave higher purification factor than DEAE.

Though techniques, such as centrifugation, affinity, aqueous two-phase systems can be applied, scale-up and cost is usually a problem (Ujam 2000, Lyddiatt 1998). In fact some authors have argued that for large scale purification of phage, column chromatography offers significant potential (Huyghes 1995). Chromatographic methods are easier to scale up (a larger column is used) and remain the practical choice for product fractionation (Lyddiatt 1998). Expanded bed chromatography offers the advantage of handling unclarified feedstock hence this makes it an ideal process for phage purification. The aim of **Chapter Five** was therefore to explore new, cheaper but effective ways of purifying phage particles using expanded bed adsorption chromatography.

Chapter Six concludes the thesis and looks at possible areas of research that this project could not address conclusively within its scope.

CHAPTER TWO

OPTIMISATION OF BED STABILITY.

2.0 INTRODUCTION.

It is of paramount importance to the bioprocess engineer to preserve molecular integrity and obtain high yields during protein purification processes. Both of these can be accomplished by eliminating unnecessary steps. Longer exposure of proteins to atmospheric oxygen increases the risk of proteolytic degradation and fewer unit operations decrease the loss of product. Thus operation cost is reduced (Chase 1994, Chang 1995, Thommes 1995). The cost of operation can also be reduced by scale up. Indeed it can be shown that for chromatographic separations production cost in large amounts is lower than in small amounts (Heaton 1996). In fact production in small batches is not just more costly but also more time consuming than production in large amounts (assuming that market size is not a constraint). However exploratory experiments are conducted at laboratory scale and it is then assumed that scaling up will not affect the process significantly.

In column chromatographic separations, scaling up will involve the use of wider columns and this is often the recommendation (Hjorth 1997). Longer columns can be used but the practicality may be a problem in addition to longer process times. The bed expansion characteristics may not significantly change with scale up. On the other hand, axial dispersion complicates scale up (Groen 1996). One of the reasons for this is because the wider the column, the higher the number of paths that any given solute can sample as it travels through the column (Skoog 1991). If there is not sufficient time for diffusion averaging to occur, scale up can seriously affect the performance of a column. Extensive work has been carried out on the effect of scale up on axial dispersion in three-phase (gas-liquid-solid) bioreactors and fluidised columns (Kim 1992, Groen 1996). Little work has been done in expanded bed chromatography. Nonetheless some researchers (Frej 1994) have compared the performance of wider and smaller diameter columns in the purification of proteins, with encouraging results.

Most of the literature on expanded bed hydrodynamics is listed in Chapter 1, section 1.4. In brief it was mentioned that most of the research concentrated on one or a few parameters. For example Mullick (1998) concentrated on the effect of bed expansion and the bed aspect ratio at low column heights. Koh (1995) examined the effect of column diameter at low bed expansions. Chang (1996) investigated the effect of feed viscosity and degree of bed expansion on the performance of expanded beds. De Luca (1994) investigated the transient expansion characteristics of expanded beds whilst Karau (1997) reported the influence of particle size distribution on adsorption performance in expanded beds. All these aspects of expanded beds when put together may explain the behaviour and performance of expanded beds

Although various aspects of axial dispersion have been investigated, it has often been difficult to determine optimal bed stability conditions by using data from various research groups since the data are subject to specific experimental conditions. It was thus important that the hydrodynamic bed stability be optimised under the same set of experimental conditions. In this work the range of the settled bed height has been extended from 5 cm to 20 cm and the bed expansion has also been extended from 1.5 to 3 times the settled bed height. These experiments have been conducted in 12.5 mm, 25 mm and 50 mm columns. As Streamline^{TM} adsorbents are commonly used in expanded bed chromatography, Streamline^{TM} SP was used in investigating axial dispersion in all experiments.

2.1 OBJECTIVES OF THESE EXPERIMENTS.

The objectives of these experiments were to investigate axial dispersion by conducting residence time distribution (RTD) experiments at different settled bed heights. Settled bed heights (H_o) of 5 cm, 10 cm 15 cm and 20 cm were used. The second objective was to investigate axial dispersion at different flow velocities (or degree of bed expansion). The beds were thus expanded to 1.5, 2, 2.5 and 3 times the settled bed heights.

The other objective of these experiments was to investigate the effect of scale up on axial dispersion. Hence the RTD experiments were conducted in columns of different diameter, i.e.

12.5 mm, 25 mm and 50 mm (i.d.). As mentioned in section 2.0, longer columns can equally be used in scale up. Therefore RTD experiments were also set up to investigate the effect of using longer columns on axial dispersion. Finally, the experiments were also designed to investigate the expansion characteristics of the beads at different flow velocities.

2.2 MATERIALS AND METHODS

2.2.1 Materials

Peristaltic pumps (Cole-Palmer) were used through the experiment. The columns were bought from Soham scientific, Cambridge UK. The UV detector and chart recorder were a gift from Amersham Pharmacia Biotech, Sweden. Na_2HPO_4 was bought from Sigma, UK.

2.2.2 Residence time distribution experiments.

The three columns (i.d. 12.5 mm, 25 mm and 50 mm) were packed with Streamline SP to a settled bed height of 5 cm. The bed was then expanded 1.5 times with 50 mM Na_2HPO_4 buffer, pH 7. Figure 2.1 is a diagrammatic representation of the equipment used for the RTD experiments. A tracer of acetone (20% in the buffer) was injected into the loop using manual valves. When the bed was stable, flow was diverted into the loop using the manual valve at point A in Figure 2.1. The absorbance of the effluent was continuously measured with the Pharmacia UV 100, and recorded by the chart recorder. A typical UV response is shown in Figure 2.2. The experiment was done in duplicate. Acetone was used as it does not bind to nor react with the packing material.

Because the three columns were likely to have different void volumes, it was essential that the effect of the void be standardised. In this experiment therefore, RTD experiments were also performed in empty columns with the adapter position just above the distributor. The variance and mean time of the empty columns were then subtracted from the variance and mean time of the filled columns.

To investigate the effect of the settled bed height, the experiment was repeated with settled bed height of 10 cm, 15 cm and 20 cm.

To investigate the effect of flow velocity (degree of bed expansion) on axial dispersion, the whole experiment was repeated with bed expansions of 2, 2.5 and 3 times the settled bed height.

To investigate axial dispersion in columns packed with the same volume of adsorbent, the 25 mm and the 50 mm columns were used. The two columns were packed with 100 ml or 200 ml of Streamline SP. The beds were then expanded to 1.5 and 2 times the settled bed height.

2.2.2.1 Calculation of variance and mean time.

A UV response as given in Figure 2.2 was divided into discrete points on the x axis (i.e. time, t). The corresponding UV signals (i.e. concentration, C_i) were then recorded. Then variance (σ^2) and mean time ($\bar{t}$) was calculated using standard statistic methods (Levenspiel 1999) as follows:

$$\sigma^2 = \frac{\sum t^2_i C_i}{\sum C_i} - \left[\frac{\sum t_i C_i}{\sum C_i}\right]^2$$

$$\bar{t} = \frac{\sum t_i C_i}{\sum C_i}$$

The Peclet number, Pe, which is used as a measure of axial dispersion in this thesis, was then calculated as described in section 2.3.

2.2.3 Bed expansion characteristics

The column was packed with beads to a height of 15 cm. The bed was then expanded with buffer and when the bed was stable, the height was read. The flow velocity was then increased and the whole process repeated until the bed front became diffuse (less distinct).

2.3 RESULTS AND DISCUSSION.

Axial dispersion is longitudinal mixing (or backmixing) in a column. If axial dispersion is high, the efficiency of the column is low hence in chromatographic separations, axial dispersion should be at its minimum. According to Fick, molecular diffusion is defined as

$$\frac{\partial C}{\partial t} = D\frac{\partial^2 C}{\partial x^2} \qquad (2.1)$$

where D is the molecular diffusion coefficient, C is concentration, t is time and x is the direction of diffusion.

In a column where molecular diffusion is outweighed by the turbulent flow,

$$\frac{\partial C}{\partial t} = D_a\frac{\partial^2 C}{\partial x^2} \qquad (2.2)$$

where D_a is the axial dispersion coefficient, C is concentration. Since column chromatographic separations can be considered as closed systems, axial dispersion can be described by the axial dispersion model, which simplifies to

$$\frac{\partial C}{\partial \theta} = \left(\frac{D_a}{UL}\right)\frac{\partial^2 C}{\partial x^2} \qquad (2.3)$$

U is the superficial velocity $\theta = \frac{t}{\bar{t}}$; L is the column length, $\bar{t}$ is mean time.

D_a/UL (= D_v) is the axial dispersion number and is the measure of the extent of axial dispersion. If flow approaches plug flow (small dispersion) $D_v = D_a/UL \rightarrow 0$. For large dispersion, $D_v \rightarrow \infty$

The inverse of D_v (i.e. UL/D_a) is the Peclet number (Pe). In chromatographic separations, it is important that D_v be as small as possible (high Pe) for it is under these conditions that the efficiency of the column is high (i.e. there is less back mixing in the column). D_v can be obtained from RTD experiments as follows:

The variance σ^2, of a continuous distribution is defined as

$$\sigma^2 = \frac{\int_0^\infty (t-\bar{t})^2 C dt}{\int_0^\infty C dt} \tag{2.4}$$

$$= \frac{\int_0^\infty t^2 C dt}{\int_0^\infty C dt} - \bar{t}^2 \tag{2.5}$$

If the measurements are made at finite number of equidistant locations (e.g. tracer input) the variance simplifies to

$$\sigma^2 = \frac{\sum t_i^2 C_i}{\sum C_i} - \bar{t}^2 = \frac{\sum t_i^2 C_i}{\Sigma C_i} - \left[\frac{\sum t_i C_i}{\sum C_i}\right]^2 \tag{2.6}$$

For closed systems

$$\sigma_\theta^2 = \frac{\sigma^2}{\bar{t}^2} = 2\frac{D_a}{UH} - 2\left(\frac{D_a}{UH}\right)^2 \left(1 - \exp(-UH/D_a)\right) \tag{2.7}$$

Hence the dispersion number D_a/UH can be calculated. For small dispersion

$$\sigma_\theta^2 \approx 2\frac{D_a}{UH} \tag{2.8}$$

The dispersion number D_v, the Peclet number Pe, the number of theorectical plates N and the height equivalent to a theoretical plate (HETP) are related to each other as follows:

The peak variance is found to be directly proportional to column length

$\sigma^2 \alpha H$ where H is the column length.

The proportionality constant is the plate height (HETP but for convenience in this case H^* is being used for HETP) hence

$$\sigma^2 = H^* L \qquad (2.9)$$

Therefore the plate height can be worked out easily. In chromatographic separations, the band spread should be at its minimum (close to plug flow) i.e. σ should be small. Hence high efficient columns have a low HETP.

The number of theoretical plates, N, is also related to σ as follows

$$N = \frac{t_R^{\,2}}{\sigma^2} = \frac{L^2}{\sigma^2} \qquad (2.10)$$

t_R is the retention time.

It is important that both σ and t_R or σ and L are in the same units.

From equation 2.9 and 2.10 it follows that

$$N = \frac{H}{\sigma^2} \times H \qquad (2.11)$$

$$= \frac{H}{H^*} \qquad (2.12)$$

From equation 2.8 we know that

$$D_v = \frac{1}{2}\frac{\sigma^2}{t^2} \qquad (2.13)$$

hence $$D_v = \frac{1}{2N} \qquad (2.14)$$

$$= \frac{H^*}{2H} \qquad (2.15)$$

In equation 2.3, U is the superficial velocity. In equation 2.10, t_R is measured from interstitial velocity, U_i. Taking this into consideration, equation 2.13 and 2.14 respectively become

$$D_v = \frac{1}{2.\varepsilon.N} \qquad (2.16)$$

$$= \frac{H^*}{2.\varepsilon.H} \qquad (2.17)$$

where ε is the void fraction.

Axial dispersion can be quantified by calculating any of these four parameters. In this project the Peclet number is used to quantify axial dispersion.

2.3.1 The effect of settled bed height on axial dispersion.

From Figures 2.3 to 2.5, it is clear that as the bed height increases, the Peclet number is increasing, thus signifying the fact that axial dispersion is decreasing. This observation is true in all the three columns and at any degree of bed expansion.

Upon entry into a column, the liquid flow pattern is disturbed. The extent depends on the type of distributor (Finette 1996). It has been shown experimentally that this non-ideal flow pattern has more impact on the bottom end of the column than in the top end of the column. Carlos (1968) observed that particle movement was high at the bottom of the column. The particles were observed to rise at the centre and coming down at the walls. He and co-workers (He 1998) noticed that this phenomenon died down up the column. The same trend was reported by Handley and co-workers (Handley 1966). They also observed that bulk solid and channelling died down up the column but this bulk solid movement was more pronounced with smaller particles. In fact some researchers have suggested that a column height of twice the column diameter is needed to

damp the effect of flow irregularities generated by the distributor (De Luca 1994). The extent of dispersion must be so low that binding will not be limited by the dispersion. From the data generated in this project, bed heights of twice the column diameter are not sufficient to reduce the effect of flow irregularities (introduced by the distributor) to ensure optimum bed stability conditions. Thus liquid-liquid mixing will be high at the bottom of the column. Consequently, low settled bed height should show high dispersion as is the case in these results. As the bed height increases, and if the flow is left constant, the same level of non-ideal flow is experienced.

In longer beds there is also time for diffusion averaging to occur (i.e. a solute can diffuse from one flow stream to the other thereby decreasing the effect of dispersion that is brought about by the flow irregularities). Hence axial dispersion should be lower in longer columns as is the case in this experiment. Some authors (Hjorth 1995) have also suggested that in short beds there is some backmixing in the column that is not compensated for by the inherent properties of the adsorbents, i.e. particle size and density. Other authors (Koh 1995) have suggested that the observed trend is due to the high bed aspect ratio in longer columns. However this argument only holds if axial dispersion is increasing with flow velocity and this is not always the case.

According to Levenspiel (1999) a minimum Peclet number of 20 is required to ensure that adsorption is not limited by the hydrodynamic bed instability. Therefore the results obtained in these experiments have the following implications;

In the 12.5 mm column

To be certain that the bed stability is not minimising adsorption

a) If $H_o = 5$ cm, a 3 times bed expansion is needed.
b) If $H_o = 10$ cm, at least 2.5 times bed expansion is needed
c) If $H_o = 15$ cm, at least 2 times bed expansion is needed
d) If $H_o = 20$ cm, 1.5 times bed expansion is enough to ensure bed stability.

In the 25 mm column.

a) If $H_o = 5$ cm, a bed expansion of 2.5 times is required

b) At H_o = 10 cm, 15 cm and 20 cm a bed expansion of 1.5 times is satisfactory.

In the 25 mm Column

a) At H_o = 5 cm, a bed expansion of 3 times seems the least requirement.

b) For H_o = 10 – 20 cm, 1.5 H_o is satisfactory.

2.3.2 The influence of flow velocity on axial dispersion.

The effect of flow velocity (bed expansion) on axial dispersion has been a point of much debate. Some authors find that axial dispersion increases with flow velocity (Chase 1992, Finette 1998, Chang 1995) while others find it to decrease with flow velocity (Slater 1991). From a theoretical point of view axial dispersion (as determined by the Peclet number, Pe) should decrease with increasing flow velocity (assuming that D_a is independent of flow velocity) since

$$Pe = \frac{UH}{D_a}$$

where U is the superficial flow velocity, H is the bed height and D_a is the dispersion coefficient. Asif (1991) showed that D_a depends on the sampling point and the mean residence time, D_a being constant as the mean residence time increases and lower at lower mean residence times. At high flow velocities, the residence time is lower than at low flow velocities but for expanded beds (as opposed to aggregative fluidised beds) the residence times are of order of minutes hence the assumption that D_a is constant is fairly justifiable.

However in the definition of the Peclet number above (i.e. $Pe = \frac{UH}{D_a}$) it was assumed that the particles are stationary, which is true in packed beds but not strictly true in expanded beds. When liquid flows past a stationary particle, its path is altered and this leads to the formation of eddies. When flow velocity increases, particle motion increases and this contributes to mixing within the bed and hence has the effect of increasing axial dispersion. Chang (1996) attributed high axial dispersion at high voidage (high flow velocity and high viscosity) to the dispersion caused by the random motion of particles. It can be speculated therefore that if particle contribution to liquid

mixing is high enough, axial dispersion will be increasing with increase in flow velocity. The extent to which particle motion contributes to dispersion will depend on experimental conditions. Even for fixed beds (below the point of minimum fluidisation (where $U < U_{mf}$) axial dispersion has been observed to increase with increasing flow velocity (Chung 1968). Beyond the point of fluidisation, axial dispersion was also observed to increase rapidly. Cairns and co-workers observed that eddy diffusion was also increasing with flow velocity (Cairns 1960). He also noticed that both the particle density and the ratio of particle diameter to column diameter increased eddy diffusion.

The RTD results for the influence of flow velocity (degree of bed expansion) on axial dispersion are shown in Figures 2.6 – 2.8.

The general trend in all the three columns is that as the degree of bed expansion increases, the Peclet number increases. Hence axial dispersion decreases. Thus the data agree with the theoretical expectations. As discussed above, these results should not be interpreted to mean that as the flow velocity increases, axial dispersion would always be reducing. At high flow velocities, it is likely that the system will resemble a aggregative fluidised bed. Particle turbulence will increase which in turn will increase liquid-liquid mixing. Furthermore, when the flow velocity is higher than the terminal velocity of the particles, the particles themselves will be elutriated.

2.3.3 The effect of column diameter on axial dispersion.

It has been shown that expanded bed adsorption chromatography is scalable (Frej 1994, Frej 1997). The term "scalable" actually means that larger column diameters can be used in expanded bed chromatography. Here the term "scale out" is being used to distinguish this scaling from the one in which the bed height is being increased ("scale up").

The advantage of scaling out is that product processing is cheaper. For example a large plant will need a higher capital. It may also need a much manpower as a larger plant will mean a higher

capacity. However the relationship between labour costs (manpower) is less dependent on capacity. Wessel's equation (Heaton 1996) is commonly used and it states that

Manpower α *number of process steps* = (*process complexity*) *x* (*capacity*)$^{0.24}$

Other studies show even less dependence on capacity (Heaton 1996). Thus increasing the scale of production is cost effective.

A major cost in scale out will be on capital. Even here the process is still economically attractive. The relationship between capital and scale of production take the form

$$\frac{C_1}{C_2} = \left(\frac{S_1}{S_2}\right)^n \qquad (2.18)$$

where C is the capital, S is the scale of production and n is a number less than 1.

Typically n is between 0.38 and 0.88 depending on the nature of the process and the operational scale. If the increase in scale involves more unit installation, n will approach unity. In processes like chromatography, where scale up will involve installation of a single large unit, as is usually the case in column separations, n will be lower. Thus from an economic point of view, increasing the scale of production is attractive.

On the other hand increase in scale may complicate other factors that determine adsorption in an expanded bed. One of those factors is axial dispersion and hence it is necessary that this phenomenon is understood in the process under investigation so that adsorption will not be limited by the instability of the bed.

The results are summarised in Table 2.1 for easy comparison. From this table, axial dispersion is least in the 25 mm column. Dispersion seems to be similar in the 12.5 and 50 mm columns. This is rather unexpected. It has long been established that adsorption is better in small diameter columns (Skoog 1991). When a solute is injected into a column, it can sample several paths as it travels through the column. The wider the column, the higher the number of paths the solute can

take hence dispersion is expected to increase. The Van Deemter equation, which closely describes axial dispersion, shows that the plate height (*HETP*) is directly proportional to the column diameter for open columns.

$$HETP = A + \frac{B}{u} + C_S u + C_M u \tag{2.19}$$

where *HETP* is the plate height, *A*, *B* and *C* are constants. *U* is the linear velocity of the mobile phase.

$$C_M u = \frac{f(d_p^2, d_c^2, u)}{D_M} u \tag{2.20}$$

where d_p is the particle diameter, d_c is the column diameter and D_M is the diffusion coefficient in the mobile phase. Hence a larger column diameter will increase *HETP*, thereby increasing axial dispersion.

Recently by using digital equipment to align the column (Bruce 1998), it was experimentally observed that a small diameter column is highly sensitive to deviations from a vertical position. Small deviations (0.15 degrees) from the vertical position caused a significant increase in axial dispersion in a 10 mm column (Pe of 140 to 50). When the same deviation was applied to a 50 mm column, the increase in axial dispersion was reduced (75 to 45). In earlier work (Van der Meer 1984, Horvath 1982) a similar experiment was conducted. Van der Meer especially observed that as little as 0.30 caused severe irregularities in flow pattern in fluidised beds.

From the experiments conducted by Chase's group (Bruce 1998), it was also revealed that even if the column seemed vertical to the naked eye, it was far from perfect. In the experiments reported in this project, the column was aligned by using suspended weights and a spirit level. These are not as accurate as purpose-built digital equipment. Thus the sensitivity of the 12.5 mm column to deviations from the vertical position may be responsible for the high axial dispersion that is

observed. As the 25 mm column is not as sensitive to deviations from the vertical position as the 12.5 mm, it is showing less axial dispersion than the 50 mm column as is normally expected.

2.3.4 The effect of the bed aspect ratio.

The results from this experiment were ranked in terms of the H/D_c (Table 2.2). The data was then analysed by plotting the Peclet number against the bed aspect ratio (H/D_c) for each column. The plots are shown in Figures 2.9- 2.11.

From Figures 2.9-2.11, it is apparent that axial dispersion decreases with increase in the bed aspect ratio. However as stated earlier (section 2.3.2), this is only true in this case because axial dispersion is also decreasing with increase in the degree of bed expansion.

The slope of the linear fit for the three plots is proportional to the diameter of the column, i.e. the slope is steeper in the 50 mm diameter followed by the 25 mm diameter and then the 12.5 mm diameter. On the other hand if Pe is plotted against column height H, the slope (U/D_a) of the linear fit lines is inversely proportional to the dispersion. Thus the linear fit in the 25 mm column which shows the least dispersion is steeper than the 50 mm column than the 12.5 mm column.

The RTD data were ranked in terms of the bed aspect ratio from which Table 2.2 was produced. From this table, the following trends are also observed.

1) At the same H/D_c, the Peclet number is generally higher at higher degree of bed expansion irrespective of the settled bed height (H_o) and the column diameter. Hence plotting Pe against H/H_o, the distribution of the data points should show a general increase as H/H_o increases. This plot is shown in Figure 2.12.

2) At the same bed expansion and H/D_c, the Peclet number is generally higher in longer columns (higher H_o) irrespective of the column diameter. Thus if Pe is plotted against H_o,

the distribution of the points should show a general increase as H_o increases. This plot is shown in Figure 2.13.

3) At same H/D_c and in the same column, the Peclet number seems to be identical as long as the product of H_o and H/H_o (i.e. the column height) are the same. This observation has an interesting application. It means that from a hydrodynamic point of view, a 20 cm column (settled height) expanded to 30 cm, is as stable as a 15 cm column expanded twice and as stable as a 10 cm column expanded thrice. It therefore means ideally, adsorption should be the same. However, the high flow velocity used to expand the 10 cm column three times, will reduce contact time between the adsorbent and the adsorbate and thus affecting the binding capacities. Chang and Chase (1996) reported that at the same expanded height (using solutions of different viscosity), the number of theoretical plates was similar. Since the experiment was conducted in the same column, the bed aspect ratio was the same. Thus this observation has been reported before although not in this context and probably unintentionally so.

Although Figure 2.3 actually shows a greater bed stability at H_o = 20 cm, Figure 2.13 reveals a much broader picture. Figure 2.13 also shows that bed stability at H_o = 20 cm is not significantly greater than bed stability at H_o = 15 cm. If the same data (Table 2.2) are plotted with the column diameter as the x-axis, then the distribution of the data should show a general increase in the 25 mm column. The plot is shown in Figure 2.14.

2.3.5 Axial dispersion in columns containing the same volume of adsorbent.

Scaling up in chromatographic separations involves use of wider columns. Usually it is recommended to leave the bed height unchanged (Hjorth 1997) so that such parameters as the retention time and hence the process time remain unchanged. Section 2.3.3 addressed axial dispersion in columns of different diameters. For a certain volume of adsorbent, a choice of column diameter has to be made. If the column diameter is too small, the bed height will be too high, and operation in an expanded mode may not be practical. A wider column may be practical but the bed height will be shorter and from section 2.3.1 it was seen that low bed heights are associated with high liquid-liquid mixing. This experiment was conducted to investigate the

effect on axial dispersion if the same volume of adsorbent was packed in columns of different diameter. The results are summarised in Table 2.3.

The superiority of small diameter columns needs no emphasis. From Table 2.3, it is clear that axial dispersion in the 25 mm column is far less compared to axial dispersion in the 50 mm column. In general axial dispersion in the 50 mm column is as much as 5-6 times that in the 25 mm column. Thus from a hydrodynamic point of view, a smaller diameter column should show better adsorption. Consequently the binding capacities obtained from a small diameter column should be significantly higher than those obtained from a larger diameter column that contains the same volume of adsorbent.

In Section 2.3.1 it was discussed that towards the low end of the column, flow is erratic. As such the inherent properties of the beads do not compensate for the axial dispersion caused by this turbulent and erratic flow in low beds. This same trend is also observed in these results. Axial dispersion in the 50 mm column is very low both at 5 and 10 cm column height. This seems to suggest that a column height of higher than 10 cm is needed for bioseparations in the 50 mm column if it is desired that adsorption should not be limited by the instability of the bed.

2.3.6 Bed expansion characteristics

When a bed is fluidised with, say, a liquid, it expands. It is desirable that this expansion is in a predictable fashion hence this experiment. The bed expands linearly with flow velocity (Figure 2.15). This is typical of streamline adsorbents and this trend agrees with what has been observed elsewhere (Chang 1993). The beads were also observed to follow the Richardson-Zaki correlation. This correlation states that

$$U = U_t \varepsilon^n$$

where U is the superficial velocity, U_t is the terminal velocity of the beads, ε is the bed voidage and n is the Richardson-Zaki index which depends on the Reynolds number (Re) where

$$\mathrm{Re} = U_t d_p \rho / \mu$$

where d_p is the diameter of the beads, ρ is the liquid density and μ is the liquid viscosity.

By plotting ln (U) against ln (ε), n and U_t can be obtained from the slope and the y intercept respectively. The expanded void fraction, ε, was calculated as

$$\varepsilon = 1 - \frac{H_O(1-\varepsilon_o)}{H}$$

where ε_0 is the settled bed void fraction which is equal to 0.4.

The plot for Streamline™ SP which was used for the RTD experiments, is shown in Figure 2.16. The values of n and U_t were calculated as 5.2 and 1974 cm/h respectively. The theoretical value of n is 5.1 and this value agrees with the experimentally determined value.

2.4 CONCLUSIONS

The success of expanded bed chromatography in bioseparation depends partly on the performance of the bed. As opposed to packed columns where the particles are in a stationary state, the particles in an expanded bed move about an equilibrium position. This movement of particles may be turbulent and hence contributes to axial dispersion within the bed. The ideal situation is when the particles are stationary and the flow of the liquid through column approaches plug flow. In this chapter it has been established that axial dispersion within the bed depends on the settled bed height, flow velocity, column diameter and the bed aspect ratio.

Axial dispersion was observed to be higher in short beds than in longer beds. Thus the bed was hydrodynamically less stable if the settled bed height was low than when the settled bed height was high. This implies that longer columns should be used to be certain that adsorption is not limited by the hydrodynamic instability of the bed.

Theoretically, axial dispersion should decrease with increasing flow velocity (bed expansion). This was indeed the case in the experiments conducted in this project. It was also observed that in short beds, a bed expansion of at least two should be used since in some cases the Peclet number was less than 20. Thus higher flow velocities are better from a hydrodynamic point of view. However high flow velocities would lead to short contact time between the adsorbent and the target molecule. Since adsorption is time dependent, high velocities are not desirable. Hence an optimum situation has to be investigated experimentally. Usually a bed expansion of two is good enough for bed stability without compromising too much on the contact time.

Axial dispersion is expected to increase with increase in column diameter if the bed height is left unchanged. This trend was not well developed in this project. This is probably because of the high sensitivity of the 12.5 cm column to slight deviations from the vertical position. When axial dispersion was investigated in columns of different diameter but containing the same volume of adsorbent, the superiority of small diameter columns is unquestionable. Hence it can be concluded that from a hydrodynamic perspective, small diameter columns are better than large diameter columns. However the scale of production needs to be taken into consideration. As

discussed in section 2.3.3, "scale out" is cheaper and bioseparations in small diameter columns may not be practical.

The effect of the bed aspect ratio on axial dispersion is the sum of the three parameters, i.e. column height, flow velocity and column diameter. In general as the bed aspect ratio increases, axial dispersion decreases. The analysis of the data with respect to *H/Dc* has made it easier for the following trends to be observed:

- Flow velocity exerts more impact on axial dispersion than does the bed height.
- At the same *H/Dc* and same flow velocity, axial dispersion increases with increasing *Ho*.
- At the same *H/Dc* and in the same column axial dispersion is a function of the expanded bed height, *H*.

The hydrodynamic stability of the bed is an important parameter in expanded bed operations. It can grossly affect adsorption and hence should be optimised before bioprocess separations commence. To the experienced operator, eye inspection is good enough to ascertain, though qualitatively, the extent of bed stability. Other instruments may be helpful to align the columns so that they are 0° with respect to the *z*-axis and 90° with respect to both *x* and *y* axes.

CHAPTER THREE

OPTIMISATION OF PRODUCT CAPTURE IN EXPANDED BEDS

3.0 INTRODUCTION

Beside the low process times, low operation cost and high yield, it has been reported that the power of expanded bed chromatography is that clarification, product capture, product concentration and initial purification are combined in a single step (Hjorth 1997, Karau 1997, Chang 1996b). Depending on the intended use of the product, it is not always true that all of these parameters will be as important as each other in every separation process. For example the dynamic binding capacity and the product concentration may be more important if the product is found in very low concentrations naturally. The purification factor will be important if the product is to be used for obtaining biochemical data of the protein or if any contaminants have side effects to patients as is the case for antivenoms. Thus it could be helpful if information were available on how the dynamic binding capacity, the product concentration and the purification factor respond to changes in the same set of experimental conditions. This would be useful in making informed decisions when optimising product purification.

It is very likely though that all these four parameters will respond differently to different experimental conditions. For example, ion exchange adsorbents have a larger capacity than affinity adsorbents yet the purification factor achieved on ion exchange adsorbents is less than that achieved on affinity adsorbents. For example Streamline DEAE was found to bind 10.4 U of Glucose-6-Phosphate Dehydrogenase (G6PDH) per ml of adsorbent with a purification factor of 4.9 at pH 8 (Chang 1996). Working in the same laboratory and at pH 8 but this time with an affinity adsorbent, McCreath (1994) obtained a dynamic binding capacity of G6PDH of 2.1 U/ml of adsorbent and a purification factor of 16.8. Thus if product capture is the main objective, then ion exchange adsorbents would be ideal for G6PDH purification. However if the purification factor were more important, affinity

adsorbents would be a natural choice. A comparative study on how the purification factor, the dynamic binding capacity and product concentration vary with changing experimental conditions is missing. Although it is possible to collect data from different authors, using such data could be misleading since the experimental conditions under which the data were obtained are likely to be different.

With ion exchange resins, the reported purification factors are usually low (Alamilo 1991, Osterlund 1997, Apeler 1997) as feed viscosity, conductivity, cell debris and the background proteins are expected to affect the product capture in one way or the other. The increased viscosity reduces the diffusivity of the protein thereby affecting the rate of mass transfer from the bulk of the liquid to the surface of the adsorbent. The conductivity reduces the electrostatic forces between the protein and the matrix in ion exchange chromatographic separations. The cell debris may compete with the target molecule for sites on the matrix. Indeed it has been shown that cells and cell debris can bind to ion exchangers (Ujam 2000).

The dynamic binding capacity (Q_d) is usually determined from batch experiments and often with pure proteins (Weaver 1996, Guan-Sajonz 1996, Chang 1996b). This is then taken as a measure of the maximum capacity expected from the purification procedure. This is indeed the best way to calculate the maximum dynamic binding capacity for two reasons: Using pure proteins ensures that there is no competitive adsorption from other proteins. Secondly doing the experiment in a batch mode ensures that equilibrium conditions are met. However this is not entirely a good approximation for expanded bed experiments. Although the dynamic binding capacity is a thermodynamic quantity, it will require longer contact times for the same level of binding to be achieved if the feed used was a typical expanded bed feed (i.e. unclarified and viscous feedstock). Thus the use of pure proteins oversimplifies the adsorption phenomena. It has also been shown (Fernandez-Lahore 1999) that the stability of expanded beds depends on matrix and the type of feed. For example Streamline SP was found to be more stable than Streamline DEAE when whole cells were used. However when cell homogenates were used, the two matrices were observed to be equally stable. Since the dynamic binding capacity is a function of the bed stability, it is therefore more meaningful

to approximate the binding capacity by using typical expanded bed feedstock. Manufacturers of devices that are used for concentrating solution indicate how much the solution can be concentrated in a given time. Although product concentration is achieved in chromatographic steps (if the protein is adsorbed followed by elution) there is no detailed quantitative information on how much this is achieved. As expected it is assumed that the eluted fractions are free of cell debris. This is true to some extent. For example it has been shown that cell clearance in an industrial centrifuge is 10^2-10^3 times (i.e. number of cell before centrifugation over number of cells after centrifugation) (Hjorth 1997). Cell clearance from expanded beds is in the range of 10^4-10^5 times which is 1 – 2 orders of magnitude higher than that achieved from an industrial centrifuge. Thus even though the supernatant from centrifugation is regarded as particulate free, it is not absolutely particulate free.

The experiments reported here were conducted so that these four parameters (i.e. dynamic binding capacity, purification, concentration and clarification) could be monitored under the same conditions. Glucose-6-phosphate dehydrogenase (G6PDH) was used as a model system because of its good binding capacity on Streamline DEAE. In expanded mode, sample loading is usually done at either constant flow rate or constant bed height. These two modes have opposing advantages and disadvantages. Loading at constant flow ensures a high void volume that allows cell debris to pass freely but contact time is shorter. The opposite is true for loading at constant bed height (i.e. the void volume is less but the contact time is longer). Since yield and purification factor are commonly used as a measure of how successful an experiment has been, these experiments may help to optimise one or the other or both.

3.1 OBJECTIVES OF THE EXPERIMENT

The objectives of these experiments were three fold. The first one was to investigate how the dynamic binding capacity, the purification factor, the product concentration and feed clarification would be influenced by the concentration of glucose-6-phosphate dehydrogenase (G6PDH) in the feed. The second objective was to investigate how the same parameters would be affected by the amount of cell debris (cell density) in the feed. As mentioned in section 3.0, feed is loaded on to columns at either constant flow or constant height. Hence the final objective of these experiments was to investigate how the same four parameters would be affected by changing the loading modes.

3.2 MATERIALS AND METHODS

Yeast pellets were purchased from a local supplier. Tris-HCl, KCl, $MgCl_2$, glucose-6-phosphate(G6P), NaDP were purchased from Sigma UK. The UV unit and the fraction collector were a gift from Pharmacia Biotech, Uppsala Sweden. The 10 mm column was bought from Soham-Scientific, Cambridge. One peristaltic pump (Cole Palmer, UK) was used in the experiment. Assays were performed on a Shimadzu Spectrophotometer (UV-1201).

3.2.1 Choice of column height and bed expansion

From the bed hydrodynamic studies (Chapter 2), it emerged that a settled bed height of 20 cm showed the least axial dispersion. The Peclet number of a 20 cm column was also found to be greater than 20 for bed expansions of 1.5 to 3 times. It was then decided to use this column height and a bed expansion of 2 so that bed stability would not limit adsorption of the product. A 10 mm column was used in these experiments.

3.2.2 Choice of enzyme and adsorbent

To obtain a good trend, it is necessary that the enzyme should bind very well to the adsorbent. The binding of G6PDH to Streamline™ DEAE has been shown to be good in

terms of both the selectivity and the capacity (Chang 1996b). It is thus a perfect choice for these experiments.

3.2.3 Preparation of yeast suspensions.

12.5 g of the dry yeast was weighed and added to buffer to give a 5% (w/v) suspension. The same mass was added to appropriate volumes of buffer to give a 2% and 1% suspension. The suspensions were poured into a bench top bead mill with a holding capacity of ~250ml. About 50% of this volume were filled with glass ballotini, 0.5 mm diameter. The suspension and the beads were properly mixed to ensure that no air was trapped between the beads. The instrument has a water-ice jacket into which the mixer unit is immersed. The mixture was allowed to cool down for about 5 minutes before disruption commenced. Each disruption cycle was conducted at constant speed of the unit for 30 seconds disruption followed by cooling for a further 30 seconds. A total number of 5 cycles were conducted.

In increasing the cell density, both the G6PDH concentration and the cell debris content were increased. Thus it was necessary to set the conditions so that only the effect of increasing the G6PDH concentration (with solid concentration constant) could be investigated. On the other end, it was also important to investigate the effect of increasing the solids content (with G6PDH concentration constant). To analyse the effect of enzyme concentration and cell debris content on the adsorption, the following experiment was designed. 4/5 of a 5% yeast suspension was centrifuged at 11000g for 15 minutes. The supernatant was then mixed with the remaining uncentrifuged 1/5 of the 5% suspension. The absorbance of the mixed suspension was measured and it was found to be similar to the 1% uncentrifuged suspensions. Thus the 1% suspensions and the 5% centrifuged suspensions had the same concentration of solid but different G6PDH concentration. On the other hand, the 5% suspensions had the same G6PDH concentration as the 5% centrifuged suspension but different in cell debris concentration. Table 3.1 shows the relative concentration of each feed. In all cases, large sample loading was used so that full capacity of the column could be utilised as done in equilibrium binding capacity experiments.

In these experiments the sample volumes were different. Consequently the loading times were also different. Since enzyme activity can be lost with time, a control sample was left at room temperature until the run was finished. Its activity was then compared with the activity of the feed at the beginning of the experiment. In all cases there was no loss of activity.

3.2.4 Expanded bed chromatography.

The column (10 mm i.d.) was packed with Streamline DEAE to give a settled bed height of 20 cm. This was expanded to 40 cm with 20 mM Tris-HCl, pH 8. The flow velocity needed to expand the bed twice was 290 cm/h. When the bed was stable, the homogenate was then loaded either at constant flow (CF) or at constant height (CH). The effluent was monitored by the UV unit and fractions were collected for offline analysis.

3.2.5 Loading modes.

The homogenised suspensions were loaded onto the column either at constant flow (CF) or at constant height (CH). A typical expanded bed feed is more viscous than equilibrating buffer. As such the bed height expands more during feed application. To maintain the constant height during loading, the flow velocity was constantly reduced until the bed was observed to have stabilised. The time taken for the bed to stabilise was about 16 minutes. The adapter was positioned 2-3 cm above the bed height.

Similarly, when loading at constant flow, the adapter was position above the anticipated bed height. The increase in bed height was monitored with time. It took 14-30 minutes for the bed to stabilise.

3.2.6 Column washing and elution

When loading was complete, the column was washed by the same equilibration buffer to remove weakly or unbound molecules. This was stopped when the UV signal returned to baseline. Flow was then stopped and the bed was allowed to settle. Bound molecules were then eluted with 1 M KCl in a downward direction. The elution flow rate was the same as the loading flow rate.

3.2.7 Protein, solids and enzyme assays

The protein content was assayed using the Bradford assay as follows. 0.1 ml of appropriately diluted and centrifuged fractions was added to 3 ml of the Bradford reagent and mixed. This was left to stand for at least five minutes and analysed at 595 nm on the Shimadzu UV spectrophotometer.

The enzyme assay solution consisted of the following: 1.35 ml of 100 mM Tris-HCl pH 7.4 containing 3.3 mM $MgCl_2$, 50 µl of 6 mM NaDP and 50 µl of 100 mM Glucose-6-phosphate. The reaction was started by adding 50 µl of the enzyme solution. Increase in absorbance was monitored at 340 nm. One unit of the enzyme is defined as the amount that consumes 1µ mole of the substrate per minute at 20°C.

The solid content in the fractions (C_s) and the solid content in the feed (C_{so}) were assayed by monitoring absorbance at 630 nm.

3.3 RESULTS AND DISCUSSION

3.3.1 The dynamic binding capacity (Q_d)

It has been demonstrated that the binding capacity (from batch experiments) and the rate of protein adsorption are affected by the presence of cells (Chase 1992). In this particular case the cells were not disrupted. However in a typical expanded bed feed, the cells are disrupted hence the presence of cell debris, other proteins and the increased conductivity and viscosity of the suspension will affect the rate of adsorption and consequently the dynamic binding capacity. The cell debris and the non-specific proteins compete for binding sites with the target protein. The viscosity reduces the diffusion rate and the conductivity reduces the electrostatic attraction between the target protein and the ion exchange matrix. Thus if a typical expanded bed feed is used to obtain binding capacity in batch modes, longer contact times are needed otherwise the effect of the cell density would be significant.

In column chromatographic separations, equilibrium is never reached (Skoog 1991) hence the dynamic binding capacity is used instead of the usual equilibrium binding capacity. The dynamic binding capacity was calculated from concentration of G6PDH in the eluate. The results are summarised in Figure 3.1.

3.3.1.1 The effect of feed concentration on the dynamic binding capacity

It is tempting to compare the binding capacities of the 1%, 2% and 5% suspensions. Increasing the cell density meant that both the cell debris and the G6PDH concentration in feed were also increased. Hence the observed trend is the effect of increasing both the G6PDH concentration and the cell density. The partially centrifuged suspensions were included in these experiments so that the effect of increasing G6PDH concentration could be investigated whilst the cell debris content remains constant and vice versa. To analyse the effect of the G6PDH concentration in the feed on the dynamic binding capacity, the 1% suspensions should be compared with the partially centrifuged 5% (5%C) suspensions. These suspensions (Table 3.1) contain the same cell debris concentration, comparable total amount of background protein but their G6PDH concentration is different.

From Figure 3.1, it is observed that the dynamic binding capacity is slightly higher in the partially centrifuged suspensions than in the 1% suspensions. If the contact times were equal, it was expected that a higher G6PDH concentration would give a higher dynamic binding capacity than a lower G6PDH concentration because of the high concentration gradient between the bulk of the liquid and the surface of the adsorbent. It therefore seems that the longer contact time used to load the 1% suspensions, greatly improved the adsorption of G6PDH to rival that of the 5% partially centrifuged suspensions (5%C).

3.3.1.2 The effect of the cell debris on the dynamic binding capacity

This is obtained by comparing the partially centrifuged suspensions with the 5% suspensions. The 5% suspensions have roughly 5 times as much cell debris as the partially centrifuged suspensions (Table 3.1). However their G6PDH concentrations are similar. This being the case it was expected that the partially centrifuged suspensions would show a higher dynamic binding capacity than the 5% suspensions. This is not the case as observed from Figure 3.1. The dynamic binding capacity of the 5% suspensions is comparable to that of the partially centrifuged suspensions within experimental error. However as can be seen from Figure 3.1, there seems to be a slightly better binding in the 5% suspensions than the partially centrifuged suspensions. There are two explanations for this observation. The first suggestion is that centrifugation may have favoured more non-G6PDH protein adsorption. If this is the case, the partially centrifuged suspensions should show a lower purification factor than the 5% suspensions. This is indeed the case (although only just) as observed from Figure 3.4. The other reason might be that the cell debris concentration might not be playing a significant role in this range.

3.3.1.3 The combined effect of cell debris and G6PDH concentration on the dynamic binding capacity

To obtain this trend, the dynamic binding capacity of the 1%, 2% and the 5% suspensions are compared with each other. As can be seen from Figure 3.1, the binding capacities are within experimental error of each other. Nevertheless the general trend is that the dynamic

binding capacity is increasing slightly with increasing feed concentration. Normally, the dynamic binding capacity should have been decreasing as the feed concentration is increased since the higher feed concentrations are more viscous and their conductivity is high. The viscosity affects the adsorption rate and the conductivity reduces the electrostatic attraction between the target enzyme and the adsorbent. The viscosity values were 1, 2 and 3 mPa.s respectively. Similarly the conductivity values were 84, 99 and 133 μS. It would be expected from these values that the dynamic binding capacity would decrease as the cell density increased. The fact that this is not the case seems to suggest that these viscosity and conductivity values exert insignificant impact on the overall capacity of the columns. The cell debris should also have decreased the dynamic binding capacity in two ways. Some of the particulate material may act as point charges that would compete for sites with the product. In the other case the solids may just interfere with the movement of molecules to the surface. However neither of these is significantly affecting the adsorption. Thus it is plausible to suggest that the cell debris, the viscosity and the conductivity of the suspensions are not playing a dominant role in limiting the adsorption of G6PDH in the range investigated here.

3.3.1.4 The influence of the loading modes on the dynamic binding capacity

The samples were loaded at either constant flow (CF) or at constant height (CH). These two loading modes have advantages over each other. Loading at constant flow ensures that during sample loading the void fraction is higher. This allows the cell debris to pass more freely thus creating room for more adsorption of the target molecule. Loading at constant height ensures that the contact time between the target molecule and the adsorbent is longer. This allows for more adsorption.

From Figure 3.1, it can be seen that the values obtained are within experimental error of each other. However two patterns are apparent. In suspensions of low cell debris (the 1% suspensions and the partially centrifuged suspensions), the dynamic binding capacity is higher if loading is done at CH than at CF. Keeping in mind the fact that loading time is longer at CH than at CF, this trend suggests that the longer contact time in suspensions of

low cell debris content is more dominant than the higher void fraction. This makes sense since these suspensions have a low cell debris concentration.

In suspensions of higher cell debris content (2% and 5% suspensions), the dynamic binding capacity is higher if loading was done at CF than at CH. Thus the void fraction which allows for more freedom of cell debris removal seems to be dominant over the influence of the contact time in these suspensions. Furthermore it was shown by Finette (1998) that the amount bound is also a function of the flow velocity. Bound α_1-antitrypsin was found to decrease with increasing flow velocity while for human serum albumin the opposite was observed. Thus much as the void fraction is important, the loading flow velocity (which is higher at CF than CH) may be playing an additional role.

3.3.2 Product concentration

Although it has been mentioned repeatedly that EBA increases product concentration (Hjorth 1997, Karau 1997, Chang 1996b) there is no quantitative data on how much this is achieved. This is probably because in chromatographic separations, product concentration is not normally used as a measure of success of a bioprocess. In some processes it is required to concentrate the product for further loading. For such processes the data on product concentration will be of great value. The data obtained in these experiments are shown in Figure 3.2

The general trend of the data is that as the feed and cell debris concentration increase, product concentration (A/A_o) decreases. If CH is compared to CF, the same picture as that obtained from Figure 3.1 is visible, i.e. the values are within the experimental error of each other. Just like Figure 3.1, Figure 3.2 also shows that in suspensions of low cell debris (1% and the partially centrifuged suspensions), G6PDH is concentrated slightly more if loading is done at CH. In high density cell suspensions (2% and 5%) the enzyme is concentrated slightly more if loading is done at CF. This confirms the suggestion that the larger void fraction in 2% and 5% suspensions is important in enhancing protein adsorption.

However analysing the enzyme concentration data based on A/A_o alone is not complete. A/A_o may be high in suspensions of low cell density. However the actual enzyme activity in the eluate may not necessarily be higher. So it was necessary that this A/A_o picture be complemented with the actual enzyme activity in the eluted fraction. These data are shown in Figure 3.3. From this figure, two things are readily noticeable. The first one is that there is no significant difference in the G6PDH activity of the eluted fractions. Contrary to the A/A_o data which showed higher A/A_o values in suspensions of low cell density, the G6PDH activity in the eluted fractions are nearly the same (within experimental error). Since shorter process times are achieved if high cell density feed is used, it would seem that loading high cell density feed is economically attractive in this perspective. The second picture is that when the two loading modes are compared against each other, the same trend as was observed in Figure 3.1 and Figure 3.1 is observed. G6PDH is concentrated slightly more in suspensions of low cell density (1% and the partially centrifuged suspensions) if loading is done at CH. If loading is done at CF, G6PDH is concentrated slightly more in suspensions of high cell density (2% and 5%).

3.3.3 The purification factor (p.f.).

The purification factor has commonly been the parameter used in reporting protein purification data. This is justifiable since the purification factor is a quantitative measure of how pure a protein is. The results for this experiment are shown in Figure 3.4.

From Figure 3.4, it is apparent that there is no significant difference in the p.f. values for all suspensions. These data are similar to that which was observed in the G6PDH activity data (Figure 3.3). There is a slight improvement on the p.f. of G6PDH if loading is done at CF. Thus there is slightly more selective adsorption of G6PDH when loading is done at CF than at CH. The purification factor of the partially centrifuged suspensions is identical to that of the 1, 2 and 5% suspensions if loading was done at CH, but it is slightly less than that of the 1, 2 and 5% suspensions if loading was done at CF. This is similar to what was observed in Figure 3.1. Thus centrifugation favoured more non-G6PDH binding as was mentioned in section 3.3.1.2.

3.3.4 Stepwise elution of G6PDH.

Throughout these experiments, 1M KCl was used to elute bound proteins. This was so to ensure that all bound G6PDH weas eluted. However because of its high ionic strength, more of the non-G6PDH proteins were eluted as well. Since the purification factor values are generally low, it was found necessary to find ways of improving the purity of the eluates. The obvious way was to use step elution with salt solution of different concentration. 0.1M, 0.2M, 0.4M and 1M salt solutions were used. The elution profile of the proteins and G6PDH are shown in Figure 3.5. From this figure it was calculated that with 0.1M KCl, 58% of the bound G6PDH were eluted against 40% of the proteins. If 0.2M KCl was used, a total of 99% of the bound G6PDH would be eluted against 65% of the background proteins. Thus it is a trivial matter to see that by eluting the bound G6PDH with 0.2M KCl, the purification factor would be improved.

3.3.5 Feed Clarification

It is generally expected that the eluted fractions would be free of cell debris. This was also observed in these experiments. The feed had been clarified $10^2 - 10^3$ times. However this is in the same order of magnitude as that achieved by industrial centrifuges so EBA was not superior in this respect.

3.6 CONCLUSIONS

Process optimisation in expanded bed chromatography also depends on experimental conditions in addition to the hydrodynamic bed stability. A thorough understanding of experimental conditions that affect specific parameters is important especially when the eluates have a specific use. Only three parameters, namely feed concentration, cell debris concentration and loading modes, were investigated in these experiments. Perhaps it is important to recap the experimental setting. Ideally it was hoped to load the same G6PDH activity, protein and solid content in which case the volumes loaded had to be different. From these experiments one fundamental conclusion can be made. On one extreme there are low cell density suspensions with low G6PDH concentration, low viscosity and low conductivity but the contact time during loading was long. On the other extreme are high cell density suspensions with high G6PDH concentration, high conductivity and high viscosity.

In the low cell density suspensions, the low viscosity and conductivity and the longer contact time should aid adsorption. The low G6PDH concentration would however give low mass transfer rates. In high cell density suspensions, the high viscosity, conductivity and cell debris would be counter-productive for G6PDH adsorption but the high G6PDH concentration is favourable. From the experiments reported in this chapter, it was observed that there is no remarkable differences in the dynamic binding capacity, purification factor and product concentration when working with low cell density suspensions compared to high cell density suspensions. Thus it would seem that the longer loading times used in low cell density suspensions is as effective as the effect of using a higher concentration feed. In spite of this general trend, there are minor trends that should be mentioned in brief. Figure 3.6 is a summation of all the experiments conducted in this chapter.

1) The dynamic binding capacity was slightly higher in high cell density suspensions if loading was done at constant flow. In low cell density suspensions the dynamic binding capacity is higher if loading was done at constant height.

2) The purification factor was observed to be higher if the sample was loaded at constant flow than at constant height. However the feed concentration and the cell density did not have a significant impact on the purification factor as it was observed to be effectively constant.

3) Partial centrifugation of the feed did not improve the dynamic binding capacity or the purification factor. It was suggested that this might be due to the fact that centrifugation favoured more non-G6PDH binding.

4) Enzyme concentration (A/A_o) was high in low cell density suspensions. A/A_o at CF was higher than at CH in high cell density suspensions and vice versa in low cell density suspensions. On the other hand there was no significant difference in the actual enzyme activity in the eluted fractions.

5) Qualitatively the eluates were free of cell debris as they had been clarified $10^2 - 10^3$ times.

With these observations, the following conclusions on product optimisation can be suggested.

1) As mentioned in paragraph two of this section, there are no substantial differences in the dynamic binding capacities, purification factor and product concentration. However the major difference is in the process times. The process time for the 1% suspensions is roughly 5 times more than that of the 5% suspensions. From an economic point of view, working with higher cell density suspensions is thus advantageous.

2) If however low cell density suspensions are preferred, loading at constant height is better than loading at constant flow. The dynamic binding capacity and product concentration are slightly higher but the purification factor is slightly lower than loading at constant flow. This implies that if the purification factor is the prime concern, then loading should be done at CF.

It has to be mentioned here that the observed trends act as a guide only. This is true specifically for G6PDH and in the experimental conditions investigated in this project. For other enzymes the trends may change. It is hence important that similar method scouting experiments be conducted for other enzymes.

CHAPTER FOUR

THE APPLICATION OF EBA TO CAPTURE MOLECULES: THE PURIFICATION OF GLUTATHIONE-S-TRANSFERASE.

4.0 INTRODUCTION

Glutathione-S-Transferases GSTs (EC 2.5.1.18) are a family of ubiquitous enzymes that catalyse the conjugation of reduced glutathione with reactive electrophiles (He 1998). These enzymes are associated with the detoxification of xenobiotics (e.g. drugs, carcinogens, and certain environmental pollutants in man (Habig 1974)). They are also responsible for pesticide and herbicide resistance in insects, parasites, fungi and plants (Nishida 1994). Other researchers in this area have shown that GSTs also protect termites from toxic effects of plant chemicals and pesticides (Haritos 1996).

On the basis of primary structure, immunological properties and substrate specificity, GSTs have been divided into six groups. These have been designated alpha, mu, pi, theta, zeta and sigma (Angelucci 2000). X-ray crystallography reveals that these GSTs have two domains joined together by a short linker of amino acid residues. Domain 1 (called the GSH binding site) is located in the N-terminal region of the protein and is made up of alpha and beta helices. Domain 2 (the H binding site), is composed solely of alpha helices and provides most of the hydrophobic binding (Angelucci 2000).

Literature has implicated oxidants or electrophilic stress in the pathogenesis of human atherosclerosis (Buczynski 1993, Darley-Usmar 1990, Sharma 1992). Such compounds as allyamine and acrolein (a highly electrophilic alpha, beta unsaturated aldehyde, a component in cigarette smoke and an industrial pollutant) have been implicated as inducers of atherosclerosis through their toxic actions on blood vessel walls and vascular smooth muscle cells. Because GSH is a nucleophile, it can conjugate to these toxic electrophiles. However this reaction is catalysed

by GST. He (1998) have shown that administration of Sulfasalazine, an inhibitor of GST, increased the cytotoxity of acrolein and allyamine by two-three fold.

GSTs are also involved in the intracellular binding and transport of hydrophobic molecules (heme, bile acids and polycyclic hydrocarbons), prostaglandin and leukotriene biosynthesis, and elimination of toxic hydroperoxides (Angeluccis 2000). They also act as binding proteins to hydrophobic endogenous and exogenous compounds (Hiratsuka 1997)

Research has also revealed that consumption of cruciferous vegetables (mustards, cabbage, broccoli, and Brussels sprouts) has been associated with a lower incidence of cancers of the pancreas, (Olsen 1991) colon and rectum (Lee 1989, Graham 1983) and urinary bladder (Graham 1983). These vegetables have significant amounts of compounds such as crambene (1-cyano-2-hydroxy-3-butene) which induce the production of either GST or GSH (March 1998). As pointed out above, the GST or GSH will then detoxify any potential carcinogenic substances.

GST is mainly used as a fusion partner for the purification of recombinant proteins (Guan 1991, Nishida 1994, Sehgal 2000). This technology has been applied for the production of different types of proteins for various end uses including antibodies and DNA binding proteins. The fusion complex comprising the target recombinant protein is attached to GST through a protease cleavage site. The process has chiefly been done in batch (Guan 1991) and packed columns (Simons 1977, Reade 1999). The adsorbent consists of glutathione as a ligand which selectively binds to the GST portion of the complex. The GST is then chopped from the target protein by using an enzyme.

The work reported in this chapter involved application of the EBA to capture glutathione-s-transferase. A commercially available ion exchange matrix (Streamline™ DEAE) and two affinity matrices tailor made for use in expanded bed chromatography were used. The affinity adsorbent developed was an in-house synthesised glutathione coupled to the Streamline™ quartz base matrix.

4.1 OBJECTIVES.

The first objective of these experiments was to prepare an affinity matrix based on the Streamline™ base matrix that would be efficient in purifying GST. The Streamline™ matrix was important as the experiments were to be conducted in an expanded mode. Once the suitable matrix had been prepared, the second objective was to compare the performance of the matrix with a commercially available one. GSH-Sepharose 4B was the matrix of choice as it is widely used. The final objective was to compare the purity of GST from the single EBA step to a two step process of Streamline™ DEAE followed by packed bed adsorption.

4.2 MATERIALS AND METHODS

Streamline quartz base matrix was a gift from Pharmacia Biotech (Sweden). Its density was 1.2 g/ml and mean diameter of the beads was about 200 μm.

4.2.1 Cell disruption

The GST used in these experiments was expressed in E.coli. The Pharmaceutical Sciences Department at Aston University kindly grew the cells, expressed the GST and harvested the cells for this project. The harvested cells were first suspended by diluting in the appropriate buffer which was either 20 mM carbonate/bicarbonate at pH 10 for ion-exchange runs or 10 mM PBS at pH 7.4 for the affinity based adsorption experiments. A dilution factor of 10 fold was usually taken as standard.

The cell suspension was then poured into a bench top homogeniser with a holding vessel volume of ~250 ml. Approximately 50% of the volume was dry 0.5 mm diameter glass ballotini. The suspension was filled to the brim and the contents uniformly mixed to ensure that no air was trapped in the void spaces of the glass ballotini. The design of the apparatus allowed for the mixer unit to be immersed in an iced water bath. Each disruption cycle was conducted at the fixed speed of the unit for a 30 seconds interval followed by a 1 minute period to allow the suspension to cool. A total of 5 cycles were conducted.

4.2.2 PROTEIN ASSAY

The protein content was assayed according to the Bradford method. 0.1 ml of the sample to be assayed was mixed with 3 ml of the Bradford reagent, mixed well and left standing for approximately 5 minutes. Absorbance of the final solution was measured at 595 nm using a Shimadzu model 1201 UV-spectrophotometer. The protein assay was calibrated against a standard using BSA in the concentration range between 0.2 and 1.0 mg/ml.

4.2.3 Measurement of solids

The concentration of the solids in the form of cell debris was measured by recording the absorbance of the sample at 630 nm. Readings were taken on the Shimadzu 1201 UV-spectrophotometer. For calculation purposes, the solids concentration in the feed was taken as unity and all other concentrations were expressed as a fraction of the feed concentrations.

4.2.4 GST assay

Glutathione-S-transferase (GST) activity was measured using the 1-chloro-2, 3-dinitrobenzene (CDNB) method. 1 unit of activity catalyses the formation of 1 μmol/min of CDNB-glutathione complex at 20°C. The assay buffer consisted of 880 μl water, 100 μl 1M phosphate buffer, pH 6.5, 10 μl of 100 mM CDNB in ethanol and 10 μl of 100 mM reduced glutathione. The mixture was allowed to stand for 5 minutes to allow the temperature to equilibrate. 10 μl of the enzyme solution was added to initiate the reaction which was followed by observing the change of absorbance at 340 nm.

4.2.5 SDS-PAGE

Reagents

12% Resolving gel : This was made by mixing the following:

H_2O(4ml), 3.3 ml of 40% acrylamide (19:1 acryl:bis acryl), 2.5 ml of 1.5M Tris-HCl pH8.8, 100 μl of 10% SDS, 100 μl of 10% ammonium persulphate (AMPS), and 10 μl of TEMED.

5% Stacking gel: This was made by mixing the following
3.6 ml of H_2O, 0.65 ml of 40% acrylamide (19:1 acryl:bis acryl), 0.63ml of 1 M Tris-HCl pH6.8, 10% SDS(50 µl), 10% AMPS (50 µl) and TEMED (5 µl))

Loading buffer:
50 mM Tris-HCl pH 6.8, 100 mM DTT, 2% SDS, 0.1% bromophenol blue, 10% glycerol, stored at room temperature, DTT was added just before use.

The resolving gel was added to a well. When the gel was set, excess water was removed. The stacking gel was then added and the comb was set.

About 6 µl of sample and marker (Sigma, UK) were pipetted and an equal volume of loading buffer was added. The samples were then heated to 95°C for 3 minutes prior to loading. The electrophoresis was ran at 200 V on the Biorad Min Assay kit until the dye front reached the bottom of the gel. The running buffer was made up of 25 mM Tris base, 94 g glycine, 50 ml 10% electrophoresis-grade SDS in 1L.

The samples were then stained using the Coomassie blue stain (40% methanol, 10% glacial acetic acid, 0.4% w/v Coomassie blue) preheated to approximately 70°C. De-staining was achieved using a mixture of 40% methanol and 10% glacial acetic acid.

4.2.6 Activation of Streamline™ Base Matrix

Epoxy activation of the Streamline™ base matrix was carried out based on the method described by Porath (1974). 60 ml of the Streamline™ quartz base matrix was washed extensively with distilled water. Excess water was removed by vacuum filtration. The adsorbent was then washed with 30 ml NaOH containing 60 mg $NaBH_4$ and 30 ml of 1,4- Butanediol diglycidyl ether. The mixture was placed in an incubator and shaken gently for 7 hours at 25°C. The activated adsorbent was finally washed once again with an excess (> 10 volumes) distilled water and equilibrated by re-suspending in the appropriate buffer for the coupling.

4.2.7 Coupling of Glutathione to the activated matrix

The attachment of the glutathione ligand to the epoxy-activated matrix was carried out at two separate pHs which resulted in the synthesis of Matrix A and Matrix B. Glutathione has mainly two reactive nucleophiles, the thiol group and the amino group. The reactivity of these functional groups depends on pH. It was thus hoped that by using two different pHs, the glutathione molecule would attach to the activated matrix *via* the thiol group in one case and *via* the amino group in the other.

4.2.7.1 Synthesis of Matrix A

This was performed at pH 7.0 using phosphate buffer. Nitrogen gas was bubbled through 120 ml of 50% slurry of the activated matrix in 0.1 M phosphate buffer for 5 minutes. The liquid was then decanted and the beads were incubated with 120 ml of the glutathione solution (0.1 g/ml in the phosphate buffer) at 37°C for 22 h. The matrix was then washed exhaustively with distilled water followed by the phosphate buffer.

The liquid was once again decanted and 120 ml of 1M thiourea were added. The flask was put in an incubator with shaking at 25°C for 4hrs. This step was necessary to block unreacted epoxy groups. After blocking of unreacted groups, the adsorbent was first washed with water and then packed into a column in which it was further washed with 500 ml of 0.5M KCl in 100 mM sodium acetate buffer, pH 4.2. The affinity matrix was unpacked from the column and refrigerated until used.

4.2.7.2 Synthesis of Matrix B

This was performed at pH 8.5 using 100 mM Sodium bicarbonate. 40 ml of activated matrix was first vacuum filtered to remove excess interstitial liquid. To this was added 80 ml of glutathione solution (0.1 mg/ml in 100 mM $NaHCO_3$ buffer, pH 8.5). The mixture was incubated for 24 hrs at 37°C in an incubator with shaking (250 cycles/min). The beads were then washed exhaustively with water. Unreacted epoxy groups were blocked in the same manner as in the previous case using 1M thiourea. The beads were packed into a column and washed with at least 10 column

volumes of the following: 100 mM $NaHCO_3$, pH 9.5 containing 1M NaCL and 1 mM EDTA; 100 mM sodium acetate, pH 4.3 containing 1M NaCL and 1 mM EDTA. The matrix was subsequently unpacked from the column and stored in the refrigerator.

4.2.8 Tween coated beads

Tween 20 was diluted 20 times in water. 20 ml of this dilute Tween was added to a flask containing 2 ml of the Matrix B. The flask was then left in an incubator with shaking for two hours at room temperature. The beads were then washed exhaustively with water. For a larger volume of beads, the ratio of Tween 20: beads was kept constant.

4.2.9 Ligand leakage

1 ml of 1:1 slurry of Matrix B and the base matrix were put in test tubes. The pH was then adjusted to 3, 5, 7, 9, and 11. The test tubes were put on a spiral mixer for one hour at room temperature. The beads were then allowed to settle and the supernatant was carefully decanted. The supernatant was then assayed for GSH by measuring absorbance at 235 nm. In a parallel experiment, 2 ml of NaCl (0.25 M, 0.5 M, 0.75 M and 1.0 M) were put into test tubes. 1 ml of 1:1 slurry of Matrix B and the base matrix were added. The process was continued as above.

4.2.10 Batch adsorption of GST to Glutathione-Streamline™ adsorbents

4 ml of cell free GST were added to 0.5 ml of adsorbent in test tubes. The mixture was contacted under mixing for 60 minutes and then centrifuged at 4000 g for 5 minutes. The supernatant was removed and the gel washed three times with 0.2M phosphate buffer, pH 7. Bound GST was eluted using either 20 mM glutathione in 100 mM Tris-HCl pH 9.6 (for affinity adsorbents) or 1M KCl (for anion exchange) in 20 mM carbonate buffer pH 10.

The batch experiments were also conducted with used Matrix B to determine the stability for this matrix after first use.

4.2.11 Ligand density quantification.

4.2.11.1 Thiol content determination. Dithiol dipyridine (dipyridyl disulphide) is sparingly soluble. The first stage was to prepare a saturated solution, filter off the undissolved solutes and calculate the concentration of dithiol dipyridine in the supernatant. 2 ml of this dithiol dipyridine were then added to 1 ml of a 1:1 slurry of adsorbent. The mixture was hand shaken to mix. Absorbance at 343 nm was taken immediately after mixing and after one hour. The readings were very similar. Calibration curves were prepared by reacting GSH with the dithiol dipyridine.

4.2.11.2 Ninhydrin test. 2 ml of a 50 mM Ninhydrin solution was added to 1 ml of a 1:1 slurry of Matrix B and the base matrix (as control) in a test tube. The test tube was placed on a spiral mixer for 1 h for the colour to develop. The beads were allowed to settle and the absorbance of the supernatant was measured at 564 nm. Standards were prepared in a similar manner by reacting GSH with ninhydrin.

4.2.12 Expanded Bed Chromatography

Expanded bed chromatography was conducted in a 1.0 cm diameter column made to order by Soham Scientific Ltd. (Ely, UK). The columns were made of precision bore glass with a length of about 45 cm. The bottom of the column incorporated a sintered glass frit as flow distributor. The nominal pore size of the sintered frits was 100-160 μm with a thickness of 2 mm. The adjustable top adaptor had no sinter to allow free passage of the solid debris and was fixed in position by a screw connection at the top of the column. The equilibrating buffer was either 10 mM PBS, pH 7.4 for affinity adsorbents or 20 mM Carbonate buffer pH 10 for ion exchange.

The liquid was pumped by a Cole-Parmer Masterflex peristaltic pump situated immediately before the column and the appropriate liquid flow was directed through the use of an arrangement of manually operated valves. A Pharmacia single channel UV detector was used for continuously monitoring of the outlet stream at 280 nm and absorbance recorded on a Pharmacia single channel chart recorder. Fractions were collected for off-line analysis for protein, solids and

GST. The settled bed height was 20 cm and the beds were expanded to 40 cm. Bound proteins were eluted with either 20 mM GSH in 100 mM Tris-HCl pH 9.6 or 20 mM Carbonate buffer pH 10. Eluted fractions were assayed for their GST activity, solid and protein content.

4.3 RESULTS AND DISCUSSION

When new adsorbents are developed, it is necessary to characterise their properties. These include their binding capacity, their selectivity, stability in various conditions and of course the density of the immobilised ligand. It was thus necessary in this project that similar properties be characterised for the in-house Streamline-GSH affinity matrix.

4.3.1 Batch adsorption experiments.

Batch adsorption experiments are ideal for method scouting. They are relatively easy and fast to carry out compared to column experiments. As such several batches can be handled at the same time. Here batch experiments were conducted to determine the selectivity of the matrices for GST. The experiments have been grouped depending on which matrices were being investigated. Table 4.1 show the selectivity of Matrix A, Matrix B and the quartz base matrix for GST. Table 4.2 compares the selectivity of Matrix B and the Tween-coated matrix for GST. Table 4.3 shows the data for Matrix B, used Matrix B and the GSH-Sepharose 4B.

4.3.1.1 Matrix A, Matrix B and the Quartz base matrix.

In discussing these results, much emphasis will be placed on the purification factor and the recovery, as these are commonly used parameters in biochemical processes. From Table 4.1, it can be seen that some proteins were adsorbed by the non-functionalised Streamline™ quartz base matrix. However no GST binding was detected. This is a desirable state for it implies that only the attached ligand is involved in the interaction with GST.

GST can also bind to the epoxy groups on the matrix. The bond formed is too strong and hence the binding of the GST to the epoxy groups is irreversible (Porath 1974). To avoid this, after coupling the GSH to the epoxy-activated matrix, reacting with thiourea blocked the reactive epoxy groups.

From Table 4.1, Matrix B shows a higher purification factor and a higher recovery than Matrix A. Matrix B has a purification factor of 2.5 and a GST recovery of 48% compared to a p.f. of 1.4 and a GST recovery of 3.2% from Matrix A. Thus Matrix B is an obvious choice for GST

purification. A purification factor of 2.5 may seem too low for an affinity matrix. This should not be taken to mean a low specificity. In fact GST has been purified to a homogeneous state and this will be discussed further in section 4.3.5. The reason for this is also discussed in the light of the SDS-PAGE results. Also a recovery of 48% is not economically encouraging. It must be noted however that this being batch experiments a longer contact time is necessary to improve the yield. The elution protocol was also not optimised for these batch experiments.

4.3.1.2 Matrix B, Tween Coated Matrix B and GSH-Sepharose 4B.

Usually non-specific binding can occur with affinity adsorbents. These include ion exchange if the coupling arm has charges on its surface (e.g. CNBr), and hydrophobic interaction. In this case ion exchange interaction was excluded by using an epoxy group during the activation stage. However hydrophobic interactions were not entirely excluded. It is known from the work of others (Abel 1996, Siddiqui 1996, Shmanai 1999) that Tween 20 reduces non-specific binding without interfering with the binding of the target protein. It was decided to use this Tween 20 to reduce non-specific binding in the synthesised matrix should there be any.

It is also normally not good enough to judge the success of immobilising a ligand by its recovery and specificity. Comparison with a commercially available matrix may give relative efficiency of the immobilisation. Batch experiments were hence conducted to compare Matrix B, the Tween-Coated Matrix B and the commercially available GSH-Sepharose 4B from Amersham Pharmacia Biotech. The results are shown in Table 4.2. From this table it is clear that the two in-house synthesised matrices are better than the commercially available GSH–Sepharose 4B from Amersham Pharmacia Biotech in terms of both the recovery and the specificity (purification factor). This was rather unexpected. However there is more GST binding in the Sepharose 4B than in both Matrix B and the Tween coated Matrix B. In the elution stage though, the least amount of GST is eluted from this Sepharose 4B. It is obvious therefore that some GST from the Sepharose 4B could not be eluted. This might be due to some irreversible binding or due to the fact that the elution has not been optimised at this stage. The Tween-coated matrix shows slightly less recovery and purification factor compared to Matrix B. However, more GST is bound to the coated matrix than on Matrix B.

4.3.1.3 Characterisation after first use.

If affinity adsorbents are reusable, the cost of a purification process is reduced. Some matrices lose their binding properties, hence must be reactivated (Scouten 1985); others may lose their physical properties such as size or expansion properties. In this case, it was decided to investigate how well Matrix B retained its capacity and specificity after first use. The data are shown in Table 4.3. From this Table, Matrix B has a p.f. of 2.7 and a recovery of 72%. The used matrix has a p.f. of 2.5 and a recovery of 63%. Thus there has been little change in the original properties of Matrix B after first use. Matrix B was cleaned with just 1M NaCl and hence it can be concluded that few GSH molecules were lost during the cleaning-in-place procedure. Just like in Table 4.2, there is more adsorption on the Sepharose 4B than the others, yet in the elution stage the amount of GST eluted is least, yielding the least recovery. Its purification factor though is comparable to that of Matrix B.

4.3.2 Ligand leakage

GSH has a strong absorption peak at 235 nm. Although this is rarely used to quantify GSH, it was verified in this project that GSH absorbance at 235 nm has a linear relationship with concentration. This is easier and quicker to carry out as it does not involve reaction with other reagents hence this method was employed in the detection of any GSH, which may have leaked from Matrix B. The results are summarised in Table 4.4. The matrix was exposed to different pH condition and salt concentrations to investigate their effect on ligand leakage.

Ideally, adsorbents should be used repeatedly to reduce the cost of operation. However no adsorbent can be used indefinitely without losing its binding capacity and selectivity. How long an adsorbent can remain in use depends in part on the cleaning-in-place procedures. It is thus important that when a new adsorbent has been synthesised, its stability in various solvents is characterised. It was anticipated in this case that NaCl and NaOH would be used during the cleaning-in-place procedure. This is because they are cheap and do not contain harmful trace contaminants. NaOH also sanitises the system and destroys pyrogens. It was however found proper to analyse its stability with pH as this would also determine best storage pH.

From Table 4.4 it is evident that high pH increases ligand leakage or degradation. From these results it was determined that NaOH should not be used in the cleaning-in-place procedure as a significant amount of GSH would be lost into the media. Ligand stability in the salt solutions does not follow any order. It seems the loss of ligands is the same in all salt concentrations. Since this is low, it was decided that NaCl up to 1M could be used in the cleaning-in-place procedures. From this table it is obvious that the best storage pH should be between 5 and 7 where ligand leakage is low.

4.3.3 Ligand density.

It emerged from the batch adsorption experiments that Matrix B was superior to the commercially available GSH-Sepharose 4B from Amersham Pharmacia. One of the suggested reasons for this was that there may be more GSH ligands immobilised on Matrix B than on Sepharose 4B. It was apparent therefore that a ligand density determination be carried out.

In earlier work by Simons (1977) it was suggested that at neutral pH, the lone amino group of glutathione is protonated. It is then presumed that when coupling of the glutathione molecule is conducted at pH 7, it is the thiol group that conjugates with the epoxy group on the activated matrix as is shown in Figure 4.1. At higher pH, both the lone amino and thiol groups are free and Simons suggested that addition of the glutathione to the epoxy activated matrix may be *via* the amino group (Figure 4.2). This then leaves the thiol group free to react with any thiol containing group such as GST.

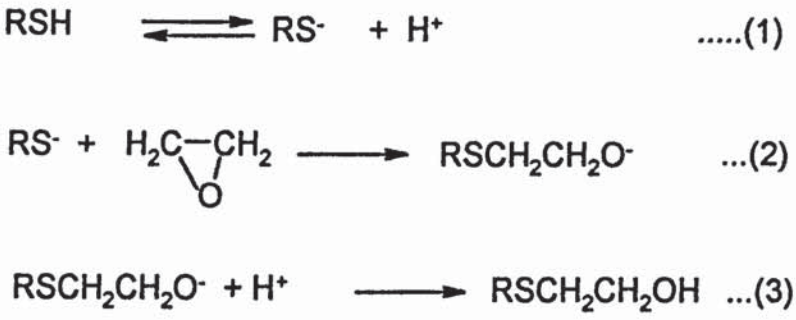
According to the arguments of Simons et al. (1977) the matrix produced at pH 7 (Matrix A) has better selectivity and capacity for GST than the one produced at pH 8.5 (Matrix B). Other authors have argued that the binding of GST to GSH-agarose depends on a free thiol group on the matrix (Scouten 1985). It is this free thiol group that interacts with the GST as is shown in Figure 4.3.

However as can be seen from the batch adsorption experiments, it is clear that the matrix obtained at higher pH (Matrix B) is superior in terms of both selectivity and capacity for GST

than the one obtained at pH 7 (Matrix A). These results seem to agree with expectation if indeed the binding of GST to the matrix depends on a free SH group.

Although the work of Simons et al. has been a point of reference for some time now, it seems the process may not be as straightforward as they supposed. To begin with the glutathione molecule exists as shown in Figure 4.4 as a function of pH. From Figure 4.4, it seems that the nitrogen of the alpha amino group (the one on the left terminal) will not take part in any reaction until the pH of the solution is at least 9.4. This implies that the immobilisation of GSH to the epoxy activated matrix will be *via* the thiol group. This has been supported by Danehy (1960), Rueben (1976) and Bruice (1976).

From the research carried out by Danehy and Noel (Danely 1960) and confirmed by Reuben and Bruice (Rueuben 1976, Bruice 1976) it is now known that the reaction between thiol groups (including glutathione) and epoxy groups proceeds *via* the thiolate ion. Danehy and Noel proposed a reaction scheme for the reaction of thiol compounds with ethylene oxide as below.



Reaction 1 is instantaneous and reaction 2 is the rate limiting step. If this is indeed the case it may be concluded that the immobilisation of GSH to the epoxy activated agarose will solely be *via* the thiol group of GSH since the thiolate ion is the strongest of all available groups including the amino group (Bruice 1976). Danehy was able to show that the reaction of thiols with epoxides increased with increasing pH and temperature. In fact the pKa of the thiol group and the amino group of glutathione were calculated as 8.72 and 9.47 (Rueben 1976). Surprisingly these values are not significantly different from the pKa values that are obtained from the constituent

amino acids, i.e. 8.3 and 9.5 (Rodwell 1996). In the light of this information it would seem that the reaction of thiols with epoxides would not be very efficient until the reaction pH is about that of the pKa of the thiol.

So far it has been assumed that the carboxyl groups on glutathione are not involved in any reaction. It has been shown that these groups can also react with ethylene oxide. There is no strong reason as to why such reactions can not take place with 1,4-n-butanediol diglycidyl ether used in these experiments since the epoxide group is at the terminal where stearic interaction is low. It is therefore possible that in conditions where the reactivity of the thiol group is low, the carboxyl group may participate in the reaction with the epoxide on the matrix. Since the pKa of the amino group is high, the likelihood of the reaction going *via* it is low and it has been suggested that solvolysis (hydrolysis in this case) will be faster than the reaction with the amino group (Bruice 1976). This makes sense as three ring epoxides are under strain hence can be opened by water.

One of the methods used for ligand quantification is the thiol content determination (Grassetti 1967, Brocklehurst 1973). Free thiol groups are reacted with dithiol dypyridine to yield thiol pyridine which absorbs at 343 nm. The reaction scheme is shown in Figure 4.5. As well as quantifying the ligand density, this method also shows which matrix has free thiol groups. The results are shown in Table 4.5 (upper part), from which, it is apparent that Matrix A has a higher thiol content than Matrix B. Since Matrix A has a low capacity for GST, it suggests that the thiol group may not be critical in the selectivity for GST. Since both matrices have thiol functional groups, the attachment of GSH to the epoxy-activated matrix produces a mixture of products.

The unique feature of GSH immobilisation to epoxy activated agarose is that either the SH group or the NH_2 group is free. Thus the thiol test results could be complemented if another method was used that would detect the presence (or absence) of free amino groups. Ninhydrin reacts with free amino groups to give a distinct purple colour which has a maximum absorbance at 564nm. At this wavelength, neither ninhydrin nor GSH absorbs. GSH was used to prepare the standard

curve and absorbances of both the standards and the samples (matrix A, matrix B and the GSH-Sepharose) were read at 564 nm. The results are shown in Table 4.5 (lower part).

Using this ninhydrin assay (Table 4.4 lower section), Matrix B has a higher ligand density than Matrix A. This is in agreement with the batch adsorption results which show that Matrix B has a higher binding capacity than Matrix A. It also explains why Matrix B showed a higher binding capacity than the sepahrose 4B. This ninhydrin test also shows that Matrix B has more free amino groups than Matrix A. It means therefore that at pH 8.5, immobilisation of the GSH molecule was dominantly *via* the thiol group. As pointed out by Simons (1977) the free amino group is critical for GST binding. The only difference though is the pH at which the thiol group takes part in the immobilisation of GSH. According to Simons this takes place at pH 7 but in this work, this took place at pH 8.5. This makes sense since the pKa of the SH group is about 8.5. As in the thiol assay, the ninhydrin assay confirms that immobilisation of the GSH yields a mixture of products.

The ligand density results lead to the following suggestions:

1) Since Matrix B showed better selectivity and capacity for the GST enzyme, and since this has less SH groups but more free NH_2 groups, binding between this matrix and the GST is more likely *via* the amino group.

2) Immobilisation of the glutathione molecule can more likely take place *via* the thiol group but other groups such as amino or carboxyl are involved. The carboxyl group is more likely than the NH_2 group as the pKa of the amino group is 9.5. The dominant product is a function of pH and in this case, immobilisation was predominantly *via* the thiol group at pH 8.5.

3) The total ligand density is the sum of the ligand density quantified by both the thiol and the ninhydrin tests.

It has been argued that the binding of GST to glutathione is accompanied by a conformation change of the protein (Mannervik 1995). Mannervik hypothesised that the observed specificity of GSH to GST is in part a conformational transition elicited by the binding of the peptide structure.

4.3.4 Expanded Bed Adsorption Chromatography

GST has on many occasions been purified using packed column chromatography (Habig 1974, Simons 1973, March 1998, He 1998, Reade 1999). This is in part due to the scale of operation (i.e. for analytical purposes EBA is cumbersome to run), and partly since EBA is a relatively new technology. Many of the purification protocols involved GSH affinity columns (Angelucci 2000, Pastore 1998, Sehgal 2000, Guan 1991, Nishida 1994). However there are a few in which ion exchange (Habig 1974, Reade 1999) and chromatofocusing (Nishida 1994, Angelucci 2000) were employed. The use of ion exchange matrices for the purification of GST has meant that other purification steps had to be included in downstream processing. This, as discussed earlier, has the disadvantage of increasing product loss, process times and cost of production. On the other hand, it has been pointed out repeatedly that affinity matrices are more expensive and hence less attractive economically (Scouten 1985). The application of GST purification has been reported elsewhere using immobilised metal affinity and histidine tagged GST (Clemitt 1998, Clemitt 2000). The aim of carrying out these expanded bed experiments was three fold. First was to purify the GST to homogeneity using the in house made affinity adsorbents. This was important as only pure GST can be used as a fusion partner and for biochemical analysis. The second aim was to investigate whether using an ion exchange matrix followed by GSH-affinity (in a packed mode) would offer advantages over a one-step GSH-affinity on EBA in terms of purity of the product. Finally it was necessary to investigate if coating the matrices with Tween 20 offers any advantages over plain GSH affinity matrix. The results for the EBA chromatographic runs are shown in Figures 4.6-4.8.

4.3.4.1 Breakthrough pattern from the Streamline™ DEAE column.

The solids which are non-adsorbing are expected to elute first followed by a slowly rising breakthrough of non-absorbing proteins. If the selectivity for GST is good, the GST would breakthrough after the proteins. This is not what is happening in Figure 4.6, which shows the breakthrough pattern of GST on Streamline™ DEAE. The solids breakthrough is as expected. However the total protein and the GST breakthrough at the same time. This shows that Streamline™ DEAE has poor selectivity for GST. This resulted in a low purification factor as confirmed by Table 4.6 and Figure 4.9.

The breakthrough curve in the elution fraction is as expected. The eluted fractions are free of cell debris since the solid absorbance is zero. Because of its low selectivity, GST has been concentrated slightly more than the background proteins.

4.3.4.2 Breakthrough curves from the affinity columns.

Affinity matrices are expected to be more specific than the ion exchange matrices. As such the breakthrough pattern for the proteins and GST is expected to be different from that observed in the ion exchange matrices. The solids breakthrough is similar to that observed in the ion exchange matrix since in both cases there is no adsorption. The background proteins should breakthrough at the same time as the solids since they too are not adsorbed. However as will be discussed in the SDS-PAGE section, most of the proteins are GST hence from Figures 4.7 and 4.8, the protein breakthrough curve rise rather slowly.

The GST breaks through last. This confirms that GSH-agarose is highly selective for GST. This is supported further by the fact that in the eluate, the GST curve lies well above the protein curve. Thus the GST has been concentrated more than the other proteins. There is no significant difference between the breakthrough curves from Matrix B and the Tween-coated Matrix B. Table 4.6 is a summary of the purification data from all the matrices. As can be seen from this table, the same amount of background protein and GST activity were loaded in all the matrices except for the Sepharose 4B whose feed was the eluate from the Streamline™ DEAE column.

The specific activity of the feed is approximately the same in each experiment. Thus in spite of the fact that the feed to the Sepharose 4B is the eluate of the Streamline™ DEAE column, their specific activities are nearly the same. This shows that little purification was achieved in the Streamline™ DEAE column as confirmed by a purification factor of 1.1 in Table 4.6

4.3.4.3 Flow through pattern

Few proteins flowed through the Streamline™ DEAE column compared to the amount of protein that flowed through the affinity columns (Table 4.6). This is expected as the ion exchange column has less selectivity than the affinity columns. The Tween coated matrix adsorbed less protein and GST than Matrix B. This suggests that Tween might increased stearic interaction thus preventing the GST to interact effectively with the glutathione. This phenomenon is the basis for coating surfaces of plates in enzyme linked immunosorbent assay (ELISA) tests. The Tween coats the surface of the plates preventing interaction between the antibody and the plates.

4.3.4.4 Elution pattern

There is a remarkable difference in the elution data from the matrices. First there is relatively more proteins eluted from the ion exchange than from the affinity matrices. This was expected as more proteins were adsorbed. Furthermore elution with a salt solution is not specific. Matrix B has a higher purification factor and capacity for GST than any of the matrices including the Streamline™ DEAE. These data agree with what was observed in the batch experiments. Matrix B and the Tween coated Matrix have the same ligand density (since the Tween-coated-matrix is actually Matrix B coated with tween). The difference in the binding capacity for GST can only be explained by the fact that the Tween may be involved in some stearic interaction with the GST.

The Tween coating step was included in the process to reduce non-specific interactions and it seems this has been achieved. From the recovery data, it can be seen that there was more GST recovered from the Tween coated Matrix (100%) than from Matrix B (92%). The 100% recovery suggests that all bound GST was eluted. Thus any hydrophobic interaction or any other non-specific interaction which would have made it impossible to elute the GST with a GSH solution

were prevented. Although the purification factor is lower compared to Matrix B, GST has been purified to homogeneity as can be seen in Figure 4.9.

4.3.5 SDS-PAGE

This step was included to check the purity of the samples from the expanded beds. In SDS-PAGE, proteins are separated based on their molecular weight in an electric field. Thus if a sample is homogeneous, it would appear as one band in an SDS-PAGE gel. The net charge on a protein is a function of the constituent amino. Since different proteins have different amino acid combination, they have different net charge. When two proteins of different net charge are brought into an electric field they migrate to the electrodes at different speeds which leads to the eventual separation of the proteins. In SDS-PAGE, the proteins have same charge density per unit length due to the sodium dodecyl sulphate used to coat the proteins. Thus separation is based on size and not charge. The small molecules move freely through the gel while large molecules are slowed down by the porosity of the gel. Hence small molecules move down the gel faster than larger molecules. A homogeneous sample will appear as one band whilst a heterogeneous sample will show several bands corresponding to the constituent components.

The feed and the eluates from the columns were all analysed farther by SDS-PAGE to check their purity. As was observed from Table 4.6, the purification factor is rather low for affinity matrices. It was thus decided to investigate the homogeneity or heterogeneity of the eluates and compare them to the feed. The SDS-PAGE results are shown in Figure 4.9.

The feed shows the presence of a number of other proteins but the biggest band is at about 29 KDa. This corresponds to the molecular weight of GST monomers. As was pointed out in section 4.3.1.1 this figure also shows that most of the protein is GST. The Tween coated matrix and Matrix B eluates have a single band. This shows that these eluates are homogeneous in nature. Thus although the purification factor is only 3.7 and 5.1 respectively, the eluates are pure.

The Streamline™ DEAE eluate shows similar bands to those found in the DEAE feed although they are less concentrated. This is a result of some purification that has been achieved.

4.4 CONCLUSIONS

The purification of glutathione-s-transferase can be achieved using the traditional methods and by using expanded bed chromatography. In this chapter it has been shown that GST can be purified to homogeneity in a single step. This would otherwise not be possible has it not been for the development of expanded bed chromatography. Obviously the choice of adsorbent plays a great role. As was observed from both the purification factor and the SDS-PAGE results, it would require a number of steps to purify GST to homogeneity using ion exchange adsorbents.

The two affinity matrices prepared (Matrix A and B) showed remarkable differences in terms of their binding capacity and purification factor. Matrix B, prepared at pH 8.5, showed higher specificity and recovery than Matrix A, which was prepared at pH 7. Matrix B was shown to have more free amino groups than Matrix A. Its ligand density was also higher than that of Matrix A. Thus it can be concluded that coupling the glutathione molecule at higher pH results in more glutathione molecules being immobilised. More importantly this results in the right group (the amino group) being made available to participate in the interaction with the GST. Matrix B was also found to retain its initial characteristics after first use and hence could be used over again.

Coating a surface with a surfactant such as Tween prevents non-specific adsorption onto that surface. Coating Matrix B with Tween 20 improved the recovery but not the binding capacity. It was suggested that the Tween as well as reducing non-specific binding may also increase stearic hindrance between the matrix and GST.

The stability of Matrix B was tested at different pH and in salt solutions. It was found that the matrix is more stable near the neutral pH. At higher pH, glutathione molecules leaked out of the matrix. The salt solution had negligible effect on the stability of the matrix. This meant that up to 1M NaCl could be used in the cleaning and regeneration of the matrix without affecting its performance.

Affinity matrices are often labelled "expensive" when compared to ion exchange matrices. This is in general true. However the reagents used to prepare glutathione agarose are reasonably cheap. The process for preparing this affinity matrix is not energy demanding. Weighing the cost of preparing this affinity matrix and comparing it to the standard procedure for purifying proteins incorporating ion exchange, one would come to the conclusion that for GST, affinity adsorbents are economically attractive.

CHAPTER FIVE

THE APPLICATION OF EXPANDED BED ADSORPTION CHROMATOGRAPHY TO CAPTURE PARTICLES: THE PURIFICATION OF BACTERIAL PHAGE T7.

5.0 INTRODUCTION

Bacteriophages (or simply phages) are viruses that infect bacteria. As typical of all viruses, they are made up of protein (94.4%) and nucleic acid (RNA or DNA, 5.6%) (Adams 1959). They lack all enzymes and they can not reproduce in their extracellular form (virion) unless they infect a host cell where they use the host cell's machinery for replication. Lytic phages are very common (Douglas 1975) and their size ranges from 20-100 nm wide and 80-200 nm long. The molecular weight of a midsize phage is 58 million Daltons (Voet 1995). Thus, as opposed to proteins, phages are regarded as particles.

Phages are generally stable in their lysates as long as they do not contain inactivating agents. Pure phages are normally stable in neutral buffered solutions and as expected low temperature favours stability. It has been said that phages can be stored indefinitely at 4^0C (Douglas 1975).

Phages have economic importance although to a lesser extent than the viruses that infect man. These have been detailed elsewhere (Adams 1959). A few of them are mentioned here. Some industries consider bacterial phages economically disastrous. Such industries as the cheese making, baking and brewing, and antibiotics industries have good reasons in avoiding any phage contamination as they adversely affect both yield and quality of product.

The recombination of genes would not have advanced this far had it not been for the phages. This novel technology needed new organisms with minimum genetic characters, was haploid so that no gene could escape detection through recessiveness, one in which rare events were

readily detectable by the rapid screening of populations numbering billions. Phage met these requirements (Adams 1959).

Phages are also used in radiation dosimetry (Adams 1959). The radiation sensitivity of certain phages is known accurately, e.g. T2. By including a known amount of phage into the sample to be irradiated, the radiation dosage may be calculated from the residual infectivity. This is a very valuable technique, giving a direct measure of the biological effectiveness of a radiation treatment that may defy calculation from physical and chemical data in complex systems.

Phages are used as diagnostic tools (Adams 1959). They are routinely used for identification of pathogenic bacteria of which staphylococci and typhoid bacilli are good examples. When this technique is properly used it can distinguish strains of bacteria indistinguishable by other methods. For example, Salmonella typhi can be distinguished from other bacteria by infecting the sample with Phage Vi - phage which will only infect this type of bacteria. The specificity of phage to bacteria is possible because of the characteristic protein, carbohydrate and lipopolysaccharide molecules found on the surface of the bacteria cells. These molecules are involved in forming cells, in motility or in binding to particular surfaces. Each of these molecules can act as a receptor for a particular phage.

Water contamination is usually indicated by the levels of contaminating bacterial. However using phages that are specific to that bacteria, may act as additional indicators of water quality (Farber 1983).

There is increasing interest in using phage as "antibiotics" due to increasing resistance of bacteria to antibiotics. Using phages for therapeutic use gave impetus to phage research in the 1930's but with the discovery of penicillin, research interests were diverted to recombinant DNA. Phages (especially lytic ones) seem to be advantageous over antibiotics. For example when a single phage infects a cell, it multiplies inside the host. Thus only small dosages are needed. Phages are specific to the host because of the specific antibody found on the cell surface. Antibiotics on the other hand are not evenly distributed and may decay with time.

Phage related enzymes have been used in the depolymerisation of capsular polysaccharides as a convenient method for the purification of oligosaccharides (Dutton 1981, Elsasser-Beile 1981, Reiger-Hug 1981, Geyer 1983, Niemann 1978, Stirm 1994). Oligosaccharides are being developed for therapeutic use (Simon 1996). The use of phages for the production of oligosaccharides requires a highly pure sample containing at least 10^{10} particle forming units.

With these developments it is likely that in the near future demands for phage therapy will increase. Demand for pure and large amounts of phage would be global. It has been projected that global demand for viral therapy could be as much as 10^{20} particles (Lyddiatt 1998). Typical infective viral particles are in the range of 10^{6}-10^{11} (Lyddiatt 1998). Thus meeting this projected demand requires better methodologies which can generate highly purified products. Much as other techniques such as centrifugation, affinity and aqueous two-phase systems can be applied, scale up and cost is usually a problem (Ujam 2000, Lyddiatt 1998). Chromatographic methods are easier to scale up (a larger column is used) and remain the practical choice for product fractionation (Lyddiatt 1998). Expanded bed chromatography offers the advantage of handling unclarified feedstock hence this makes it an ideal process for phage purification. The aim of these experiments was therefore to explore new, cheaper but effective ways of purifying phage particles using expanded bed adsorption chromatography.

5.1 OBJECTIVES

The objectives of the experiments were to optimise phage capture in expanded beds by investigating the effect of loading pH, elution pH, loading temperature, type of adsorbent and effect of divalent cations.

5.2 MATERIALS AND METHODS

5.2.1 E.coli Culture.

LB broth was autoclaved at 121°C for 15 minutes to sterilise it. This sterile LB broth base was incubated at 37°C until temperature equilibration occurred. The medium was then inoculated with *E.coli* (C_{600}) (using a loop) and incubated overnight at 37°C for the bacteria to reach their logarithmic phase of growth. This overnight bacterial culture was subsequently used for Phage T7 propagation.

5.2.2 Phage Propagation

An overnight culture of bacteria was used. 10 µl of phage was added to 20 ml of E.coli culture and incubated for 2-3 hours. This culture was added to a litre of an overnight culture of *E.coli* (as prepared in section 5.2.1) and incubated for a further 3-4 hours. During the incubation the absorbance of the culture was measured every 30 minutes. After the incubation, NaCl to a final concentration of 1 M, and PEG_{6000} to a final concentration of 6% w/v were added and the culture was incubated for a further one hour. The NaCl was added to stabilise the phages and the PEG_{6000} was added to clarify the culture. Part of the supernatant was centrifuged for 20 minutes at 11000 g. The phage suspensions were then stored at 4°C until used.

5.2.3 Particle assay

The total number of phage particles (infectious and non-infectious) was assayed by the method of Huyghe (1995). The phage suspensions was diluted (10x, 15x or 20x) in 0.1% Sodium Dodecyl Sulphate (SDS) in 10 mM PBS, pH 7.4, in a test tube. The test tubes were centrifuged for 1 minute at 11000 g to remove any precipitates. The phage suspension was scanned between 310 nm and 320 nm. If the absorbance was less than 0.02 units, the sample was then read at 260 and 280 nm. If the ratio of A_{260}/A_{280} was between 1.2 and 1.3, the samples were considered pure enough for particle assay. Number of particles was estimated as

$$Number\ of\ particles = A_{260} \times 1.1 \times 10^{12}$$

5.2.4 PFU assay.

The phage suspensions were serially diluted typically by a factor of 10. 100 µl of these diluted suspensions was pipetted out into another tube containing 200 µl of E.Coli C_{600}. This mixture was incubated for about 20 minutes to allow the phage to adsorb to the bacteria. 3 ml of top agar maintained at 47°C were added to the incubated mixture, briefly vortexed and quickly added to a petri dish containing bottom agar. The plates were allowed to cool for 5 minutes before being placed in an incubator at 37°C equipped with a CO_2 vent. Plaques were counted after 5-6 hours.

5.2.5 Adsorption Isotherm of Phage T7 on Streamline™ DEAE.

A Phage T7 suspension of known total particle content was used. The suspension was serially diluted with 20 mM Tris-HCl, pH 7.5 by a factor of two. 10 ml of the diluted samples was added to 1 ml of Streamline™ DEAE in duplicates. The samples were put on a roller mixer for an hour after which the supernatant was assayed for phage content.

5.2.6 Expanded bed adsorption.

A 12.5 mm column (i.d.) was used for the expanded bed experiments unless stated otherwise.

5.2.6.1 Effect of loading pH.

Centrifuged phage suspensions were diluted 10x with 20 mM Tris-HCl at a pH of 6.2, 7.5 or 9.2. The column was initially equilibrated with 20 mM Tris-HCl at the respective pHs. When the bed was stable, the sample was loaded at constant flow. This was followed by washing with the buffer to remove unbound particles. Elution was done in a packed bed mode with 1 M NaCl in the respective buffers. Elution flow rate was 2 ml/min.

5.2.6.2 Effect of Elution pH.

The centrifuged samples were diluted 10x with 20 mM Tris-HCl, pH 7.5. The column was equilibrated with this buffer until the bed was stable. Then the sample was loaded at constant flow followed by washing. Elution was done with 1 M NaCl in 20 mM Tris-HCl, at pH of 7.5, 9.1 or 6.4. The elution flow rate was 2 ml/min.

5.2.6.3 Effect of loading temperature.

The column was jacketed with a polystyrene pipe and sealed watertight. An inlet tubing was inserted at the top and the outlet tubing was inserted at the bottom of the pipe. For adsorption at 1°C, water in ice was used as a reservoir while for 30 and 25°C, a water bath maintained at these temperatures was used as a reservoir. The jacket was then filled with water to a steady state. Thus the column was maintained at the required temperatures by counter current flow. The column was equilibrated and expanded to twice the settled bed height with 20 mM Tris-HCl pH 7.5. When the bed was stable, 100 ml of centrifuged Phage T7 suspensions were then loaded at constant flow. This was followed by washing to remove weakly bound and trapped substances. The bound Phage T7 was eluted with 1 M NaCl in 20 mM Tris-HCl, pH 7.5.

5.2.6.4 Effect of Divalent Cations.

Divalent cations have been observed to increase adsorption of phage although the reasons for this are not well understood (Romanowska 1976). In this experiment the effect of Mg^{2+} ions was investigated. The centrifuged phage suspension was diluted 10x with 20 mM Tris-HCl, pH 7.5 containing 10 mM magnesium sulphate. The column was also equilibrated with the same buffer. Elution was effected with 1 M NaCl in the same buffer in packed mode at 2 ml/min.

5.2.7 Solids assay.

Cells can be quantified by manual counting under a haemocytometer. However E.coli cells are so small that a magnification of about 3000 is required. This is beyond the reach of light microscopes. As a result the solids were assayed as follows. The absorbance of the samples were read at 600 nm. The samples were then passed through a 0.2 μm filter. The phages should pass through this filter. The absorbance of the filtrate was read and compared to the absorbance of the original sample. This assay is qualitative and gives only relative values.

5.2.8 Preparation of Polylysine Agarose

5.2.8.1 Activation

50 ml of the Streamline™ quartz base agarose were washed with 2 M phosphate buffer, pH 11.5 made by adding 2M K_2HPO_4 to 2M K_3PO_4. The beads were then vacuum filtered to remove excess liquid. They were then re-suspended in 75 ml of the phosphate buffer. This was left standing in ice cold water and transferred to a fume cupboard. 50 ml of CNBr was then added slowly to the re-suspended beads. The reaction was allowed to go on for 10 minutes after which the beads were washed exhaustively with water on a glass sinter.

5.2.8.2 Coupling.

The method of Sundberg (1973) was employed. The beads were washed with 250 mM $NaHCO_3$, pH 9.0. 50 ml of the $NaHCO_3$ buffer containing 100 mg of the polylysine were added. The beads were then left in an incubator with shaking (250 cycles/min) at room temperature for 20 hours. This was followed with washing with water after which they were left to stand in 1 M Tris-HCl, pH 8 for 2 hours to block excess groups. Finally the beads were washed with water, 0.25 M $NaHCO_3$, pH 9.0 containing 1 M NaCl, 0.25 M Sodium acetate, pH 4 containing 1 M NaCl and 0.25 M $NaHCO_3$, pH 9 buffer.

5.2.9 Phage T7 purification from unclarified feedstock.

Unclarified phage suspension was diluted 10x with 20 mM Tris-HCl, pH 7.5. The column was equilibrated with this buffer until it had reached a stable bed configuration. 100 ml of the unclarified feedstock were then loaded on the column at a constant flow of 4 ml/minute. This was followed by washing with the equilibration buffer at 4 ml/min to get rid of unbound or weakly bound particles. Washing was stopped when the UV signal returned to base line. Elution was done by 0.2 M, 0.5M, and 1M NaCl in 20 mM Tris-HCl, pH 7.5 at a flow rate of 2 ml/min.

5.3 RESULTS AND DISCUSSION

5.3.1 Phage propagation.

It is important that when carrying out phage propagation, the harvesting should be done when the phage have completely lysed the bacteria. This is generally well documented in the literature (Sambrook 1989). For lytic phage, a clarification of the bacterial suspension should be noticed visually. In the experiments reported here however, the cell culture clarification was not readily noticeable to the naked eye. A standard experiment was used to approximate the time required for lysis to be complete. This was done by monitoring the absorbance of the cell culture at 600 nm. Since Phage T7 is lytic, the cell culture clear with time. Using a UV spectrophotometer, this clarification could be monitored quantitatively. The results are shown in Figure 5.1. From this figure, it can be seen that it took about 2 hours for the E.coli to be lysed. This is about the same period as that documented in the literature (Sambrook 1989). Thus although with visual inspection the cell culture clarification was not distinct, the phage had actually lysed the E.coli.

5.3.2 Adsorption Isotherm.

Adsorption isotherms play a significant role in the design of experiments aimed at optimising product capture. The equilibrium position in chromatographic separations is described by the adsorption isotherm. In turn this can be used to approximate feed concentrations needed for maximum binding (Gosling 1989). Adsorption isotherms are also useful in determining the strength of binding. A steep slope of the isotherm means strong binding. Adsorption isotherms for proteins are well documented though for viruses such information is scarce. It was important therefore that the adsorption isotherm for the Phage T7 on Streamline^{TM} DEAE be established. This adsorption isotherm is shown in Figure 5.2.

From the isotherm it can be seen that the maximum binding capacity is about 10^{11} particles/g of adsorbent. This is equivalent to 10^{12} particles/ml of adsorbent. This value is equal to the one obtained by theoretical calculations for viruses (Braas 1996, Lyddiatt 1998) and a similar calculation for this phage is shown in Appendix 1. The concentration of feed needed to achieve maximum binding under the experimental conditions is about 10^{11} particles/ml. For typical

feedstock used in these experiments, if the total number of particles was 10^{11}, the number of infective phage particles was about 10^6-10^7 PFU/ml.

From the adsorption isotherm it can also be seen that the affinity of the phage to the DEAE is medium (since the isothermal line lies near the 45° line). This is desirable since it means that the phage can be eluted with mild conditions. However as will be discussed later, questions arise as to why under some conditions, very little phage is recovered.

5.3.3 Phage T7 purification.

The purification of particles, especially cells, has been an area of interest for some time (Scouten 1985). The main problem with particles is their three-dimensional nature. Whereas in molecules, the whole surface area is available for interaction, in particles it is only the exposed functional groups on the surface. The other problem is that viruses being larger than proteins, their diffusivities are an order of magnitude lower than that of proteins (Braas 1996, Lyddiatt 1998). Thus mass transfer from the bulk of the liquid to the surface will limit the adsorption processes. This is made worse by the fact that in molar terms viral suspensions are dilute (10^{-13} M), 10^7 times more dilute than proteins (Lyddiatt 1998). Because of the problems of particle adsorption on surfaces, other methods which do not involve adsorption such as CsCl density centrifugation have found widespread use (Hirsh 1982, Sain 1981, Hyughe 1995)

Using expanded bed chromatography to capture particles is still in its infancy. Recently, (Ujam 2000) it has been demonstrated that expanded beds could be used to separate cells using ion exchange adsorbents. The application of expanded beds to purify phages is yet to be published. Phage purification is more challenging than cell purification as the suspensions do not just contain the phage particles but also the host cells and cell debris. Hence there is need for a better selective system for phage adsorption. In these experiments, Streamline™ DEAE was used unless stated. Both infective and non-infective phage can bind to the Streamline™ adsorbent. The data were analysed in terms of both the Plaque Forming Unit (PFU) and the total particle assay. The phage lysates contains the virions, intact and broken bacterial cells, proteins

and nucleic acids. Unless otherwise stated, the cells were removed by centrifugation so that they could not contribute to the observed increase in absorbance at 280 nm.

The concentration of proteins in the lysates was quantified by the Bradford method. Surprisingly this was found to be less than 0.05 mg/ml. The absorbance of a 0.05 mg/ml solution at 280 nm is negligible. Thus it would be a near perfect approximation to assume that the change in absorbance at 280 nm was solely due to the presence of phage particles.

5.3.3.1 Effect of elution pH on phage recovery.

Phages are sensitive to the pH of the surrounding environment (Gerba 1978, Douglas 1975). As for proteins, pH determines the net charge on the surface of the virus. It is also known that most viruses are stable within pH 5-8 (Douglas 1975). Phage T7 though is not as widely studied as T4 or lambda (Voet 1995). It was thus important that the effect of pH on Phage T7 adsorption and elution be investigated. The samples were loaded at pH 7.5 but eluted either at pH 6.4, 7.5 or 9.2. The results on the influence of elution pH on phage recovery are shown in Figure 5.3 and Table 5.1.

From the PFU assay, Table 5.1, there is no significant differences in the recovery of active phage. These results seem to suggest that once the loading pH has been set, eluting the phage at different pH will not affect the recovery as long as the phage are not inactivated in the solution. It is worth mentioning though that it does seem that elution at a slightly acidic pH gives better recoveries of the active phage (Table 5.1). This was also observed in the purification of phage SKVI (Romanowska 1976). Elution at pH 6.0 gave a recovery of 62% and at pH 7.3 gave a recovery of 52%.

The total particle assay also shows similar trend (Figure 5.3). The breakthrough patterns of the particles are similar. During loading, it was expected that the breakthrough should be the same since all were loaded under the same conditions. If the pH of the elution buffer has any effect on the bound phage, this would be observed in the elution profile. As can be seen from Figure 5.3, total number of particles is similar (calculated as $2.0x10^{12}$, $2.5x10^{12}$, and $2.3x10^{12}$ at pH

7.5, 6.4, and 9.1 respectively). Thus the pH of the elution buffer has negligible effect on the bound phage particles (active and non-active).

5.3.3.2 The influence of loading pH.

Whilst the elution pH influences the elution of the bound phage, the loading pH may influence the number of phage particles that actually get bound. The samples were loaded and eluted at pH 6.4, 7.5 or 9.2. From Table 5.1, active phage recovery was 86% at pH 6.4, 61% at pH 7.5 and 4% at pH 9.2. Thus just as in the elution pH results above (section 5.3.3.1), slightly acidic pH favours more active phage binding and recovery. This also seems to suggest that the pI of the phage is below 6.4. The low recovery at pH 9.2 may be due to the extreme pH and the long time the phage was exposed to. As opposed to the above elution pH where the phage was only exposed to pH 9.1 for less than 30 minutes, in these experiments the phage was exposed to pH 9.2 for at least 1.5 hours. Thus it could be possible that some phage were inactivated by exposure to this pH for such a long time. Another explanation is that this high pH simply does not favour phage adsorption.

From Figure 5.4 it can be seen that the total number of phages that flowed through are roughly the same at the three loading pHs. This was calculated to be 4.8×10^{12}, 5.0×10^{12} and 5.5×10^{12} for pH 7.5, 6.4 and 9.2 respectively. However the number of particles that could be eluted at pH 9.2 is only half as many particles as could be eluted at pH 7.5 or 6.4. These results seem to suggest that alkaline pH increase irreversible binding. Thus there is a possibility that the low recovery of the active phage may be due to irreversible binding.

5.3.3.3 Effect of temperature on phage recovery

For proteins it has been found that loading at elevated temperatures (up to 37°C) increases binding without affecting the hydrodynamics of the bed (Hjorth 1995). The argument for this observation is that diffusivities are higher at elevated temperatures. Phages being larger than proteins, their diffusivity may not be significantly affected by temperature changes. However phages are stable at 37°C hence this stability may favour adsorption. The effect of temperature

on Phage T7 purification was investigated at 1°C, 25°C and at 30°C. The results are summarised in Figure 5.5 and Table 5.1.

When loading was done at 1°C, 85% of the active phage were adsorbed. From table 5.1, nearly all (99.6 %) of the adsorbed phage were eluted. This is a good recovery and is better than at the other two loading temperatures. When the loading was done at 25°C and 30°C, 99% of the loaded phage were adsorbed. Thus high temperature favours more adsorption. However the recovery was only 61% and 7% respectively. Thus a significant percentage of the phage could not be eluted. It seems logical to attribute these low recoveries to two factors: irreversible binding and degradation. However it should be remembered that phage propagation is conducted at 37°C. Hence degradation due to temperature is ruled out and this leaves the irreversible binding as a plausible explanation. Phages are often associated with irreversible binding even at room temperature (Romanowska 1976). From the results in these experiments however, it seems that it is chiefly the active phage that binds irreversibly. From Figure 5.5 which shows the elution profile of the phage loaded at different temperature, the area under the curves are in the ratio of 74:59:55 for 1°C, 25°C and 30°C respectively. Thus indeed more phage particles were recovered at 1°C. However the ratio is not as much as it is for the PFU assay (99:61:7 respectively). Thus the active phage seems to bind irreversibly more than the non-active phage.

5.3.3.4 Phage T7 purification on Polylysine agarose

Although the adsorption of phage onto polylysine agarose is listed under affinity chromatography (The Sigma Catalogue), the binding is not based on group specificity. It is actually based on ion exchange (Sundberg 1973). That is why elution is effected by using a high salt concentration solution. However, as opposed to ion exchange matrices which have point charges, on polylysine agarose, the charge is distributed over an area. Thus the contact area is larger, hence polylysine should show better binding characteristics than matrices with point charges such as DEAE. Also ion interaction varies with the type of phage. Sundberg (1973) observed that up 0.5 M NaCl had no effect on phage elution. This was not the case in the results reported here. The results for Phage T7 adsorption on polylysine agarose are shown in Table 5.1

and Figure 5.6. 70% of the loaded active phage were bound. From Table 5.1, only 43% of the bound active phage could be eluted. This figure is lower compared to the ion exchange recoveries under the same experimental conditions (pH 7.5 at room temperature). The same irreversible binding could explain this observed low recovery.

The number of particles eluted from polylysine agarose (10^{11}) is an order of magnitude less than that eluted under the same experimental conditions using the Streamline™ DEAE column. On the other hand, the total number of active phage is as much as that obtained from the Streamline™ DEAE. Thus it is easy to visualise that there is more selective adsorption of active phage on the polylysine agarose than on the Streamline™ DEAE.

The purification factor of phage is obtained from the ratio of total particles (P) to active (or infectious, I) phage. For polylysine agarose, P/I = 100000. Under the same experimental conditions on the DEAE column, the P/I was 125000. Thus indeed the polylysine agarose is more selective for active phage than the Streamline™ DEAE. In spite of this, ion exchange resins will still be of widespread use in phage purification because polylysine is very expensive. It must be said though that in either case P/I is low.

5.3.3.5 The influence of Divalent cations.

Divalent cations are known to increase the adsorption of phage especially with affinity adsorbents (Romanowska 1976). The reasons for this are not so clear and hence it was hoped that a similar effect could be observed with ion exchange adsorbents. Mg^{2+} ions were used in this case. The loading was done at room temperature and at pH 7.5. 85% of the loaded active phage became bound. This is somewhat less than the other samples loaded without Mg^{2+} ions under the same conditions. The recovery was also poor (only 17%). Thus it would seem that Mg^{2+} ions offer no improvement in the purification of Phage T7.

5.3.3.6 Phage purification from unclarified feedstock

Expanded bed chromatography is economically attractive only if unclarified feedstock is used. So far, centrifuged samples have been used. The problem with ion exchange in purifying

phages from unclarified feedstock is that the intact bacterial cells and the cell debris can bind to the adsorbent. Indeed it has been shown that E.coli cells and yeast cells can bind to ion exchangers (Ujam 2000). Thus ion exchange would only be useful if the non-phage particles can be eluted at a different salt concentration than the phage particles. Ion exchange would even be more useful if it can selectively bind or elute active phage particles. In this experiment the phage suspension was loaded at pH 7.5 and at room temperature. Elution was effected by 0.2 M, 0.5 M and 1 M NaCl in 10 mM PBS, pH 7.5. The results are shown in Table 5.2 and Figure 5.7

From Table 5.2, most of the cells and cell debris (75%) were in the 0.2 M eluate. The remaining 25% were in the 0.5 M. The 1 M fraction contained no non-phage particles. This suggests that there was some selective elution of the phage from the non-phage particles.

The eluted fractions were then filtered to remove the cells and the cell debris. The absorbance of the filtrate which contained the phage particles was measured. The absorbance of the 0.2 M filtrate was found to be higher than the absorbance of the 0.5 M filtrate. This implies that the 0.2 M fraction contained more phage particles than the 0.5 M fraction and as can be seen from Table 5.2, the 0.2 M fraction contained more than twice the number of particles than the 0.5 M fraction. These two fractions also contained roughly the same amount of protein although it was small. The 1 M fraction contained neither non-phage particles nor proteins. It also contained the least number of phage particles (about 10% that of the 0.2 M fraction).

From Table 5.2, most of the active phage were in the 0.5 M fraction (62%) followed by the 0.2 M fraction (16%). The 1 M fraction contained the least number of active phage particles. The P/I ratio of the 0.5 M fraction is about an order of magnitude higher than in the other two fractions (Table 5.2). Thus there is also selective elution of the active and non-active phage particles. This also implies that most of the non-active phage bind weakly to the Streamline™ DEAE matrix since most of them could be eluted with the 0.2 M NaCl solution. Despite the fact that the 1 M fraction has the least number of particles, its P/I value is comparable to the 0.2 M fraction. It was pointed out in section 5.3.3.3 that more of the active phage binds irreversibly

than does the non-active phage. This view is supported by the fact that most of the non-active phage could be eluted with a low ionic strength solution whilst the active phage needed a higher salt solution. Overall, most of the phage particles (67%) were in the 0.2 M fraction.

5.4 CONCLUSIONS

The purification of phage by expanded bed chromatography opens a new chapter in bioseparation processes. Expanded bed chromatography has often been used to bind the target molecules whilst the cell debris and particulate material pass through the bed freely. This work has demonstrated that particles can also be selectively adsorbed using expanded bed chromatography. The implication of this is that experimental design can be tackled from two fronts. The strategy can be such that either the target molecule is bound and the particles flow through or *vice versa.*

Phage purification by expanded bed chromatography can be accomplished by looking at the same parameters that affect protein adsorption. In this project it was observed that phage T7 purification is affected by the loading pH, elution pH, temperature, divalent cations and the ionic strength of the eluting buffer. The recovery of active phage is better if the loading buffer is slightly acidic as at high pH the recovery is very low. The elution pH does not significantly affect the recovery of active phage. However elution with neutral to slightly acidic pH is desirable. High temperatures favour more binding but unfortunately the recovery is low as some phage are bound irreversibly. Mg^{2+} reduced both the binding and the recovery of phage T7. Although phage purified on polylysine agarose shows a better purity than that from Streamline™ DEAE, this is off set by the low recovery, low capacity and the high cost of this ligand.

Phage binding to the Streamline™ Matrix shows substantial differences from protein binding. Whilst a particular protein is expected to elute at a particular salt concentration, phage need a wide concentration range. Even with 1 M NaCl, some phage could not be eluted.

There is also a difference in the binding strength between the active and non-active phage. Most of the non-active phage binds weakly to the ion exchange matrix. The active phage shows intermediate binding strength. This resulted in selective elution of the active from the non-

active phage. Most of the active phage were in the 0.5M fraction whiles the most of the non-active phage were in the 0.2M fraction.

Expanded bed chromatography can be used in the purification of phage directly from unclarified feedstock. It was observed from these experiments that most of the non-phage particles (cells and cell debris) were in the 0.2M fraction (just like the non-active phage particles). Thus both the cells/cell debris and the non-active phage could be eliminated in the down stream processing of phage particles.

Perhaps it is worth mentioning that better adsorbents need to be developed for viral particles. The porous beads developed for proteins are not ideally suited for viral particles. The pore size ranges from 30-400 nm in diameter (Lyddiatt 1998). Phages may get trapped in these pores. But perhaps a major problem is that contaminating proteins and DNA, being smaller than the viral particles, can bind in the pores of the adsorbent (Lyddiatt 1998). The phage on the other hand should bind mainly on the surface. Thus selective elution of the surface-bound phage or selective purging of the pores could improve purity of eluates.

CHAPTER SIX

CONCLUSIONS AND FUTURE WORK

6.0 CONCLUSIONS

The successful application of chromatography in the purification of biological molecules and particles requires a thorough understanding of the factors that influence product capture and its subsequent purification. As opposed to packed beds purification processes carried out by expanded beds are complicated by the hydrodynamic stability of the bed. Whereas particles in a packed bed are in a stationary position, in expanded beds, the particles are supported sorely by the upthrust of the liquid flowing through the column. This makes the particles stay in a dynamic state – moving about an equilibrium position. The expanded bed principle is to minimise this movement and make the system acquire the chromatographic properties of a packed bed (high number of theoretical plates) whilst maintaining the good properties of a fluidised bed (ability to handle unclarified feedstock).

It is a trivial matter to make a system handle unclarified feedstock, for what one needs to do is to increase the void fraction. Making the expanded bed maintain the good contacting efficiency of packed beds needs careful analysis of parameters that influence liquid-liquid mixing or axial dispersion. These parameters are physical in nature and some of these have been investigated in this project.

The settled bed height: Axial dispersion was observed to be higher in shorter beds and *vice versa*. This was in agreement with work carried out by other researchers. There are two reasons for this. Flow of liquid is always erratic towards the low end of a column and hence dispersion is expected to be highest. The second reason was that there is always back-mixing in a column. However in short beds the inherent properties of the beads cannot compensate for this backmixing. Thus use of longer columns would be better for product capture.

The flow velocity (degree of bed expansion): The axial dispersion was found to decrease with increasing flow velocity, just as would be expected theoretically. It was pointed out in

the discussion that further increases in the flow velocity (assuming the terminal velocity is not exceeded and the column is long enough) would eventually lead to high dispersion when particle motion becomes more turbulent. The conclusion was that a two fold bed expansion is good enough for most expanded bed operations.

Column diameter: The influence of column diameter on axial dispersion (for the same settled bed height) was not conclusive. The 12.5 mm column was found to have comparable axial dispersion as the 50 mm column. This was attributed to the fact that small diameter columns (12.5 mm column in this project) are very sensitive to small deviations from a vertical position. Thus an electronic device would make all the difference if accurate data are of vital importance. For the same volume of beads, a small diameter column was found to be far superior compared to a larger diameter column. In this project a 25 mm column was found to be at least five times as stable as a 50 mm column. It would seem natural to use small diameter columns therefore. However packing 1L of beads into a 10 mm column would mean a settled bed height of about 13 metres!! This is not a useful height to work with especially if the bed is to be expanded. The other disadvantage is that elution times will be agonisingly long. Thus it would be prudent to use a moderately larger column that would also take advantage of better bed stability in longer columns.

The bed aspect ratio: Using the bed aspect ratio as one of the parameters has revealed some of the hidden trends of axial dispersion. With the bed aspect ratio it is possible to analyse the data irrespective of the column diameter, bed height or bed expansion used. In this project it was observed that as the bed aspect ratio increased, axial dispersion decreased.

There are also others factors that affect the performance of chromatographic separations. These relate to the selectivity and capacity of the separation process. Some of these factors are chemical in nature such as pH, types of buffers and ionic strength of the buffers. Others are physical in nature. In this project the chemical parameters were fixed and efforts concentrated on how the physical parameters affect chromatographic processes. Glucose-6-phosphate dehydrogenase was used as a target molecule and yeast cell suspension as an ideal expanded bed system.

Loading at constant flow yielded slightly higher purification factors than loading at constant height. This was the case irrespective of the cell density. The effect of the loading modes on the binding capacity was varied. In suspensions of low cell density, the binding capacity was better if the feed was loaded at constant height. In suspensions of high cell density, the binding capacity was higher if the feed was loaded at constant flow.

Increasing the concentration of G6PDH in the feed (although in doing so the cell debris and the viscosity were also increased) increased the binding capacity. The purification factor also increased with G6PDH concentration if the sample was loaded at constant flow. At constant height, there was no difference in the purification factor values even if a higher G6PDH concentration was used. It was also observed that partial centrifugation did not improve the binding capacity or the purification factor. It was suggested that this might be due to the fact that centrifugation favoured more non-G6PDH binding.

The activity of G6PDH in the eluted fractions was about the same. Thus the samples were concentrated to the same level although the feed characteristics and the loading modes were different. This seems to imply that product concentration (at least for G6PDH) is independent of experimental conditions. However the ratio of G6PDH activity in the eluates (*A*) to that of the feed (*Ao*) i.e. *A/Ao* was higher in low cell density suspensions. It was also observed that loading at constant height gave higher *A/Ao* values in low cell density suspensions. In higher cell density suspensions, *A/Ao* was higher when the sample was loaded at constant flow than at constant height. The level of solid clarification was comparable to that obtained from industrial centrifuges.

Having optimised the hydrodynamic bed stability and investigated the conditions that affect the selectivity and capacity of adsorption, it was time to apply these findings in the purification of a target molecule and particle. A recombinant protein, glutathione-s-transferase and Phage T7 were used.

The purification of GST involved the use of a commercially available Streamline^{TM} DEAE matrix and an in-house-made GSH affinity matrix. Two affinity matrices were synthesised.

One was coupled at pH 7 (Matrix A) and the other was coupled at pH 8.5 (Matrix B). From method-scouting experiments employing batch adsorption, Matrix B was found to be superior to both Matrix A and the Streamline™ DEAE in terms of both the binding capacity and the purification fold. Matrix B was also found to give better recovery and purification than the commercially available GSH-Sepharose 4B. Furthermore Matrix B retained its initial properties after first use. Coating Matrix B with Tween 20, a surfactant that reduces non-specific binding, improved the recovery of GST but the capacity was somehow less than plain Matrix B. It was suggested that this might be due to stearic hindrance introduced by the tween.

When the ligand densities were determined on the two in-house-made matrices, it was found that Matrix B had a significantly higher ligand density than Matrix A. This explained why Matrix B showed a higher binding capacity than Matrix A. By using the ninhydrin test and the thiol content determination, it was observed that Matrix B had more free amino groups than Matrix A, whilst Matrix A had more free thiol groups than Matrix B. These findings lead to the suggestion that the binding of GST to GSH-affinity matrix is chiefly effected by the free amino groups. The immobilisation of GSH to agarose gives a major product and a minor product and that the dominant product depends on the coupling pH.

Consequently Matrix A was eliminated and Matrix B was used in the purification of GST by expanded bed chromatography. Streamline™ DEAE in an expanded bed mode followed by GSH-Sepharose 4B in a packed mode was also used to investigate if doing so would have any advantages over the one step purification of GST using Matrix B. The eluates and the feed were also analysed by SDS-PAGE to determine their heterogeneity or their homogeneity.

Surprisingly the purification factor obtained by using Streamline™ DEAE followed by GSH-Sepharose 4B was less than that obtained by the single step purification using Matrix B. This is partly due to the fact that little purification (p.f. 1.1) was achieved with the streamline™ DEAE. Hence effectively the combined steps were as good as using one step purification in a packed mode with GSH-Sepharose 4B. However both eluates from the sepharose and Matrix B were homogeneous as determined from the SDS-PAGE results.

There was no obvious advantage in using the two step purification compared to the one step purification with Matrix B.

The application of expanded bed chromatography to the purification of particles opened up new avenues in which bio-product purification could be tackled. There is little work in phage purification these days and since applying expanded beds in phage purification is novel, some of the chemical factors that influence phage adsorption had to be investigated.

The pH of the elution buffer (from pH 6.2-9.2) had little effect on the recovery of both active and non-active phage although a slightly acidic pH showed an improved recovery. The loading pH on the other hand showed profound effect on phage purification. Slightly acidic pH gave far better recoveries of active phage than alkaline pH. It was also observed that at higher pH (pH 9.1), little of both the active and non-active phage could be recovered.

Temperature affected both the binding and recovery of phage. It was observed that at low temperature binding was slightly less than at room temperature or at 30°C. However the recovery of active phage at low temperature was nearly 100% whilst at 30°C the recovery was less than 5%. Thus higher temperature influence irreversible binding.

Eluting phage with different salt concentration showed that non-phage particles, active phage and non-active phage could be selectively eluted thus giving better purification values of the more important active phage. Most of the non-phage particles and the non-active phage could be eluted with a low salt concentration (0.2 M). Most of the active phage on the other hand were eluted with a 0.5 M solution. The 1 M fraction contained no non-phage particles and the ratio of active to non-active phage in this fraction was equal to that of the 0.2 M fraction.

Overall this project has shown that there are other areas that are yet to be explored in the use of expanded beds for the purification of bio-products. Although expanded beds are better understood in the purification of enzymes, there will always be need to optimise the capture of individual products. Hence optimising separation process is an on-going activity. Using

expanded beds to purify particles has just begun. More light will be shed upon this problem as different types of particles are investigated.

6.0.1 Disadvantages of expanded bed chromatography.

In the literature, nearly all users of expanded bed chromatography speak highly of it. This seems to suggest that EBA has no drawbacks. Indeed the advantages of expanded bed chromatography outweigh its disadvantages, but this depends on the nature of separation that one is conducting.

1) Exploratory experiments are always time consuming in expanded beds. Usually even with EBA, clarified suspensions are used. Such experiments will be faster with packed beds.
2) Volume consumption in expanded beds is very high. The actual volume used depends on how quick bed stabilisation was attained and how easily the cell debris were washed. On average a volume of at least 5 times that used in packed beds is consumed.
3) The savings in operation costs may depend on nature of a process. Synergen reported a cost of 109% compared to conventional methods (McCormick 1993). One of the reasons for this may be due to the fact that the process was not optimised. However a saving of 40% as was reported by Amersham Pharmacia Biotech is questionable.

Nevertheless, expanded bed adsorption chromatography remain a viable technique for bioseparation and will remain so for decades to come.

6.1 FUTURE WORK

There will always be research into the optimisation and applications of expanded beds. Of particular relevance in this project is that some aspects could not be investigated further within the scope of the project. For example when the residence time distribution data were analysed by the bed aspect ratio, it was found that dispersions were equal if the bed aspect ratios were equal, (i.e. a 20 cm column expanded to 30 cm and a 15 cm column expanded to 30 cm had equal degree of dispersion). This observation is important and should be investigated further. Two sets of experiments should be conducted. The RTD experiments should be repeated but with the help of an electronic device to fix the alignment of the column. Then with a suitable enzyme, binding capacities should be determined to verify if this would correlate to the RTD trend.

How does the phage adsorb to the beads? It is well documented in the literature that phage particles attach to cell surfaces with their tail. This is because of specific sites on the cell surface that can only interact with the tail of the phage. However phage purification by, say, ion exchange does not depend on specific sites. In fact the likelihood is that adsorption is *via* the head. Unless this is visualised, only speculations can be made. On the other hand this information is vital in designing 21st century adsorbents. If, for example, it can be established that the phage bind to the adsorbents via their head, then this can be manipulated to use immobilised phage in column chromatography.

In this project, an experiment was conducted to find the orientation of phage when they bind to ion exchange adsorbents. An electron microscope was used to scan through Streamline™ DEAE containing bound Phage T7. It must be acknowledged here that this was rather too ambitious. Looking for a phage particle on a 200 μm bead is as good as looking for a needle in a hay stack. Luck was not on our side. A simpler experiment could be set up. The phage could be bound as is normally done followed by washing to remove unbound particles. The beads with the bound phage could then be contacted with bacteria culture and after 3 hours, the bacteria suspension assayed for phage activity. If any lysis be detected or a PFU assay yield positive results, this would prove that the tail is not involved in the immobilisation.

The use of phage-related enzymes in the depolymerisation of capsular polysaccharides is another area that needs further investigation. The standard procedure is to incubate a highly active phage suspension with the polysaccharides. This is done in a batch mode. However it can also be done in a column if the phage can be immobilised onto beads. However it is now known that these phage related enzymes are associated with the tail of the phages (Stirm 1994). Thus if immobilised phage were to be used, it is imperative that the immobilisation be *via* their heads.

Expanded bed chromatography is yet to be used in the purification of milk proteins. The standard procedure is to precipitate out the caseins followed by centrifugation to separate the precipitate from the supernatant. If expanded bed chromatography is used for example, this centrifugation step may be skipped. The problem of lipids may be solved by using organic solvents that do not denature the product of interest.

FIGURES AND TABLES

The figures and tables are grouped in accordance with the chapter they relate to. For example all figures and tables for chapter two will be found under subsection *Chapter Two* of this section.

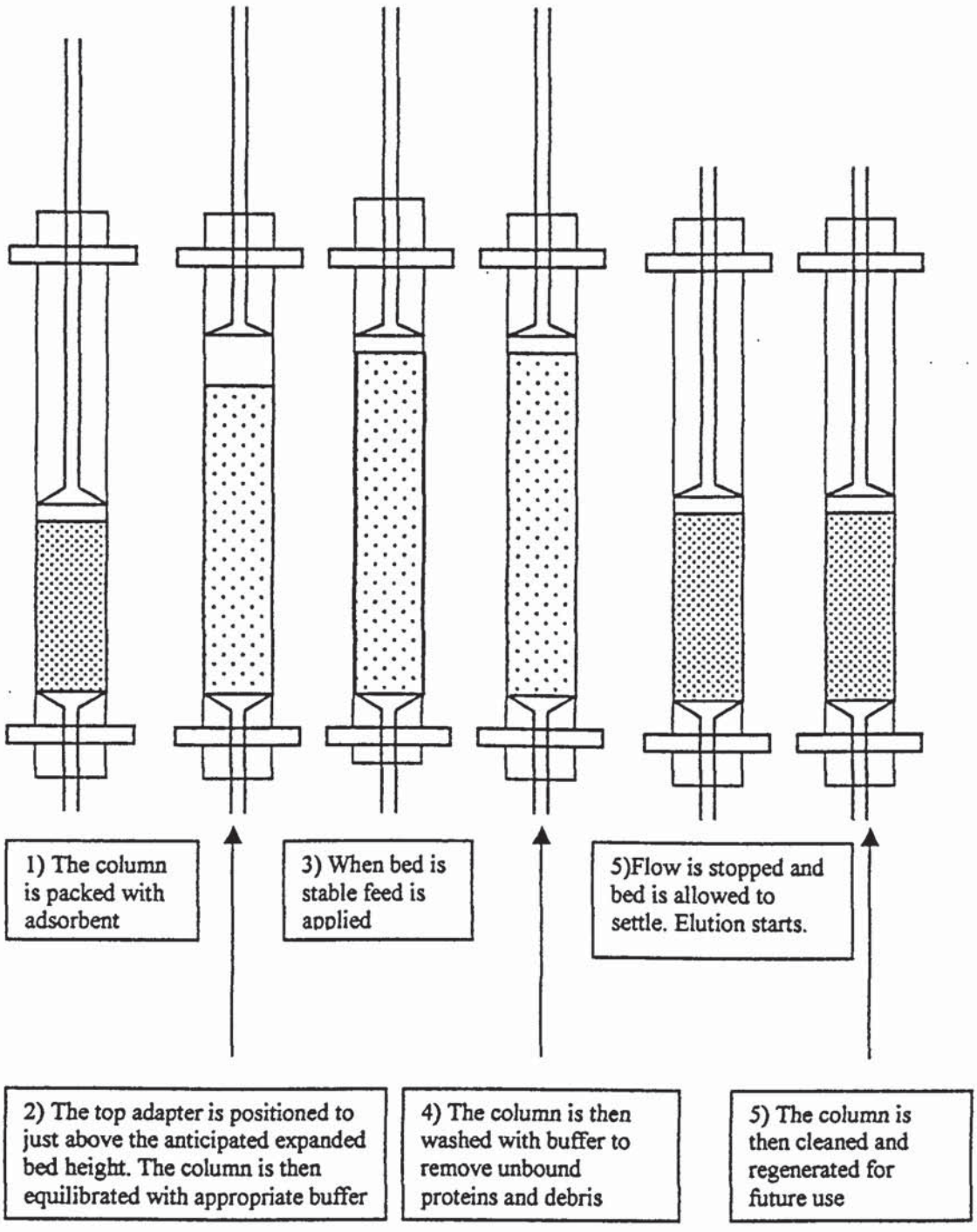


Figure 1.1: The operation principle of expanded bed adsorption chromatogrphy.

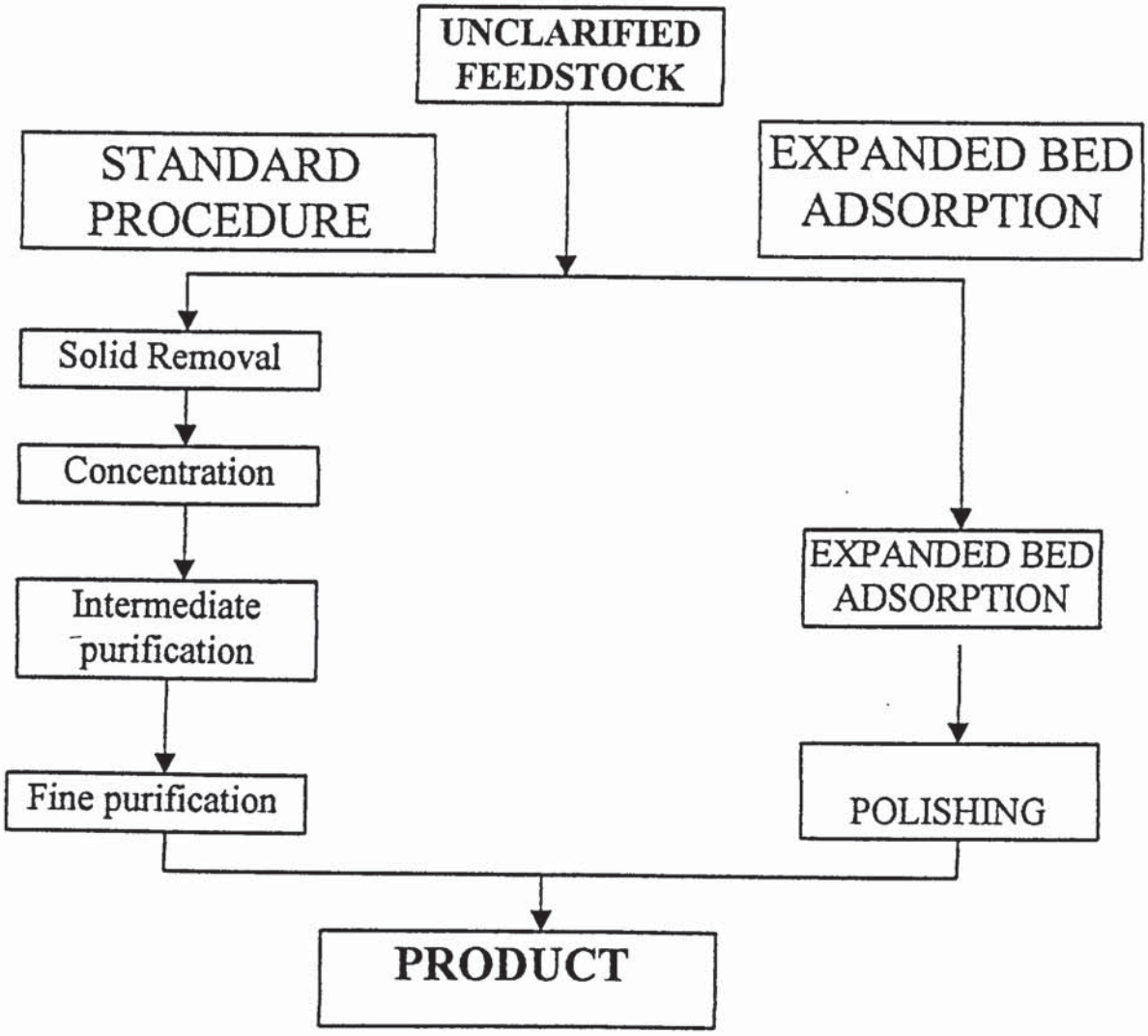


Figure 1.2: Comparison of the standard and EBA routes for protein purification.

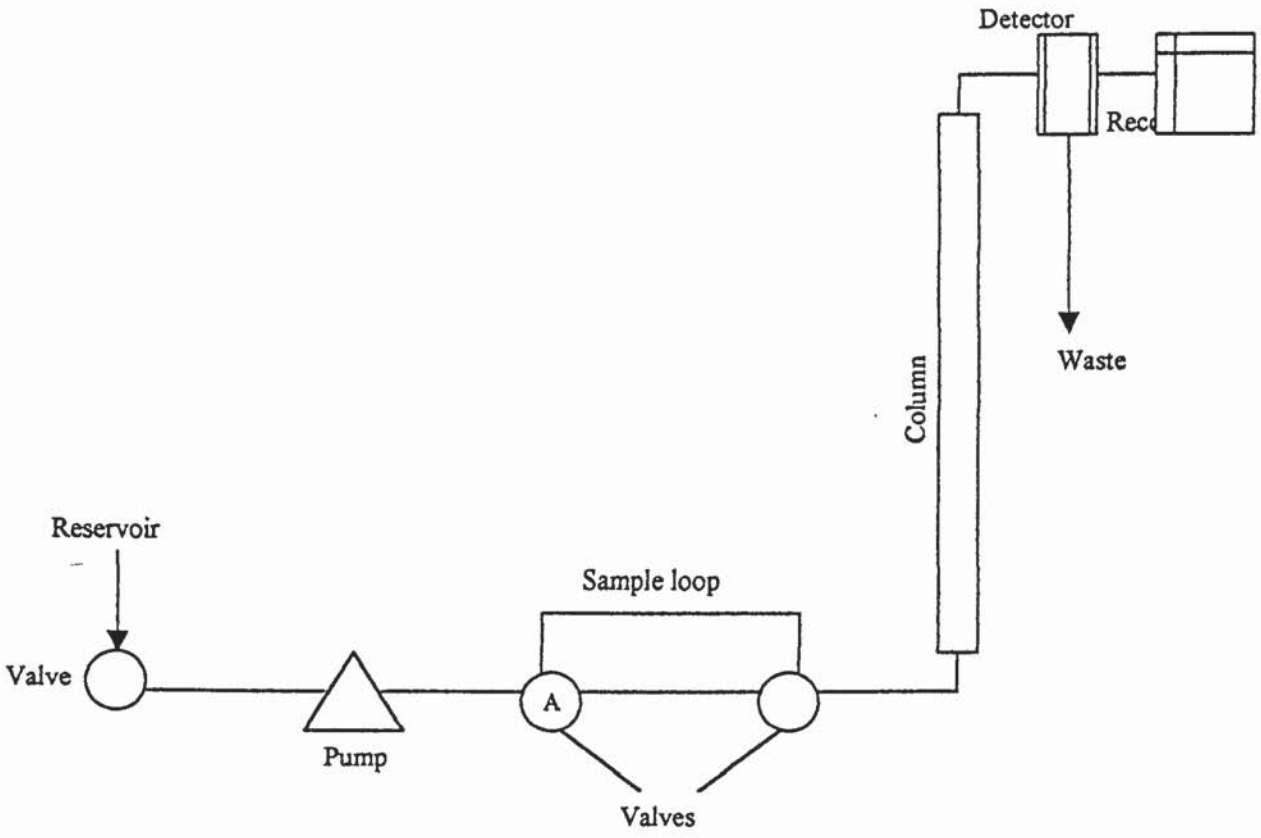


Figure 2.1: Diagrammatic representation of the equipment used in the residence time distribution experiments.

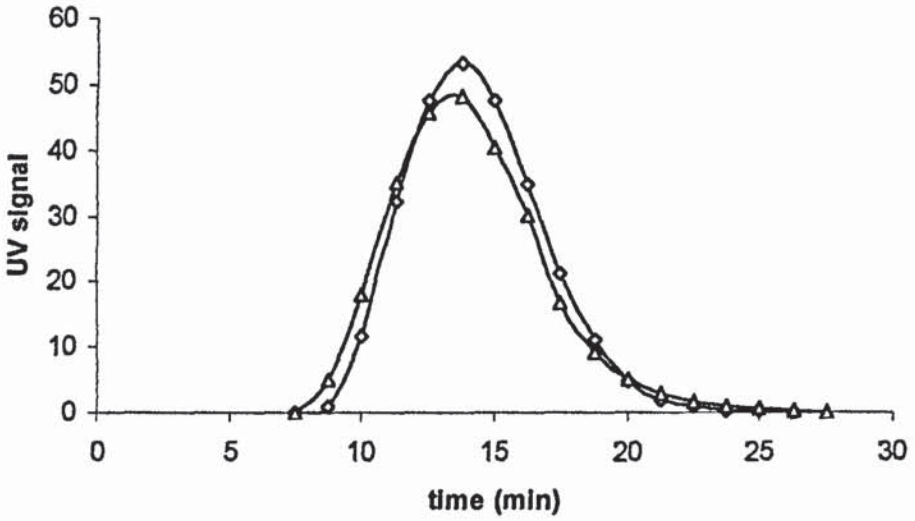


Figure 2.2: A typical curve for the residence time distribution experiments. In this case H_o was 15 cm. Bed expansion was 2 times and the experiment was done in duplicate. The experiment was conducted in the 12.5 mm column.

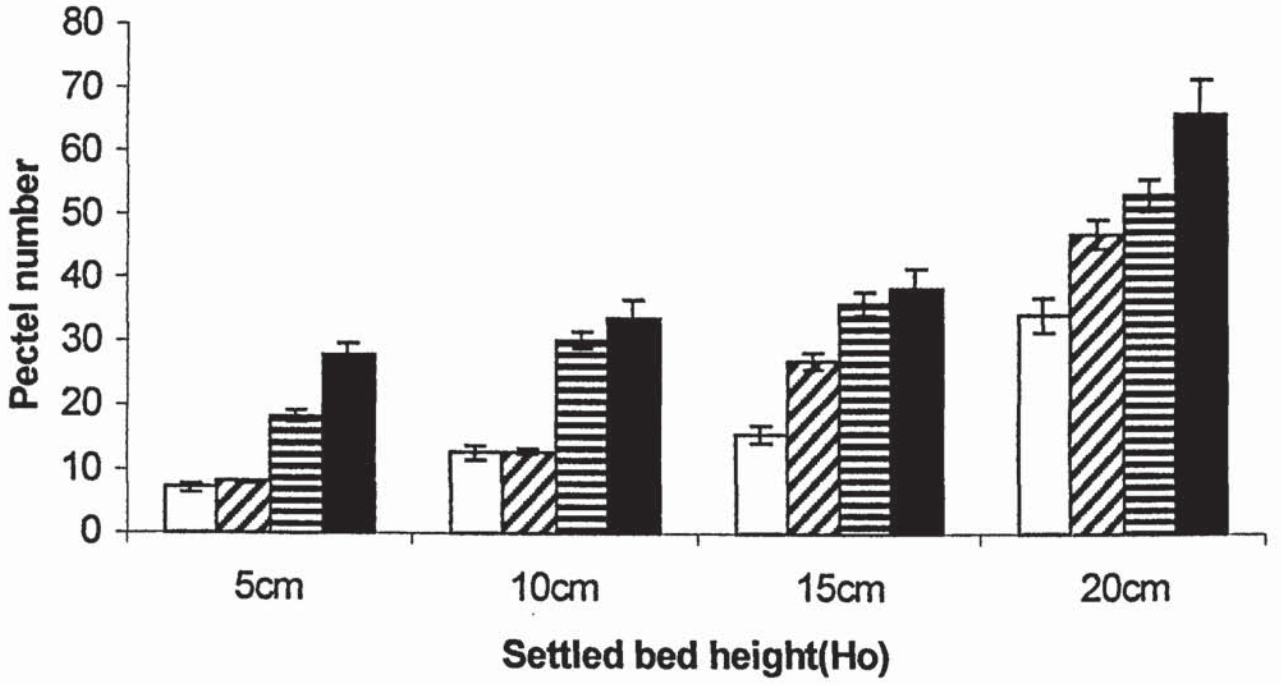


Figure 2.3: The effect of the settled bed height on axial dispersion in the 12.5 mm column. A 12.5 mm column packed with 5 cm, 10 cm 15 cm or 20 cm of Streamline DEAE was equilibrated with 50 mm Na_2HPO_4 pH 7. When the bed was stable an acetone tracer was injected into the column. The acetone concentration of the effluent was measured from which the Peclet number was calculated. The bed expansion was 1.5 (□), 2 (▨), 2.5 (▤) and 3 (■) times the settled bed height.

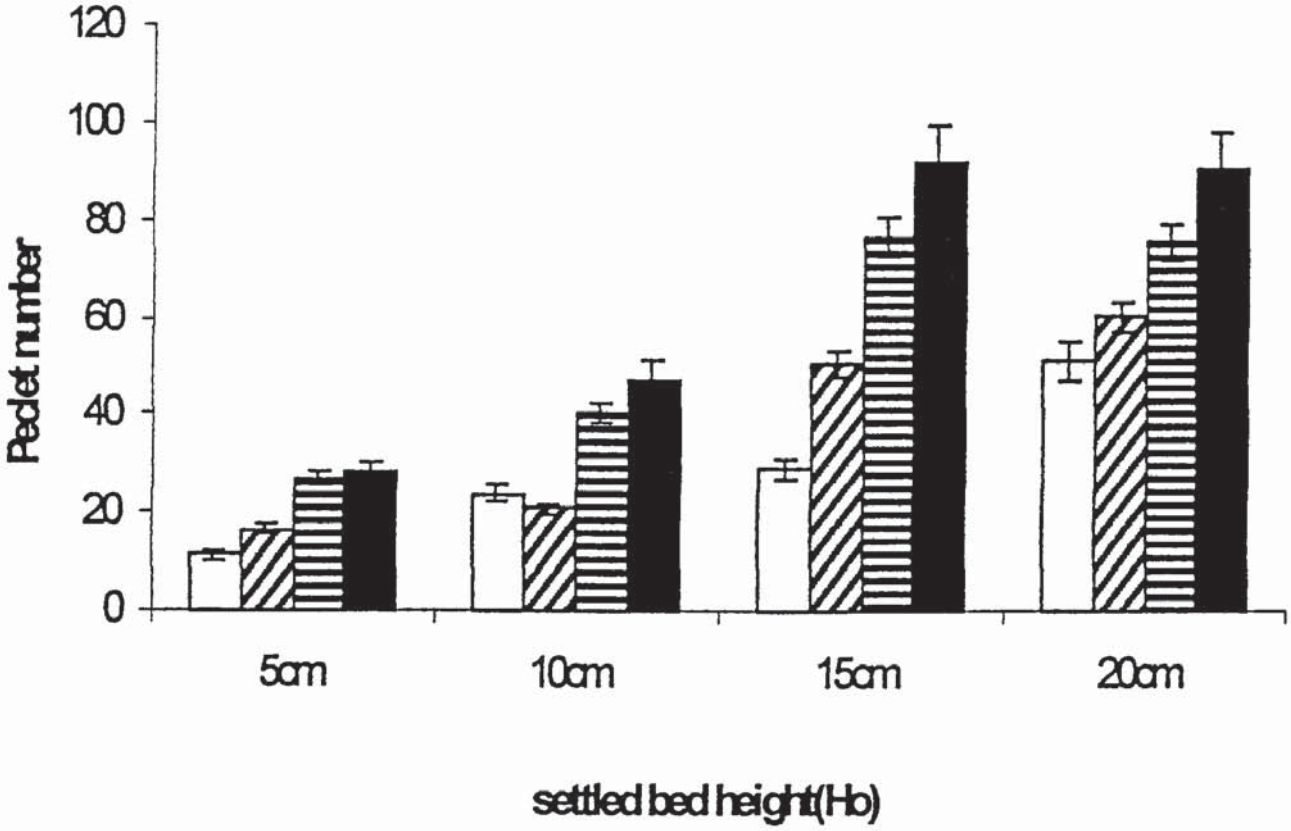


Figure 2.4: The effect of the settled bed height on axial dispersion in the 25 mm column. A 25 mm column packed with 5 cm, 10 cm 15 cm or 20 cm of Streamline DEAE was equilibrated with 50 mm Na_2HPO_4 pH 7. When the bed was stable an acetone tracer was injected into the column. The acetone concentration of the effluent was measured from which the Peclet number was calculated. The bed expansion was 1.5 (□), 2 (▨), 2.5 (▩) and 3 (■) times the settled bed height

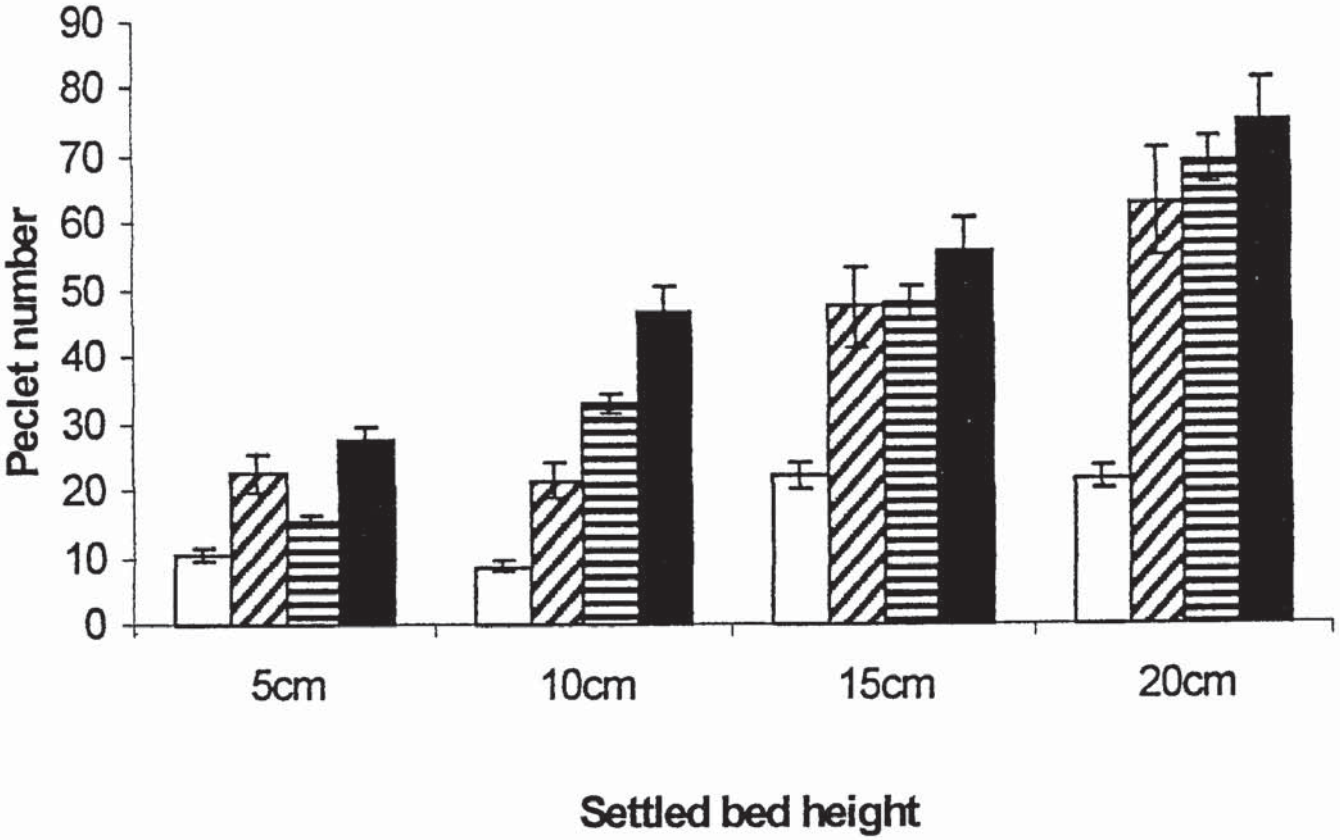


Figure 2.5: The effect of the settled bed height on axial dispersion in the 50 mm column. A 50 mm was column packed with 5 cm, 10 cm 15 cm or 20 cm of Streamline™ DEAE and equilibrated with 50 mm Na_2HPO_4 pH 7. When the bed was stable an acetone tracer was injected into the column. The acetone concentration of the effluent was measured from which the Peclet number was calculated. The bed expansion was 1.5 (□), 2 (▨), 2.5 (▤) or 3 (■) times the settled bed height

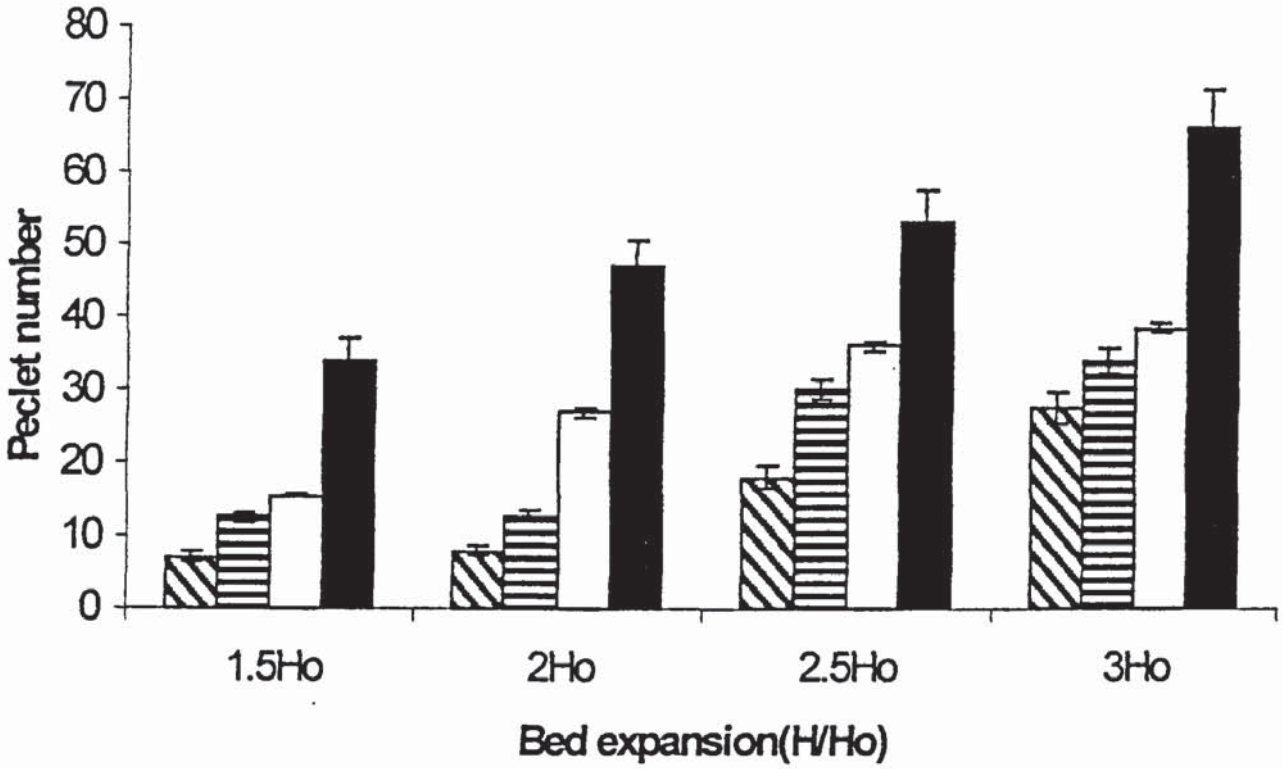


Figure 2.6: The effect of flow velocity (degree of bed expansion) on axial dispersion in the 12.5 mm column. The column was fluidised with 50 mm Na_2HPO_4, pH 7. When the bed was stable an acetone tracer was injected. The concentration of acetone in the effluent was being monitored. From the data collected, the Peclet number was calculated. The settled bed height of the column was 5 cm (▨), 10 cm (■), 15 cm (□) and 20 cm (■).

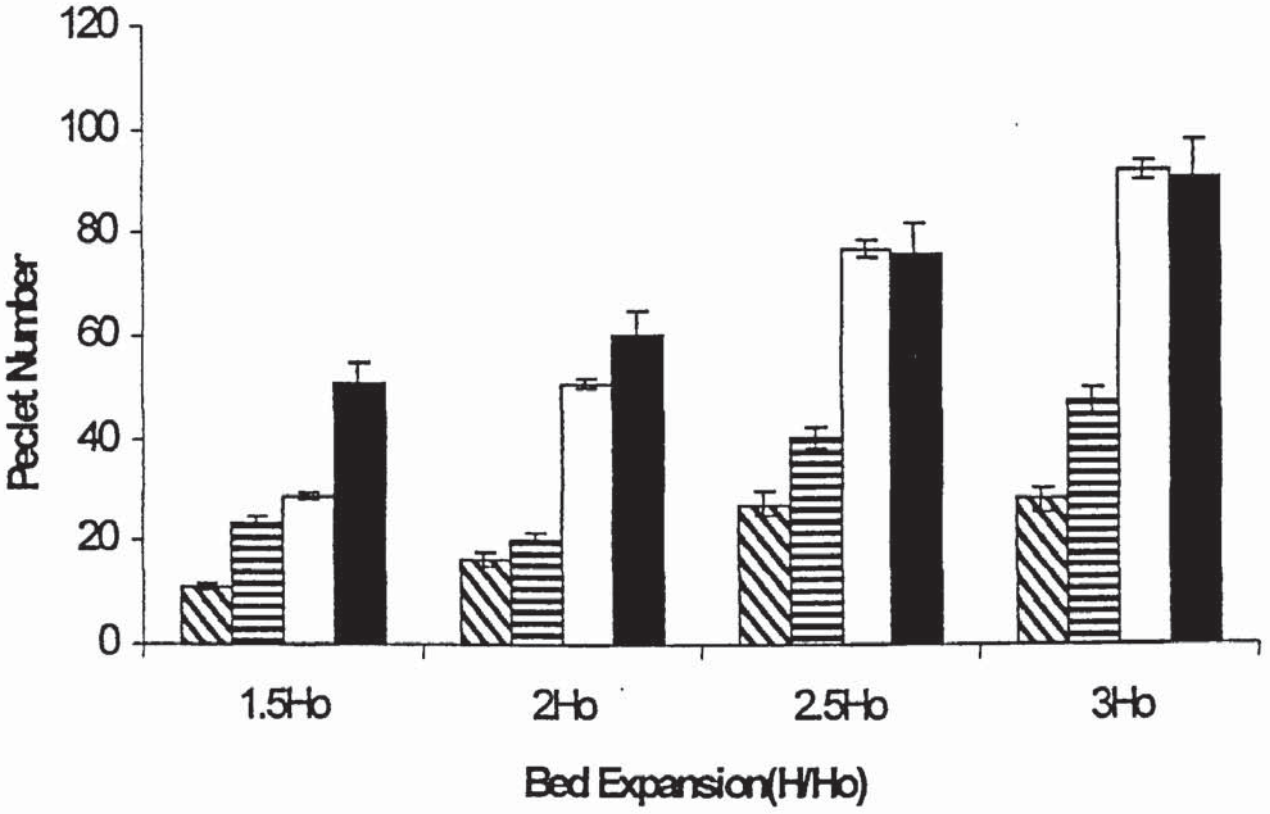


Figure 2.7: The effect of flow velocity (degree of bed expansion) on axial dispersion in the 25 mm column. The column was fluidised with 50 mm Na_2HPO_4, pH 7. When the bed was stable an acetone tracer was injected. The concentration of acetone in the effluent was being monitored. From the data collected, the Peclet number was calculated. The settled bed height of the column was 5 cm (▨), 10 cm (▬), 15 cm (□) and 20 cm (■).

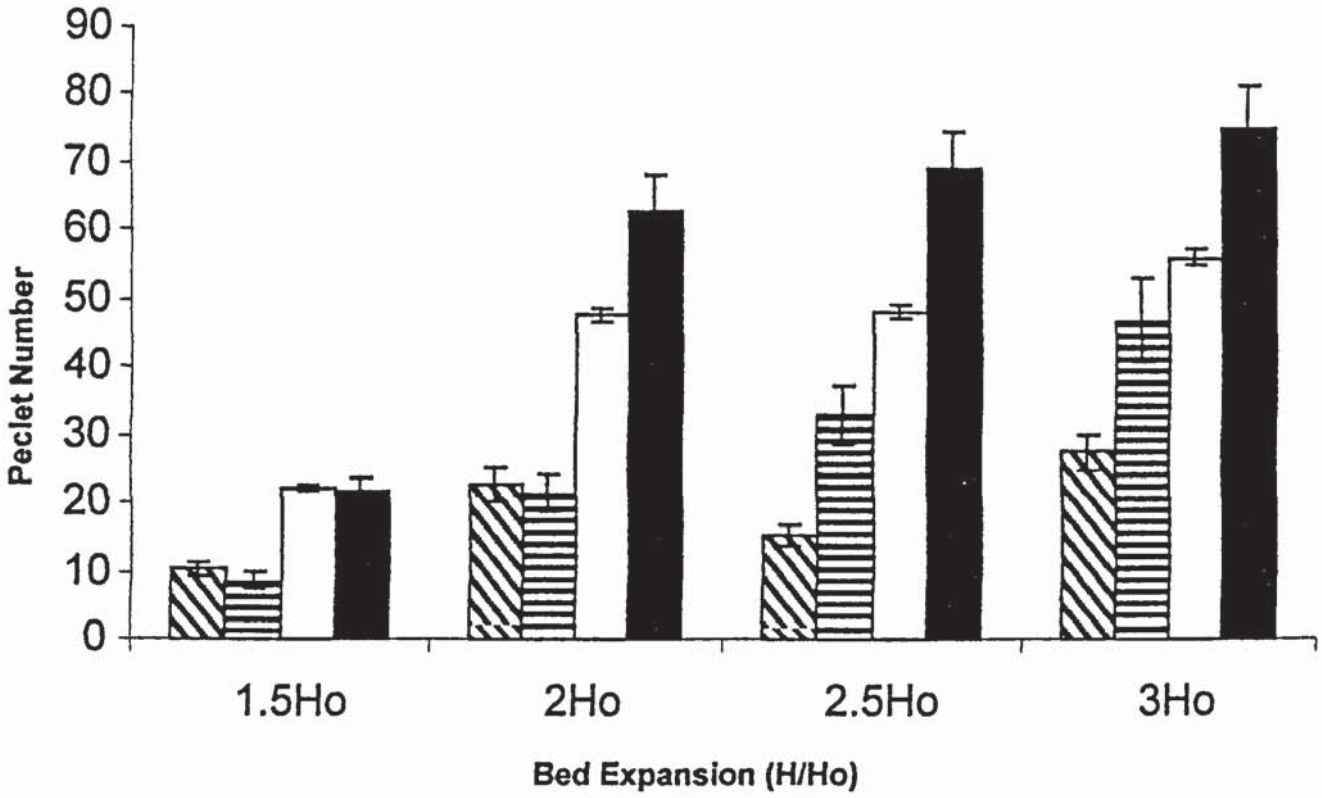


Figure 2.8: The effect of flow velocity (degree of bed expansion) on axial dispersion in the 50 mm column. The column was fluidised with 50 mm Na_2HPO_4, pH 7. When the bed was stable an acetone tracer was injected. The concentration of acetone in the effluent was being monitored. From the data collected, the Peclet number was calculated. The settled bed height of the column was 5 cm (▨), 10 cm (▬), 15 cm (□) and 20 cm (■).

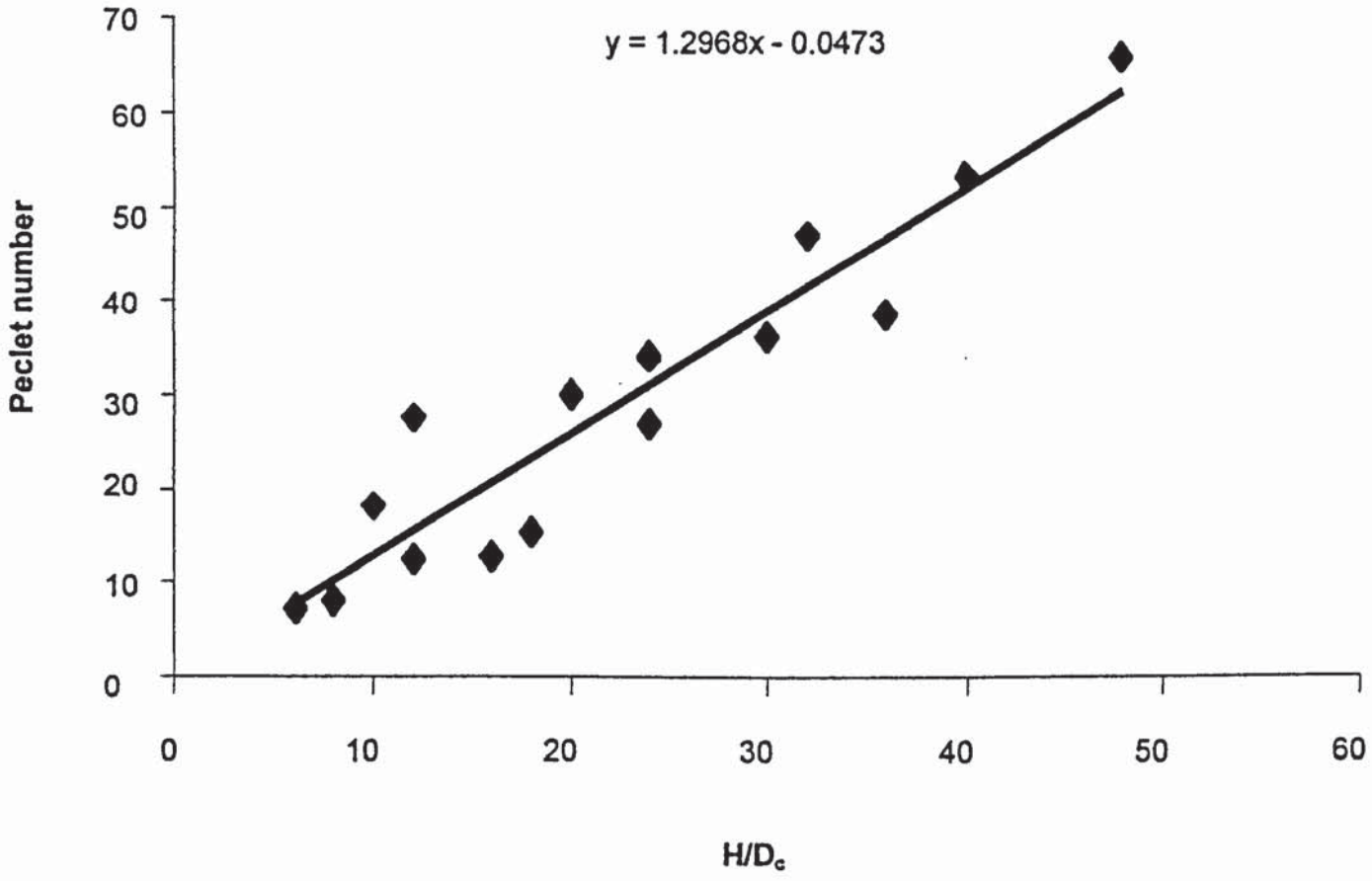


Figure 2.9: The influence of the bed aspect ratio (H/Dc) on axial dispersion in the 12.5 mm column. The column was packed with adsorbent to a settled bed height of 5 cm, 10 cm, 15 cm and 20 cm. The beds were then expanded with 50 mm Na_2HPO_4, pH 7 to a height of 1.5, 2, 2.5 and 3 times the settled bed height. The RTD were calculated from the concentration of acetone tracer in the effluent. The data were ranked in order of the bed aspect ratio (H/Dc) where H is the expanded column height and Dc is the column diameter.

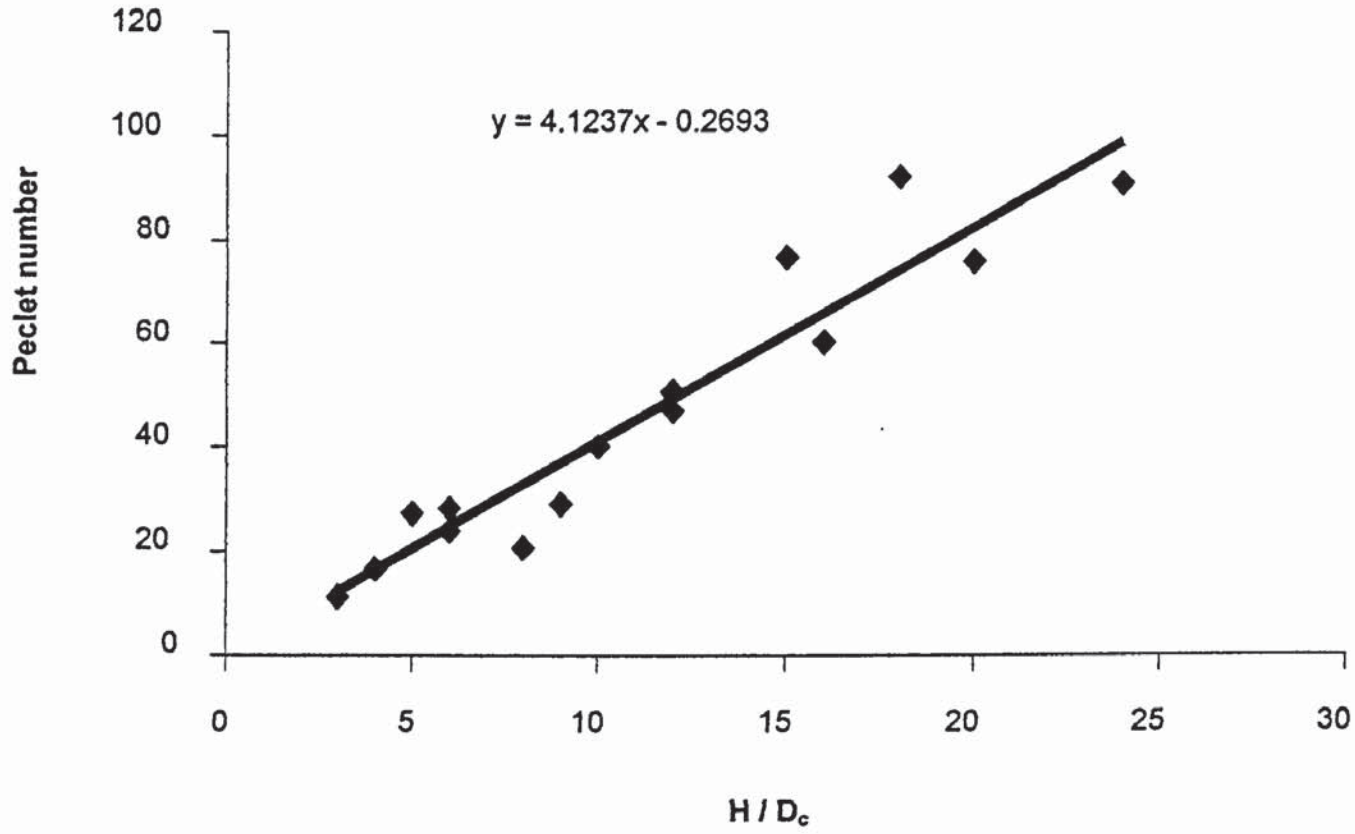


Figure 2.10: The influence of the bed aspect ratio (H/Dc) on axial dispersion in the 25 mm column. The column was packed with adsorbent to a settled bed height of 5 cm, 10 cm, 15 cm and 20 cm. The beds were then expanded with 50 mm Na_2HPO_4, pH 7 to a height of 1.5, 2, 2.5 and 3 times the settled bed height. The RTD were calculated from the concentration of acetone tracer in the effluent. The data were ranked in order of the bed aspect ratio (H/Dc) where H is the expanded column height and Dc is the column diameter.

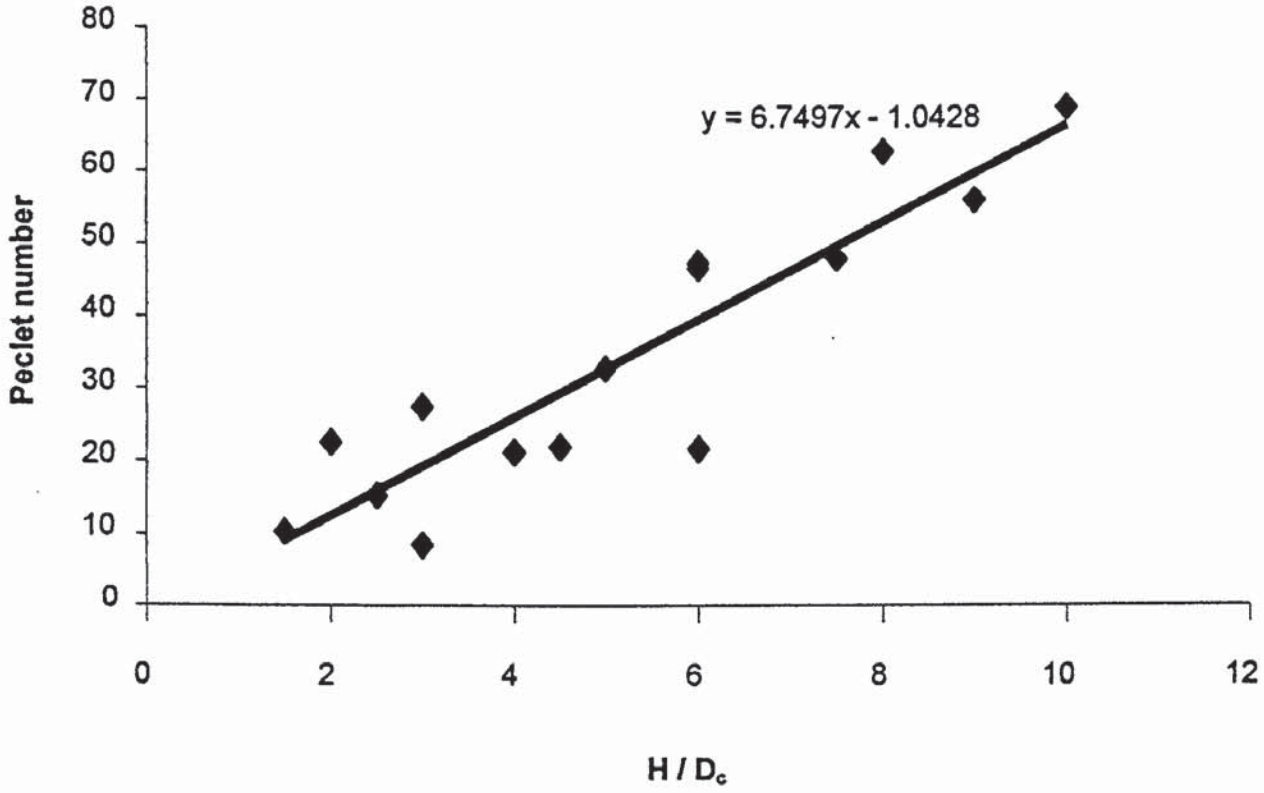


Figure 2.11: The influence of the bed aspect ratio (H/Dc) on axial dispersion in the 50 mm column. The column was packed with adsorbent to a settled bed height of 5 cm, 10 cm, 15 cm and 20 cm. The beds were then expanded with 50 mm Na_2HPO_4, pH 7 to a height of 1.5, 2, 2.5 and 3 times the settled bed height. The RTD were calculated from the concentration of acetone tracer in the effluent. The data were ranked in order of the bed aspect ratio (H/Dc) where H is the expanded column height and Dc is the column diameter.

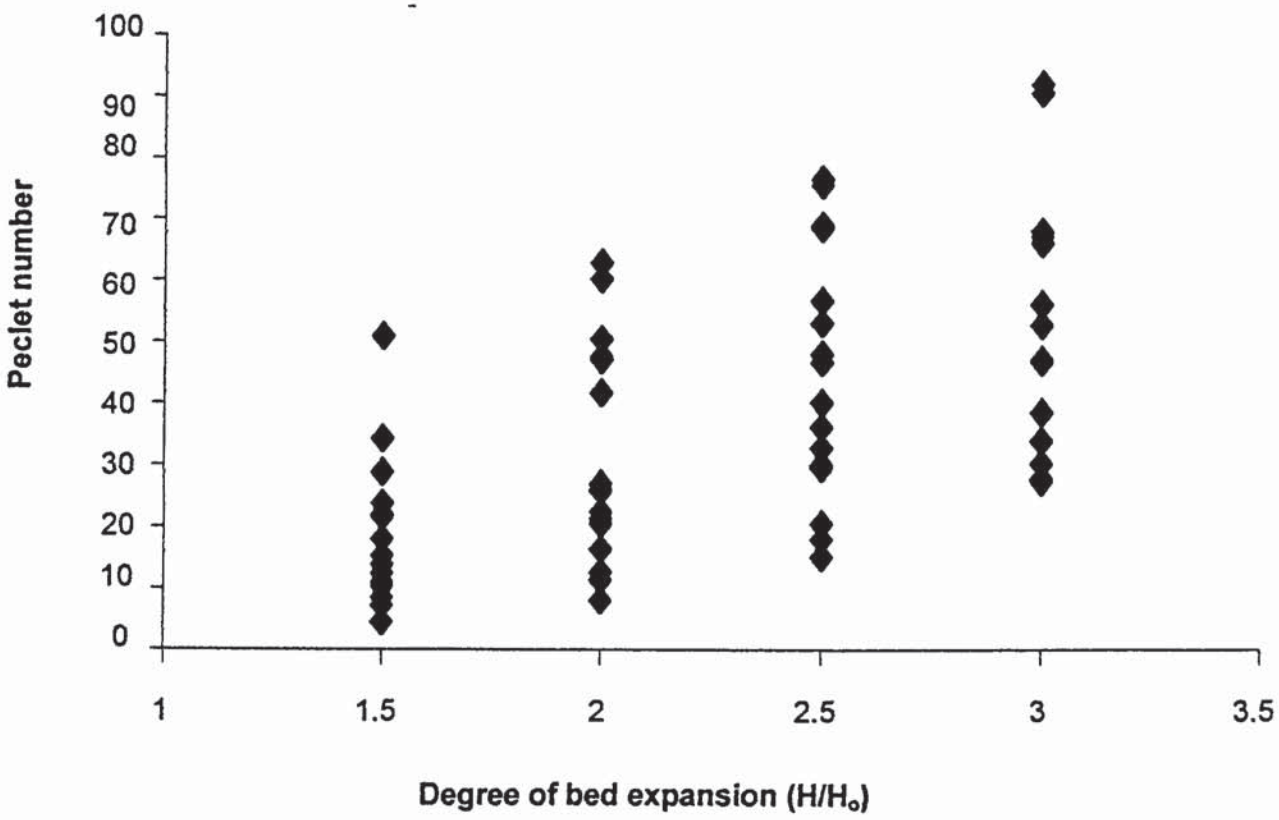


Figure 2.12: Distribution of RTD data with respect to the degree of bed expansion : Three columns of internal diameter 12.5 mm, 25 mm and 50 mm were packed with Streamline™ DEAE to a settled height of 5 cm, 10 cm, 15 cm and 20 cm. The beds were expanded to 1.5, 2, 2.5 and 3 times the settled bed height using 50 mm Na_2HPO_4 pH 7 as buffer. The data from all the columns were pooled together and plotted against the degree of bed expansion.

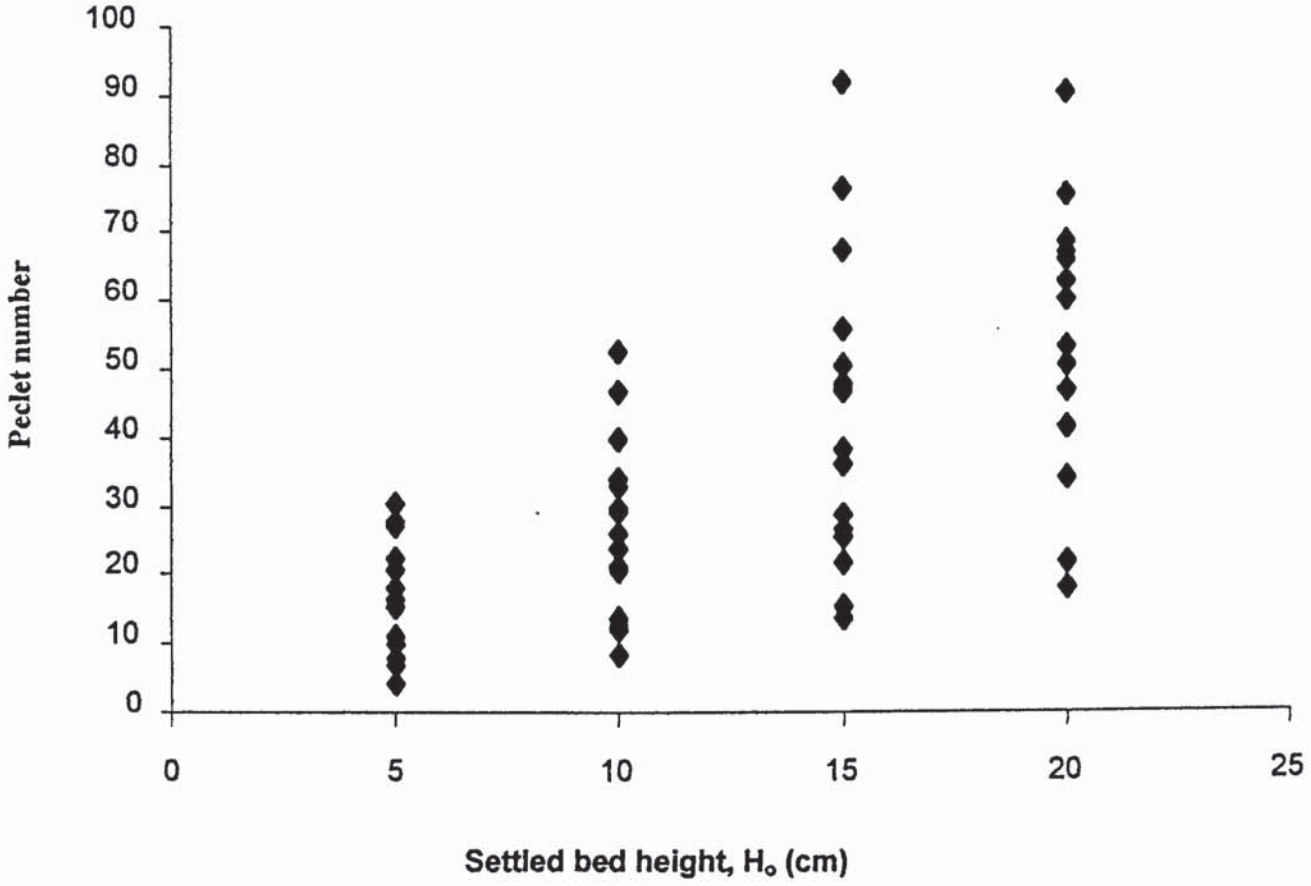


Figure 2.13: Distribution of RTD data with respect to settled bed height. Three columns of internal diameter 12.5 mm, 25 mm and 50 mm were packed with Streamline™ DEAE to a settled height of 5 cm, 10 cm, 15 cm and 20 cm. The beds were expanded to 1.5, 2, 2.5 and 3 times the settled bed height using 50 mm Na_2HPO_4 pH 7 as buffer. The data from all the columns were pooled together and plotted against the settled bed height.

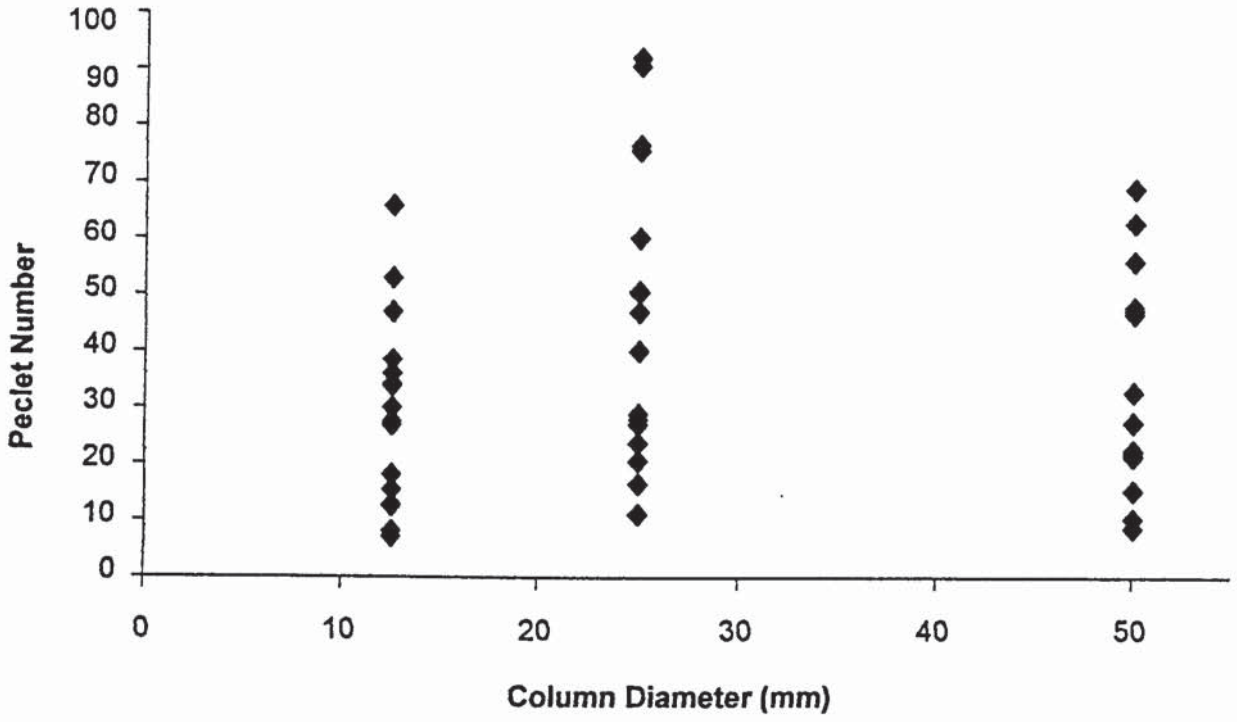


Figure 2.14: The distribution of the RTD data with respect to column diameter. Three columns of internal diameter 12.5 mm, 25 mm and 50 mm were packed with Streamline™ DEAE to a settled height of 5 cm, 10 cm, 15 cm and 20 cm. The beds were expanded to 1.5, 2, 2.5 and 3 times the settled bed height using 50 mm Na_2HPO_4 pH 7 as buffer. The data from all the columns were pooled together and plotted against the column diameter.

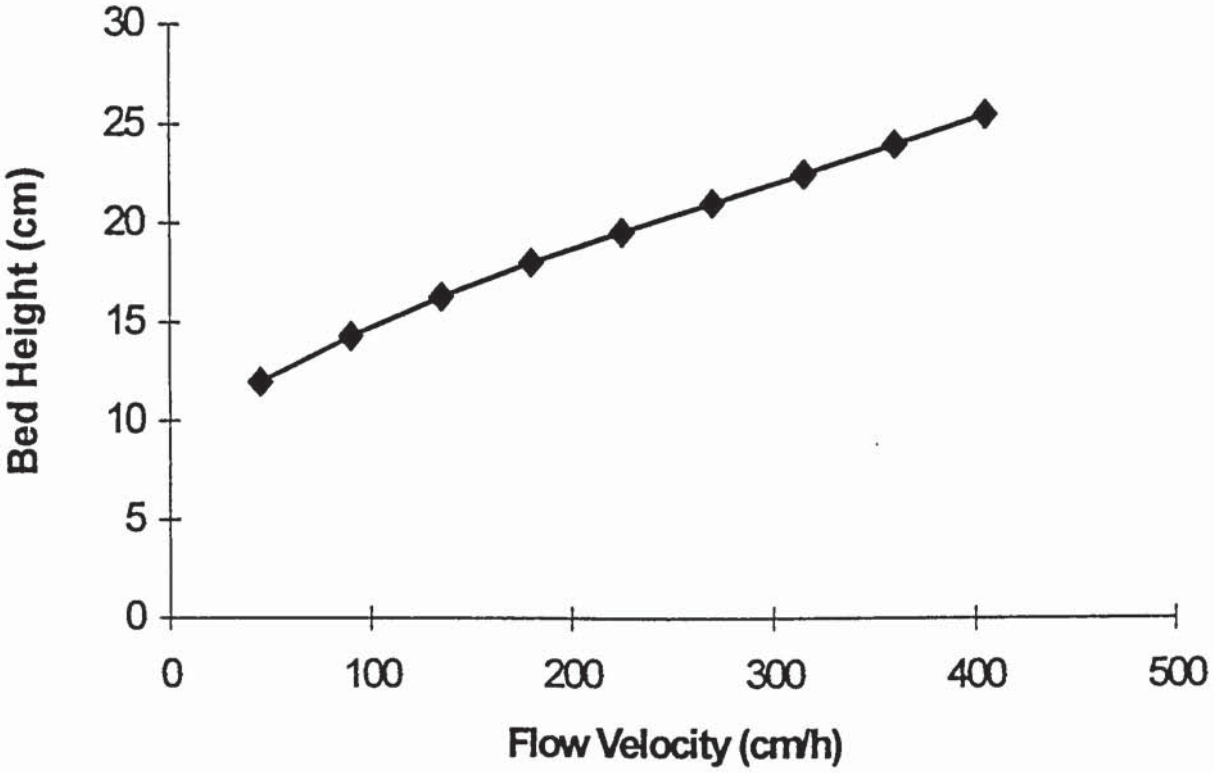


Figure 2.15: Bed expansion characteristics of Streamline™ SP as a function of flow velocity. Streamline™ SP was packed into a 12.5 mm column to a settled bed height of 10 cm. The column was then equilibrated with 50 mm Na_2HPO_4 pH 7 at flow velocity of 45 cm/h. When the bed was stable the bed height was measured after which the flow velocity was increased and the process repeated until the bed front was less distinct.

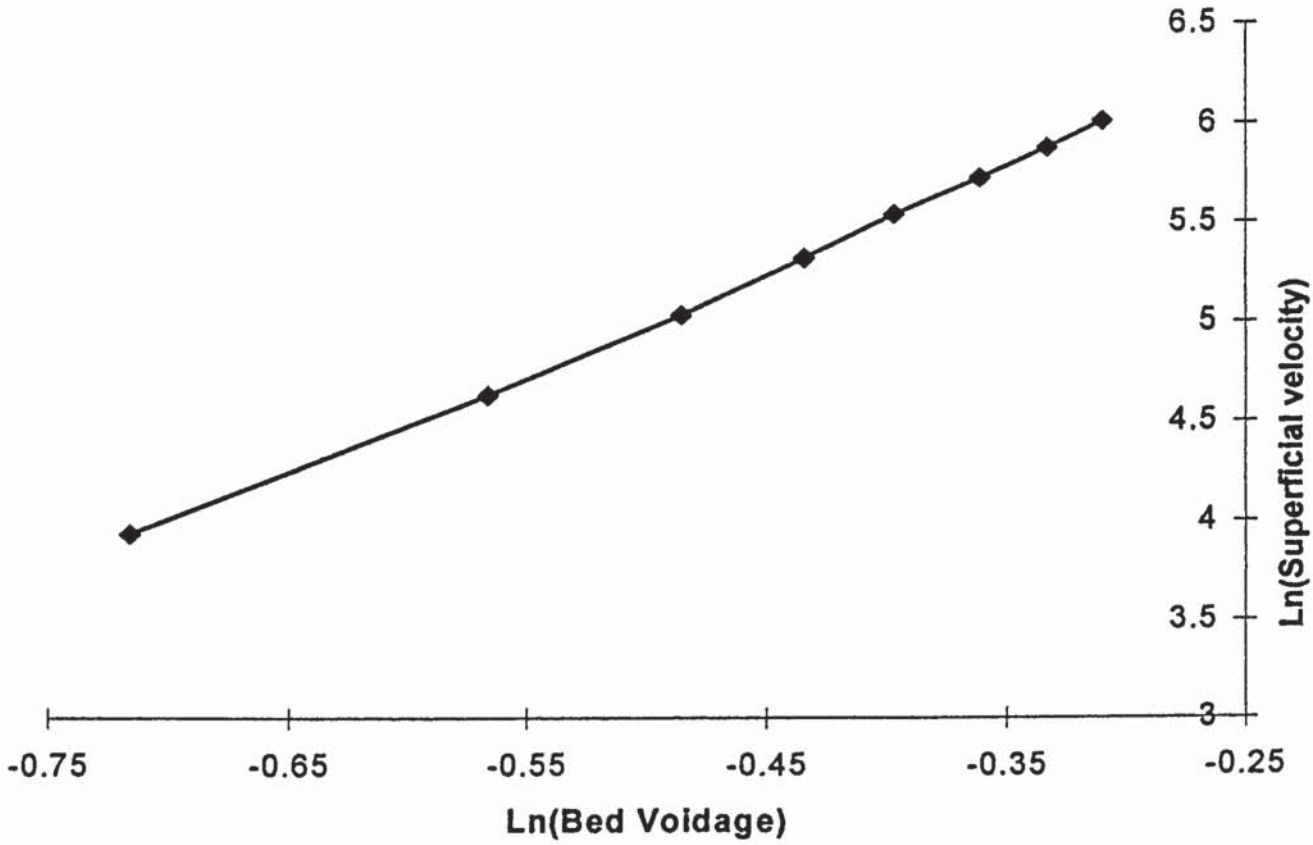


Figure 2.16: Plot of the logarithm of the superficial velocity against the logarithm of bed voidage: Streamline™ SP was packed into a 12.5 mm column to a settled bed height of 10 cm. The column was then equilibrated with 50 mm Na_2HPO_4 pH 7 at flow velocity of 45 cm/h. When the bed was stable the bed height was measured after which the flow velocity was increased and the process repeated until the bed front was less distinct. The bed voidage was calculated and used to produce the figure.

Bed Expansion (H/Ho)	Column diameter(mm)	Ho = 5 cm Pe,	σ	Ho = 10 cm Pe,	σ	Ho = 15 cm Pe,	σ	Ho = 20 cm Pe,	σ
1.5	12.5	7	1.2	13	0.2	15	0.3	34	4.7
	25	11	1.8	24	0.4	29	0.6	51	7.0
	50	10	1.7	9	0.2	22	0.4	22	3.0
2	12.5	8	0.4	13	0.5	27	0.3	47	3.5
	25	16	0.9	21	0.8	50	0.5	60	4.4
	50	22	1.2	21	0.8	47	0.5	63	4.7
2.5	12.5	18	0.9	30	1.3	36	0.4	53	2.3
	25	27	1.4	40	1.7	76	0.9	76	3.3
	50	15	0.8	33	1.4	48	0.6	69	3.0
3	12.5	28	3.3	34	3.6	38	1.1	66	5.0
	25	28	3.3	47	5.0	92	2.8	91	6.8
	50	27	3.2	47	5.0	56	1.7	75	5.6

Table 2.1: Comparison of axial dispersion in the 12.5 mm, 25 mm and 50 mm columns. The columns were packed with adsorbent to a settled bed height of 5 cm, 10 cm, 15 cm and 20 cm. The beds were then expanded to a height of 1.5, 2, 2.5 and 3 times the settled bed height. The RTD were calculated from the concentration of acetone tracer in the effluent.

Ho	H/Ho	H/Dc	Dc	Pe,	σ
5	1.5	1.5	50	10	1.7
5	2	2	50	22	1.2
5	2.5	2.5	50	15	0.8
5	3	3	50	27	3.2
5	1.5	3	25	11	0.4
10	1.5	3	50	9	0.2
5	2	4	25	16	0.9
10	2	4	50	21	0.8
15	1.5	4.5	50	22	0.4
5	2.5	5	25	27	1.4
10	2.5	5	50	33	1.4
5	3	6	25	28	3.3
10	1.5	6	25	24	0.4
10	3	6	50	47	5.0
15	2	6	50	47	0.5
20	1.5	6	50	22	3.0
5	1.5	6	12.5	7	1.2
15	2.5	7.5	50	48	0.6
10	2	8	25	21	0.8
20	2	8	50	63	4.7
5	2	8	12.5	8	0.4
15	1.5	9	25	29	0.6
15	3	9	50	56	1.7
10	2.5	10	25	40	1.7
20	2.5	10	50	69	3.0
5	2.5	10	12.5	18	0.9
10	3	12	25	47	5.0
15	2	12	25	50	0.5
20	1.5	12	25	51	7.0
5	3	12	12.5	28	3.3
10	1.5	12	12.5	13	0.2
15	2.5	15	25	76	0.9
20	2	16	25	60	4.4
10	2	16	12.5	13	0.5
15	3	18	25	92	2.8
15	1.5	18	12.5	15	0.3
20	2.5	20	25	76	3.3
10	2.5	20	12.5	30	1.3
20	3	24	25	91	6.8
10	3	24	12.5	34	3.6
15	2	24	12.5	27	0.3
20	1.5	24	12.5	34	4.7
15	2.5	30	12.5	36	0.4
20	2	32	12.5	47	3.5
15	3	36	12.5	38	1.1
20	2.5	40	12.5	53	2.3
20	3	48	12.5	66	5.0

Table 2.2 The influence of the bed aspect ratio on axial dispersion. Three columns of internal diameter 12.5 mm, 25 mm and 50 mm were packed with Streamline™ DEAE to a settled bed height of 5 cm, 10 cm, 15 cm and 20 cm. The beds were expanded with 50 mm Na_2HPO_4 pH 7 to a bed height of 1.5, 2, 2.5 and 3 times the settled bed height. When the expanded bed was stable an acetone tracer was injected. The concentration of acetone in the effluent was monitored and the data generated was ranked in order of the bed aspect ratio to produce this table.

Column diameter(mm)	H/Ho	Ho(cm)	Pe	σ
25	1.5	20	51	7.0
50	1.5	5	9	0.2
25	2	20	60	4.4
50	2	5	21	0.8
25	1.5	40	59	4.0
50	1.5	10	10	0.2
25	2	40	100	9.0
50	2	10	22	0.8

Table 2.3: Axial dispersion in the 25 mm and 50 mm columns containing the same volume of adsorbent. The two columns were packed with the same volume of Streamline™ DEAE (196 ml and 392 ml). The beds were expanded to 1.5 and 2 times the settled bed height with 50 mm Na_2HPO_4 pH 7. When the bed was stable an acetone tracer was injected into the column and its concentration was monitored in the effluent.

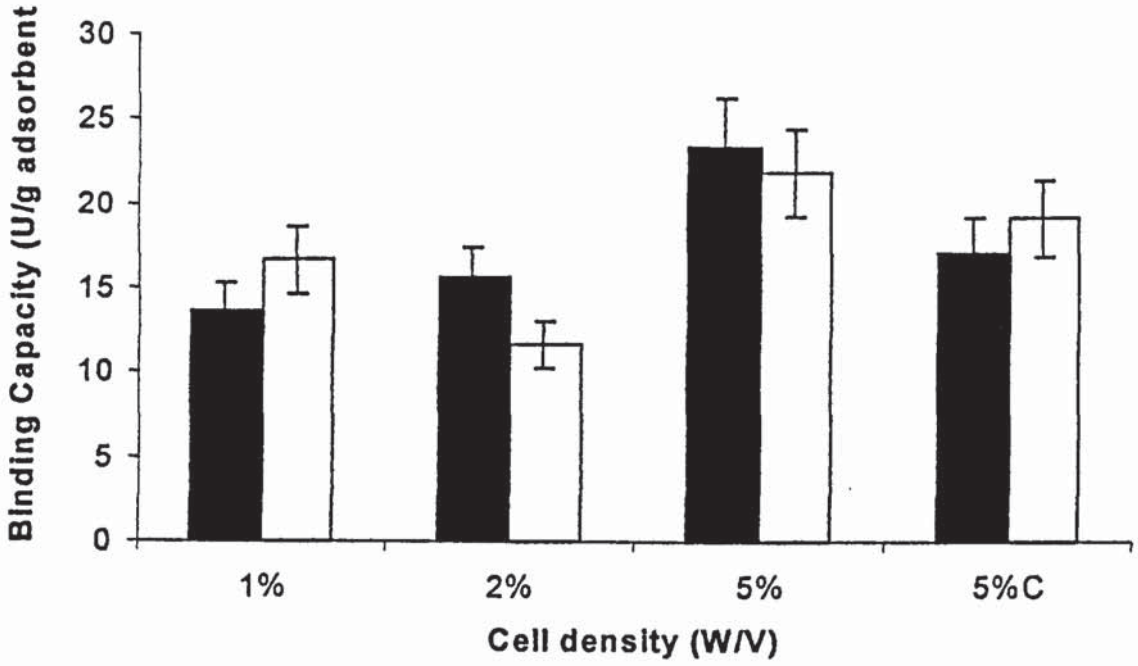


Figure 3.1: The dynamic binding capacity of G6PDH on Streamline DEAE. A 10 mm column was packed with Streamline DEAE to a settled bed height of 20cm. The column was then expanded to twice the settled bed height with 20 mM Tris-HCl pH 8. When the column was stable, yeast suspensions containing G6PDH were loaded on the column. After thorough washing, bound proteins and G6PDH were eluted with 1M KCl in 20 mM Tris-HCl pH 8 buffer. The yeast suspensions were loaded at either constant flow CF (■), or constant height CH (□). The cell density of the yeast suspensions ranged from 1% to 5% (w/v). 5%C represents the partially centrifuged suspensions.

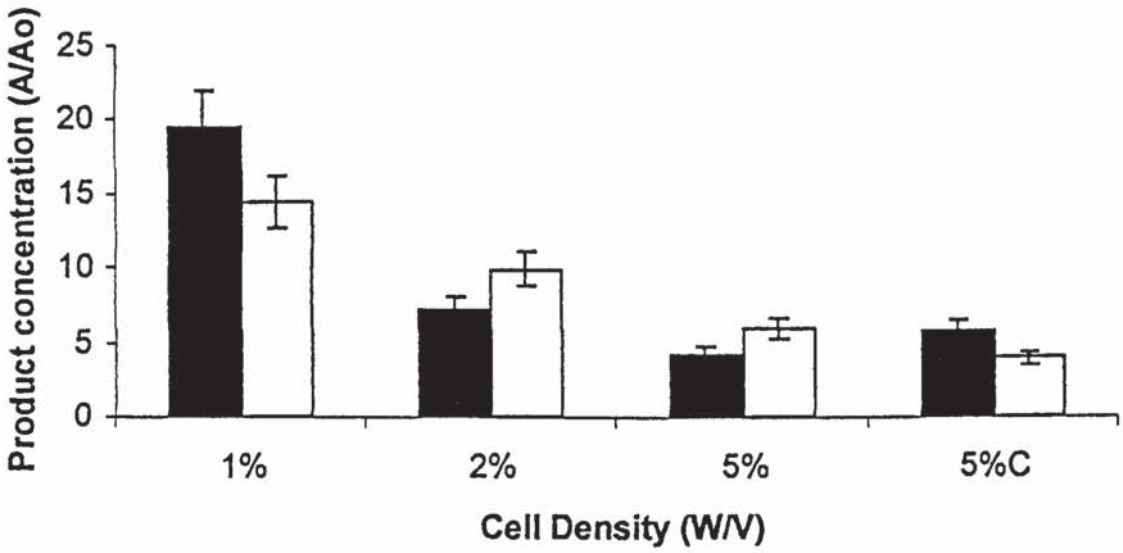


Figure 3.2: The effect of feed concentration, cell debris and loading modes on product (G6PDH) concentration. Yeast suspensions containing G6PDH were loaded onto a 20 cm bed expanded to 40 cm in a 10 mm column. After thorough washing, bound proteins and G6PDH were eluted with 1M KCl in 20 mM Tris-HCl pH 8 buffer. The yeast suspensions were loaded at either Constant height CH (■), or constant flow CF (□). The cell density of the yeast suspensions ranged from 1% to 5% (w/v). 5%C represents the partially centrifuged suspensions. Equilibrating buffer was 20 mM Tris-HCl pH 8. The G6PDH activity of both the elutes (A) and the feed (Ao) were measured.

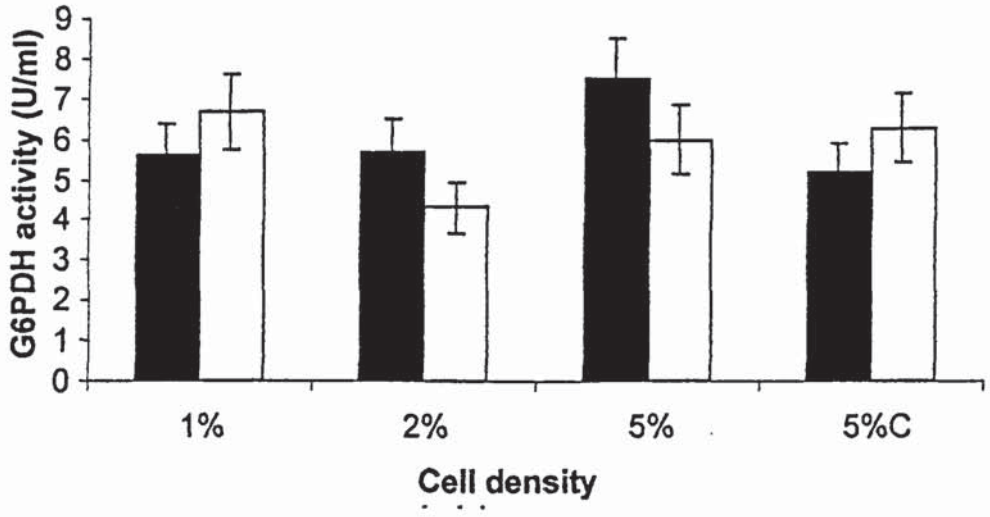


Figure 3.3: The effect of feed concentration, solid content and loading modes on product concentration. Yeast suspensions containing G6PDH were loaded on a two times expanded bed of Streamline DEAE (settled bed height 20 cm) in a 10 mm column. After thorough washing, bound proteins and G6PDH were eluted with 1 M KCl in 20 mM Tris-HCl pH 8 buffer. The yeast suspensions were loaded at either constant flow CF (■), or Constant height CH (□). The cell density of the yeast suspensions ranged from 1% to 5% (w/v). 5%C represents the partially centrifuged suspensions. Whereas Figure 3.2 looks at A/Ao values of the eluates, Figure 3.3 reports the actual G6PDH concentration in the eluates.

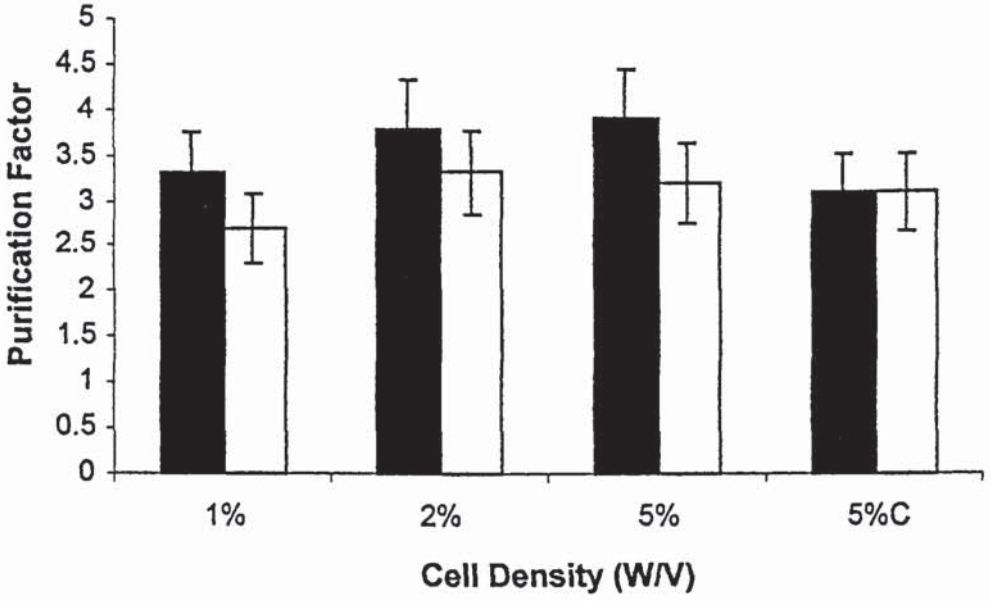


Figure 3.4: The influence of feed concentration, cell debris and loading modes on the purification factor. A 10mm column was packed with Streamline DEAE to a settled bed height of 20cm. The column was then expanded to twice the settled bed height with 20mM Tris-HCl pH 8. When the column was stable, yeast suspensions containing G6PDH were loaded on the column. After thorough washing, bound proteins and G6PDH were eluted with 1M KCl in 20mM Tris-HCl pH 8 buffer. The yeast suspensions were loaded at either constant flow CF (■), or Constant height CH (□). The cell density of the yeast suspensions ranged from 1% to 5% (w/v). 5%C represents the partially centrifuged suspensions.

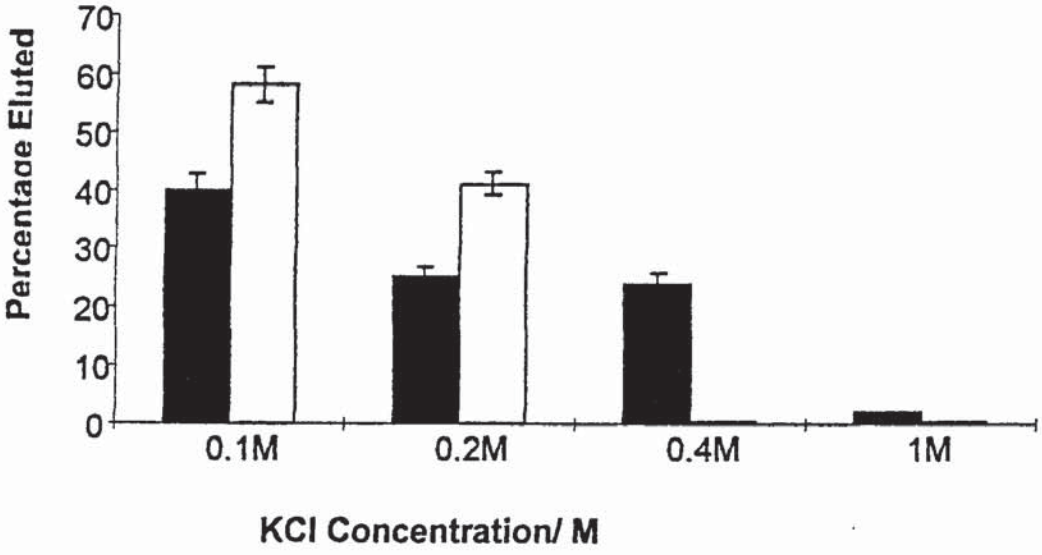


Figure 3.5: Stepwise elution of G6PDH from a Streamline DEAE column. A 10mm column was packed with Streamline DEAE to a settled bed height of 20cm. The column was then expanded to twice the settled bed height with 20mM Tris-HCl pH 8. When the column was stable, a 5% yeast suspension containing G6PDH was loaded on the column at constant flow. After thorough washing, bound proteins (■) and G6PDH (□) were eluted with 0.1M, 0.2M and 1M KCl in 20mM Tris-HCl pH 8 buffer.

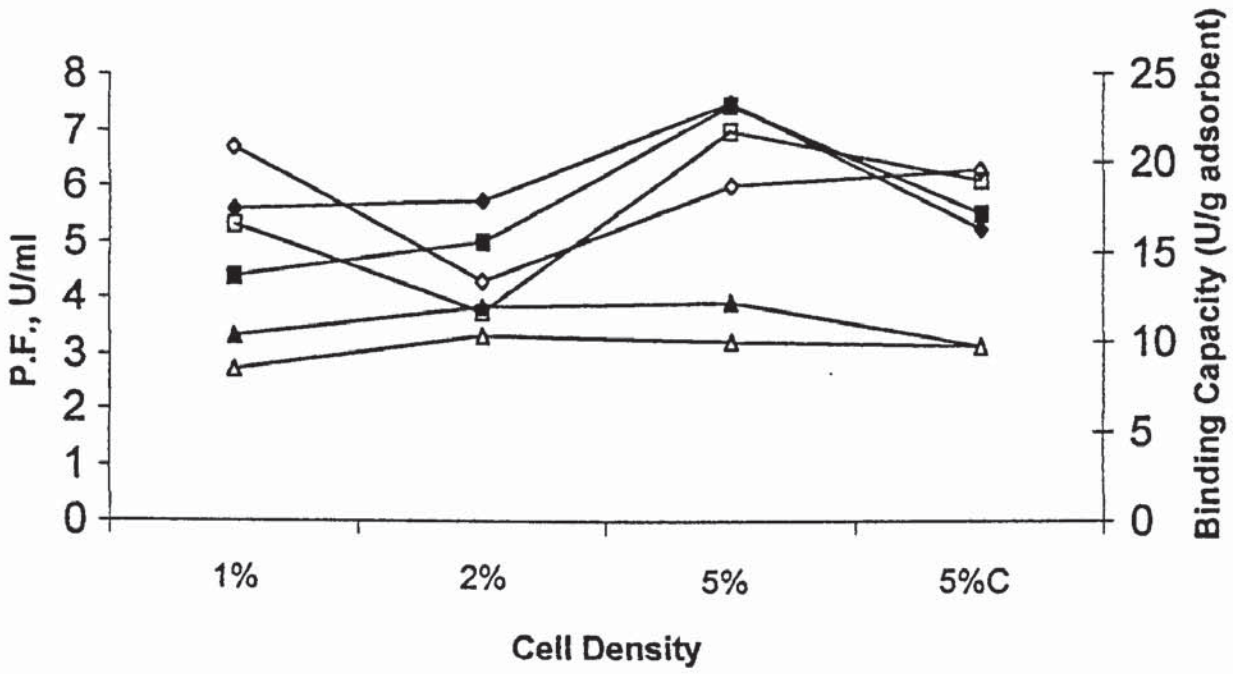
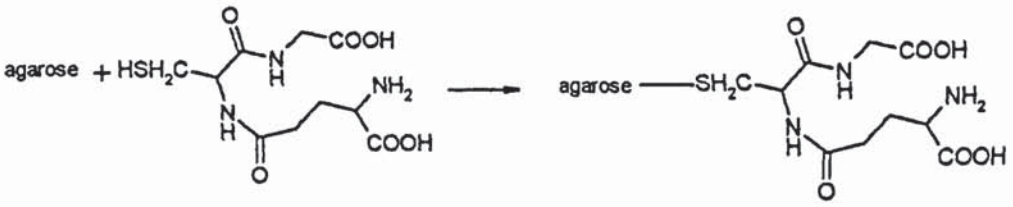


Figure 3.6: Summary of the optimisation of G6PDH purification. The dynamic binding capacity (Figure 3.1), product concentration (Figure 3.3) and he purification factor (Figure 3.4) have been plotted together in Figure 3.6. Product concentration at CF (♦), product concentration at CH (◇), Purification factor at CF (▲), purification factor at CH (△), dynamic binding capacity at CF (■), dynamic binding capacity at CH (□).

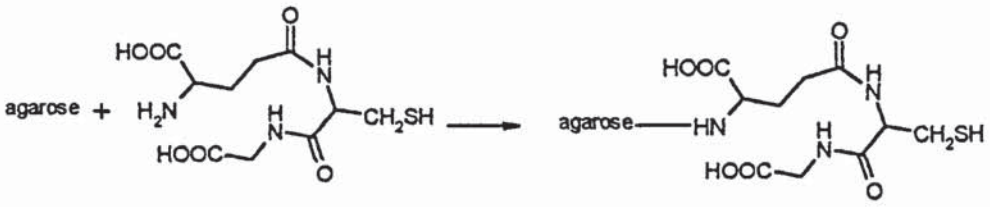
	1%		2%		5%		5%C	
	CH	CF	CH	CF	CH	CF	CH	CF
Loaded volume (ml)	1177	1175	486	562	183	210	200	187
Co (proteins (mg/ml)	2.47	2.18	4.2	4.9	15.6	13	10.3	13.6
A_o (G6PDH) (U/ml)	0.38	0.38	0.77	0.71	2.4	1.95	2.0	2.5
C_{so} (Solids) (Relative)	0.2	0.2	0.4	0.4	1.0	1.0	0.2	0.2
Total load (proteins, mg)	2907	2561	2041	2754	2855	2730	2060	2543
Total load (G6PDH, U)	447	447	374	399	439	410	400	468
Total load (solids, Relative)	235	235	194	224	183	210	40	37
Total recovered (Proteins, mg)	2409	2268	1866	2462	2661	2678	1760	1860
Total recovered (G6PDH, U)	466	502	288	373	413	421	403	366
Total recovered (Solids, %)	100	100	100	100	100	100	100	100

Table 3.1: Mass balance of G6PDH, Total Protein and Total Solids. This table shows the concentrations and volumes of loaded the samples, total amounts loaded and total amounts recovered. CH = constant height, CF= constant flow. The suspensions were prepared by dissolving 12.5g of yeast with buffer such that the cell density was 1%, 2% and 5%. The 5%C suspensions are the partially centrifuged 5% suspensions as described in Section 3.2.3



Matrix A

Figure 4.1 : Reaction of epoxy activated agarose with glutathione at neutral pH.



Matrix B

Figure 4.2: Reaction of epoxy activated agarose with glutathione at alkaline pH.

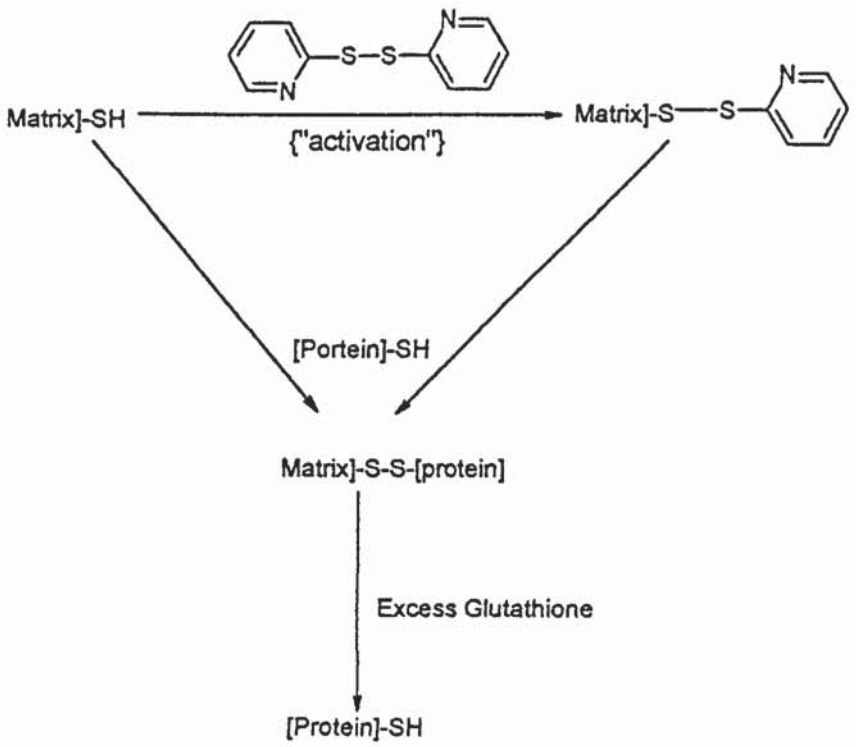


Figure 4.3: Interaction between GST and GSH-agarose. In this particular example, the glutathione molecule is attached via the amino group. The GSH agarose has been reacted with dithiol dypiridine to introduce a better leaving group.

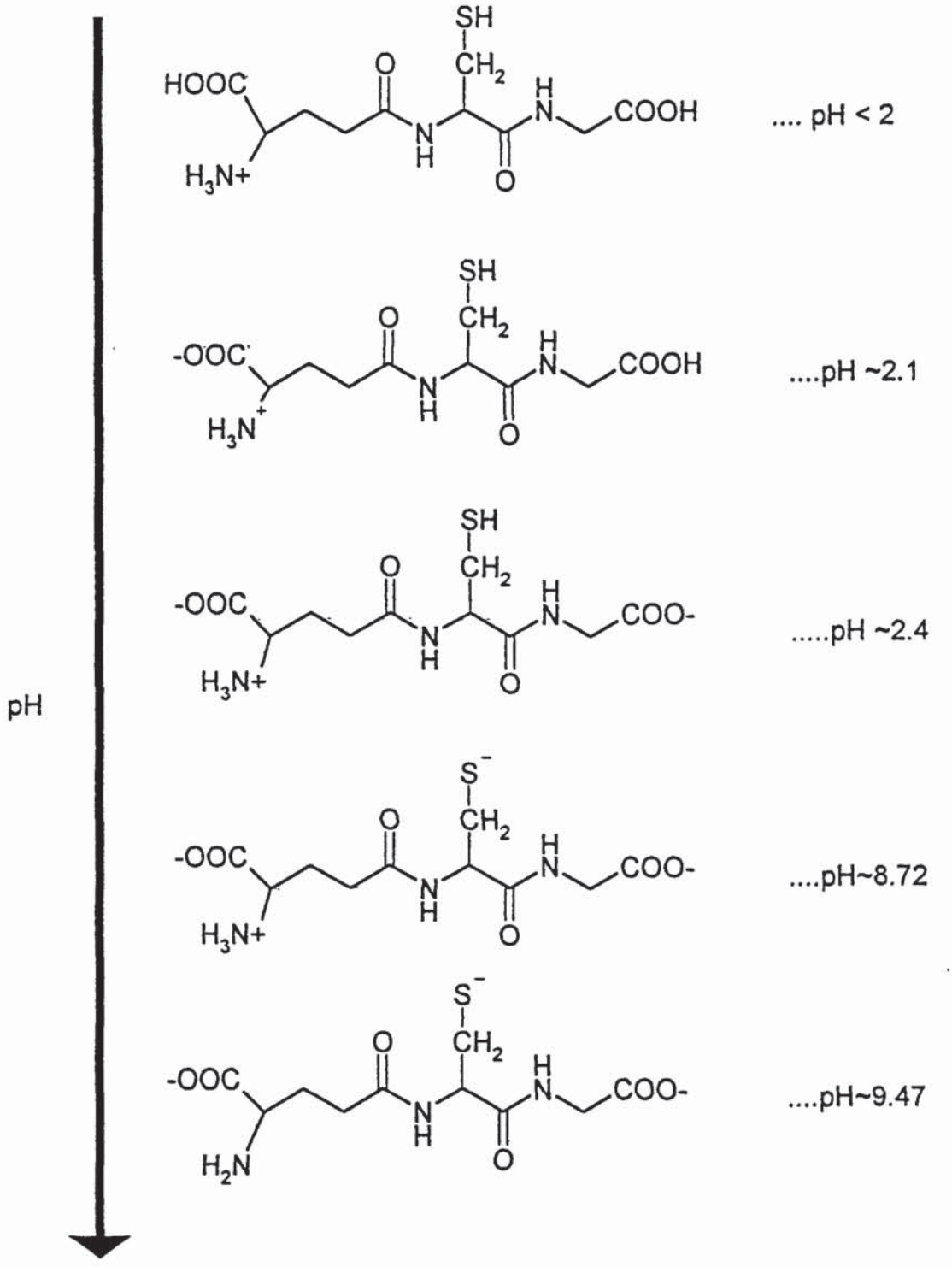


Figure 4.4: Deprotonation of GSH as pH is increased. As the pH of the medium is increased, the most acidic functional group of glutathione deprotonates. The most basic functional group (the NH_3^+) deprotonates last.

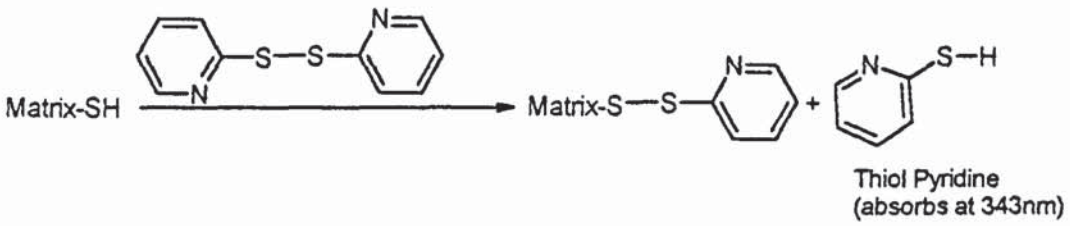


Figure 4.5 Reaction of dithiol dipyridine with a thiol containing moiety.

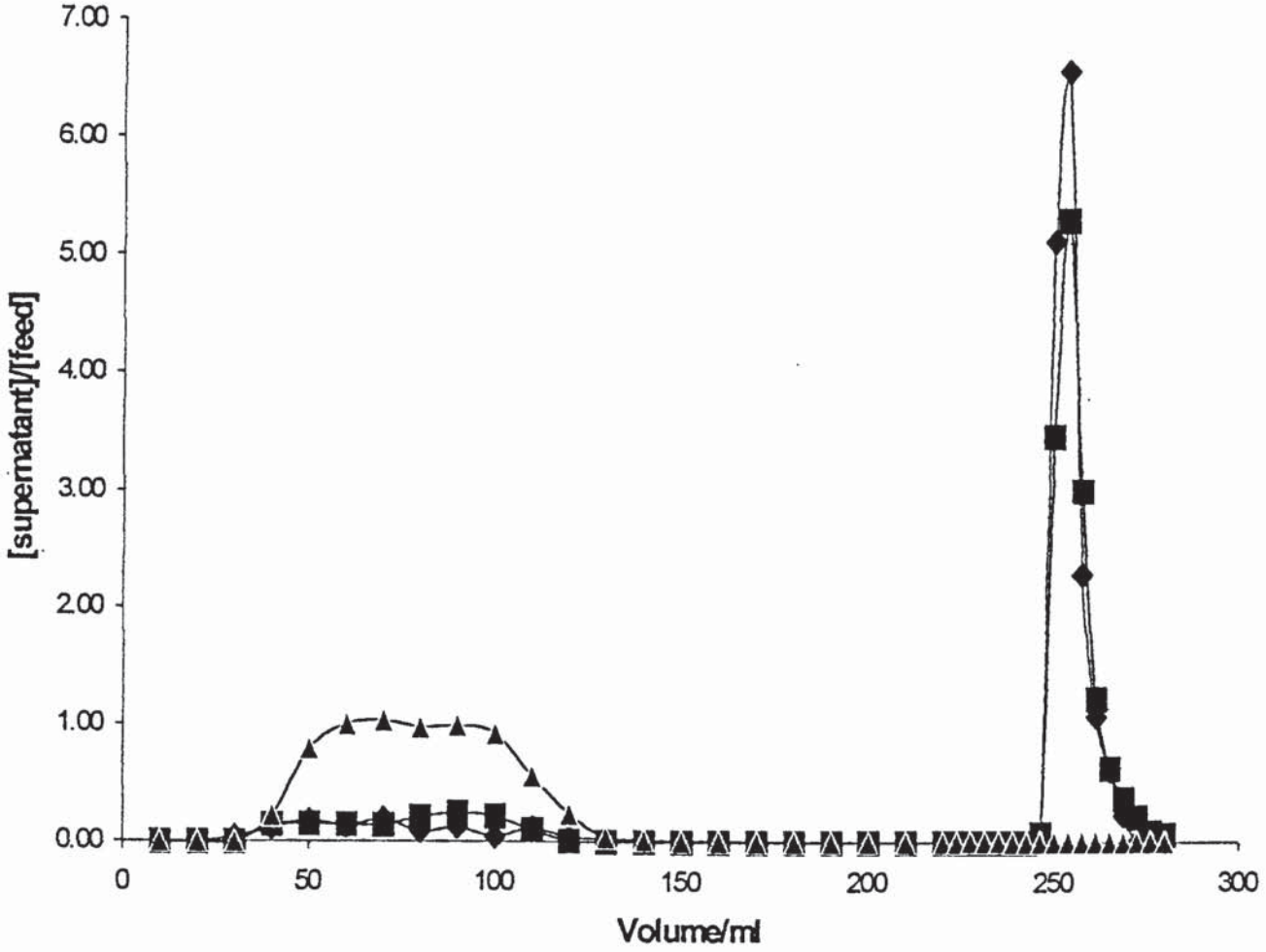


Figure 4.6: The purification of GST from a Streamline™ DEAE column. 70 ml of an E.coli suspension containing GST were loaded onto a two times expanded bed column (10 mm i.d.) of Streamline DEAE, packed height 20 cm. The effluent was measured for its solid content Cs/Cso, (▲), protein content C/Co, (■) and GST activity U/Uo, (◆). The column was equilibrated with 20 mM of sodium carbonate buffer, pH 10. Elution was effected by 1 M NaCl in the carbonate buffer.

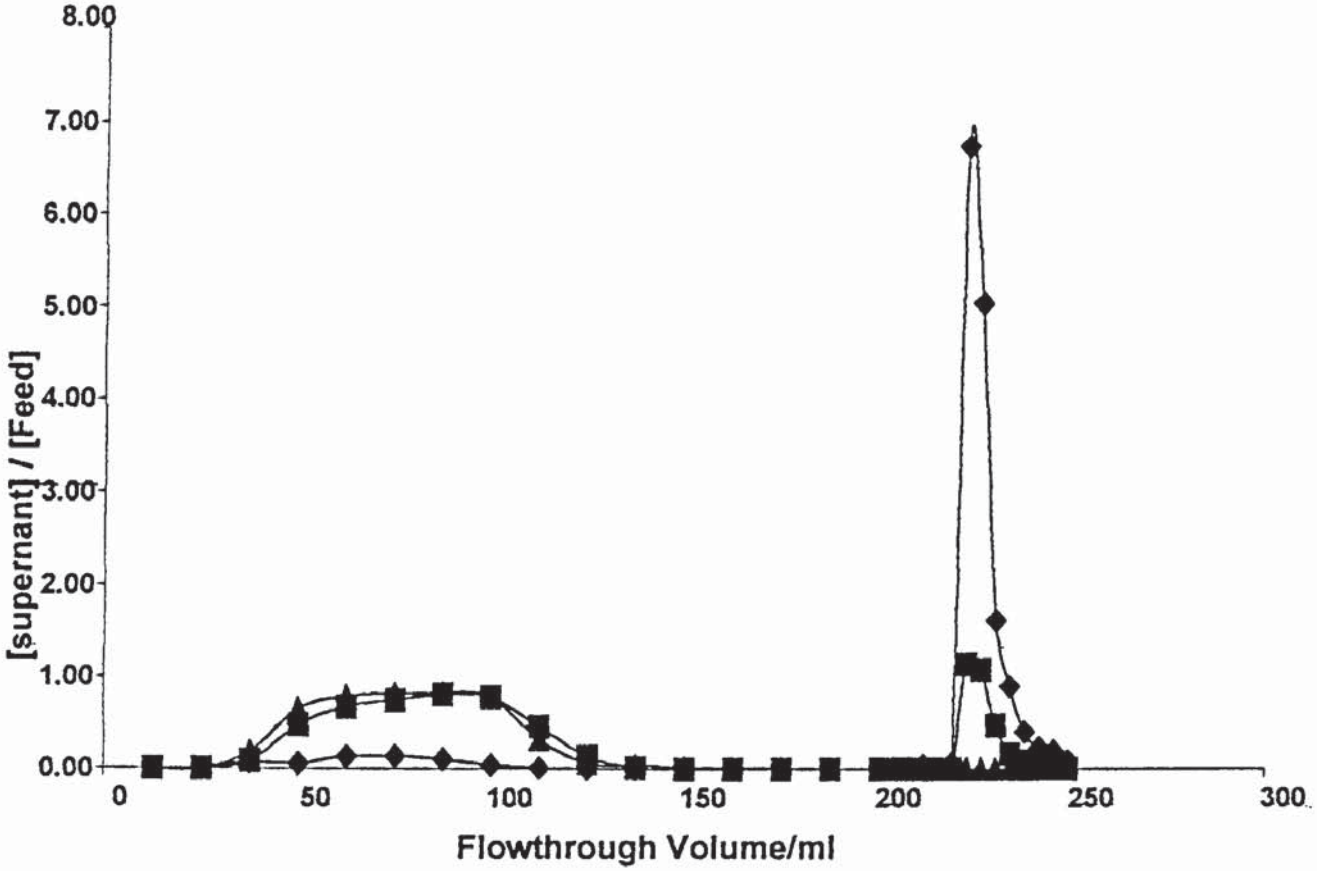


Figure 4.7: The purification of GST from Matrix B. 70 ml of an E.coli suspension containing GST were loaded onto a two times expanded bed column (10 mm i.d.) of Matrix B (prepared as described in the methodology section) packed height 20 cm. The effluent was measured for its solid content Cs/Cso, (▲), protein content C/Co, (■) and GST activity U/Uo, (◆). The column was equilibrated with 20 mM of Phosphate Buffer Saline (PBS), pH 7.4. Elution was effected by 20 mM GSH in 0.1 M Tris-HCl, pH 9.6.

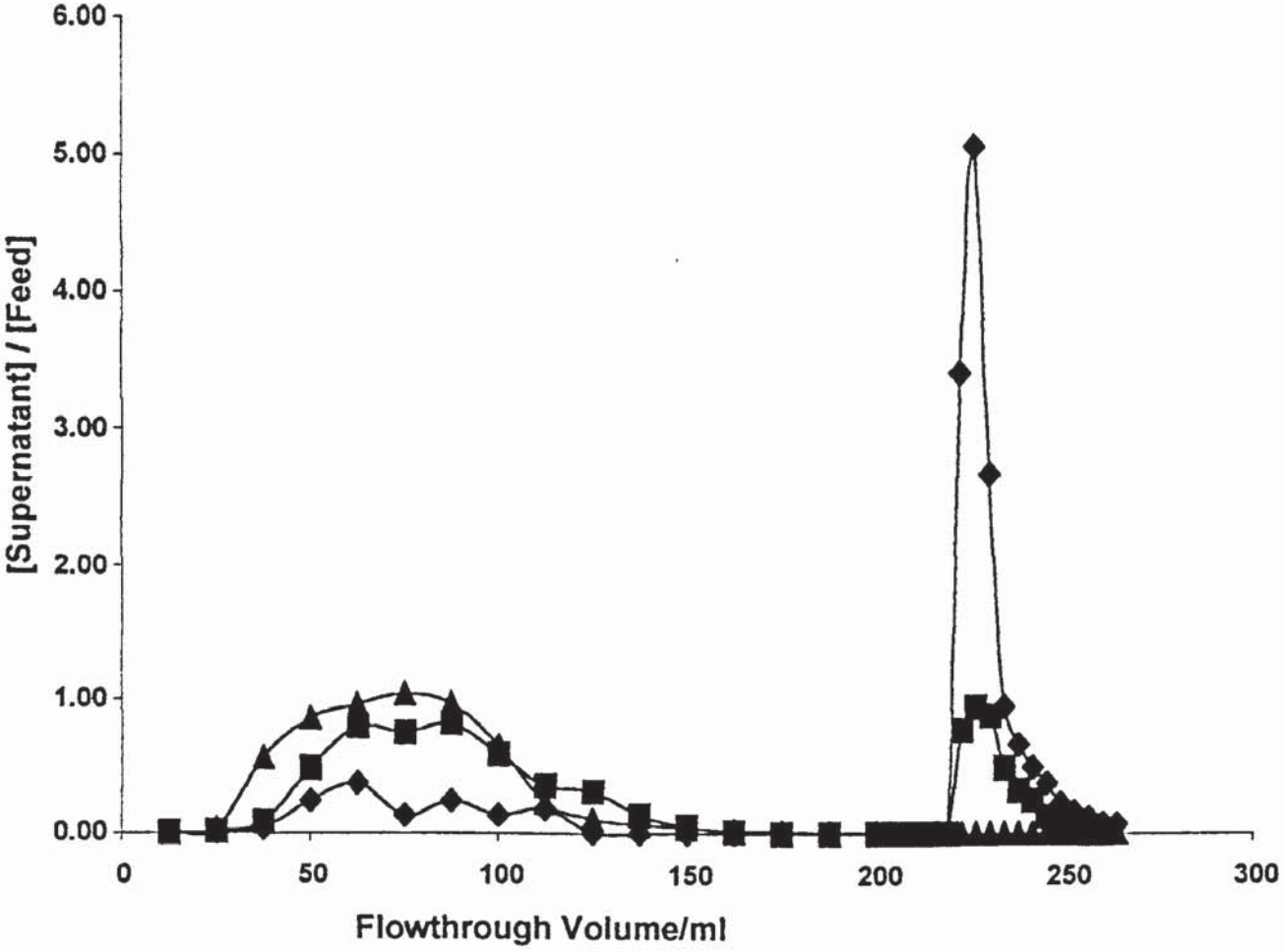


Figure 4.8: GST purification from the Tween coated column. Matrix B coated with Tween 20 waspacked into a 10mm i.d. column to a settled bed height of 20 cm. This was expanded twice with 20 mM PBS, pH 7.4. 70 ml of an E.coli suspension containing GST were then loaded onto the column at constant flow. The effluent was measured for its solid content Cs/Cso, (▲), protein content C/Co, (■) and GST activity U/Uo, (◆). The column was equilibrated with 20 mM of Phosphate Buffer Saline (PBS), pH 7.4. Elution was effected by 20mM GSH in 0.1M Tris-HCl, pH 9.6.

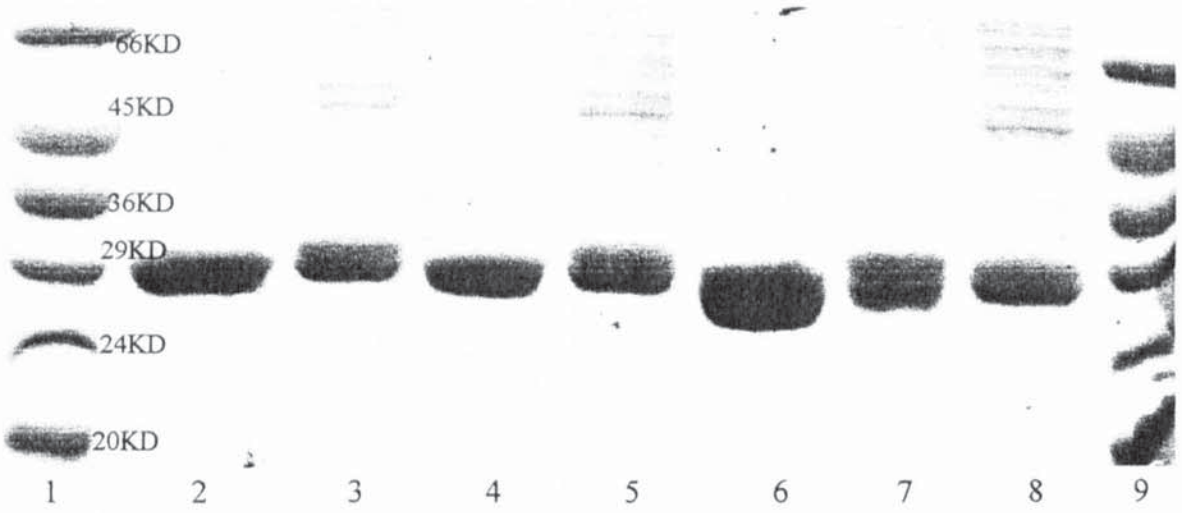


Fig 4.9: SDS-PAGE results of the feed and eluates from the expanded bed experiments . Feed and eluates from the chromatographic runs were run on an SDS-PAGE (4% stacking gel, 12% resolving gel). Lane 1: Marker, lane 2: Eluate of the Tween coated matrix, Lane 3: Feed for the Tween coated matrix, Lane 4: Eluate of Matrix B, Lane 5: Feed for Matrix B, Lane 6: GSH-Sepharose eluate, Lane 7: DEAE Eluate, Lane 8: DEAE Feed, Lane 9: Marker.

	Loaded		Specific activity U/mg	Supernatant		Elution		Specific activity U/mg	Purification factor	Recovery/%	
	Protein /mg	GST/U		Protein /mg	GST/U	Protein /mg	GST/U			Protein	GST
Matrix B	3.3	25651	7890	2.5	3759	0.52	10462	20108	2.5	67.9	47.8
Matrix A	3.3	25651	7890	2.6	11012	0.04	467	10734	1.4	7.0	3.2
Base	3.3	25651	7890	2.6	25166	0.01	0	23664	0.0	1.0	0.0

Table 4.1: Batch adsorption data for Matrix A, Matrix B and the Base matrix. 4ml of GST cell free suspension were added to 0.5ml of adsorbent in a tube. The tube was then put on a spiral mix for one hour at room temperature. The supernatant was collected. The beads were washed with phosphate buffer to remove unbound proteins. The adsorbed proteins were eluted with 20mM GSH in 0.1M Tris-HCl pH 9.6. The supernatants and the eluates were assayed for GST activity and total protein.

	Loaded		Specific activity U/mg	Supernatant		*Elution*		Specific activity U/mg	Purification factor	Recovery/%	
	Protein/ mg	GST/U		Protein/ mg	GST/U	Protein/ mg	Gst/U			Protein	GST
Tween Coated	1.8	2800	1564	1.62	627	0.24	1233	5086	3.2	15	57
Matrix B	1.8	2800	1564	1.67	1119	0.20	1111	5552	3.5	11	66
Sepharose 4B	1.8	2800	1564	1.52	21	0.17	745	4501	2.9	9	27

Table 4.2: Batch adsorption data for the Tween coated matrix, Matrix B and the GSH- Sepharose 4B. To 0.5ml of adsorbent in a test tube were added 4ml of clarified GST suspension. The tube was then put on a spiral mixer for one hour at room temperature. The supernatant was collected. Unbound proteins were washed with phosphate buffer and bound proteins were eluted with 20mM GSH in 0.1M Tris-HCl pH 9.6. The supernatants and the eluates were assayed for GST activity and total protein.

	Loaded		Specific activity U/mg	Supernatant		Elution		Specific activity	Purification factor	Recovery/%	
	Protein /mg	GST/U		Protein/ mg	GST/U	Protein /mg	GST/U			Protein	GST
Matrix B	2.3	5482	2425	1.4	736	0.53	3427	6437	2.7	65	72
Used Matrix B	2.3	5482	2425	1.4	438	0.54	3200	5967	2.5	63	63
Sepharose 4B	2.3	5482	2425	1.5	0	0.37	2268	6204	2.6	46	41

Table 4.3: Batch adsorption data for Matrix B, Used Matrix B and Sepharose 4B. 4ml of GST cell free suspension were added to 0.5ml of adsorbent in a tube. The tube was then put on a spiral mix for one hour at room temperature. The supernatant was collected. The beads were washed with phosphate buffer to remove unbound proteins. The adsorbed proteins were eluted with 20mM GSH in 0.1M Tris-HCl pH 9.6. The supernatants and the eluates were assayed for GST activity and total protein.

	GSH Concentration (mg/ml)			GSH Concentration (mg/ml)	
pH	Matrix B	Base	[NaCl]/M	Matrix B	Base
3	0.06	0	0.25	0.09	0
5	0.04	-	0.5	0.11	-
7	0.09	0	0.75	0.09	-
9	0.15	-	1	0.11	0
11	0.42	0			

Table 4.4: Ligand leakage from Matrix B. 1ml of a 1:1 slurry of Matrix B was put in test tubes. The pH of the slurry was adjusted to 3, 5, 7, 9 and 11. In parallel to this experiment, to 2ml of NaCl solutions at various concentrations were added 1ml of the 1:1slurry of the beads. The test tubes were put on a spiral mix for 1h after which the supernatant was assayed for GSH by measuring absorbance at 235nm.

THIOL CONTENT DETERMINATION		
	GSH/g of matrix	STD Deviation
Matrix A	0.23	0.02
Matrix B	0.12	0.02
NINHYDRIN TEST		
	GSH/g of matrix	STD Deviation
Matrix A	6.7	0.44
Matrix B	97.5	9.3
Sepharose 4B	60.1	3.6

Table 4.5: Ligand density quantification using the Thiol and Ninhydrin tests. 2ml of dithiol dipyridine or ninhydrin were then added to 1ml of a 1:1 slurry of adsorbent. After thorough mixing, the absorbance of the supernatant was read at either 343nm for the thiol content determination or at 564nm for the ninhydrin test.

	LOAD			FLOW THROUGH		*ELUTION*				
Matrix	Protein (mg)	GST U	spec. act U/mg	Protein mg	GST U	Protein mg	GST U	Spec. act U/mg	P.F.	GST Recovery %
DEAE	29.6	54935	1856	5.7	8668	22.2	46811	2109	1.1	101
Sepharose	19.8	38661	1953	10.3	2540	4.1	28021	6834	3.5	78
Matrix B	31.6	63699	2016	23.7	6496	5.1	52354	10265	5.1	92
Tween-Coated	30.6	63330	2070	24.1	15004	6.3	48184	7648	3.7	100

Table 4.6 GST purification data from the streamline DEAE, GSH-Sepharose 4B, Matrix B and the Tween coated matrices. The adsorbents were packed into a 10mm i.d. column. For the streamline adsorbents the bed was expanded to twice the settled bed height with appropriate buffer. 100ml of sample were loaded followed by washing to remove unbound substances. Elution was done with 20mM GSH in 100mM Tris-HCl pH 9.6 for the affinity adsorbents or 1M KCl in Carbonate buffer pH 10 for the ion exchange adsorbents. For the GSH-Sepharose 4B, the experiment was conducted in a packed mode.

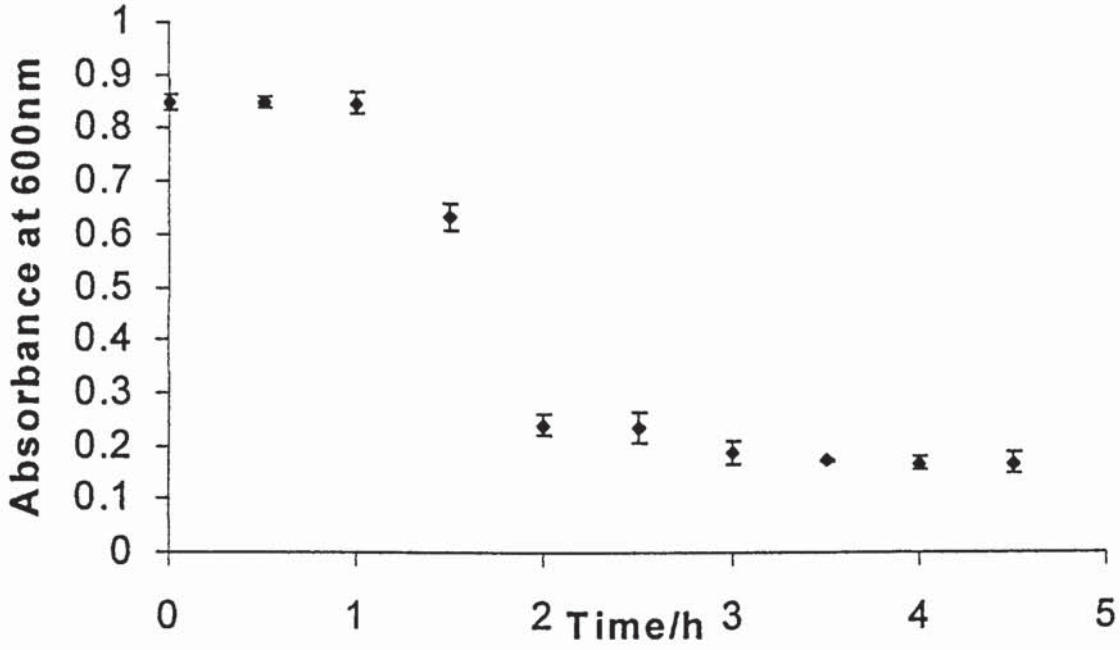


Figure 5.1: Lysis of E.coli by Phage T7 as a function of time. 5 ml of an overnight culture of E.coli (C_{600})was infected with phage T7 and incubated at 37^0C with shaking. After 3 hrs, this was transferred to a flask containing 100ml of E.coli. The absorbance at 600 nm was measured and the flask was then incubated at 37^0C. Absorbance was measured every 30 minutes.

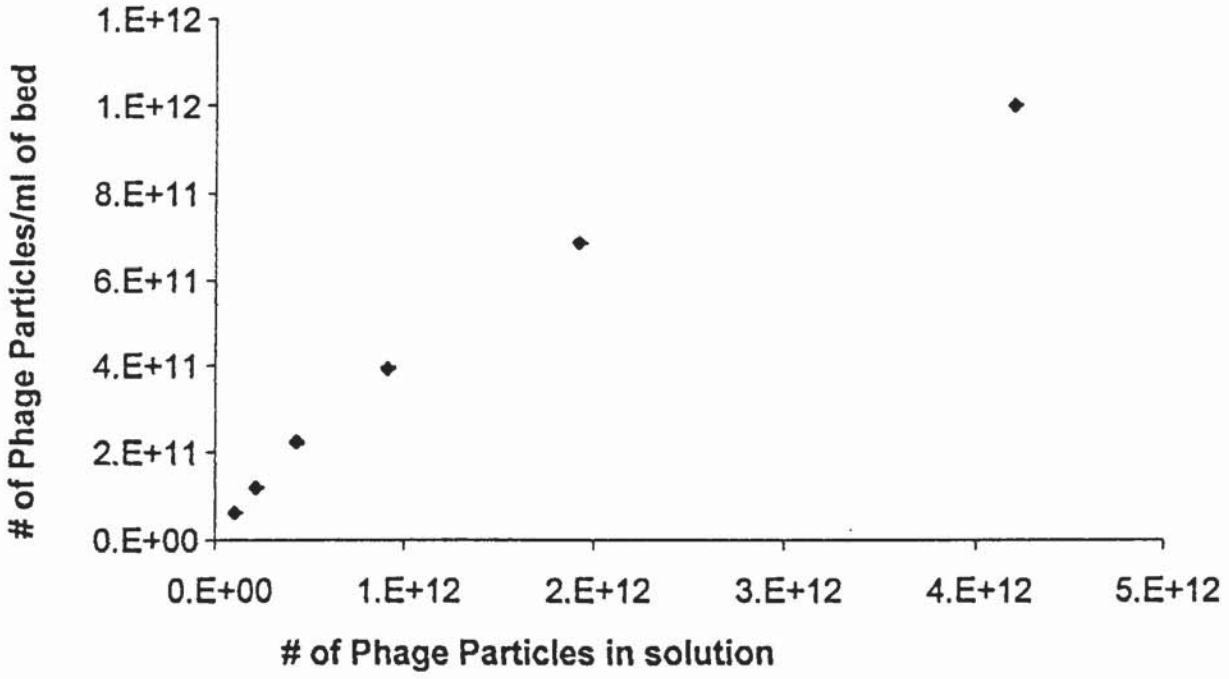


Figure 5.2: Adsorption Isotherm of Phage T7 on Streamline™ DEAE. Serial dilutions of 10 ml of phage T7 suspension were added into tubes containing 1 Ml of 1:1 slurry of Streamline DEAE. The tubes were put on a roller mix for an hour at room temperature after which the supernatant was assayed for phage particles.

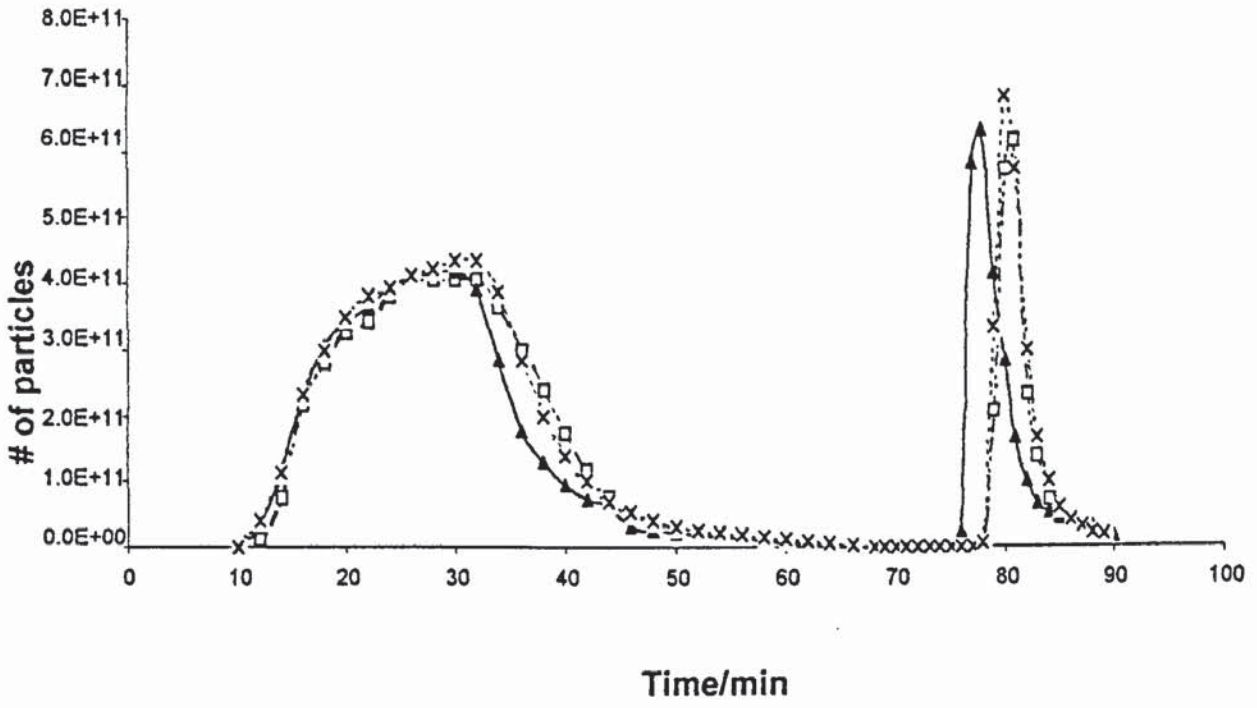


Figure 5.3: Phage elution at different pH. A 12.5 mm column was packed with Streamline™ DEAE to a height of 20 cm. The column was then expanded to 40 cm with 20 mM Tris-HCl, pH 7.5. When the column was stable, 100 ml of phage T7 suspension was loaded at 4.8 ml/min. This was followed by washing with the same buffer after which the bound particles were eluted with 1 M KCl in 20 mM Tris-HCl at pH 7.5 (▫), 6.4 (▲), and 9.1(x).

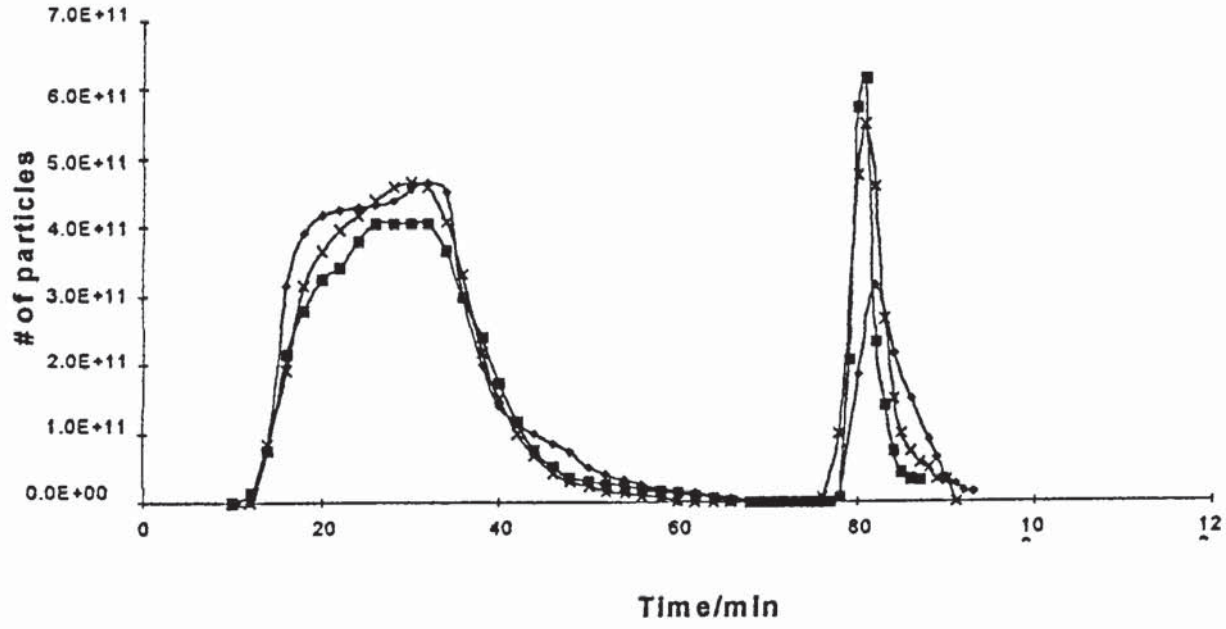


Figure 5.4: Effect of loading pH on Phage T7 purification. A 12.5 mm column was packed with Streamline™ DEAE to a height of 20 cm. The column was then expanded to 40 cm with 20 mM Tris-HCl. When the column was stable, 100 ml of phage T7 suspension were loaded at 4.8 ml/min. This was followed by washing with the same buffer after which the bound particles were eluted with 1 M KCl in 20 mM Tris-HCl. The loading and elutionwere done at pH 7.5 (▪), 6.2 (x), and 9.2 (♦). Elution was done at a flow rate of 2 ml/min.

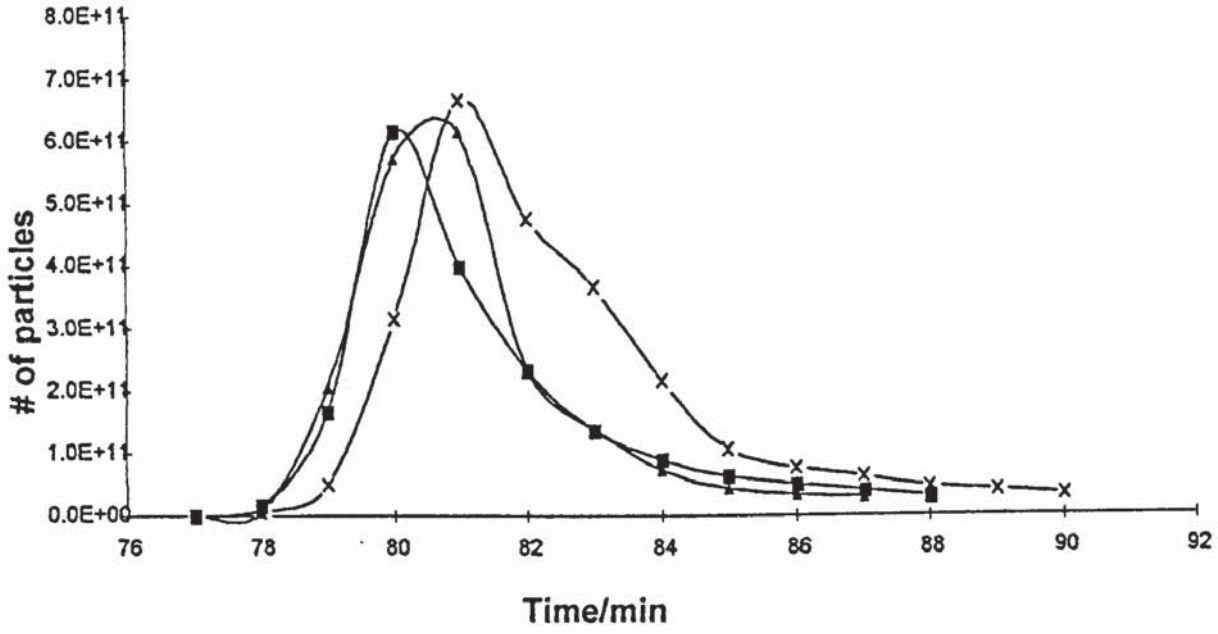


Figure 5.5: Effect of temperature on phage T7 elution. A 12.5 mm column was packed with Streamline™ DEAE to a height of 20 cm. The column was then expanded to 40 cm with 20 mM Tris-HCl, pH 7.5. When the column was stable, 100 ml of phage T7 suspension were loaded at 4.8 ml/min. This was followed by washing with the same buffer after which the bound particles were eluted with 1 M KCl in 20 mM Tris-HCl. The elution was done at 1^0C (x), 25^0C (■) and 30^0C (▲). Elution flow rate was 2 ml/min.

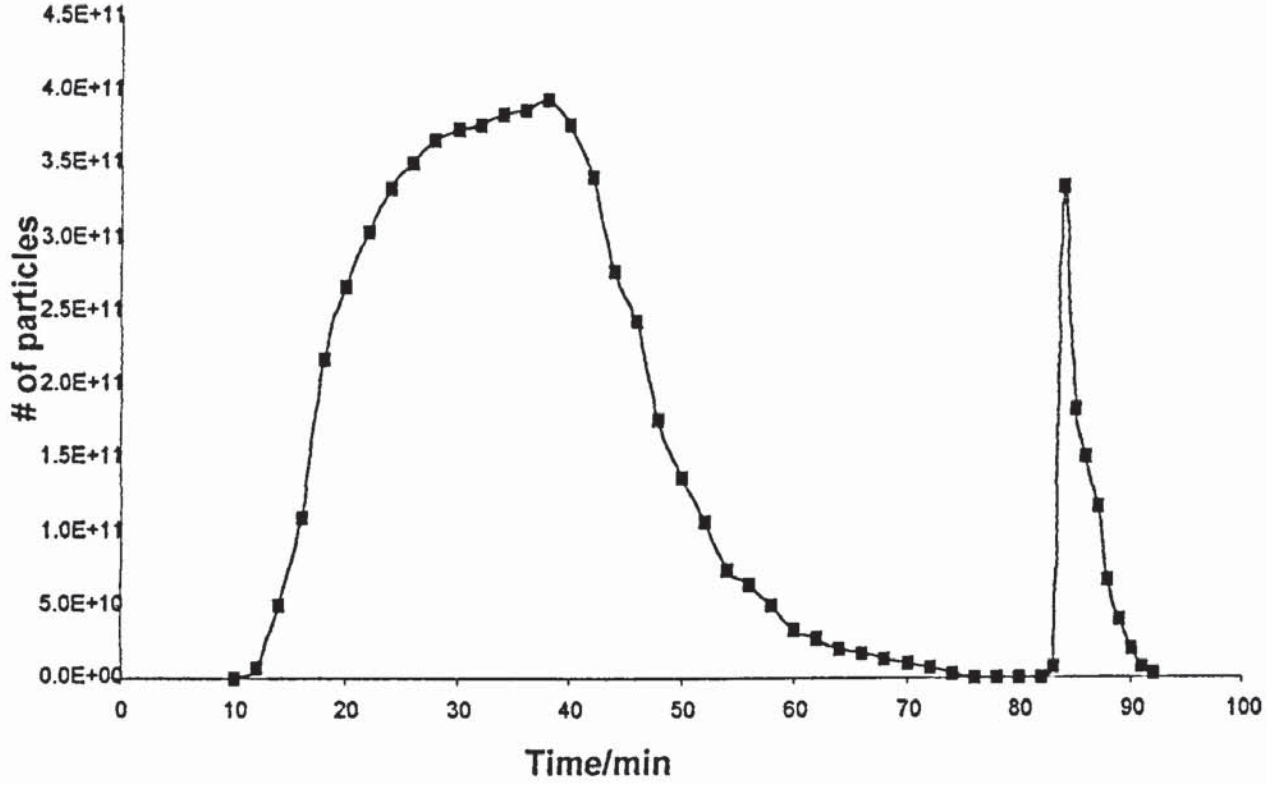


Figure 5.6: Phage purification on Polylysine agarose. A 12.5 mm column was packed with polylysine agarose (Streamline™ quartz based agarose activated by CNBr to whicg-h polylysine was coupled) to a height of 20 cm. The bed was then expanded to 40 cm with 20 mM Tris-HCl pH 7.5. When the column was stable 100 ml of phage T7 suspension were loaded at a flow rate of 4.8 ml/min. This was followed by washing. Elution was done with 1 M KCl in 20 mM Tris-HCl pH 7.5 at a flow rate of 2 ml/min.

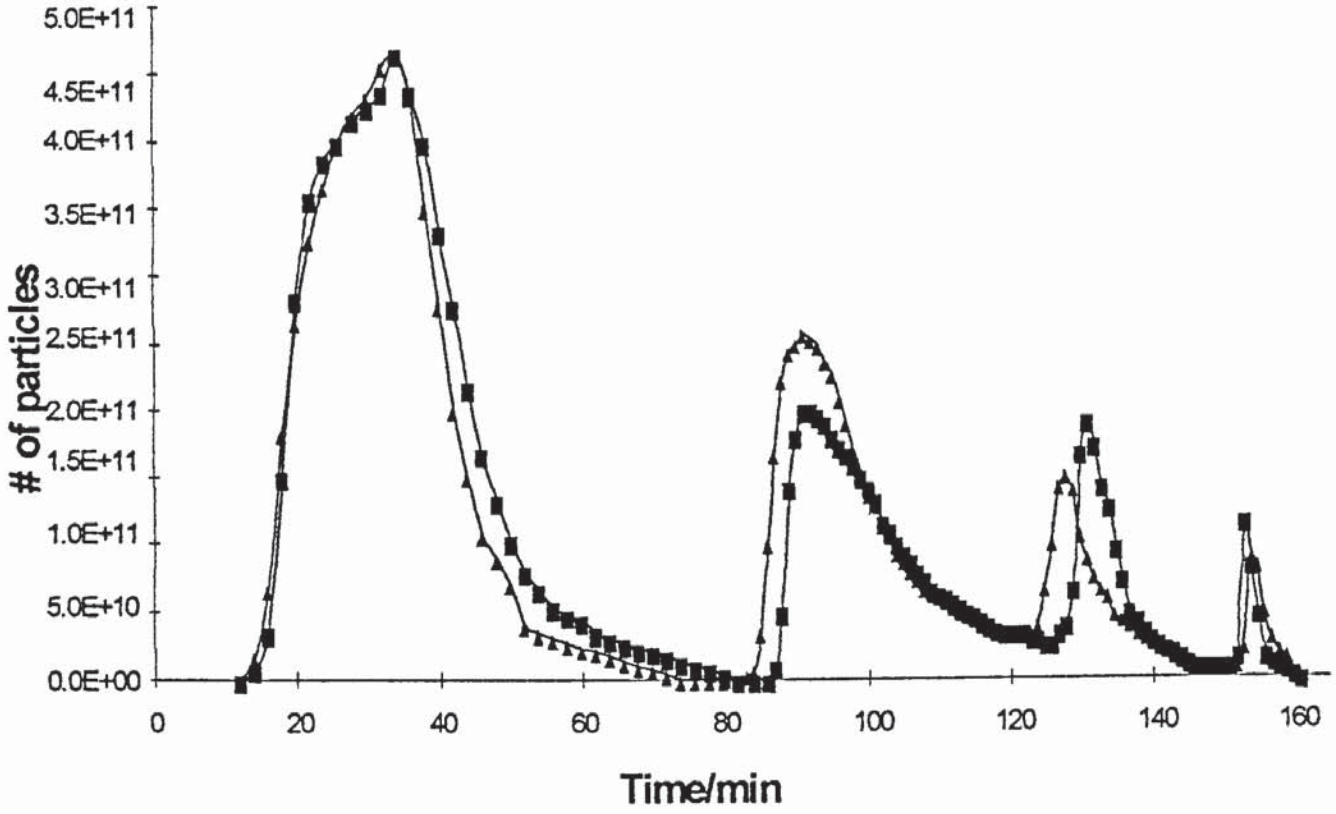


Figure 5.7: Phage purification from unclarified feedstock. A 12.5 mm column was packed with Streamline™ DEAE to a height of 20 cm. The column was then expanded to 40 cm with 20 mM Tris-HCl, pH 7.5. When the column was stable, 100 ml of unclarified phage T7 suspension were loaded at 4.0 ml/min. This was followed by washing with the same buffer after which the bound particles were eluted stepwise with 0.2 M, 0.5 M and 1 M KCl in 20 mM Tris-HCl pH 7.5. Elution flow rate was 2 ml/min. The experiment was done twice. First run (▲), second run (■).

		Loaded (PFU)	Flow through (PFU)	Eluted (PFU)	Recovery(%)
Influence of eluting pH	pH 7.5	2.5E+07	2.2E+05	1.5E+07	61.3
	pH 9.06	2.5E+07	2.0E+06	1.3E+07	54.6
	pH 6.2	2.5E+07	1.7E+05	1.6E+07	66.3
Influence of loading pH	pH 7.5	2.5E+07	2.2E+05	1.5E+07	61.3
	pH 6.4	2.5E+07	1.7E+06	2.0E+07	85.7
	pH 9.2	2.5E+07	1.6E+06	9.8E+05	4.2
Divalent cations	MgSO4	1.0E+6	1.5E+5	5.0E+4	17.4
Influence of Temperature	1°C	1.0E+06	1.5E+05	8.5E+05	99.6
	30°C	1.E+06	7.E+03	7.E+04	6.7
	RT	2.5E+07	2.2E+05	1.5E+07	61.3
"Affinity"	Polylysine	1.30E+08	3.86E+07	3.96E+07	43.3

Table 5.1 : PFU Assay results for Phage T7. 100 µl of serialy diluted phage suspensions were with 200 µl of E.Coli. This mixture was incubated for about 20 minutes after which 3 ml of the top agar maintained at 47°C were added briefly vortexed and quickly added to a petri dish containing the bottom agar. The plates were allowed to cool for 5 minutes before being placed in an incubator at 37° C equipped with CO_2 vent. Plaques were counted after 5-6 hours.

	0.2M	0.5M	1 M
Total PFU	**4E+09**	**1E+10**	**3.2E+08**
Total particles/ml	**4E+12**	**1.5E+12**	**3.4E+11**
P/I	**1000**	**140**	**1000**
% of active phage	**16.3** ± 4.7	**62.3** ± 18.1	**1.8** ± 0.28
% of Non active phage	**67.4** ± 6.6	**26.8** ± 0.33	**5.8** ± 0.01
% of Solids	**75** ± 3	**25** ± 5	0

Table 5.2: Purification of Phage T7 from unclarified feedstock. A 12.5 mm column was packed with Streamline™ DEAE to a height of 20 cm. The column was then expanded to 40 cm with 20 mM Tris-HCl, pH 7.5. When the column was stable, 100 ml of unclarified phage T7 suspension were loaded at 4.8 ml/min. This was followed by washing with the same buffer after which the bound particles were eluted stepwise with 0.2 M, 0.5 M and 1 M KCl in 20 mM Tris-HCl pH 7.5. Elution flow rate was 2 ml/min. The experiment was done twice. Both active and total particles were assayed.

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APPENDIX 1

CALCULATION OF NUMBER OF PHAGE PARTICLES PER MILLILITRE OF PACKED BED

The mean diameter of the streamline beads is 200 μm
The radius (R) = 10^{-4} m

Therefore the area of each particle $= 4\pi R^2$
$= 1.26\times10^{-7}$ m^2

Volume of each particle $= \frac{4}{3}\pi R^3$
$= 4.19\times10^{-12}$ m^3

Hence area / unit volume of beads $= 3/R$
$= 3\times10^{4}$ m^2/m^3 particles

For 1m^3 of packed bed, only 74% of this volume will be occupied by beads (assuming an hexagonal close packed structure).

Hence surface area/ unit volume of packed bed $= 0.74\times(3\times10^{4})$
$=2.22\times10^{4}$ m^2/m^3 bed

For close packing of phage particles, 91% of surface area will be covered.

Since the diameter,D of phage is 100 nm $= 10^{-7}$ m

Projected surface area of the phage $= \frac{\pi}{4}D^2$
$=7.85\times10^{-15}$ m^2

Hence number of phage particles/ m^2 $=0.91 \,/\, 7.85\times10^{-12}$

$=1.56\times10^{14}$

Hence number of phage/m^3 $=1.56\times10^{14}\times2.22\times10^{4}$
$=2.57\times10^{18}$ phage/m^3 of bed
$=2.57\times10^{12}$ phage/ml of bed

APPENDIX 2

NOMENCLATURE

Symbol	Description	SI units
CF	constant flow	m/s
CH	constant height	m/s
D	diffusion coefficient	m^2/s
D_a	axial dispersion coefficient	m^2/s
D_c	column diameter	m
D_p	particle diameter	m
D_v	dispersion number	
H	expanded bed height	m
H_o	settled bed height	m
H^*, $HETP$	Height equivalent to a theoretical plate	m
n	the Richardson-Zaki index	
N	number of plates	
$p.f.$	purification fold	
Q_d	dynamic binding capacity	Units/ml, mg/ml
t	time	s
$\bar{t}$	mean time	s
t_R	retention time	s
U	superficial flow velocity	m/s
U_i	interstitial velocity	m/s
U_t	terminal velocity	m/s

Greek Symbols

ε_o	settled bed void fraction	
ε	expanded bed void fraction	
ρ	liquid density	kg/m^3
σ^2	variance	s^2
μ	viscosity	kg/m.s

Dimensionless Groups

Pe	Peclet number
Re	Reynolds number