CONSTRUCTION, EXPRESSION, AND PURIFICATION OF A HISTIDINE-TAILED BACTERIOPHAGE T4 LYSOZYME

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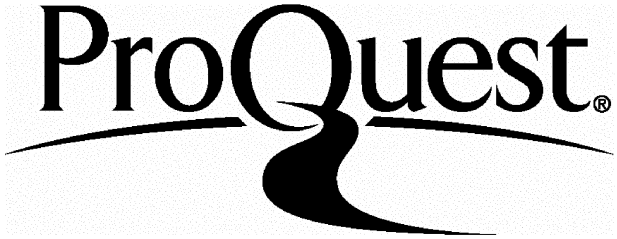

ABSTRACT

Construction, expression and purification of a histidine-tailed bacteriophage T4 lysozyme.

Many recombinant proteins are expressed in the periplasm of Gram negative bacteria. Release of these proteins can be achieved by the use of muramidases but these are expensive and can act as potential process contaminants further downstream.

A version of bacteriophage T4 lysozyme has been constructed by the addition of a His-Gln-$(His)_3$ peptide to the C-terminus of a cysteine-free protein. The expressed fusion protein can be purified using immobilized metal affinity chromatography and reused.

The protein was initially recovered (to a high level) on iminodiacetic acid (IDA) Sepharose columns charged with Zn^{2+}, Ni^{2+}, and Cu^{2+} from crude cell lysates. Since no binding to the columns was observed with the wild type protein, the interaction can be attributed to the fusion tail. The retention strength on the columns was Cu>Ni>Zn, although few differences in the levels of purity and recovery of the enzyme were observed.

In addition, the purification of the same recombinant protein from crude and clarified cell extracts using novel non-porous ferromagnetic supports (coated with IDA and M^{2+}) is described. Various parameters were investigated in order to achieve maximal recovery of purified product while maintaining enzyme activity. The capacity of Cu-charged supports was determined for both extracts (0.35 mg/ml clarified, 0.45 mg/ml crude), the buffer which provided maximal binding was investigated (20 mM sodium phosphate, 0.2 M NaCl, pH 7.2) as was that which effected maximal elution (0.1 M sodium acetate, 0.5 M NaCl, pH 3.5). It is recommended that two wash steps would eliminate unbound proteins prior to elution of the target protein where greater than 90% was recovered in two steps. The minimum time required for maximal binding under the conditions used here was 5 minutes. Other divalent metal ions were tested for their ability to bind the target protein and only Cu, Ni and Zn exhibited any significant success. The order of retention was as found in the column chromatography - Cu>Ni>Zn. Recovery and purity levels were similar to those obtained on the columns. Consecutive recycling without cleansing treatment is not recommended above two cycles.

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ABBREVIATIONS

A	adenine
AAS	atomic adsorption spectroscopy
Ala	alanine
Amp^r	amplicillin resistance
Arg	arginine
Asp	aspartic acid
bp	base pair
bT4l	bacteriophage T4 lysozyme
C	cytosine
Cm	chloramphenicol
Co^{2+}	divalent cobalt/Co(II)
C-terminus	carboxy/3' terminus
Cu^{2+}	divalent copper/Cu(II)
Cys	cysteine
ΔA	change in absorbance
DNA	deoxyribonucleic acid
e	lysozyme gene
FDA	Food and Drug Administration
$Fe^{2+/3+}$	divalent/ trivalent iron
G	guanine
Galdh	glutaraldehyde dehydrogenase
Gln	glutamine
Glu	glutamic acid
Gly	glycine
GST	glutathione *S*-transferase
HGMS	high gradient magnetic separation
His	histidine
His_6	six consecutive histidine residues
IDA	iminodiacetic acid
IgG	immunoglobulin G
Ile	isoleucine
IMAC	immobilized metal affinity chromatography
lac I^q	lactose repressor
Leu	leucine
Lys	lysine
M(II)	divalent metal ion

MCS	multiple cloning site
Met	methionine
mRNA	messenger ribonucleic acid
Ni^{2+}	divalent nickel
NTA	nitrilotriacetic acid
N-terminus	amino/5' terminus
OD	optical density
oligo	oligonucleotide
ori	origin of replication
PCR	polymerase chain reaction
Phe	phenylalanine
Pro	proline
SDS-PAGE	SDS-polyacrylamide gel electrophoresis
-SH	sulphydryl groups
T	thymine
TED	tris(carboxymethyl)ethylenediamine
Tet	tetracycline
Thr	threonine
Trp	tryptophan
Tyr	tyrosine
UV	ultraviolet
Val	valine
WT	wild type
WT*	pseudo wild type
Zn^{2+}	divalent zinc/Zn(II)

UNITS

Å	angstroms (10^{-10} m)
°C	degrees Centigrade
g	gram
h	hours
kDa	kiloDalton
l	litre
m	metre
mA	milliampere
mg	milligram
min	minutes
ml	millilitre
µg	microgram
µm	micrometer
µl	microlitre
M	molar
mM	millimolar
N	normal
ng	nanogram
nm	nanometre
psi	per square inch
rpm	revolutions per minute
v/v	volume/volume
w/v	weight/volume

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The microbe is so very small
You cannot make him out at all,
But many sanguine people hope
To see him through a microscope.
His jointed tongue that lies beneath
A hundred curious rows of teeth,
His seven tufted tails with lots
Of lovely pink and purple spots
On each of which a pattern stands
Composed of forty separate bands;
His eyebrows of a tender green
All these have never yet been seen
But scientists who ought to know
Assure us that they must be so.
Oh, let us never, never doubt
What nobody is sure about.

Hilaire Belloc.

To Darren (MLSHP)

CHAPTER ONE

INTRODUCTION

1.1 Introduction

The final goal in protein purification is to maximise the yield without incurring a cost increase or reducing the biological activity or the level of purity of the final product. Since there are, in general, many steps involved in the downstream purification process, it is necessary to try to achieve these aims at every step of the purification procedure as the loss factor is cumulative. In one large scale process for asparaginase production (Wang *et al.*, 1979) involving thirteen main recovery and purification steps, the average step yield was 91% giving an overall yield of approximately 30%. A similar situation was also found in the process isolation of penicillin acylase (Lagerlöf *et al.*, 1976) from *Escherichia coli* (*E. coli*) involving twelve steps where an average step yield of 94% was obtained which is equivalent to an overall yield of 52%. Gupta and Mattiason (1994) estimated that downstream processing costs can account for 50-80% of the total production cost. It is therefore possible to increase protein yield by decreasing the number of steps involved in the purification as a whole and there is often a compromise between high step yields and good purification.

The isolation in pure form of a given protein from a given cell or tissue can be complex especially if the protein exists in very low concentrations. However, many different proteins have been isolated in pure form. Various characteristic properties of proteins are exploited to enable separation of mixtures of proteins such as molecular size (dialysis, ultrafiltration, centrifugation, gel filtration chromatography), solubility (isoelectric precipitation, solvent fractionation), ionic charge (ion exchange chromatography, electrophoresis), or biological affinity for other molecules (affinity chromatography).

Over the past decade, new protein purification techniques have emerged as a result of recombinant deoxyribonucleic acid (DNA) technology. DNA encoding additional polypeptide or protein tags can be fused to the gene of interest and expression of these gene fusions results in protein fusions which may be purified by techniques using the properties of the additional polypeptide fusion/affinity tag (affinity, hydrophobic, covalent and ion-exchange chromatography). These techniques have eliminated the need for extensive screening and optimization procedures which were previously required for purification. An enzymatic or chemical cleavage site may be included to permit removal of the purification tag which may be necessary especially if the purification fusion is antigenic. Affinity ligand techniques have grown rapidly and represent one of the most powerful tools available for downstream purification both in terms of recovery and selectivity.

1.2 Protein expression and purification systems

Several affinity purification tags have been used in purifying proteins as diverse as interleukin 2 from yeast (Hopp *et al.*, 1988), to alkaline phosphatase from *E. coli* (Ong *et al.*, 1989). The tags can vary in size from a few amino acid residues to whole genes such as β-galactosidase and are usually positioned on either the amino- or the carboxy-terminus (N- or C-terminus respectively) of the protein of interest. Tails with a variety of characteristics have been used such as entire enzymes (Germino and Bastia, 1984; Ullmann, 1984; Smith and Johnson, 1988; Dykes *et al.*, 1988), peptide-binding proteins (Moks *et al.*, 1987; Nilsson and Abrahmsén, 1990), biotin-binding domains (Cronan, 1990), and antigenic epitopes (Hopp *et al.*, 1988). Many fusion tail systems have been constructed in specific expression vectors that contain convenient cloning sites for the insertion of the target protein gene in one or more reading frames such that the protein of interest is fused to the fusion tag. Some of the commonly available systems are outlined below.

A specific interaction exists between staphylococcal protein A and the constant regions of immunoglobulin G (IgG) (Nilsson *et al.*, 1985). When the gene of interest is fused to either the intact protein A or the region coding for the Ig binding domain and expressed in *E. coli* or *Staphylococcus aureus* the fusion protein can be purified using an IgG-Sepharose column and the protein A tag can be cleaved by treatment with formic acid or hydroxylamine. Moks *et al.* (1987) used a modified system to isolate insulin-like growth factor.

Hopp *et al.* (1988) developed a tag, Flag™, which was designed specifically for immuno-affinity chromatography. This IBI FLAG® System is commercially available from Kodak Scientific Imaging Systems and is based on the fusion of an eight amino acid FLAG marker peptide to either the N- or C-terminus of a cloned protein. The twenty-four base pair (bp) FLAG coding sequence is cloned adjacent to the appropriate coding sequence for expression in a vector. The FLAG peptide is then recognised by monoclonal antibodies on an affinity gel. After purification the tag can then be removed from the fusion protein by proteolytic treatment with enterokinase. Amongst other proteins successfully isolated with this system, interleukin 2 Flag™ fusion protein has been expressed, secreted from yeast and purified from a crude yeast broth (Hopp *et al.*, 1988). Although highly selective, binding capacities for small molecules are low, making scale-up a costly process. Since the tag was designed to be immunogenic, it must be removed from therapeutic proteins.

Another purification tag is based on the enzyme glutathione *S*-transferase (GST) which has a specificity for glutathione. This system was developed by Smith and Johnson (1988) and is commercially produced as the GST Gene Fusion System from Pharmacia Biotechnology. This provides a complete system for expressing and purifying

fusion protein in *E. coli*. The 26 kiloDalton (kDa) enzyme from *Schistosoma japonicum* acts as the affinity handle for the purification of the fusion protein. pGEX vectors are used to clone the protein of interest and the expressed fusion proteins can be purified from bacterial lysates by affinity chromatography using glutathione sepharose columns. The presence of the enzyme also provides a colourimetric assay for detecting expressed fusion proteins. It is possible to separate the affinity tag from the protein of interest by proteolysis with thrombin or factor Xa. This system has been used successfully in many applications such as the production of vaccines (Johnson *et al.*, 1989; Fikrig *et al.*, 1990) and the purification of recombinant antigenic epitopes of a human antigen (Berthold *et al.*, 1992). Glutathione-agarose is available commercially and is reported to have a capacity of 8 milligrams of fusion protein per millilitre agarose (Smith and Johnson, 1988).

Another fusion system available from Pharmacia Biotechnology is the pEZZ 18 Protein A Gene Fusion Vector which contains the Protein A signal sequence and two synthetic "Z" domains which are based on the "B" IgG binding domain of Protein A. This construction allows "ZZ" fusion proteins to be secreted from *E. coli* into the culture medium. Purification of the expressed protein is possible as the "ZZ" domain is tightly bound by IgG sepharose.

The PinPoint™ Xa Protein Purification System (Promega) also purifies recombinant proteins expressed in *E. coli*. The DNA coding for the protein of interest is cloned into a PinPoint™ Vector downstream of the sequence encoding a peptide which becomes biotinylated *in vivo*. The fusion protein can then be isolated by passing the cell lysate through a column of avidin Softlink™ Resin. A Factor Xa protease cleavage site is located between the biotinylated peptide and the target protein, allowing cleavage and purification of the protein of interest.

The pET vectors were originally constructed by Studier and colleagues (Studier and Moffatt, 1986; Rosenberg *et al.*, 1987; Studier *et al.*, 1990) and the newer pET derivatives have been developed by Novagen. A series of vectors are available which enable the addition of a His•Tag™ sequence (consecutive stretch of either 6 or 10 histidine residues) that can be expressed at the N- or C-terminus of the target protein. The His•Tag sequence allows the target protein (soluble or insoluble in the host) to be purified in one step by metal chelation (nickel) chromatography using Novagen's His•Bind™ Resin available in 2.5 ml columns.

Qia*express*™ Ni-NTA Protein Purification System (Qiagen) is based on the selectivity of metal chelate affinity chromatography using a nickel-nitrilotriacetic acid (Ni-NTA - see section **1.3.1.1**) resin for proteins carrying a tag consisting of six consecutive histidine residues (His_6). The Qia*express* pQE expression vector series provides multicloning sites in all three reading frames and the tag can be expressed at the N- or C-terminus of the protein. The resin consists of Ni-NTA covalently linked to

Sepharose and supplied as a slurry for column chromatography. Düring (1993) used this Ni-chelate affinity chromatography technique to purify bacteriophage T4 lysozyme (bT4l) which had the histidine residues attached to the N-terminus.

Raupach *et al.* (1994) described a general approach for the cloning, expression and purification of heterologous proteins in *E. coli* host strains using the technique of the polymerase chain reaction (PCR). The gene of interest is cloned into a vector and the oligonucleotide primers (oligos) are specifically designed to contain either 3' or 5' additional nucleotides coding for a short amino acid sequence which is fused to either end of the protein. The technique was named affinity expression cassette PCR (AEC-PCR) and was applied to the cloning, production and purification of ADP-ribosyltransferase of the pertussis toxin. In this example, a string of six histidine residues were engineered to either the N- or C-terminus to serve as a handle for the isolation of the recombinant protein by immobilized metal affinity chromatography (IMAC - see section **1.3.1**).

Skerra (1994), introduced a generic technique to enable single-step purification of bacterially expressed Ig fragments independent of their antigen-binding properties. A one-step IMAC purification of a functional recombinant F_{ab} fragment-His_6 fusion produced in *E. coli* using the vector pASK84 is described in this paper. A C-terminal His_6 tail was fused to the heavy chain and conferred sufficient affinity to immobilised metal ions (iminodiacetic acid-Sepharose charged with zinc) in order to enable the affinity purification of the functional recombinant F_{ab} fragment to homogeneity in a single step.

When choosing a fusion tail system for purification, several issues must be addressed. It is necessary to consider whether the protein will be expressed in a prokaryotic or eukaryotic system (for protein folding and post-translational modification), if expressed in *E. coli* will it be expressed in the periplasm (where there would be the advantage of decreased proteolysis and low level of contaminating proteins), will the tail interfere with the biological activity of the desired protein and if so, what method of cleavage will be employed.

1.3 Affinity chromatography

Affinity chromatography is a type of adsorption chromatography in which the molecule to be purified is specifically and reversibly adsorbed by a complimentary binding substance (ligand) immobilized on an insoluble support (matrix). It enables purification of almost any biomolecule on the basis of its biological function or individual chemical structure. Using this technique many separations have been achieved in a single step allowing time-saving over less selective multi-stage procedures. Highly selective

separations can be obtained and hence this technique has been used to remove small amounts of biological material from large amounts of contaminating substances. Since affinity chromatography has a concentrating effect it enables large volumes to be conveniently processed.

The principle of affinity chromatography can be described as follows. A successful separation requires that a biospecific ligand is available and that it can be covalently attached to a matrix. It is also important that the immobilized ligand retains its specific binding affinity for the substance of interest and that methods are available for selective desorption of the bound substance in an active form after washing away any unbound material. This purification technique has been used to isolate enzymes, antibodies, hormones, nucleic acids and even whole cells.

A common matrix material is Sepharose which is a bead-formed agarose gel and is widely used for immobilizing biologically active molecules providing excellent flow properties with minimal channelling in the bed thus ensuring that rapid separations are obtained. Sepharose is stable under the wide range of conditions commonly used in the laboratory and is chemically and biologically inert. The ligand which is attached to the matrix should exhibit selective and reversible binding for the substance to be purified and its binding activity should not be destroyed when attached to the matrix.

1.3.1 Immobilized metal ion affinity chromatography (IMAC)

A type of affinity chromatography which has proved useful is immobilized metal affinity chromatography which was introduced by Porath *et al.* (1975) to fractionate human serum proteins on chromatography columns containing several heavy metal ions. The principle of this technique is the coordination between electron donor groups on certain amino acids (histidine, cysteine and tryptophan) at the protein surface and first row transition metal ions and zinc which are immobilized on an adsorbant. The metal ions most frequently used are copper (Cu^{2+}), nickel (Ni^{2+}), zinc (Zn^{2+}), and cobalt (Co^{2+}). In aqueous solution the metal ions are electron-pair acceptors and can be considered as a Lewis acid, and the water molecules can be considered as electron-pair donors or a Lewis base. The metal ion is immobilized (usually chelated) onto a solid support. The incoming protein binds to the metal ion(s) via electron donor grouping(s) resident on its molecular surface.

The coordination selectivity in the binding of proteins and other solutes is strongly pH dependent (Porath and Olin, 1983). Protons can affect the immobilized metal ion as well as the surface properties of proteins in the sample. Thus, when the pH is varied, the absorption and/or desorption process can easily be assessed. An increase in the efficiency of separation can be obtained by a pH gradient.

Due to advances in recombinant DNA technology, this principle of binding has now been extended by the ability to introduce fusion tails into many proteins. In 1987 Smith *et al.* investigated the ability of many di- and tri-peptides to bind immobilized metal ions. The next step was to show that the metal ion affinity of a small peptide could be transferred to a larger polypeptide. This was accomplished by the same group (Smith *et al.*, 1988) using luteinizing hormone-releasing hormone analogues. Many groups have now used recombinant DNA techniques to enable extraction of a desired protein from crude cell lysates.

The first experiment in which a protein's affinity toward a chelated metal was altered involved fusing the sequence coding for histidine-tryptophan to that of proinsulin (Smith *et al.*, 1988). Review articles on the use of engineering metal binding sites into many recombinant proteins for facile purification have been written (Smith, 1991; Ford *et al.*, 1991).

IMAC offers a variety of chromatographic conditions that can be used to carry out selective isolation - recombinant proteins can be expressed as either soluble or insoluble products (denaturants can be used when the expressed product is insoluble), the bound proteins can be eluted by several methods (pH gradient, competitive binding), and the columns are resistant to microbial contamination due to the high metal content. The small inexpensive metal complexes are stable under a wide range of conditions and can be recycled many times without the loss of activity. In addition, the columns can be cleaned and easily regenerated without reduction in the protein binding capacity. Therefore, the technique of IMAC proves to be a versatile technique which is becoming more popular as the crystal structures of proteins are discovered.

1.3.1.1 Choice of chelating ligand

As mentioned in the above section, the metal ion is attached to a solid support/matrix via an immobilized chelating ligand. A ligand with one donor atom with a free pair of electrons is monodentate and can only form metal complexes. Polydentate ligands provide two or more metal coordination sites and are capable of forming a metal chelate with an ion. An ideal chelate resin for the purification of biopolymers must on the one hand strongly complex the metal ions and on the other hand must permit reversible interactions between metal ions and the desired protein.

There are several ligands in use but the three common types are iminodiacetic acid (IDA), a nitrilotriacetic acid (NTA) derivative and tris(carboxymethyl)ethylenediamine (TED). The coordination number of the metal ion and the amino acids affects the choice of the chelator.

Perhaps the most commonly used chelating ligand is IDA. It is a tridentate chelator which binds the metal ion through its nitrogen atom and two carboxylate

oxygens (Arnold, 1991). Its small size and hydrophilic nature, as well as the overall neutral charge of the complex with divalent ions, minimise secondary interactions between the metal chelate and the protein. IDA holds metals such as copper, zinc and nickel tightly but still leaves coordination sites available for protein binding. The metal ions have six sites (with the exception of copper which it is thought can have four or six), three of which bind to the resin while the remaining three are occupied by water molecules which can be displaced by appropriate protein functional groups. Adsorption of a protein to a IDA-divalent metal ion (IDA-M(II)) gel has to be performed at a pH at which an electron donor grouping(s) on the protein surface is at least partially unprotonated.

Hochuli *et al.* (1987) reported a new tetradentate metal chelate adsorbent which occupies four positions in the metal coordination sphere leaving the remaining two positions for protein interactions. NTA was found to be selective for proteins and peptides containing neighbouring histidine residues. The group reported that copper and nickel metal ions were retained more strongly on the NTA than on IDA adsorbant. NTA was found to be more stable to metal leaching than IDA adsorbant. (Although NTA is a potential candidate we have chosen not to use it in our system)

TED is a pentadentate chelator and has the ability to bind metal ions strongly. However, only one site remains free for interaction with an amino acid and therefore only a weak interaction with a protein could exist. While only a single free site is likely to be available on a hexacoordinate metal ion bound to the TED gel, there are at least two, usually three, free coordination sites on an IDA gel.

Since most information available is based on the tridentate chelating ligand IDA, we have chosen to use this chelator in this study.

1.3.1.2 Choice of metal ion

Proteins can make use of metal ions to bind substrates, to maintain structure, to effect catalysis and for allosteric control and regulation (Arnold and Haymore, 1991). Metal recognition can be engineered into proteins for applications such as protein purification. The apparent affinity of a protein for a metal chelate depends strongly on the metal ion involved in coordination.

Metals frequently exhibit characteristic affinity behaviour independent of their oxidation state (Porath and Olin, 1983). An example of a metal which behaves in such a way is mercury which has an affinity for sulphur. However, this is not always the case and it should be noted that iron-containing (Fe^{2+}- and Fe^{3+}-containing) adsorbents can show differences in their adsorption properties.

Metals most often used in IMAC are first row transition metals chelated to IDA. The small metal chelates generally used tend to be stable under a wide range of solvent

conditions and temperature and hence are usually recyclable. Transition metal ions are immobilized to chelating groups and allowed to interact with proteins in the mobile phase. Only those proteins with a high affinity for the particular immobilized metal ion are chemically adsorbed by forming coordinate bonds with unoccupied coordination sites on a metal. The adsorbed protein is washed free of other proteins in the sample and then it is eluted from the column.

Andersson *et al.* (1991) made an interesting discovery when trying to isolate serum albumins with Zn-, Cu-, Ni- and Co-IDA. Although serum albumins may bind Zn^{2+} and Co^{2+} ions when both are in solution, they are not retained on IDA columns containing these metal ions. Therefore, the ability of a peptide or protein to bind a metal ion in solution is not an indication of the binding abilities when the metal ion is immobilized when on a column. This was also noted by El Rassi and Horvath (1986) with respect to protein binding to free and chelated copper.

When Cu^{2+} is chelated to tridentate IDA, the metal ion has three coordination sites free for interaction with biopolymers (Hochuli *et al.*, 1987). Nickel also has three such sites but is not bound as tight and may be released from the adsorbent during chromatography. The affinity of IDA for Cu^{2+} was found to be fifteen times that for Ni^{2+}, whose affinity in turn is more than three times that for zinc (Lilius *et al.* 1991).

1.3.1.3 Choice of amino acid

Stability constants of first row transition metals with amino acids indicate that the amino acids can act as bidentate ligands through their amino and carboxylate groups, but only a few contain side chains that are capable of tridentate coordination such as aspartic acid (Asp), histidine (His), cysteine (Cys), glutamine (Gln), methionine (Met), tyrosine (Tyr) and lysine (Lys) (Smith *et al.*, 1987).

Porath *et al.* (1975) suggested that certain amino acid residues such as His, Cys and tryptophan (Trp) can participate in binding immobilized metal ions. Further studies by Hemdan and Porath (1985) investigated the retention of natural amino acids on immobilized Ni-IDA at neutral pH. L-Cys, L-His and L-Trp were retained most strongly. L-arginine (Arg) and L-Lys displayed higher retentions than the other amino acids while L-glutamic acid (Glu) and L-Asp were not retained at all. The retention of the aromatic amino acids (phenylalanine (Phe), Tyr, and Trp) and glycine (Gly) increased with pH between 7.5 and 9.0 and an increase in the ionic strength further enhanced that retention due to the contribution of the hydrophobic interactions.

His and Cys are known to form stable complexes with Zn and Cu ions in nearly neutral aqueous solutions by virtue of the imidazole and thiol groups respectively. Studies with avidin by Hemdan *et al.* (1989) indicated that when Trp was present on the surface of a protein it acted as a weak ligand and therefore its contribution to retention

was insignificant. Andersson *et al.* (1991) investigated the interaction of serum albumins and Zn-, Cu-, Ni-, and Co-IDA. There appeared to be no evidence for any significant contribution of Trp or Cys residues to the chromatographic event. Practical difficulties arise in maintaining accessible Cys residues in their reduced state. Surface-exposed Cys residues rapidly oxidise in the presence of Cu^{2+} to form disulphide-linked dimers (Arnold, 1991). It is also possible that free thiols can scavenge metal ions from the IDA ligand.

Positively charged polyArg tails have been developed for the recovery and purification of target proteins by ion-exchange chromatography (Brewer and Sassenfeld, 1985). Hydrophobic interactions have been used by Persson *et al.* (1988) for the purification of β-galactosidase which contained eleven Phe residues at the N-terminus (Superose column), and covalent chromatography was used when four Cys residues were attached to the N-terminus of the galactokinase gene (Sepharose column). The native forms of either of these proteins were not isolated on the same columns.

In order for binding of the amino acids to the immobilized metal ions to occur, the residues must be located on the surface of a protein molecule and be accessible to the ligand-metal ion chelate (Hemdan *et al.*, 1989). Histidine accounts for approximately 2% of the amino acids in globular proteins (Arnold, 1991) and the occurrence of surface residues is therefore rare, hence this amino acid is a good candidate for IMAC. As a result most proteins do not exhibit high affinities for IDA-metal ion complexes. Secondary contributions from other amino acids can affect apparent metal affinity (Hutchens and Yip, 1991). It is possible that nearby side chains can interfere with the accessibility of the histidyl nitrogens of the imidazole ring - Smith *et al.* (1987) and Arnold (1991) suggested that aromatic side chains (Trp, Phe and Tyr), provided that they lie near the accessible His residues, appear to contribute to protein retention on an IDA-M(II) column but the mechanism is not yet understood. Hutchens and Yip (1991) suggest that, in addition to the protein surface environment, physical and chemical variables such as temperature, degree of solvation, and buffer composition can alter the degree of His proton ionization at a given pH.

In His, cations are found to lie in the imidazole plane along the lone pair on the nitrogen atom (Chakrabarti, 1990). There are thought to be two tautomeric forms of the imidazole ring, one form is adopted when the metal ion is bound. The orientation of the His residues is usually stabilized through hydrogen bonding to other amino acids in the polypeptide.

Since many studies have involved the use of His as an affinity tail to aid protein purification thereby providing a wide knowledge, and also the fact that surface accessible His residues are a rare occurrence in native proteins, it seems an ideal candidate for the

choice of amino acid for the selective purification of proteins by IMAC. A selection of reported purifications using His are outlined below.

Hemdan *et al.* (1989) studied several families of proteins and cytochromes and found that the retention of a protein molecule on an IDA-Ni(II) column required at least two His residues while the retention on a IDA-Cu(II) column occurs with a minimum of one His available for coordination. To the first approximation, proteins are retained on the metal affinity columns according to the number of accessible His residues (Yip *et al.*, 1989; Arnold, 1991; Todd *et al.*, 1991). Metal-ligand binding can be far stronger than the sum of the binding strengths of the individual monodentate ligands - known as the "chelate effect" (Arnold and Haymore, 1991). However, not all histidyl residues are fully accessible to the metal ions and therefore vary in their ability to bind.

Several avian and mammalian chicken-type (c-type) lysozymes were chromatographed on chelated and immobilized transition metal ions (Co^{2+}-, Ni^{2+}-, Cu^{2+}- and Zn^{2+}-IDA) under a variety of experimental conditions (Zhao *et al.*, 1991). These lysozymes were chosen for this study as there is a variable number of His residues in natural variants of c-type lysozymes and although several Trp are present in a lysozyme molecule, they are for the most part sequestered in the interior of the molecule. It was found that varied affinity of the lysozymes for the chelated metal ions, IDA-M(II), can be rationalized primarily in terms of the presence, multiplicity and microenvironments of His residues. The proteins which did not contain His residues interacted weakly with the columns and from the results obtained it is clear that all proteins containing one His were recovered earlier from IDA-Cu(II) columns compared to those proteins containing two or four His. However, bovine lysozyme c2 (four His residues) does not bind significantly more strongly to IDA-Cu(II) than California quail lysozyme (two His residues). It is thought that this is due to the fact that not all four residues of the bovine lysozyme are exposed on the surface of the molecule. The chromatographic resolution of some of these closely related proteins attests to the analytical power of immobilized metal-ion affinity chromatography.

Lilius *et al.* (1991) chose galactose dehydrogenase (Galdh) as a prototype target protein to investigate the capability of metal affinity precipitation to facilitate the purification of genetically engineered proteins. A DNA fragment encoding five His residues was fused to the 3'-terminal end of the Galdh gene from *Pseudomonas fluorescens* which was then expressed in *E. coli*. The additional His residues functioned as an affinity tail enabling the modified protein to be purified by IMAC. After purification, the tail could be removed enzymatically by carboxypeptidase A. The Galdh was recovered completely from the Zn-charged column and the purification factor for this step was calculated to be as high as 1030. When a protein solution containing native Galdh was loaded onto the column using the same conditions, no enzyme was adsorbed.

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) analysis, protein determination and enzymatic analysis after this step showed that the recombinant Galdh enzyme was at least 70% pure.

Loetscher *et al.* (1992) was able to isolate a monoclonal antibody on Ni-NTA by the fusion of a chelating peptide containing Lys, Gly and His in the form of Lys-Gly-$(His)_6$, to the carbohydrate side chains of the antibody. Martin (1994) used a series of five His residues fused to the carboxyl terminus and hinge region of an antibody to enable purification of single-chain antibody fragments on Zn-IDA.

Carlsson *et al.* (1993) also used a peptide fragment containing His residues (four) fused in-frame to the 5'-end of the *Bacillus stearothermophilus* lactate dehydrogenase gene and the gene product could be purified to homogeneity using either Zn^{2+}-affinity chromatography or affinity chromatography on oxamate agarose.

Waine *et al.* (1994) purified a recombinant *Schistosoma japonicum* antigen as a putative vaccine candidate against schistosomiasis. This was achieved by synthesising the schistosome antigen with a short polyHis tag fused to the N-terminus which was then used for subsequent affinity purification under non-denaturing conditions using Ni-chelate affinity chromatography. The 22.6-kDa antigen was recovered in purified, non-denatured, recombinant form, and in sufficient quantity to assess the protective value of the molecule in vaccination/challenge experiments.

There are many other examples of the successful use of five or six His residues attached to either the N- or C-terminus of proteins (including single chain antibodies and viral proteins) which were expressed in *E. coli* (e.g. Laukkanen *et al.*, 1994; Doyle and Tolan, 1995; Evans *et al.*, 1994; Kipriyanov *et al.*, 1994) and some of which have been expressed in a Baculovirus expression system (e.g. Wang *et al.*, 1994; Meyer *et al.*, 1994).

It can therefore be concluded that the composition and conformation of a protein, in particular the proximity and surface exposure of His residues, dictate the affinity a protein may have toward a given metal chelated to a stationary phase (Sulkowski, 1985; Hemdan and Porath, 1985; Hemdan *et al.*, 1989; Todd *et al.*, 1991). Results from Johnson and Arnold (1995) show a fundamental relationship between the density of binding sites on the surface (the metal ions) and the number of target groups on the protein (surface His) and state that the protein will adsorb with the highest binding energy to those arrangements of metal ions which most closely match its pattern of His residues.

From the examples of the purifications outlined above it can be seen that the addition of tails containing His residues to either the N- or C-terminus of proteins provides an ideal purification "handle" for proteins.

1.3.1.4 Possible histidine configurations

As mentioned above, His is a preferred choice of amino acid for IMAC with divalent metal ions as it is highly selective due to the low occurrence of His residues in proteins. However, these residues must be accessible for binding and one way in which this can be brought about is by the addition of, or substitution with, His residues. The total binding capacities for proteins with two surface His residues are approximately 30% greater than capacities for single-His variants, while that for variants with three His is estimated to be at least an order of magnitude stronger than the two-site binding (Todd *et al.*, 1994). Vosters *et al.* (1992) recommended that three or more alternating His residues should result in the isolation of chimeric proteins from crude mixtures in one step.

It is possible to manipulate the amino acid sequence according to the specific requirements for binding and the following is a rough guide as to the conformations which can arise. Dihistidine configurations are referred to as follows; for example His-X_3-His, His-X_2-His or His-X-His where X indicates any amino acid. When two His residues are in an α-helix, they can only chelate a metal ion if they are separated by X_3; if a reverse β-turn is required they must be separated by X_2; when the conformation is a β-strand structure, the two residues must be separated by X (Ghadiri and Choi, 1990; Arnold and Haymore, 1991).

Smith *et al.* (1987) conducted an initial survey of di- and tripeptides containing His, Lys, and Asp which exhibited a wide range of affinities for immobilized metal ions. Of the forty-nine peptides examined, three (His-Trp, His-Tyr-NH_2, and His-Gly-His) had unusually high affinities for Co(II), Ni(II), and Cu(II) IMAC columns and were identified as potential chelating peptide purification handles. They tested fourteen peptides with the general formula His-X and all bound tightly to IDA-Cu(II). The seven X-His peptides, on the other hand, did not bind under the same conditions. His-X peptides can form six-membered rings with the metal ion, N-terminal amine, and the nitrogen on the imidazole which appeared to confer stability on the formed complexes.

Hochuli *et al.* (1988) fused a polyHis peptide (containing 2-6 adjacent His residues) to mouse dihydrofolate reductase at both the N- and C-terminus. The fusion proteins were purified on Ni(II)-NTA and subsequently treated with carboxypeptidase A to remove the polyHis tail. However, the efficiency of the fusion peptide was found to be dependent on the solvent system used in the chromatography.

The fusion peptide should include an optimal sequence for binding M(II) ions. Ljunquist *et al.* (1989) investigated β-galactosidase and a synthetic IgG binding protein were each extended with copies of the sequence (Ala-His-Gly-His-Arg-Pro) where Ala is alanine and Pro is proline - thereby including a His-X-His configuration - and purified the proteins by IMAC. The addition of two copies of this sequence did not enhance affinity

toward chelated Zn but when 4 or 8 copies were added, the recombinant proteins bound Zn(II)-IDA. The reason given for the requirement of multiple copies of the fusion peptide is that the interaction between histidines and Zn^{2+} is relatively weak and suggests that multivalent binding is needed to achieve efficient recovery of the recombinant protein.

An example of a His-X_2-His configuration was reported by Beitle and Ataai (1993) using angiotensin I as the affinity tag. This decapeptide containing valine (Val), isoleucine (Ile) and leucine (Leu) in the form of (Asp-Arg-Val-Tyr-Ile-His-Pro-Phe-His-Leu) displays strong metal-binding properties (Yip *et al.*, 1989) and an octapeptide derived from it (Val-Tyr-Ile-His-Pro-Phe-His-Leu) was placed at the N-terminus of a β-lactamase. The fusion protein was recovered from periplasmic inclusion bodies to high purity in one step by Zn(II)-IDA IMAC.

The metal-binding affinity conferred by a surface His-X_3-His site has been utilized to make possible a single-step purification of engineered protein, bovine somatotropin, which was efficiently recovered (up to 95%) in a relatively pure and biologically active form (97 to 98%) from a crude cell lysate using an IDA-Cu(II) matrix (Arnold and Haymore, 1991). Suh *et al.* (1991) also engineered variants of bovine somatotropin to contain synthetic metal-binding sites consisting of two solvent-exposed His residues separated by a single turn of an α-helix formed by His-X_3-His variants, and a His-X_2-His variant. It was found that the imidazole groups from the two His from consecutive turns in the α-helix could coordinate a single metal (also Chakrabarti 1990; Todd *et al.*, 1991) while the His-X_2-His conformation did not increase the retention of the protein above that when two unassociated surface-exposed His residues were present. The metal affinity of the His-X_3-His site was found to depend on the rigidity of the helix into which the site is engineered. Kellis *et al.* (1991) substituted residues at positions 4 and 8 for His in iso-1-cytochrome *c* of *Saccharomyces cerevisiae* thereby forming an N-terminal configuration of His-X_3-His. This variant binds Cu(II)-IDA with an affinity twenty-four times higher than a protein with a non-chelating histidyl residue. As mentioned above, the ability of the two His in an α-helical arrangement to coordinate a single metal gives rise to a substantial increase in the binding free energy compared with two His that are independent of one another.

Recently it has been suggested that proteins containing multiple accessible His appear to interact with the support by simultaneous coordination to more than one metal ion, the result of which is to increase the apparent binding by as much as a factor of 1000 (Todd *et al.*, 1994). Protein binding was found to depend on the availability of copper sites and the fact that binding was found to be significantly weaker at low surface concentrations of copper was perhaps due to the fact that multiple interactions could not

take place. At copper loadings less than 50% of the maximum, the protein binding capacity drops to zero.

When designing an affinity tail, the amino acid composition, the surface exposure and the accessibility of the His tail to the chelated metal ion all influence the degree of selectivity. Ideally the affinity tag should protrude from the protein of interest so that it is accessible for binding. Hochuli (1990) outlined a scenario with a ligand that has two free positions for binding. If two His are available, one complex is possible. With three adjacent His and two free ligand positions, two different complexes are possible. In a likewise fashion, four adjacent His residues could be bound in three isomeric forms. This explains why the greater the number of surface His residues available for binding, the stronger the binding strength. However, it is important not to have too many interactions occurring as noted by Skerra *et al.* (1991) when the fusion of a series of nine His residues onto a protein resulted in binding that was too strong to allow significant elution. Taking all the above data into consideration, it was decided to add a polyhistidine tail to the terminus of the chosen protein in the form of an α-helix (His-X_3-His). By the addition of five His residues it was thought that binding would be maximised and still allow elution of the protein from the support. It was also believed that an additional tag of this nature would be small enough so as not to necessitate its cleavage.

1.3.1.5 Choice of protein

As mentioned above, the test protein must contain His, Cys, or Trp residues to enable purification by immobilized metal affinity chromatography. His would be the amino acid of choice as it is quite rare and also it has been used by other workers.

It was important to use a protein for which there was a lot of data available. Bacteriophage T4 lysozyme (bT4l) was the chosen protein for several reasons - there is extensive knowledge about its three dimensional structure, its DNA sequence has been resolved, it is known that the active site is far away from the N- and C-termini (both possible sites for the addition of the proposed polyhistidine tag), it is produced intracellularly so it is possible to confine and release it in smaller volume/more concentrated, it is an assayable protein, and it was available as a clone in an expression vector (kindly donated by B.W. Matthews). Hen egg white lysozyme (has the same mode of action as bT4l) is a commercially available enzyme and is used for the disruption of cells. It could be used on a wider scale but for the fact that it is relatively expensive. Therefore, in theory, if it could be recovered from crude cell lysate for its own purification, it could be potentially recovered from other fermentations and recycled thereby reducing the cost of other purification processes. The protein also contains a single His residue which is not on the surface of the molecule and therefore cannot take

part in IMAC. Therefore, if the addition of any His residue(s) enables purification by IMAC, it can be attributed to the presence of this extra residue(s).

The bT4l gene system has been widely used for the study of basic problems in molecular biology, such as the relationship between gene and protein sequences, the mechanisms of mutagenesis and the mechanism of enzymatic catalysis.

1.4 Bacteriophage T4 lysozyme - introduction

A wide variety of both prokaryotic and eukaryotic enzymes have bacterial cell walls as their site of action. In 1915, Twort first described a filterable "lytic agent" which causes lysis of staphylococci and in 1917, d'Herelle reported his independent discovery of "bacteriophage" (Tsugita, 1971).

Bacteriophage T4 lysozyme is an endoacetylmuramidase, cleaving the β(1-4) glycosidic bond between the *N*-acetylmuramic acid and *N*-acetylglucosamine (part of a long complex sugar molecule which is found in the cell wall of many bacterial cells). The normal host of the T4 bacteriophage is *E. coli* and the lysozyme is produced late in the bacteriophage life cycle to allow the release of the progeny bacteriophage particles.

The protein is strongly basic (Tsugita, 1971) and has an enzyme specificity similar to egg white lysozyme which has been extensively studied. The enzyme proved to be stable when incubated at 37°C for 20 hours in 0.1 M phosphate buffer, pH 6-6.5 but was inactivated by prolonged incubation at more acidic or alkaline pH values. Fifty per cent inactivation occurred at 53.5°C.

1.4.1 Bacteriophage T4 lysozyme - structure

In order to modify a protein it is necessary to ensure that any manipulations that are carried out will not structurally alter the active site, or have a negative effect on the biological activity or expression of the amended protein. It is therefore necessary to look at the primary, secondary and tertiary structure of the native protein.

Tsugita and Inouye (1968) published the complete amino acid sequence of bT4l and found it to consist of a single peptide chain of 164 amino acids, with two Cys residues which existed as sulphydryl groups without forming a disulphide bridge. The sulphydryl groups (-SH) are shown to have no direct contribution to the enzymatic activity, since the enzyme was fully active even after oxidation of the -SH groups. The molecular weight of the structure was calculated to be 18,635. **Appendix 1** contains the complete amino acid sequence of this protein. Inouye *et al.* (1970) used the method of dilute acid hydrolysis to confirm the primary structure of bT4l.

Owen *et al.* (1983) determined the nucleotide sequence of the lysozyme (*e*) gene and also approximately 130 additional nucleotides on each side. **Appendix 2** contains the complete DNA sequence of bT4l.

The three-dimensional structure of the lysozyme from bacteriophage T4 was determined by Matthews and Remington (1974) from a 2.5 Å resolution electron density map. About 60% of the molecule was found to be in a helical conformation and the overall dimensions of the molecule were estimated at approximately 50 x 30 x 30 Å. There are two quite distinct lobes with the C-terminal part lying in the upper domain, while the lower domain contains most of the amino-terminal portion of the molecule. The most obvious connection between the two domains is a long helix of twenty residues which extends from one extremity of the molecule to the other. From this article it is inferred that the active site must open to allow substrate to enter. A perspective drawing illustrating the polypeptide backbone of bacteriophage T4 lysozyme can be seen in **Appendix 3**.

Remington *et al.* (1978) determined the three-dimensional X-ray crystal structure (at 2.4 Å resolution) to provide a detailed view of the interactions of the folded protein. The N-terminal domain (residues 1-60) contains all of the β-sheet structure and two α-helices. The C-terminal domain (residues 80-164) is like a barrel and is composed of seven α-helices. Residue 67 is thought to form a "hinge" which enables substrate binding at the active site contained within the deep cleft between the two domains.

Narang *et al.* (1987) chemically synthesised a 495 bp DNA sequence encoding T4 lysozyme. The synthetic bT4l exhibited conformation essentially identical to the wild type bT4l as determined by X-ray crystallography with the two domains held together by a rigid α-helix (60-79) which appears to provide the constructural constraint that prevents loss of activity by maintaining the relative spatial positions of the two domains. The active residues were found to be located at residues 11 (Glu) and 20 (Asp).

Many mutation/substitution studies have taken place to determine the role of certain amino acids in the bT4l. In general, residues identified by structural or chemical data as involved in substrate binding and/or catalysis are usually critical for function. Many mutant variants have formed crystals of high quality and it has thus been possible to examine closely the structural effects of the numerous amino acid substitutions in these proteins. By comparing the properties of mutant and wild type proteins, the contributions of individual amino acids can be estimated.

Tsugita (1971) suggested that residues 20 (Asp), 22 (Glu), 105 (Glu), 138 (Trp), 140 (Asp) and 141 (Glu) are all essential for full catalytic activity. Remington *et al.* (1978) suggested that deletion of the Phe at residue 4 substantially reduces the catalytic effectiveness of the enzyme, and also indicated that Asp20 has an important role in

enzymatic activity. Weaver and Matthews (1987) and Narang *et al.* (1987) also indicated that Asp20 was catalytically important.

To test the role of the rigid α-helix that links the two domains, residues 60-79, each of the residues 73-75 (Ala-Ala-Val) were replaced by helix-breaking Gly residues (Narang *et al.*, 1987). This mutant was found to lack enzymatic activity. The loss of activity was thought to be as a result of the change of the relative two domain positions.

Several studies have focused on Cys residues. Perry and Wetzel (1984) substituted Ile at position 3 (Ile3) with Cys and the protein retained full enzymatic activity. Mild oxidation generated a disulphide bond between Cys3 and Cys97. Perry and Wetzel (1986) substituted Cys54 with threonine (Thr) and Val. Neither substitution greatly affects the enzymatic activity although either together with Cys3 showed a 50% decrease in the activity. Matsumura and Matthews (1988) engineered disulphide bonds into the active site cleft of Cys-free lysozyme at positions 21 and 142 which are located on opposite sides of the active-site cleft. Protein with substitutions Thr21Cys and Thr142Cys spontaneously formed a disulphide bond under oxidative conditions *in vitro* and the enzymatic activity was switched off. On exposure to reducing agent the disulphide bond is broken and full activity is restored. The cross-link prevents substrate from binding to the active site, leading to complete inactivation.

Studies involving multiple substitution of Ile3 enabled Matsumura *et al.* (1988) to show how hydrophobic interactions at this site contribute to the overall stability of the protein and that this Ile residue contributes to the major hydrophobic core of the C-terminal lobe and helps to link the N- and C-terminal domains.

Alber *et al.* (1988) investigated the role of Pro residue at position 86 (Pro86). This residue is on the surface of the protein at the beginning of the C-terminal domain and not near the active site. The substitutions caused more obvious changes in enzymatic activity than in protein stability. The residues within the substituted region altered to accommodate the new residue and the helix became distorted.

Rennell *et al.* (1992) investigated the functional role of amino acids which formed nine α-helices by amino acid substitution to identify those crucial to enzyme function and reported that those at the C-terminal positions of the helices were found to be critical.

1.4.2 Bacteriophage T4 - DNA

bT4l was chosen for study of the genetic code because it is an easily isolated protein of low molecular weight. Studies by Terzaghi *et al.* (1966) confirmed the general nature of the genetic code and unravelled the direction of translation of the messenger ribonucleic acid which was also studied by Streisinger *et al.* (1968). The mechanism of frame-shift mutations was also investigated using bT4l as the model (Streisinger *et al.*, 1966).

Most restriction enzymes do not cut bacteriophage T4 DNA as it contains glycosylated hydroxymethylcytosine in place of cytosine (Kaplan and Nierlich, 1975). Soon after infection, T4 makes endonuclease, coded by genes *denA* and *denB* which break down cytosine-containing DNA (Owen *et al.*, 1983). Another gene (56^{am}) encodes a dCTPase whose action reduces incorporation of cytosine into DNA. T4 mutations in these three genes makes DNA containing cytosine instead of hydroxymethylcytosine and the resulting DNA is sensitive to restriction enzymes and DNA fragments can thus be cloned.

The DNA in mature bacteriophage T4 is a single linear molecule with a region of terminal redundancy (Streisinger *et al.*, 1966). Owen *et al.* (1983) reported a relatively low guanidine (G) and cytosine (C) content is found both within the T4 DNA lysozyme gene (36%) and in the flanking regions (35%). Also G and C are less frequently used (23%) than adenine (A) and thymine (T) in the third position of the codons.

Okada *et al.* (1972) showed by comparisons of mutant and wild type amino acid sequences that a frameshift hotspot was a run of five A nucleotides in the wild type gene. Three and possibly all four runs of five A nucleotides in the *e* gene appear to be hotspots for frameshifts. Fifteen direct repeats of eight or more bp occur within the sequence.

1.5 Expression systems for the production of bacteriophage T4 lysozyme

Two expression systems have been developed to produce and purify bT4l (Muchmore *et al.*, 1989; Perry *et al.*, 1985). In both systems the plasmid is under the control of the (*trp/lac*) hybrid *tac* promoter which is inducible. This promoter contains the -35 region of the *trp* promoter and the -10 region of the *lac* promoter (which also contains the *lac* operator and hence is regulated by the *lac* repressor). When the *lacI* repressor binds to the *lac* operator, RNA polymerase is prevented from binding to the operator thus halting transcription of any gene downstream of the -10 region. Both systems are inducible with isopropyl-β-D-thiogalactoside (IPTG).

The system of Muchmore *et al.* (1989) contains the lysozyme gene flanked by tandem *lacUV5* and *tac* promoters and the *trp* terminator. Tight control of expression is provided by the presence of the $lacI^q$ gene on the plasmid. The *trp* terminator eliminates selection against cells harbouring the expression plasmid. The plasmid is referred to as pHSe5. Using this system they were able to produce 10-25 mg/10-20% of protein from litre-scale growths of host *E. coli*. Alber and Matthews (1987) reported typical yields of 50-70 mg of lysozyme per litre of induced cell culture.

The second expression system is that of Perry *et al.* (1985). The phage T4 gene coding for the lysozyme was cloned into a plasmid controlled by the *tacII* promoter and is known as the pT4lystacII plasmid. *E. coli* strain D1210 ($lacI^q$) transformed with this

plasmid produced active T4 lysozyme at levels up to 2% (or 10 mg/l) of the cellular protein after induction.

The gene has been cloned into a λ vector for DNA sequence analysis (Owen *et al.*, 1983) but no attempt to express this cloned gene has been reported. Knight *et al.* (1987) introduced mutations into the lysozyme gene which was present on a P22 hybrid phage (substituting for the P22 lysozyme) to isolate secondary site revertants. This construct was not used as an expression vector.

1.5.1 Choice of expression system - pHSe5

The expression system used throughout this thesis was a modified version to that of Muchmore *et al.* (1989). As mentioned above, the wild type (WT) protein contains two Cys residues at positions 54 and 97 which do not form a disulphide bridge. In order to eliminate the possibility of bonds forming between molecules of the protein, these two residues have been replaced. Cys at position 54 has been substituted with Thr (designated Cys54Thr) and the Cys at position 97 has been amended to Ala (Cys97Ala). The protein thus produced is Cys-free and designated pseudo wild type or WT*. A diagrammatic representation of this plasmid is shown in Chapter 3 **Figure 3.3** indicating the relative positions of the genes and their direction of transcription, the promoters, the origin of replication and the termination sequence. This Cys-free protein was known not to have reduced enzymatic activity. The uncharged polar Cys residues were replaced with uncharged polar Thr and non polar Ala.

1.6 Possible sites for mutation

Remington *et al.* (1978) produced several mutant lysozymes with an insertion in the amino-terminal region of the molecule which were found to have essentially normal activity, indicating that considerable flexibility is allowed in the makeup of this region of this molecule.

Matthews (personal communication) confirmed that the C-terminus of bT4l is well away from the active site and is therefore a good candidate site for modification. It is known, for example, that up to two residues can be removed from the C-terminus without significant change in the properties of the enzyme.

Düring (1993) added a fusion tail composed of six His residues to the N-terminus of bT4l to enable purification as the first several amino acids of the protein protrude from the core protein (Weaver and Matthews, 1987).

It was therefore decided to add a polyHis tail to the C-terminus of bT4l by mutagenesis to enable extraction of the recombinant protein by IMAC.

1.6.1 How to achieve mutagenesis - the theory of Site-directed mutagenesis

Mutagenesis can be achieved in several ways including the use of chemical reagents such as acridine orange. However, this chemical produces mutants at random positions within the gene. One general method which allows specific sites to be targeted is the procedure of site-directed mutagenesis.

Site-directed mutagenesis was first described in 1978 by Hutchinson *et al.* and can be accomplished *in vitro* by the hybridization of a synthetic oligo to a single-stranded template DNA. The oligo is designed to be complimentary to the single-stranded template DNA except for a region of mismatch near the centre. It is this region of mismatch which contains the desired nucleotide alteration(s).

After hybridization of the oligo to the single-stranded template, it is extended with a DNA polymerase to create a heteroduplex double-stranded structure. The nick is then sealed with DNA ligase and the duplex structure is transformed into a bacterial host. Several methods are now available for the selective destruction of the parental strand both *in vitro* (e.g. Sculptor™ IVM system from Amersham Life Science, Taylor *et al.*, 1985; Sayers *et al.*, 1992) and *in vivo* (Kunkel, 1985; Kunkel *et al.*, 1987). After propagation in the transformed host, the heteroduplex gives rise to homoduplexes whose sequences are either that of the original wild-type DNA or that containing the mutation. The resulting colonies can be screened for incorporation of the alteration on the basis of antibiotic resistance profiles or nucleic acid hybridization where the oligonucleotide can be labelled and used as a probe.

As mentioned above, in order for site-directed mutagenesis to occur, single-stranded template DNA is required. This may be achieved either by subcloning into a single-stranded phage vector such as M13, or by the use of phagemids (a chimeric plasmid containing a phage origin of replication of a single-stranded DNA bacteriophage (e.g. f1, fd, M13)). A phagemid has the ability to produce single-stranded DNA upon infection of the host bacterium with a helper phage. The expression vector pHSe5 used in this current work is a phagemid.

1.7 Current purification methods for bacteriophage T4 lysozyme

Bacteriophage T4 lysozyme has previously been purified to homogeneity by several methods. Tsugita and Inouye (1968) purified the protein from bacteriophage by repeated concentration by ion exchange chromatography on Amberlite IRC-50 cationic resin followed by molecular sieving on Sephadex G-75 columns. The final recovery was approximately 40% and involved six steps.

More recent purifications essentially utilise weak acid ion exchange resins for chromatography and Sephadex for molecular sieving with the loading of lysates of

induced plasmid-bearing cells. Several groups have used ion exchange columns such as CM-Sephadex (Alber *et al.*, 1988), CM-Sepharose (Alber and Matthews, 1987), or CM-Sepharose followed by SP Sephadex (Griffey *et al.*, 1985; Muchmore *et al.*, 1989; Poteete *et al.*, 1991). DEAE-cellulose (basic columns) and CM-cellulose followed by a gel filtration column were used by Perry and Wetzel (1986). Several steps are involved in all these purification procedures and therefore purification by affinity chromatography in one step would be less time consuming and beneficial.

Szewczyk *et al.* (1982) described a purification method which takes advantage of the affinity inhibition of bT4l by a muropeptide. The protein was first applied to two consecutive amberlite CG 501 columns, then passed through a muropeptide-Affi-Gel 202 affinity chromatography column and finally purified on a column of CM-Sephadex C-50. After all these steps 47% of the original activity was recovered with a purification factor of approximately 1000.

Recently Düring (1993) has described the purification of bT4l in a single step by fusing a tail containing six His residues onto the N-terminus of the gene and passing the lysate through a Ni-NTA agarose column. However, when the purified protein was electrophoresed on SDS-PAGE gels, the size of the protein was dependent upon the method of elution, the reason for which was not explained in the article. The recovery and purification factors achieved by this method were not stated.

1.8 Chromatographic supports

As mentioned above, in affinity chromatography a substance is irreversibly immobilized on an insoluble support and binds molecules in solution with complimentary binding specificities. Popular solid phases include agarose, polyacrylamide and controlled pore glass, which are either packed into columns or removed from suspension by centrifugation. All chromatography mentioned so far has used columns for the purification procedure with support particles greater than 50 μm in diameter used to pack columns of various proportions. However, conventional affinity supports and ligands are not generally suitable to deal with complex protein mixtures as the presence of suspended solids and fouling components tend to decrease their efficiency and have a slow speed at which the separation takes place. Therefore it would be advantageous if technology could cope with the more complex mixtures without prior purification steps. It was therefore questioned as to whether the principles and results of IMAC on columns could be extended to other supports to overcome their disadvantages.

Several criteria are required for the ideal support such as the support should: be easy to handle (tests with polyacrylamide-Fe_3O_4 particles showed that these were not suitable as they were easily broken - Munro *et al.*, 1977), be reusable, allow rapid

extraction so the process turnaround time is minimal, lack abrasion, not clump, produce reproducible results, have the ability to work at ambient temperature (if possible), have a long shelf life, have the greatest surface area possible available for binding (spherical supports are therefore ideal), involve the use of inexpensive equipment. In addition the binding of the desired purification product must be reversible. A serious limitation with porous supports is that it is difficult to eliminate contaminating substances. Halling and Dunnill (1980) reported that non-porous supports particles appear to be more resistant to fouling and therefore are easier to clean.

Recent developments by the Tokyo Soda Company has led to the production of a resin-based metal chelate adsorbent, TSK, which can also be coupled by IDA and has a bead diameter of 10 μm. This matrix has been used by several groups (Belew *et al.*, 1987; Yip *et al.*, 1989; Todd *et al.*, 1994) with various degrees of success. Belew *et al.* (1987) found that copper leaked from the column when glycine was present in the elution buffer and that zinc and nickel also leaked if a pH of 4.5 or lower was used. However, they found that the problem was reduced if ammonia was present in the elution buffer.

It was therefore necessary to look for other possible supports which would also allow for one-step purification of proteins from crude mixtures and have the ability to work efficiently at large-scale. Instead of having a rigid support matrix as is present in columns, the possibility of a mobile support having all the ideal criteria was thought to be a practicable solution. Such supports can be small and be able to move freely in the crude liquors. Investigations into the use of magnetic supports proved promising as they could be non-porous, easily collected by the application of a magnetic field, and spherical so the surface area available for binding was maximised.

1.8.1 Magnetic supports

Magnetic supports were first used by Robinson *et al.* (1973) to immobilize enzymes which were then able to be retrieved from liquor containing colloids or undissolved solids. Since then there has been a great deal of interest in this department in the use of such supports but mainly for enzyme immobilization. Munro *et al.* (1977) reported the use of polyacrylamide-iron oxide particles as supports for bioaffinity adsorbents for use in enzyme purification. Various other supports were tested such as cellulose, nylon, nickel powder, ferrite powder, stainless steel and rock magnetite. Fine magnetic particles were prepared by the oxidation of Fe^{2+} with nitrite, activated with silane and, with the aid of glutaraldehyde, combined with enzymes (Shinkai *et al.* 1991). Depending on the ratio of the two components, particles range in size from 4-70 nanometers.

In general, four methods have been used to prepare non-porous magnetic supports. Silane coupling reagents have been used to attach an organic functional group to the surface of a magnetic particle. It is possible to adsorb biological polymers to the surface of the support (often with polyethylene glycol). Magnetic particles can be encapsulated or a thin layer of polymer can be crosslinked over the particle surface.

Non-porous magnetic supports can have several desirable properties such as resistance to corrosion in aqueous suspension, ligands can be both active and stable on the surface, they are sufficiently magnetically responsive to allow easy collection, and there is no magnetic agglomeration. Until the 1980s, extraction of magnetic supports at large-scale was difficult to achieve as most commercial magnetic separators were not strong enough (field strength below 0.5 Tesla). Since then advances in technology have provided high gradient magnetic separation (HGMS) having field strengths as high as 5 Tesla. HGMS machines use a combination of high field strength throughout the working volume and a high voidage magnetic matrix material in the working solution.

1.8.2 The use of magnetic extraction

Molecules and materials can be divided into two groups - those with unpaired electrons which are attracted to regions of high magnetic field, and those whose electrons are spin-paired and hence are repelled from high field regions. Magnetic separation techniques have been in use for several decades but their applications in technology have been limited due to the inability to generate the high magnetic fields that were necessary for large-scale protein isolation. Several systems are now commercially available and have been used to isolate molecules as diverse as messenger ribonucleic acid (mRNA), lymphocytes, and whole cells. The beads can be coated with a range of substances to allow efficient binding of the required protein to the beads to occur. Residual magnetism causes permanent aggregation of particles and has severely limited their use in biological applications. Superparamagnetism allows the particles to respond to the magnetic field but not to have any magnetic memory and hence aggregation does not occur and the particles can be reused.

Initial work by Molday *et al.* (1977) described a cell surface probe consisting of iron-containing polymeric microspheres which were tagged with fluorescent dyes and chemically coupled to antibodies or lectins and were used for the magnetic separation of red blood cells and lymphoid cells, and also in the detection of immunoglobulin receptors and wheat germ agglutinin receptors on lymphocytes and HeLa cells by scanning electron and fluorescent microscopy. The average size of the particles was 40 nm.

In the same year Mosbach and Andersson (1977) described the use of magnetic fluids containing particles of oxides as affinity chromatography gels. The ultramicroscopic particles were stabilized by a coating and the overall diameter of each was approximately

13 nm. The particles were found to behave like true homogeneous fluids and were highly susceptible to magnetic fields. The usefulness of the magnetic Sepharose beads was shown in the purification of horse liver alcohol dehydrogenase from crude extracts which was accomplished in one step. Thus pre-formed polymers already substituted with ligands could be magnetised without alteration of the affinity properties of the gel and used to specifically extract the desired protein from a crude mixture.

Little work was carried out to advance the technology until Safarík (1991) used magnetic affinity adsorbents, based on chitin, agar and agarose, to isolate hen egg white lysozyme. Hen egg white was diluted with water, and after mixing the precipitated proteins were removed by centrifugation. The supernatant was incubated with the affinity adsorbent at room temperature and the magnetic particles were collected with a permanent magnet placed outside the reaction container. The gels were washed several times with water after which the lysozyme was desorbed from the magnetic adsorbants with HCl. All three adsorbents had the ability to bind lysozyme from the hen egg white, with magnetic chitin having the highest capacity. However, all three showed adsorption of contaminating proteins. Purities in the range of 8 times with respect to the crude hen egg white were achieved.

As mentioned above, there are now several systems available commercially which use magnetic extraction as a means of purification of various molecules from crude mixtures. For example, Promega market a RNA purification system - PolyATtract® System 1000 which has the ability to isolate mRNA from total RNA by magnetic separation. Biotinylated oligo(dT) is annealed in solution to mRNA. Since there is an affinity of biotin for streptavidin, the desired protein is then selectively extracted with Streptavidin-coated MagneSphere® Paramagnetic Particles. These particles may also be used in the magnetic separation of M13 single-stranded DNA when coupled to a biotinylated oligonucleotide which is complimentary to sequences in that DNA.

Dynabeads® M-280 (Dynal International, Norway) are uniform superparamagnetic polystyrene beads for use in molecular biology having a diameter of 2.8 μm and a surface area of 5-8 m^2/g. Dynabeads $Oligo(dT)_{25}$ can be used for the isolation of polyadenylated mRNA from eukaryotic sources. The beads can be coated in streptavidin for the isolation of biotinylated compounds as in Dynabeads *lacZ* which have a biotinylated oligonucleotide complimentary to a part of the *lacZ* region in different vectors. Other ligands can be attached to the core beads such as antibodies and used for the purification of antigens.

Another company, Advanced Magnetics Inc., (Massachusetts, USA) manufacture BioMag® products which consist of suspensions of coated magnetic iron oxide particles to provide functional groups which permit covalent attachment of proteins or ligands with the retention of biological activity. The particles are available with a variety of

coatings (amine-, carboxy-, thiol-terminated, protein A, avidin) and a wide variety of biological molecules can be attached to BioMag particles (such as antibodies, antigens, lectins, enzymes, biotin). All BioMag products consist of the same core of magnetic material, between 0.5-1.5 μm in diameter having a surface area of greater than 100 m^2/g. These non-porous particles are irregular in shape and therefore have a far greater surface area than spherical or bead-like supports of the same dimensions. The speed of separation of the BioMag depends on the experimental conditions including the viscosity, the number of magnets used, and the volume used.

Of the core magnetic beads available, the BioMag® were chosen as they could be easily coated, had an extremely large surface area, were uniform in size and did not possess any magnetic memory. The technology for the production of the beads used in this thesis was developed elsewhere in this department (O'Brien *et al.*, 1996[a]). The method for preparation of the non-porous magnetic particles used in the experimental work in this thesis is outlined in the material and methods chapter (section **2.4.18**) and a diagrammatic representation of the cross-section of a bead is illustrated in **Figure 2.1**.

1.9 The aim of this project

An ideal purification procedure maximises the yield of the desired product in as pure and active a form as possible. One way in which the overall yield of the protein can be increased is to reduce the number of purification steps involved. Immobilized metal affinity chromatography is becoming a widely used technique for the one-step purification of proteins from crude cell lysates. The system used is based on the affinity of certain amino acid residues (His, Cys and Trp) on the protein for specific metal ions (first row transition metal ions and zinc). Most IMAC procedures are only capable of small-scale purification due to limitation imposed by the matrix.

The aim of this thesis was to utilise the knowledge available for genetic manipulation of genes and IMAC to devise a novel system for use as a generic one-step purification system for the recovery of His-containing proteins expressed in *E. coli* by the application of the principles behind affinity chromatography. This system was to be tested at bench-scale but with the possibility of scale-up in mind.

The chosen protein was bacteriophage T4 lysozyme which was available on a plasmid vector. This Cys-free protein has been extensively studied and it is known to contain one inaccessible His residue. The N- and C-termini are also known to be away from the active site so that manipulation of the gene in these areas will not affect the activity of the enzyme. The protein was modified at the DNA level, by site-directed mutagenesis, to incorporate a polyHis affinity tail on the C-terminus of the protein. To verify that the addition of the polyHis tail did in fact allow one-step purification from

crude cell lysate as predicted, a crude liquor was applied to affinity columns. The activity of the recombinant protein was indeed unaffected by the incorporation of the tag.

Once verification of the recombinant protein structure had been achieved (by sequence analysis), the retention of the protein on Cu-, Zn-, and Ni-charged chelated Sepharose columns was determined. Using an extension of this technology, novel charged magnetic particles (charged with the same metal ions as the columns) were successfully used in the attempt to isolate the same recombinant protein to a similar degree from a similar crude mixture. The novel particles were free to mix with the solution and collected by the application of a magnetic field. As with the columns, the magnetic support could be washed and the recombinant protein efficiently eluted from the beads.

As these supports were novel, many parameters were investigated so as to maximise the yield of the recombinant lysozyme, information which is necessary to have available for possible scale-up purification procedures. However, it is appreciated that the parameters have been refined for the isolation of the bT4l and some conditions may need to be altered for their use in the isolation of other proteins. It is envisaged that the use of these novel particles may be easily extended as a generic technique to large-scale batch isolation of many polyHis-tagged recombinant proteins.

CHAPTER TWO

MATERIALS AND METHODS

2.1 Materials

2.1.1 Chemicals

All chemicals, unless otherwise stated, were supplied by BDH Ltd., (Poole, Dorset, UK) and were of AnalaR grade or of the highest grade available.

5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal) and iso-propyl-β-D-thiogalactoside (IPTG) were supplied by Northumbria Biologicals Ltd. (Cramlington, Northumberland, UK).

The dye concentrate used in the Bradford protein assays was purchased from Bio-Rad Laboratories (Hemel Hempstead, Herts., UK), and that used in the Pierce® Coomassie and the BCA protein assays were provided by Pierce & Warriner (UK) Limited (Chester, UK). All were used according to the manufacturer's instructions.

Radiochemicals / [^{35}S]-αdATP were obtained from Du Pont NEN (Stevenage, Herts., UK).

2.1.2 Bacterial strains and plasmids

For details of growth requirements and genotypic/phenotypic features of strains and plasmids used see **Table 2.1** and **Table 2.2** respectively. Strain maintenance was achieved by growing *Escherichia coli* on nutrient agar plates containing selective antibiotics as required according to plasmid phenotype. However, in order to retain the F' plasmid in cells used for transformation in *E. coli* JM107, this strain was maintained on minimal medium plates (see section **2.3.6**).

Glycerol stocks were prepared from selective agar plates by the addition of 3 ml sterile 20% (w/v) glycerol and scraping the colonies from the surface of the agar with a sterile glass rod. The cells were then stored at -70°C. A loopful of frozen glycerol stock was sufficient to inoculate a selective agar plate.

2.1.3 Antibiotics

All were purchased from Sigma Chemical Co. (Poole, Dorset, UK).

	Stock solutions	Final concentration in media
Ampicillin (Amp)	10 mg/ml (in H_2O)	100 μg/ml
	100 mg/ml (in H_2O)	100 μg/ml
Chloramphenicol (Cm)	10 mg/ml (in ethanol)	10 μg/ml
Tetracycline (Tet)	5 mg/ml (in ethanol)	15 μg/ml

Antibiotics were stored at -20°C and added to the media as required (for agar, added when it was still molten but below 60°C).

Table 2.1 Bacterial strains

Bacterial strain	Genotype	Reference
Escherichia coli JM107	*endA*1, *gyrA*96, *thi*1, *hsdR*17, *supE*44, *relA*1, λ⁻, *Δ*(*lac-proAB*), [F', *traD*36, *proAB*, *lacI*^{q}ZΔM15]	Yanish-Perron, C., *et al.*, (1985)
Escherichia coli BMH71-18*mutS*	*thi1*, *supE*, *Δ*(*lac-proAB*), [*mutS*::Tn10], [F', *proAB*, *lacI*^{q}ZΔM15]	Wallace, R.B., *et al.*, (1981)
Escherichia coli CJ236	*dut*1, *ung*1, *thi*1, *relA*1, *deo*R$^+$, pCJ105(Cm^r, F')	Invitrogen Corporation, San Diego, California, USA.

Table 2.2 Plasmids

Plasmid	Phenotype	Reference
pHSe5	Amp^r	Donation from B.W.Matthews. Muchmore *et al.*, (1989)
pUC-f1	Amp^r	Pharmacia Biosystems Ltd., Milton Keynes, UK.
pBluescript®II KS(-)	Amp^r	Stratagene, La Jolla, California, USA
pALTER™-1	Tet^r, Amp^s	Promega Corporation, Southampton, UK.

2.1.4 Restriction Enzymes

Restriction enzymes were obtained from either Northumbria Biologicals Ltd. or New England Biolabs Inc., USA., as were the T4 DNA ligase, T4 DNA polymerase, and T7 DNA polymerase. All were stored at -20°C.

2.1.5 Oligonucleotides

All oligonucleotides were obtained from Genosys Biotechnologies Inc. (Cambridge, UK) in a lyophilised form and each were resuspended with 1 ml sterile distilled water.

2.2 Buffers and solutions

All buffers and solutions were prepared with double-distilled deionized water and autoclaved at 15 psi for 20 minutes unless stated differently.

2.2.1 IPTG

Stock solution of 200 mg/ml in water was filter sterilised through 0.22 μm filters (Acrodisc, Gelman Sciences, Ann Arbor, USA) after which it was stored at -20°C. The solution was not autoclaved.

2.2.2 X-gal

Stock solution of 20 mg/ml in dimethylformamide. The universal was wrapped in aluminium foil and stored at -20°C. The solution was not autoclaved.

2.2.3 Tris Borate EDTA (TBE) buffer (x 10)

Per litre:	Trizma base (Sigma)	108 g	(90 mM)
	Boric Acid	55 g	(90 mM)
	Na_2EDTA	9.3 g	(10 mM)

Working concentration TBE x 0.5.

2.2.4 Tris Acetate EDTA (TAE) buffer (x 50)

Per litre:	Trizma base (Sigma)	242 g
	Glacial acetic acid	57.1 ml
	0.5M EDTA pH 8.0	100 ml

Working concentration TAE x 1.

2.2.5 Tris EDTA (TE) buffer (x 100) pH 8.0

Per litre:	Trizma base (Sigma)	121 g
	Na_2EDTA	38 g

Working concentration of TE x 1.

2.2.6 Universal restriction buffer x 10

Tris-HCl pH 7.5	500 mM
$MgCl_2$	50 mM

2.2.7 Final sample buffer

Bromophenol blue	0.25%
Xylene cyanol	0.25%
Ficoll Type 400	15%

The solution was filtered and stored at room temperature.

2.2.8 Solutions for the isolation of plasmid DNA (miniprep. method)

This method is from Sambrook *et al.*, 1989

Solution 1:	glucose	50 mM
	Tris-HCl pH 8.0	25 mM
	EDTA pH 8.0	10 mM
Solution 2:	Made fresh, not autoclaved.	
	NaOH	0.2 N
	SDS	1%
Solution 3:	5 M Potassium acetate	60 ml
	Acetic acid	11.5 ml
	Distilled water	28.5 ml

2.2.9 RNAase A type I

A stock solution of 10 mg/ml in TE x 1 buffer was boiled for 10 minutes, allowed to cool to room temperature, and stored at -20°C. This solution was not autoclaved.

2.2.10 Phenol : chloroform

Phenol was stored at -20°C. To equilibrate the phenol to a pH > 7.8, it was allowed to warm to room temperature and then melted at 68°C. Hydroxyquinoline was added to a final concentration of 0.1%. An equal volume of 0.5 M Tris-HCl, pH 8 was added to the melted phenol and stirred for 15 minutes. The solution was allowed to stand until the two phases had separated when the aqueous phase was aspirated and discarded. This step (addition of Tris-HCl) was repeated until the pH of the phenolic (lower) phase had a pH greater than 7.8. When the final aqueous phase was removed, 0.1 volume of 0.1 M Tris-HCl, pH 8 containing 0.2% β-mercaptoethanol was added as was an equal volume of chloroform. The resulting solution was stored in the dark at 4°C and the solution was not autoclaved.

2.2.11 Caesium-saturated isopropanol

TES:	Tris-HCl pH 8.0	50 mM
	NaCl	50 mM
	EDTA	5 mM

Ten grams of CsCl were dissolved in 10 ml TES. Forty millilitres of isopropanol were added and mixed. The solution was allowed to settle and the upper layer was always used. The solution was not autoclaved.

2.2.12 Acrylamide stock solution (36%) for sequencing gels

For 200 ml:	Acrylamide	68.4 g
	N,N' Methylenebisacrylamide	3.6 g
	Distilled water to 200 ml.	

4.5 g Amberlite MB1 beads (Bio-Rad Laboratories, Hemel Hempstead, Herts., UK) were added and the mixture was stirred gently for 30 minutes. Whatman 3MM chromatography paper was used to filter the solution and it was stored at 4°C. The solution was not autoclaved.

2.2.13 Sequencing gel (6% acrylamide)

For 80 ml:	TBE x 10	8 ml
	Urea	33.6 g
	Acrylamide stock solution	13.3 ml

Made up to 80 ml with distilled water.

0.17 ml 10% ammonium persulphate and 0.054 ml TEMED (Bio-Rad) were added to crosslink the matrix and the gel was poured as quickly as possible.

The sequencing gels were preelectrophoresed (usually at 1400 volts for 1 h) prior to loading the samples and run at a constant 1400 volts for 2.5 h (or until the first dye/bromophenol blue has just run off the bottom of the gel).

2.2.14 Sequencing gel fixer

Acetic acid : methanol : water

1 : 1 : 8

The gel was soaked in fixer for approximately 20 minutes at room temperature and rinsed with distilled water before drying at 80°C for 90 minutes.

2.2.15 30% Acrylamide/bis solution for SDS-PAGE gels

For 100 ml:

Solution 1 : Acrylamide	30 g in 50 ml distilled water
Solution 2: *N,N'*Methylenebisacrylamide	0.8 g in 20 ml distilled water with gentle heating.

The above two solutions were mixed and the volume was made up to 100 ml with distilled water. The solution was then filtered through Whatman 3MM chromatography paper and stored at 4°C. This solution was not autoclaved.

2.2.16 SDS-PAGE Tank buffer

x 5 :	Tris base (Sigma)	30 g
	Glycine	144 g

Distilled water to 1 litre.

x 1 :	5 x tank buffer	200 ml
	Distilled water to	990 ml
	10% SDS	10 ml

2.2.17 SDS-PAGE Sample buffer

2-mercaptoethanol	0.2 ml
SDS	0.2 g
Glycerol	4 ml
Bromophenol blue	8 mg

Made up to 10 ml in 0.1 x stacking gel buffer. An equal volume of sample buffer was added to the sample and the mixture was heated at 95°C for 5 minutes before loading onto the gel.

2.2.18 SDS-PAGE Gel

This method is based on that of Laemmli (1970).

For 10 ml Resolving gel:	12%	15%
Resolving buffer (Tris-HCl pH 8.8)	2 ml (2 M)	2.5 ml (1.5 M)
30% Acrylamide/bis	4 ml	5 ml
10% SDS	0.1 ml	0.1 ml
Distilled water	3.85 ml	2.26 ml
10% Ammonium persulphate	0.05 ml	0.1 ml
TEMED (Bio-Rad)	0.005 ml	0.006 ml

The gel was poured as quickly as possible, and allowed approximately 1 hour for gel to set. An overlay of water or water-saturated butanol was applied to the top of the

poured gel while polymerisation took place. When the gel had set, the overlay was rinsed away and a 5% stacking gel was poured above.

For 10 ml 5% Stacking gel:

Stacking gel buffer (1 M Tris-HCl pH 6.8)	1.25 ml
30% Acrylamide/bis	1.67 ml
10% SDS	0.1 ml
Distilled water	6.75 ml
10% Ammonium persulphate	0.1 ml
TEMED (Bio-Rad)	0.01 ml

The gel was poured as quickly as possible, the comb inserted and allowed to set.

The gel was run at a constant current of 20 mA until the dye-front was at the bottom of the gel. It was then fixed for approximately 20 minutes at room temperature with gentle shaking (see section **2.2.20** below).

2.2.19 Coomassie blue stain

20% Methanol

10% Glacial acetic acid

0.2% Coomassie blue R250 (LKB), made up in distilled water.

The gel was stained in this solution for approximately 1 h at room temperature with gentle shaking.

2.2.20 Destain/fixative for SDS-PAGE gels

30% Methanol

7% Glacial acetic acid

Gels were fixed and destained with gentle shaking at room temperature. The solution was changed as and when necessary during the destaining process.

2.2.21 Silver stain for SDS-PAGE gels

Silver Stain Plus kit (Bio-Rad Catalogue No. 161-0449) was used when silver staining gels according to the manufacturer's instructions.

2.3 Media

Media were supplied by the following companies:

Nutrient agar	Oxoid Ltd., Basingstoke, Hampshire
Nutrient Broth No. 2	Oxoid Ltd., Basingstoke, Hampshire
Yeast extract	β-lab, East Molesey, Surrey
Bacteriological agar	Gibco BRL, Paisley, Scotland
Bactotryptone	Difco, Detroit, USA

All media were prepared in distilled deionised water and autoclaved at 15 psi for 20 minutes.

2.3.1 Nutrient broth and nutrient agar

Used according to manufacturer's instructions.

2.3.2 Luria-Bertani (LB) broth and agar

Broth per litre :	Bactotryptone	10 g
	Yeast extract	5 g
	NaCl	10 g

Agar: as above and added 1.5% bacteriological agar prior to autoclaving.

2.3.3 TYP broth

Broth per litre :	Bactotryptone	16 g
	Yeast extract	16 g
	NaCl	5 g
	K_2HPO_4	2.5 g

2.3.4 Lysozyme activity plates - media

LB bottom agar (1.5% agar) contained ampicillin (100 μg/ml) and 5 mg IPTG/plate. Top agar contained 2 ml LB top agar (0.7% agar) and 0.5 ml of an overnight culture of pUC18 in *E. coli* JM107. See method in section **2.4.7**.

2.3.5 *Lac Z* indicator plates

Nutrient agar or LB agar incorporated with X-gal to final concentration of 40 μg/ml, and IPTG to a final concentration of 20 μg/ml.

2.3.6 Minimal media plates

(For the preparation of competent cells)

M9 salts x 5	$Na_2HPO_4.2H_2O$	6.4 g
	KH_2PO_4	1.5 g
	NaCl	0.25 g
	NH_4Cl	0.5 g

Distilled water was added to yield a final volume of 100 ml.

1.5 g bacteriological agar was dissolved in 78 ml distilled water and autoclaved. The following components (all of which had been individually sterilised) were added when the media temperature reached approximately 60°C:

5 x M9 salts	20 ml
20% D- glucose	2 ml
thiamine (2 mg/ml)	0.1 ml (filter sterilised through a 0.22 μm Acrodisc)
1 M $CaCl_2$	0.01 ml
1 M $MgSO_4$	0.2 ml

2.4 Methods

2.4.1 Isolation of plasmid DNA

2.4.1.1 Small-scale method -

This method is the alkaline lysis method found in Sambrook *et al.* (1989). Typical DNA yields from 1.5 ml overnight culture were 15-20 μg.

2.4.1.2 Large-scale method

An overnight culture was set up with 5 ml broth and appropriate antibiotic(s). 2 ml of this overnight was used to inoculate 400 ml broth (plus antibiotic) and the 2 l flask was shaken at 37°C overnight.

The culture was harvested by centrifuging at 8,000 rpm (Sorvall® Superspeed RC2-B centrifuge, GSA rotor) for 10 minutes. The pellet was resuspended in a total of 20 ml TE x 1. 50 ml alkaline SDS (0.2 M NaOH, 1% SDS) was added and the contents were mixed thoroughly by inversion. 40 ml of 4 M NaAcetate pH 6.5 was mixed well with the viscous solution and then the tube was stored on ice for a minimum of 15 minutes. The tube was then centrifuged at 9,000 rpm (Sorvall® Superspeed RC2-B centrifuge, GSA rotor) for 25 minutes at 4°C.

The supernatant was poured into a clean centrifuge pot, 85 ml isopropanol added, mixed well, and placed on ice for 15-30 minutes. The tube was centrifuged at 9,000 rpm (Sorvall® Superspeed RC2-B centrifuge, GSA rotor) for 10 minutes at 4°C.

The pellet was resuspended in 5 ml TE x 1 containing RNAase I (20 µg/ml). The contents were incubated at 37°C for 15-30 minutes, phenol chloroform extracted, and the top layer ethanol precipitated (1 ml 5 M NaCl and 10 ml ethanol) by standing on ice for 5 minutes. The tube was then centrifuged at 10,000 rpm (Sorvall® Superspeed RC2-B centrifuge, GSA rotor) for 10 minutes and the supernatant discarded.

The pellet was dried well before resuspension in 9 ml TE x 1 containing 9.9 g CsCl. Ethidium bromide solution was added to a final concentration of 100 µg/ml. The tube contents were placed into an ultracentrifuge tube (Beckman Quick-Seal™) and centrifuged at 40,000 rpm (Beckman L7 Ultracentrifuge, 70.1 Ti rotor) for 36 h.

The ultracentrifuge tube was observed under long-wave UV light and two bands were observed (top band is chromosomal DNA, and bottom band is plasmid DNA). The bottom band was therefore removed from the tube and the ethidium bromide extracted using caesium-saturated isopropanol. The bottom layer was repeatedly extracted with the isopropanol until the pink colouration had disappeared. The volume was made up to 5 ml with TE x 1, 0.5 ml 5 M NaCl and 10 ml ethanol added, mixed and placed at -20°C for a minimum of 30 minutes to precipitate the DNA. It was then centrifuged at 10,000 rpm (Sorvall® Superspeed RC2-B centrifuge, SM34 rotor) for 10 minutes, the pellet dried and resuspended in 2 ml TE x 1. A 10 µl sample was run on an agarose gel to test the concentration of the DNA and the remaining DNA was aliquoted and stored at -20°C.

2.4.2 Restriction enzyme digestions

Typically 0.5 µg DNA was digested with 10 units of the required restriction enzyme with 1/10 total digest volume of 10 x restriction buffer and the volume made up to 20 µl with sterile distilled water. The digests were then incubated in a 37°C waterbath for 2 h.

2.4.3 Ligation reactions

All ligation reactions were performed as described by Sambrook *et al.* (1989). Typically equimolar amounts of vector and insert DNA were mixed in as small a volume as possible and the volume made up to 8 µl with sterile distilled water. To this were added 1 µl 10 x ligase buffer and 1 µl T4 DNA ligase (10 units/µl). The ligation reactions were carried out at 4°C overnight.

2.4.4 Agarose gel electrophoresis

The agarose was obtained from Sigma Chemical Co. (St. Louis, USA) and was Type V: High Gelling Temperature. The working concentration was usually 1% in TBE buffer x 0.5, and the running buffer was TBE x 0.5. The electrophoresis apparatus was an Horizon™ 58, supplied by Gibco BRL Life Technologies Ltd. (Paisley, Scotland).

Gels were run at a constant 100 volts for 1 h, and then soaked in a bath of ethidium bromide solution (0.5 μg/ml) for 10 minutes.

After staining, the gel was viewed under a UV transilluminator and photographed using Ilford HP5 Plus film (10.2 x 12.7 cm), and the film developed and fixed with Ilford Microphen Film Developer and Ilford Microphen Film Fixer, respectively (according to the manufacturer's instructions).

Molecular size markers were run simultaneously to samples on the agarose gels and were typically λ DNA (Promega Corporation, Southampton, UK) digested to completion with *Pst*I.

2.4.5 Preparation of competent *Escherichia coli* cells

An *E. coli* colony was taken from a minimal media plate and used to inoculate 5 ml nutrient broth overnight. 1 ml of this culture was used to inoculate 200 ml nutrient broth containing 20 mM $MgCl_2$. This was grown until it had reached an optical density absorbance value at 600_{nm} of 0.4-0.5. The cells were spun down, washed once with 50 ml ice-cold 75 mM $CaCl_2$, 15% (v/v) glycerol, and finally resuspended in 5 ml of the same solution. This was aliquoted into 0.5 ml amounts and stored at -70°C.

2.4.6 Transformation of *E. coli* competent cells with plasmid DNA

For each transformation reaction one aliquot of frozen competent cells was thawed and the circular plasmid DNA was added (typically 0.2-1 μg). The tube was vortexed gently to mix the contents and left on ice for 45 minutes. The cells were then heat-shocked by placing them in a 37°C waterbath for 10 minutes, ice for 5 minutes and then added the contents to 5 ml nutrient broth and grown at 37°C for 1 h. The transformation mixture was plated out onto an appropriate selective agar plate, and incubated overnight at 37°C.

2.4.7 Lysozyme activity plates - method

This method is similar to that used by Alber and Matthews, 1987. Glass petri dishes were used. LB bottom agar (1.5% agar) contained Ampicillin (100 μg/ml) and 5 mg IPTG/plate. Top agar contained 2 ml LB top agar (0.7% agar) and 0.5 ml of an overnight culture of pUC18 in *E. coli* JM107. Plates were incubated at 37°C for 5-8 hours to allow lawn to grow. The lawn was exposed to UV light (UV Transilluminator, Ultra-Violet Products, Inc., San Gabriel, CA, USA) for two minutes by inverting the petri dish and placing it directly on top of the transilluminator. To ensure that the lawn was killed, a piece of filter paper impregnated in chloroform was placed in the lid of the petri dish, the plate was inverted and then placed in a fume cupboard for 30 minutes. The bacteria for testing were spotted onto the surface of the lawn and the plates were

incubated overnight at 30°C. The colonies yielding the largest zones of clearing of the lawn were taken to be the best lysozyme producers.

2.4.8 Preparation of phagemid single-stranded DNA for the pALTER™-1 mutagenesis system

This is the method used to isolate single-stranded DNA using the successful pALTER-1 mutagenesis system as described in Chapter 3 section **3.3.4**.

A colony of *E. coli* containing pQR195 was used to set up a 2 ml overnight culture in TYP broth (15 μg/ml tetracycline) at 37°C. 0.1 ml of the overnight culture was used as a starter culture to inoculate 5 ml TYP broth (with tetracycline) in a 50 ml flask, which was then shaken vigorously for 30 minutes at 37°C. The culture was then infected with M13K07 helper phage (at a multiplicity of infection of 10) and also added K_2HPO_4 (to a final concentration of 20 mM to improve single-stranded DNA yields). The flask was then vigorously shaken overnight, after which time the supernatant was harvested by centrifugation at 10,000 rpm (Sorvall® Superspeed RC2-B centrifuge, SS34 rotor) for 15 minutes, the supernatant respun (to ensure that all cells were pelleted), and then the phage was precipitated (0.25 volume of a solution of 3.75 M ammonium acetate, pH 7.5, and 20% polyethylene glycol [MW 8,000]). After leaving the mixture on ice for 30 minutes, it was then centrifuged at 10,000 rpm for 15 minutes and the pellet thoroughly drained, and resuspended in 0.4 ml TE buffer. To remove excess polyethylene glycol, 0.4 ml chloroform : isoamyl alcohol (24 : 1) was added and the mixture was vortexed, and centrifuged at 12,000 rpm (Eppendorf microfuge) for 5 minutes. The aqueous phase was transferred to a fresh tube and phenol : chloroform extracted followed by several phenol extractions. The phagemid DNA was then ethanol precipitated and the dried pellet resuspended in 20 μl sterile distilled water.

2.4.9 Mutagenesis procedure for the pALTER™-1 mutagenesis system

This step is a follow on step to that of section **2.4.8** above used in the mutagenesis procedure of the pALTER-1 system found in Chapter 3 section **3.3.4**.

Having obtained single-stranded DNA it was then possible to carry out the mutagenesis procedure. The ampicillin repair oligo was used at a 5 : 1 oligo : template ratio and the mutagenic oligo at a 25 : 1 ratio, with approximately 100 ng template DNA. The annealing reaction was heated to 70°C for 5 minutes, allowed to cool very slowly to room temperature, placed on ice, and T4 DNA polymerase and T4 DNA ligase added along with synthesis buffer. In order for mutant strand synthesis and ligation to occur, the reaction was maintained at 37°C for 90 minutes. The entire contents of the reaction tube was used to transform competent cells of strain BMH 71-18 *mut S*. After allowing the cells to recover in broth for 1 h at 37°C, ampicillin was added (125 μg/ml), and

the incubation was continued overnight. The DNA was then extracted from 1.5 ml of overnight culture and approximately 0.1 μg of plasmid DNA was used to transform JM107. Selection was carried out using LB agar containing ampicillin (125 μg/ml). Ampicillin resistant colonies were also tested for tetracycline resistance before DNA was isolated and checked by restriction enzymes. All positive clones were then analysed by direct sequencing.

2.4.10 Routine growth and expression of the recombinant protein

Cultures were routinely grown up from colonies from a fresh, selective master plate. Colonies from such plates were inoculated into 5 ml Nutrient broth No.2 containing ampicillin (100 μg/ml) and the culture was shaken on a reciprocal shaker overnight at 37°C. The following morning 200 ml of prewarmed Nutrient broth (containing the selective antibiotic) in a 1 litre Erlenmeyer flask was inoculated with 2 ml of the overnight culture and shaken at 37°C, 300 rpm (orbital shaker, Model G25, New Brunswick Scientific Co. Inc., Edison, New Jersey USA) until OD_{600} of around 0.8-0.9 (late logarithmic/early stationary phase) was achieved (typically 3 hours). The cultures were then induced with IPTG to a final concentration of 1 mM and cell growth continued at 30°C, 150 rpm. After incubating for 2.5 hours post-induction, the culture was harvested (see sections **2.4.11** and **2.4.12**).

2.4.11 Harvesting of culture for column separation

Due to the leaky nature of the induced culture (see Chapter 3 section **3.4.2**), the cells were disrupted directly into the media and prepared as outlined below. After incubating the culture for 2.5 hours post induction, the entire culture (cells plus medium, total volume approximately 200 ml) was directly sonicated on ice (Soniprep 150, Fisons, England) for three 1 minute cycles at 8 μ amplitude. The debris was spun out by centrifugation at 8,000 rpm, 10 minutes (Sorvall® RC-5B centrifuge, GSA rotor), dialysed against the column start buffer at 4°C, two times for a period of two h each. The sample was stored at 4°C and filtered through a 0.22 μm filter (Acrodisc) before loading onto the column. On average the sample contained approximately 0.28 mg of total protein /ml as determined by the Bio-Rad Bradford assay.

In all column experiments, the sample loaded onto each column was freshly prepared and used within 48 h.

2.4.12 Harvesting of culture for use on charged magnetic supports

After incubating the 200 ml culture for 2.5 hours post induction, the cells were harvested by centrifugation at 8,000 rpm for 10 minutes (Sorvall® Superspeed RC2-B centrifuge, GSA rotor) and washed with one fiftieth the volume of the experimental

binding buffer (usually 20 mM Na_2HPO_4, 0.5 M NaCl, pH 7.2) before sonication in eppendorf tubes (three times on ice for 20 seconds, 8 μ amplitude). Unclarified (crude) samples were then spun at 13,000 rpm in a bench microfuge to pellet the debris and the supernatant was aspirated into a fresh tube thereby constituting the clarified sample. On average the crude sample contained approximately 3.8 mg total protein/ml, while the clarified sample contained approximately 2.75 mg total protein/ml (as determined from the Bio-Rad Bradford assay).

In all experiments with the magnetic supports, a fresh culture was set up for each experiment. Samples were stored at 4°C for a maximum of 24 h before use.

2.4.13 Double stranded sequencing

Double stranded sequencing was performed with the aid of the Pharmacia T7 Sequencing™ Kit (Catalogue number 27-1682-01, Pharmacia LKB Biotechnology AB, Uppsala, Sweden).

Approximately 2 μg DNA were placed into a sterile 1.5 ml eppendorf tube and denatured by the addition of two-fold volume of 0.2 M NaOH, 0.2 mM EDTA, vortexed and incubated at 37°C for 5 minutes. The DNA was ethanol precipitated by adding one tenth of the volume of 2 M ammonium acetate pH 4.5 and two volumes of ethanol, vortexing and placing tube at -20°C for 20 minutes. The tube was then spun at full speed for 5 minutes, the ethanol removed carefully, and the pellet washed with 70% ethanol, and recentrifuged for a further 5 minutes with the tube in the same orientation. The supernatant was removed and the pellet dried in a speedyvac (Gyrovap DNA Concentrator, V.A. Howe & Co. Ltd., Oxon, UK). The pellet was then resuspended in 20 μl sterile distilled water and aliquoted into 2 x 10 μl amounts. Forward or reverse primer, 0.8 pmol, was added to one aliquot followed by 2 μl annealing buffer. The second aliquot could be stored at -20°C or used with the reverse primer and thereby sequence the second strand.

2.4.13.1 Sequencing all 4 bases

The tube containing the DNA, primer, and the annealing buffer (14 μl in total) was incubated in a 65°C waterbath for 5 minutes, 37°C for 20 minutes and room temperature for 10 minutes. The enzyme premix cocktail for each primer/template to be sequenced contained a mixture of 10 μCi α-^{35}S-dATP (10 μCi/μl), 5 units T7 DNA polymerase (4 units/μl), and 3 μl A-labelling mix. When the primer/template mixture had cooled to room temperature, 5.25 μl of the enzyme premix were added to the tube, mixed by vortexing and spun for 1 second. The tube was incubated at room temperature for 5 minutes for the extension reaction to take place. For each DNA sample to be sequenced 4 microfuge tubes were labelled A, T, C, G, and 3 μl of the appropriate

"short" reaction mix were added to the corresponding tubes. 4.5 µl post extension mix were added to each of the four tubes containing the reaction mixes using a fresh tip for each transfer. The contents of the tubes were vortexed and spun down before incubation at 37°C for 5 minutes after which time the termination reaction was completed. 3 µl of sequencing stop mix was then added to each tube, the lids pierced and the tubes placed into a simmering waterbath for 3 minutes. The samples (usually 4 µl) could then be loaded onto the sequencing gel and electrophoresed or stored at -20°C for future analysis.

2.4.13.2 Sequencing one base

This method allows rapid screening of a number of samples and is especially useful if screening for mutants as they can be directly compared to the wild type track. The method is similar to that in section **2.4.13.1** above with the following exceptions :- only 3 µl of the primer/template/annealing buffer mixture is used, and correspondingly only a quarter of the enzyme premix is made and used per template, and only one of the four short reaction mixes.

2.4.14 Lysozyme assays

The lysozyme assay used was an adaptation to that of Locquet *et al.* (1968). 25 mg lyophilised *Micrococcus lysodeikticus* (*M. lysodeikticus*) cells (Sigma Chemical Co., ATCC No. 4698) were resuspended in 100 ml 67 mM sodium phosphate buffer pH 6.2 and kept at room temperature (22°C). A standard curve using chicken egg white lysozyme (Sigma Chemical Co., EC 3.2.1.17, 46,200 units/mg solid or 48,000 units/mg solid) in 67 mM sodium phosphate buffer, pH 6.2, with concentrations 0-10 µg/ml was established for each new assay. 0.1 ml test sample was added to 0.9 ml resuspended cells in a 1 ml cuvette, mixed briefly, and the change in absorbance at 450 nm was measured for 60 seconds (Philips PU 8720 UV/VIS spectrophotometer). In this buffer system the specific activity for chicken egg white lysozyme was found to be 30,000 units/mg.

The unit definition for the T4 bacteriophage lysozyme is that 1 unit will produce a ΔA_{450} = 0.001/minute at pH 6.2 at 22°C, using a suspension of *M. lysodeikticus* as substrate (as above), in a 1 ml reaction mixture (1 cm light path).

Tsugita (1971) noted that when checking the decrease in absorbance at various wavelengths at every 50 nm from 350 to 650, no practical reason of preference was found between 450 and 650 nm.

2.4.15 Precipitation of proteins with trichloroacetic acid (TCA)

In Chapters 4 and 5 some protein samples were concentrated before loading onto SDS-PAGE gels and stained with Coomassie by the method described below. The sample volume was adjusted to 1 ml by the addition of distilled water (usually 400 µl of sample was used) and 333 µl 100% (w/v) TCA (Sigma) was added to the eppendorf tube (giving a final concentration of 25%). The tube was then vortexed carefully in order to mix the tube contents and then placed at -20°C for 15 minutes. The eppendorf was then spun at 14,000 rpm for 5 minutes at room temperature to collect the precipitate. The supernatant was carefully discarded and the pellet was washed with 1 ml acetone/HCl (5 mM HCl in acetone) by vortexing. The tube was once again spun at 14,000 rpm for 5 minutes and the supernatant discarded. The pellet was washed in 1 ml acetone by vortexing and spun at 14,000 rpm for 5 minutes, after which the pellet was dried in a 55°C oven and then resuspended in one tenth the starting volume with 2 x sample buffer (**2.2.17**). The sample had thereby been concentrated 10-fold. If the sample was not a blue colour, the pH was adjusted by the addition of Trizma base crystals (Sigma) one at a time and mixing by vortexing until the familiar Coomassie blue colour was present.

2.4.16 The use of HiTrap™ Chelating Sepharose High Performance® columns

These columns were purchased from Pharmacia LKB Biotechnology, Sweden. All columns were run at room temperature and each solution was first filtered through a 0.22 µm sieve before it passed through the columns in order to avoid clogging and unnecessary flocculation. Sodium chloride was used in all of the chromatography buffers at a concentration of 0.5 M to eliminate ion exchange effects. It is recommended by Pharmacia that the composition of the sample should be adjusted to the pH and ionic strength of the start buffer and this was achieved by dialysis as seen in section **2.4.11**. All chromatography was performed using a simple Pharmacia system (Pharmacia Biotech, St. Albans, Herts., UK) comprising of a P1 peristaltic pump connected to a FRAC100 fraction collector. The pH of individual fractions was measured using a micro-pH probe attached to a Beckman pHI12 meter.

Three different metal ions were used in the chromatographic runs - nickel, zinc, and copper. The columns were prepared as follows: firstly they were charged by passing 2.5 ml of a 0.1 M metal chloride solution (at a flow rate of 2 ml/min.) through the column. The column was then flushed with distilled water to eliminate excess unbound metal ions. A blank run was then performed in order to elute any metal ions that were unspecifically bound which might otherwise be eluted during desorption and this was achieved by passing through 25 ml of start buffer (20 mM sodium phosphate, 0.5 M NaCl, pH 7.2) which was followed by 25 ml of elution buffer (20 mM sodium

phosphate, 0.5 M NaCl, pH 3.5) all at a flow rate of 2 ml/min. The columns were then equilibrated by washing with 25 ml start buffer (2 ml/min.).

In all cases samples were then pumped directly onto the columns at a flow rate of 2 ml/min. The columns were then washed with 15.6 ml (approximately 3 column volumes) of start buffer at a flow rate of 1.3 ml/min. to wash through all unbound material. The slower flow rate was used in order to give a greater contact/reaction time of the buffers with the matrix of the column. Elution of the protein from the metal ions was carried out by a pH adjustment which was achieved by the addition of the elution buffer (also at 1.3 ml/min.) and thereby forming a gradient of decreasing pH. At-line measurements of the pH of individual fractions were then made, and in order to ensure that a low pH would not effect the biological activity of the protein, 100 µl of 1 M Tris-HCl, pH 7.0 was added to each ml of eluted sample which had a pH below 7.0 in an attempt to restore the pH to neutral. Collected fractions were assayed for lysozyme activity (see **2.4.14** for method), protein estimations (Bio-Rad Bradford assay using bovine serum albumin (BSA, from Sigma) as the standard), and protein composition (by SDS-PAGE analysis on 12% gels - see **2.2.18**).

In order to regenerate the columns, five column volumes of a solution containing 0.05 M EDTA in start buffer was passed through the column to strip the metal ions from the IDA. The column was then washed with 25 ml distilled water, and it was then ready for recharging as outlined above. If the column was to be stored, 25 ml start buffer was passed through the column after stripping and then it was washed in distilled water and stored at 4°C.

2.4.17 Preparation of magnetic chelator particles

Amine terminated superparaferromagnetic iron oxide particles (BioMag®) from Advanced Magnetics Inc. (Cambridge, Massachusetts, USA) were supplied by Metachem Diagnostics Ltd. (Piddington, Northampton, UK). These particles have an average diameter of 1 µm. Also obtained from the same company were two magnetic separation units capable of holding microcentrifuge tubes (2 ml), 15 ml and 50 ml tubes.

The magnetic particles were firstly coated with a layer of polyglutaraldehyde according to the procedure described by Haling and Dunnill (1979), this was carried out as follows: The vial of amine-terminated BioMag® ferric oxide particle suspension was vortexed well and 750 µl (equivalent to 37.5 mg) was pipetted into a clean pH-stat vessel containing 36.75 ml 2.04% (v/v) glutaraldehyde solution (Sigma Chemical Co., USA), thereby giving a final glutaraldehyde concentration of 2% (v/v). The pH was rapidly adjusted to 11 by the addition of 0.5 M sodium hydroxide using an autoburette titration instrument (Radiometer, Copenhagen, Denmark) and held there for 60 minutes. The reaction mixture was then transferred to a clean Falcon tube, the supports were

recovered by magnetic separation, and the supernatant aspirated off and discarded. The particles were washed 10 times with distilled water, twice with 1 M sodium chloride and twice more with distilled water, by cycles of resuspension, vortex mixing and magnetic recovery using successively decreasing wash volumes (after 4 washes transferred into a microcentrifuge tube). The polyglutaraldehyde coated particles were finally resuspended in distilled water to a concentration of approximately 37.5 mg/ml and stored at 4°C.

On the second day the beads were coated with an epoxy layer as follows: The polyglutaraldehyde coated particles were recovered from suspension by magnetic separation and resuspended to 400 µl with distilled water. To this 1070 µl 70% 1,4-butanedioldiglycidyl ether (Sigma) were added, followed by 15 µl 10 M sodium hydroxide and 15 µl sodium borohydride (Sigma)(60 mg/ml). The reaction mixtures were agitated vigorously on a vibrax shaker (IKA Model VXR/VX2E, Germany), setting 1400, for 6 hours at room temperature. The epoxy-activated supports were recovered magnetically and washed eight times with distilled water and twice with 20 mM sodium phosphate buffer, pH 6.8 containing 1 M sodium chloride. The particles were then resuspended in 1 ml phosphate buffer and stored overnight at 4°C.

Epoxy-activated supports were recovered by magnetic separation and resuspended to 600 µl with distilled water. To this, 400 µl 75% iminodiacetic acid (IDA, Sigma) in 2 M sodium carbonate was added, followed by 10 µl sodium borohydrate (60 mg/ml). The reaction mixture was vortexed and incubated at 60°C for 24 hours with gentle shaking.

The IDA-coupled particles were magnetically retrieved from solution, washed twice with distilled water and once with 0.1 M sodium acetate buffer pH 3.8, containing 0.5 M sodium chloride. The beads were resuspended with 1 ml ethanolamine (Sigma) and incubated at 4°C for 48 hours.

The blocked support preparation was washed three times with distilled water to remove excess ethanolamine and finally resuspended in 20 mM sodium phosphate buffer pH 6.8 containing 1 M sodium chloride, and stored for use at 4°C.
A diagram showing the relative positions of the layers on the core particles can be seen in **Figure 2.1** (not to scale).

Each batch of beads was tested for the ability to bind copper ions and bovine haemoglobin from which various parameters could be calculated and hence the quality of the supports could be assessed. Description of the tests used are described below, and **Table 2.3** gives a summary of the parameters for each batch.

2.4.17.1 Test 1 - The ability of the magnetic supports to bind copper.

Initially 37.5 mg beads were added to the pH-stat vessel. Assuming that there was approximately 10% loss (O'Brien, personal communication) in production and that the final volume is 1 ml, therefore 29.6 µl of suspension should contain approximately 1 mg support. For each batch, a specified volume (see **Table 2.3**) of IDA-coated support was placed into a microfuge tube and washed three times with 0.1 M sodium acetate, 0.5 M sodium hydroxide, pH 3.8, by vortexing, separation on a magnetic rack, followed by aspiration of the liquid (being careful not to aspirate off any beads).

The support was then charged once with a solution of copper chloride (5 mg/ml in the sodium acetate buffer used in the wash steps above) by constant shaking on a vibrax shaker (setting 1800) for 2 hours at room temperature. The particles were then washed twice with 1 ml of the same sodium acetate buffer in order to eliminate any unbound metal ions.

The bound copper ions were then stripped from the supports with 1 ml 0.1 M EDTA in 20 mM sodium phosphate, 0.5 M NaCl, pH 8.0 and the supernatant aspirated off from the beads for direct atomic absorption spectrometer (AAS) studies using a copper detection lamp (Perkin Elmer Atomic Absorption Spectrometer 3100, The Perkin-Elmer Corporation, Norwalk, CT, USA). This provides information on the IDA surface binding capabilities of the magnetic supports.

The remaining beads were dissolved by the addition of 1 ml concentrated hydrochloric acid, samples diluted one hundred fold with distilled water and these samples were passed through an AAS with an iron detection lamp to provide information on the amount of beads present in the specific volume of suspension.

From standard curves (parts per million (Cu or Fe) against atomic absorption values) it is possible to calculate the concentration of metal present ([Cu] µg/ml, *[Fe] µg/ml). Since the atomic weight of copper is 63.5, therefore 1 µmole of Cu is equivalent to 63.5 µg. Hence it is possible to calculate the µmoles Cu bound.

After all the coating and coupling steps the actual weight of Fe per gram of support drops. O'Brien *et al.* (unpublished results) found that 1 mg dry support gives a "measured by atomic absorption spectrometer" Fe content of 1.15 mg, and hence a conversion factor of 1.15 is used to modify the converted Fe AAS readings for mg / ml support (corrected values in "# [Fe] µg/ml" column in **Table 2.3**).

Having obtained this data, it is now possible to calculate the Ligand Density (µmoles Cu / g support).

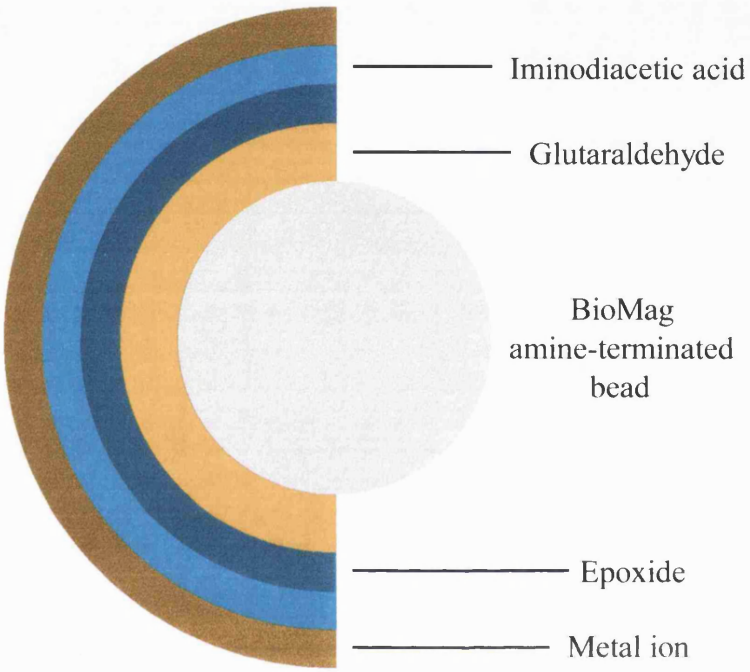


Figure 2.1 Diagrammatic representation of the coating layers on the core BioMag® particles (not to scale).

2.4.17.2 Test 2 - The ability of the magnetic supports to bind bovine haemoglobin (Sigma Chemical Co., St. Louis, USA).

Bovine haemoglobin has 20 accessible histidine residues and is commercially available hence it is a good choice to use when testing the binding ability of these charged magnetic supports. Several concentrations of bovine haemoglobin solution were made in 20 mM sodium phosphate, 1 M NaCl, pH 6.8, ranging from 50-500 μg/ml. Each batch of beads were tested for the ability to bind the haemoglobin at the different concentrations in order to determine their capacity.

The following procedure was carried out for each batch used: - into each microfuge tube was placed 10 μl of support. The magnetic bead supports were washed three times with 0.1 M sodium acetate, 0.5 M sodium chloride, pH 3.8 and charged with a solution of copper chloride as in test 1 above (section **2.4.17.1**). A control tube containing 20 mM sodium phosphate buffer and no beads was set up. To each tube was added 100 μl of one of the bovine haemoglobin solutions. The tubes were shaken on a vibrax for 2 hours at room temperature after which the beads were magnetically retrieved. The protein content of the aspirated supernatant was then determined using the Bio-Rad Protein Assay (Bio-Rad Laboratories Ltd., Hemel Hempstead, Herts., UK) in order to assess the amount of protein that had been bound by the beads. The beads are then destroyed with concentrated hydrochloric acid and samples passed through an AAS containing an iron lamp to assess the amount of beads present in 10 μl of suspension. From these two data sets (protein estimation and iron content) it is possible to calculate the binding capacity of each batch of beads.

From test 1 above it is also possible to calculate the Fe content in 10 μl support. The result of protein estimations from test 2 yields information on the amount of bovine haemoglobin bound to the charged supports. Therefore the binding capacity of the supports is the amount of protein bound (in μg) divided by the amount of support present in the experiment (in mg) to yield a value given in μg/mg or mg/g.

2.4.17.3 Experimental protocol for usage of magnetic supports.

The typical procedure for use of the magnetic supports is outlined below and variations from this are stated in the text in Chapter 5. For each experiment, 1 mg beads were used per microfuge tube.

1. The beads were washed three times with 1 ml of a solution of 0.1 M sodium acetate, 0.5 M NaCl, pH 3.8 by vortexing for 30 seconds, allowing the beads to be collected to the side of the tube by applying a magnetic field (placing tubes in a magnetic rack for approximately 5 minutes), and carefully aspirating away the supernatant.

2. The beads were then charged with a metal chloride solution (5 mg/ml in the sodium acetate buffer in step 1 above) by continual shaking typically on a vibrax setting 1800 for two hours at room temperature.
3. The beads were then washed as in step 1 above.
4. The particles were then washed three times in binding buffer to increase the pH to neutral.
5. The protein and binding buffer were added to the tubes (typically 0.3 mg total protein in a total volume of 750 µl) and mixing occurred on a vibrax shaker (setting 1400) at 4°C.
6. The unbound fractions were collected and stored at 4°C.
7. The beads were washed twice with 1 ml of the binding buffer in order to wash away any loosely bound protein.
8. The recombinant T4 bacteriophage lysozyme was then eluted from the beads in a smaller volume (350 or 500 µl) and if the pH of the buffer used for elution was below 7.0, 1 M Tris-HCl pH 8.0 was added at one tenth of the elution volume to restore the pH to approximately 7.
9. The beads were then stripped with a solution of 0.1M EDTA in 20 mM sodium phosphate, 0.5 M NaCl, pH 8.0.
10. All fractions were tested for protein content and lysozyme activity. SDS-PAGE gels were run with some samples and photographs can be seen in Chapter 5.

Table 2.3 Magnetic support data

Batch	Volume of suspension	[Cu] µg/ml	µmoles Cu	*[Fe] µg/ml	#[Fe] µg/ml	Ligand Density	Support in 10 µl	Bound haemoglobin	Binding capacity
1	32.6 µl	2.74	0.043	460	400	107.5	0.123 mg	33.6 mg	273 mg/g
2	37.0 µl	2.28	0.036	414	360	100.0	0.097 mg	29.2 mg	301 mg/g
3	35.5 µl	2.7	0.043	415	361	119.1	0.102 mg	25.4 mg	249 mg/g
4	35.5 µl	2.35	0.037	437	380	97.3	0.107 mg	31.1 mg	291 mg/g
5	29.6 µl	2.97	0.047	674	586	80.2	0.198 mg	40.0 mg	202 mg/g
6	29.6 µl	2.32	0.037	679	590	62.7	0.199 mg	39.9 mg	201 mg/g
7	29.6 µl	2.96	0.047	653	568	82.7	0.192 mg	39.9 mg	208 mg/g
8	29.6 µl	2.67	0.042	655	570	73.6	0.193 mg	41.5 mg	215 mg/g
9	29.6 µl	3.78	0.059	653	568	103.8	0.192 mg	39.9 mg	208 mg/g
10	30.0 µl	2.44	0.038	537	467	81.3	0.156 mg	34.6 mg	222 mg/g
11	30.0 µl	3.18	0.050	551	479	104.3	0.160 mg	36.3 mg	227 mg/g
12	30.0 µl	1.89	0.030	515	448	66.9	0.149 mg	34.2 mg	230 mg/g
13	30.0 µl	1.8	0.028	523	455	61.5	0.152 mg	33.4 mg	220 mg/g

*[Fe] µg/ml from atomic absorption spectrometer standard curve
#Corrected [Fe] µg/ml by applying the correction factor of 1.15 to * above (see text section **2.4.17.1**)

CHAPTER THREE

CONSTRUCTION AND EXPRESSION OF A RECOMBINANT BACTERIOPHAGE T4 LYSOZYME

3.1 Introduction

This chapter will describe the methods used to construct a recombinant bacteriophage T4 lysozyme by the addition of a polyhistidine tail to the C-terminus of the gene using the technique of site-directed mutagenesis. It will also describe the expression of the recombinant protein in *E. coli* and give a brief account of the factors which effect the enzyme activity.

3.2 Design of oligonucleotides

As mentioned in Chapter 1 (section **1.5.1**), the bacteriophage T4 lysozyme gene in the expression plasmid pHSe5, is a pseudo wild type in that two residues in the wild type have been replaced (Cys54Thr and Cys97Ala), to produce a cysteine-free protein. It is known from modelling studies that the C-terminus of the gene is well away from the active site and is therefore a good candidate site for modification. It is also known that up to two residues can be removed from the C-terminus without significant change in the properties of the enzyme (B.W. Matthews, personal communication).

As mentioned in section **1.6.1**, in site-directed mutagenesis the desired alteration(s) is contained in the central region of a synthetic oligo. The desired alteration in this case was not a simple one base deletion or addition but contained a 5 amino acid (15 base) insertion as discussed in Chapter 1 (section **1.3.1.4**). Therefore, in order to make the hybridization of the oligo to the template as stable as possible it is desirable to have longer flanking regions and it was decided that 12 bases either side of the proposed insertion site (just prior to the stop codon) was adequate. An explanation of the oligo composition can be seen in **Figure 3.1**. The mutagenic oligo was 5' phosphorylated (as recommended by Promega).

The His-containing tail was designed to contain 5 His (5 codons of CAT) residues. However, on obtaining and sequencing a recombinant plasmid of the correct phenotype, it was noticed that the second incorporated residue was glutamine (a codon of CAG). It is not known if this error was due to an inaccuracy in the original oligo or if the third base was mutagenised in some manner during the incorporation of the oligo. The recombinant protein still contained the desired His-X_3-His conformation as discussed in Chapter 1 and had the ability to be adequately expressed and purified (first tested on columns - see Chapter 4) and hence was used in all following studies.

To enable sequencing across the stop codon area of the lysozyme gene, two oligos were designed to act as primers (one forward and one reverse). **Figure 3.2** shows the relative binding regions of these primers to the template plasmid DNA. The DNA sequence of the lysozyme gene is from Owen *et al.* (1983).

(a) C-terminus of wild-type bacteriophage T4 lysozyme

amino acid 5'.. Tyr Lys Asn Leu Stop 3'
base TAT AAA AAT CTA TAA AGC TGT TTA

(b) C-terminus on incorporation of histidine-containing primer

5'.. Tyr Lys Asn Leu **His Gln His His His** Stop 3'
TAT AAA AAT CTA **CAT CAG CAT CAT CAT** TAA AGC TGT TTA
←12 base flanking region→ ←——**15 base insertion**——→ ←12 base flanking region→

Figure 3.1 Site-directed mutagenesis used to construct the alteration in the C-terminal region of the lysozyme gene. a) The C-terminal amino acid composition of wild-type T4 lysozyme with the corresponding bases underneath, b) The amino acid composition of the histidine-containing oligonucleotide and the corresponding base composition illustrating the position of the additional 5 amino acids prior to the stop codon.

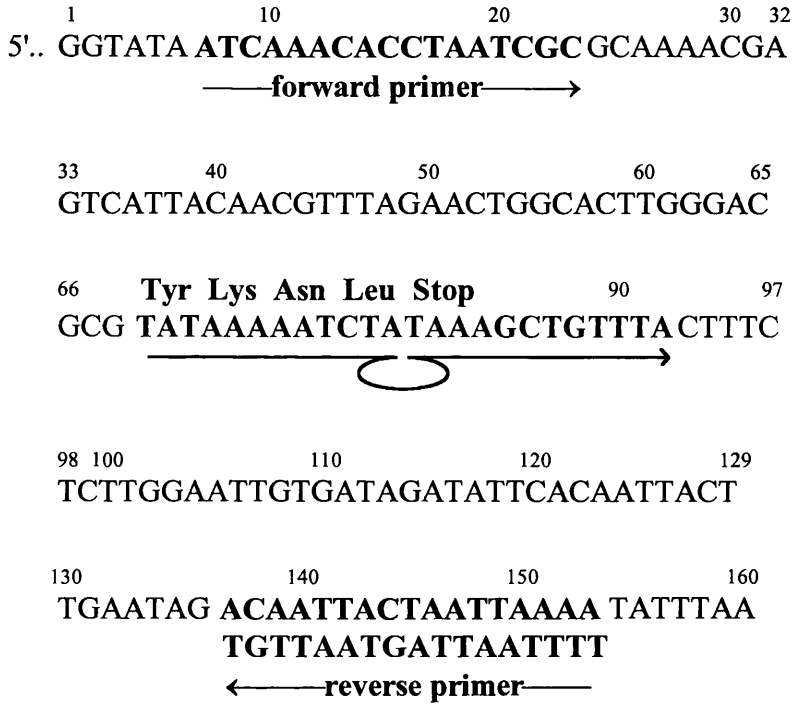


Figure 3.2 The three arrows indicate the position of annealing of the oligos to the lysozyme gene DNA showing their base composition, direction, and location relative to the region to be sequenced (just prior to the **Stop** codon region). There were two oligos used for sequencing (the forward and reverse primers) while the middle arrow indicates the position of the mutagenic oligo. (Sequence data of the lysozyme gene from Owen *et al.*, 1983)

3.3 Site-directed mutagenesis - experimental

Despite the use of several methods to isolate a fusion-tailed version of bT4l, only one method was successful. Here follows a brief description of the methods used. It should be noted that in all the following methods, the starting point was pHSe5 as described by Muchmore *et al.* (1989), and shown diagrammatically in **Figure 3.3**.

3.3.1 Insertion of f1 ori into pHSe5

The first approach used was to attempt to put a single-strand origin of replication (*ori*) into pHSe5 in order that single-stranded DNA could be produced for subsequent mutagenesis procedures.

The f1 origin of replication was cut out of pUC-f1 (Pharmacia Biotech Limited, Milton Keynes, UK) on a *Eco*RI/*Hin*dIII fragment, the ends made flush with the Klenow fragment of DNA Polymerase I, and the fragment was purified from an agarose gel using Geneclean®II kit (Stratech Scientific Ltd., Luton, UK) according to the manufacturer's guidelines. The fragment was then blunt-end ligated into pHSe5 at the *Alw*NI site which had also been treated with Klenow polymerase I, transformed into *E. coli* JM107 and expressed at 37°C for three hours. The bacteria were then superinfected with M13K07 bacteriophage overnight (multiplicity of infection = 10). The cells were harvested and the phage-containing supernatant (concentrated with Polyethylene glycol/NaCl) was used to infect a fresh overnight culture of *E. coli* JM107. Transformants were plated out on agar plates containing ampicillin. However, of the transformants screened, none was of the correct construction and this was probably due to the difficulty experienced with blunt-end ligation as all transformants had a reconstituted *Alw*NI site. The structure of the resulting plasmids, as revealed by restriction enzyme mapping, proved rather complex and not a simple insertion of the f1 origin into pHSe5. It was decided not to continue with this approach.

3.3.2 *Escherichia coli* CJ236

This method takes advantage of a strong biological selection against the original DNA template which is preferentially destroyed on transfection (Kunkel, 1985; Kunkel *et al.*, 1987). It uses standard procedures for site-directed mutagenesis in conjunction with an unusual template. It involves the use of a second strain of *E. coli* - CJ236. This strain is commonly known as a *dut*$^-$ (dUTPase deficient and hence the cell is unable to convert dUTP to dUMP and some of the dUTP is incorporated into the DNA at sites normally occupied by thymine) *ung*$^-$ strain (is not able to produce uracil-N-glycosylase and hence uracil which has been incorporated into the DNA is not removed). The presence of uracil in the template is not inhibitory to *in vitro* DNA synthesis and after completion of the *in vitro* reactions of mutagenesis the uracil can be removed from the

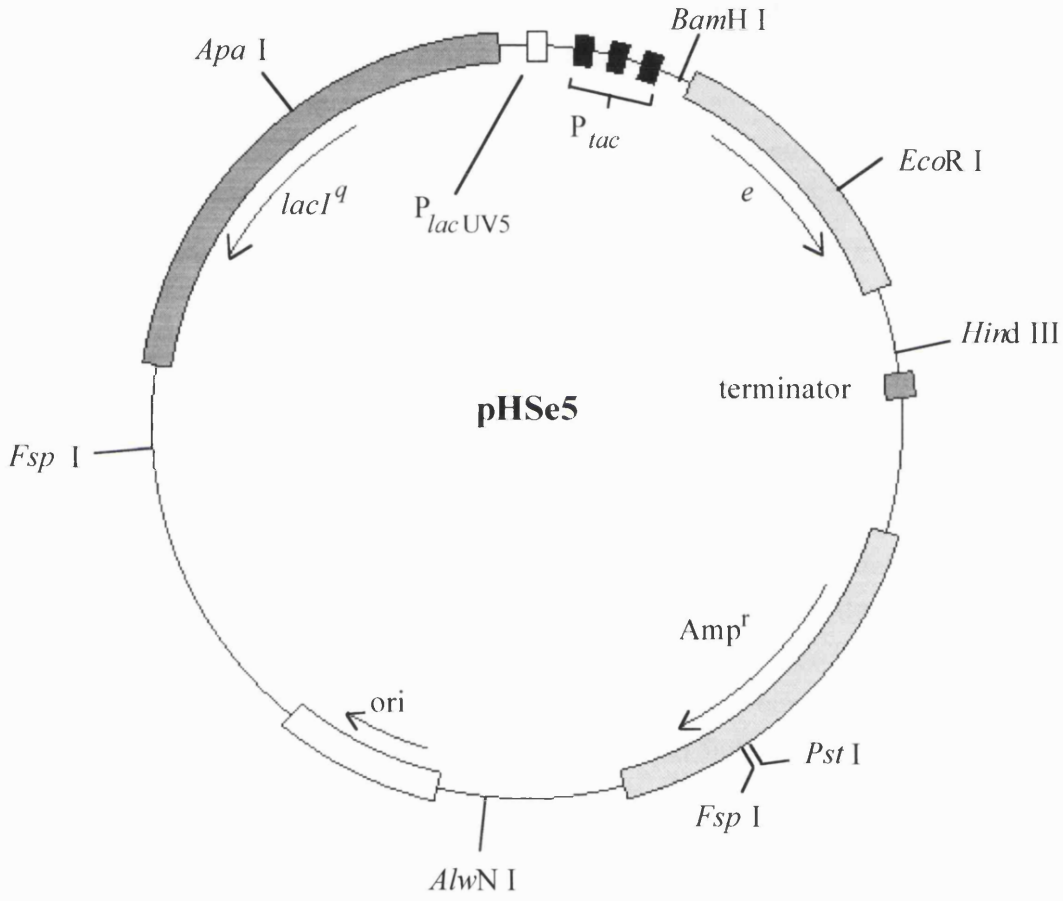


Figure 3.3 Diagrammatic representation of pHSe5 showing the relative positions of the genes and their direction of transcription [lysozyme (*e*), ampicillin resistance (Amp^r), Lac repressor (*lacI*^{q})], the promoters (P_{tac} and P_{lacUV5}), the transcription termination sequence downstream of the *e* gene, and the origin of replication (*ori*). The restriction enzyme sites used in the construction of the recombinant plasmid are also indicated.

template strand with uracil N-glycosylase (most commonly achieved using competent *ung*$^+$ *E. coli* cells). This renders the original template strand inactive and the infective complimentary strand containing the alteration is used as the template for *in vivo* replication. This strain also contains a F' and therefore can be used for the isolation of single-stranded DNA.

The method used for site-directed mutagenesis in this case was that of gapped-duplex. Each of the two strands of DNA were digested with different restriction enzymes so that different regions were excised from the two strands. Upon denaturation and reannealing the two digests would reanneal to give a molecule which was single-stranded only in the region of the *e* gene.

The pseudo wild type plasmid pHSe5 was transformed into strain CJ236 and hence some thymine residues were replaced by uracil. The DNA was isolated and cut with *Pst*I, a unique site on the plasmid. pHSe5 DNA was also cut with both *Eco*RI and *Hin*dIII to excise the C-terminal end of the *e* gene. Both "species" of DNA were then heat denatured to obtain single-strands of nucleic acid and equimolar amounts were mixed along with the oligo containing the polyhistidine tail. The mixture was cooled slowly to allow annealing to take place, and T7 DNA polymerase was added. The DNA was then transformed into a *dut*$^+$*ung*$^+$ strain (JM107), and transformants selected on ampicillin. In total, forty transformants were sequenced and all proved to be wild type.

A second approach using the CJ236 strain was used and is similar to that above except that it did not involve gapped duplex formation. Again the wild type pHSe5 DNA was transformed into the *dut*$^-$ *ung*$^-$ strain and the DNA isolated. The uracil-containing DNA was then denatured along with the oligo containing the polyhistidine region, polymerised, ligated, ethanol precipitated, and finally transformed into JM107. However, on sequencing some transformants, no desired constructs were found.

3.3.3 pBluescript® II KS(-)

This is a phagemid with a multiple cloning site (MCS), a blue/white colour system which allows the identification of the presence of inserts (a cloned insert interrupts the *lac*Z α-peptide and recombinant colonies appear white), and T3 and T7 bacteriophage promoters (which allow efficient *in vitro* synthesis of strand-specific RNA).

The *e* gene was to be excised from the pHSe5 vector and placed into the MCS of pBluescript. It was difficult to find a restriction enzyme which could cut a) both pBluescript and pHSe5 once, and b) pHSe5 outside of the *e* gene and preferably within the *lacI*^{q} gene. One possible way was a double digest with *Apa*I/*Hin*dIII and this would provide a sticky end ligation. However, *Apa*I was unable to cut the pBluescript DNA. The only other alternative was a ligation with one blunt and one sticky end, by cutting pHSe5 with *Fsp*I / *Hin*dIII and purifying the largest (2.3 kilobase) fragment, cutting

pBluescript with *Hin*dIII/*Hin*cII, and transforming the resulting construct into JM107 and selecting on Ampicillin, X-gal, IPTG agar. Many white colonies were selected and their DNA cut with restriction enzymes to verify their structure. However, all contained DNA similar in size to that of pBluescript.

3.3.4 pALTER™-1

The Altered Sites™ *in vitro* mutagenesis system is based on the use of a second mutagenic oligo to confer antibiotic resistance to the mutant DNA strand. As with the pBluescript system above, the pALTER-1 is a phagemid and it contains two antibiotic resistance genes. The MCS is within the *lac* Z gene so again there is a blue/white selection for screening for recombinants. The tetracycline resistance gene is always functional while the ampicillin resistance gene has been inactivated. Ampicillin resistance (Amp^r) can be restored by the annealing of an oligo to the single-stranded DNA template at the same time as the mutagenic oligo. The annealed DNA is firstly transformed into a repair minus strain of *E. coli* BMH 71-18 *mutS* (which suppresses *in vivo* mismatch repair (Zell, R. and Fritz, H.J., 1987)) and selection is by Amp^r. The second round of transformation uses JM107 and Amp^r to ensure proper segregation of mutant and wild type plasmids and mutants are screened by sequencing. (The oligos should be phosphorylated to allow them to be ligated into circular products)

The lysozyme gene was excised from approximately 5 μg of pHSe5 DNA on a *Bam*HI/ *Hin*dIII fragment, and purified from a 1% agarose gel using a Geneclean®II Kit (Stratech Scientific Ltd., Luton, UK). Approximately 0.25 μg of the fragment was then directly ligated (an equimolar amount) into the corresponding region of the MCS of pALTER-1. The resulting construct, pQR195, was transformed into *E. coli* JM107 and selected on Tet (15 μg/ml), X-gal (40 μg/ml), IPTG (0.5 mM) LB agar. After overnight incubation at 37°C, recombinant clones/white colonies were apparent among blue background colonies. Structures of the recombinants were verified by restriction enzyme digests and the DNA of a positive clone was isolated at large-scale.

Single-stranded DNA was then prepared (method described in Chapter 2 section **2.4.8**) for use as a template in the mutagenesis procedure (section **2.4.9**), where the mutagenic oligo was annealed to the template (at the same time as the Amp^r oligo), transformed into competent cells of strain BMH 71-18 *mutS*, and the DNA extracted and transformed into strain JM107. The desired constructs were tested for Amp^r and Tet^r resistance.

In all 120 colonies were screened by restriction enzyme profiles. Ten clones were further investigated by direct sequencing, firstly by sequencing using one nucleotide/dideoxynucleotide (section **2.4.13.2**), and if appeared promising, a standard sequencing reaction was then carried out (section **2.4.13.1**). Only one clone, designated

pQR751, was found to contain the desired mutation, and a photograph of the autoradiogram showing the resulting sequence compared to the pseudo wild type *e* gene may be seen in **Figure 3.4.** The screening process was laborious as a 15 base insert cannot be determined by restriction enzyme mapping alone, and direct sequencing is the only method which can realistically prove incorporation of the polyhistidine region. The low frequency of mutation may be attributed to many factors including annealing conditions or inadequate number of bases in the flanking regions on the oligo (Promega state that oligos of 26 bases in length have been successfully used to perform 4 base alterations and suggest that larger changes should have 20-30 matched bases on either side).

3.4 Expression of the recombinant protein

In order to be able to directly compare the wild type and recombinant proteins, the C-terminal His-containing region of the *e* gene from pQR751 was excised on a *Bam*HI/*Hin*dIII fragment and used to replace the corresponding region of the original expression vector, pHSe5, thus forming pQR752 where the polyhistidine tailed C-terminus of the *e* gene is placed back into the vector with the powerful promoters for protein expression. Glycerol stocks were made of the three constructs in *E. coli* (pQR195, pQR751 and pQR752) and stored at -70°C.

3.4.1 Finding good lysozyme-producing colonies

Good lysozyme producing cells were identified by streaking out some of the glycerol stock onto selective agar and then testing individual colonies on lysozyme activity plates, by a method similar to that of Alber and Matthews (1987) (Chapter 2 section **2.4.7**).

3.4.2 Routine expression of pQR752 in *E. coli*

Cultures were routinely grown according to the method described in Chapter 2 section **2.4.10**. In all experiments, a fresh culture was grown up and treated as described in sections **2.4.11** (column sample preparation) and **2.4.12** (magnetic support sample preparation).

Due to the four strong promoters upstream of the *e* gene, lysozyme is produced within 30 min post induction (only very low uninduced levels of production are seen). While expression is not lethal (studies here and J. Wozniak, personal communication), lysozyme has been found to leak from the cells into the culture medium and can lead to

lysis of the cells as seen from OD_{600} readings - see **Figure 3.5** - and lysozyme activity measurements - **Figure 3.6**.

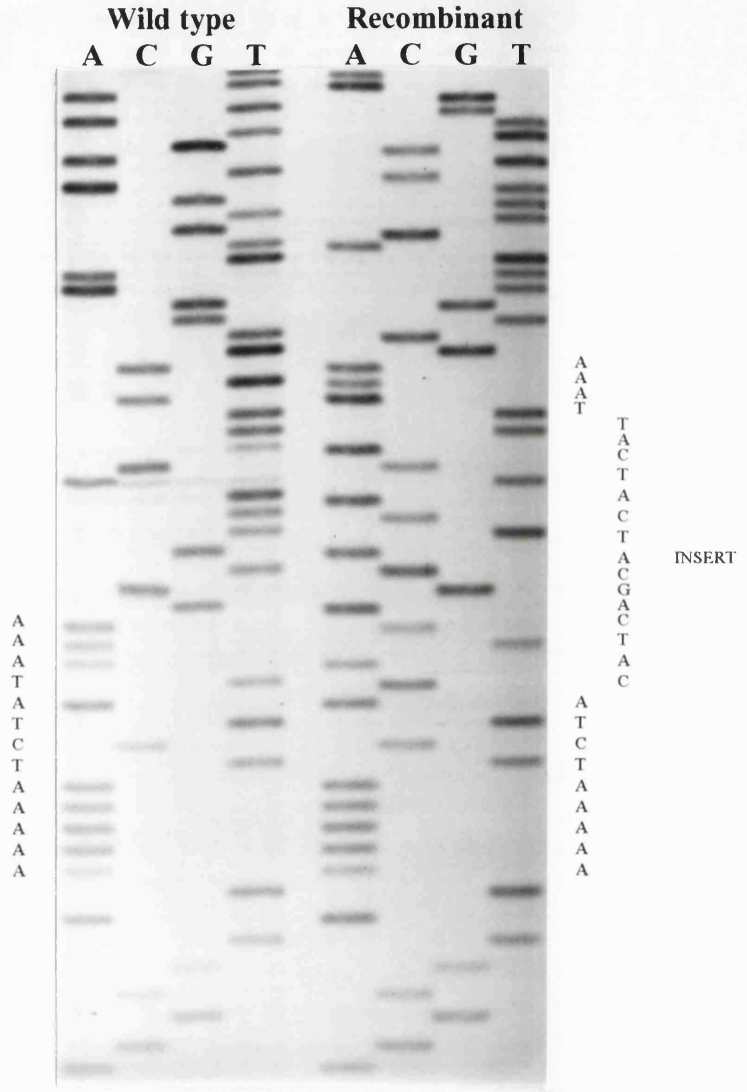


Figure 3.4 Photograph of an autoradiogram of a sequencing gel (with forward primer) showing the base composition of the pseudo wild type lysozyme sequence (left side) compared to that of the recombinant protein (right side).

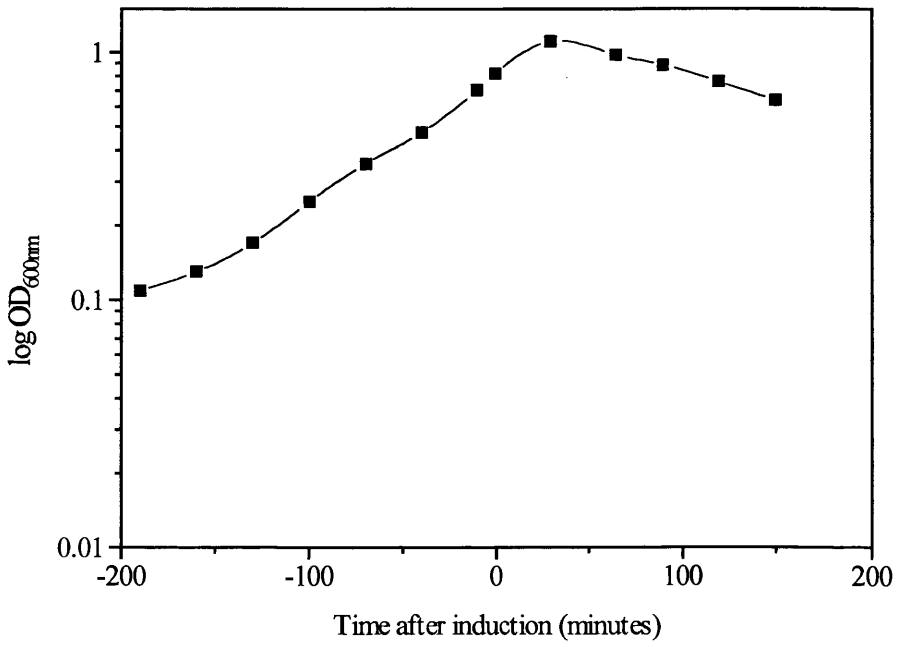


Figure 3.5 Typical growth curve of pQR752 in *E. coli.*

The growth curve shown in **Figure 3.5** was typical for the routine growth of pQR752 in *E. coli.* The OD_{600} readings were seen to decrease from the maximum value between 30 and 60 minutes after induction with IPTG and this was due to the lysing of some of the cells in the culture. This finding is verified by the lysozyme activity measurements in the media - see **Figure 3.6**.

The data presented in **Figure 3.6** shows the distribution of (a) lysozyme activity, and (b) total protein plotted against time after induction of the culture with IPTG. These results were obtained by extracting 1 ml culture from the fermentation at a specified time. The sample was spun down in a high speed microfuge (13,000 rpm) for 5 minutes to pellet the cells. The supernatant was aspirated into a fresh tube and tested directly for lysozyme activity and total protein content. The pellet was resuspended in 1 ml of 67 mM sodium phosphate, pH 6.2 (assay buffer) and sonicated on ice (3 x 30 seconds) at 8 μ amplitude. The sonicated mixture was then spun at 13,000 rpm for 5 minutes to pellet the debris and the resulting supernatant was tested for lysozyme activity and total protein content. It can be seen from **(a)** that there is a significant amount of lysozyme produced within the cells 30 minutes after induction with IPTG. At 1.5 hours after induction the activity is greater in the medium than in the cells and this is due to the lysozyme leaking into the culture medium as a result of overexpression. It can be seen from the total protein estimations in part **(b)** of the same figure that protein leaks out from the cells into the culture medium as the cells lyse.

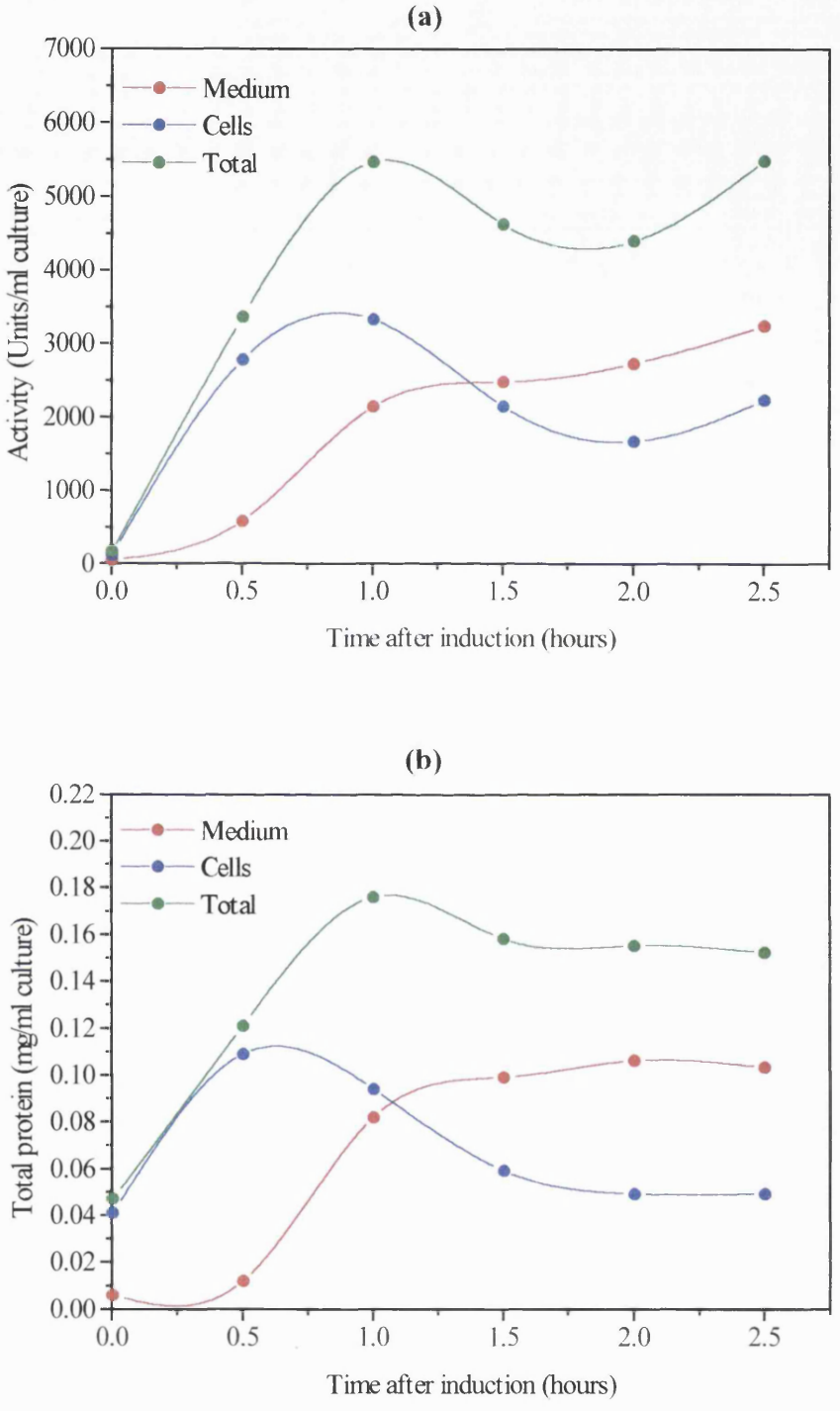


Figure 3.6 Distribution of: a) lysozyme activity and; b) total protein in culture.

Other results indicate (data not shown) that there is no significant difference between the enzyme activity and total protein content of the pseudo wild type and the cultures containing the polyhistidine-tailed protein. Changing the broth from Nutrient No.2 to LB had little effect on the distribution of activity between the broth and the cells (data not shown). However, independent studies by O'Brien (1996[b]) have indicated that leakage of the lysozyme into the medium can be decreased by growth of the culture in a high biomass medium. Maximum lysozyme production is achieved 2.5 hours post induction and comprises between 10-20% total protein produced (as seen in SDS-PAGE gels e.g. **Figure 5.7**).

3.5 Factors effecting the activity of T4 lysozyme

In all experiments within this thesis, many different buffers were used to test the binding and the elution of the tailed lysozyme from chelated supports. It will be seen throughout Chapters 4 and 5 that the enzyme can be activated or deactivated depending on the type of buffer used. It has also been shown that reactivation of the protein can occur under certain conditions, indicating that the enzyme is resilient.

Tsugita and Inouye (1968) purified T4 bacteriophage lysozyme and studied several characteristics of the enzyme. Using a 30 mM phosphate buffer (pH range 5.0-7.2) and a 50 mM Tris-HCl buffer (pH 7.2-8.9), they found that the optimal pH for enzyme activity was 7.2-7.4. The enzyme appeared to be stable when incubated at 37°C for 20 hours in 100 mM phosphate buffer, pH 6.0-6.5. The enzyme was found to be inactivated by this treatment at more acidic or more alkaline conditions. Magnesium and sodium ions were found to exert a stabilising effect but concentrations were not specified.

Becktel and Baase (1987) found that attempts to correlate activity and thermodynamic stability were not possible as they found the enzyme to be essentially inactive at pH values below approximately 5. Replacement of either of the Cys residues in the wild type produced a variant that was significantly more resistant to irreversible thermal inactivation than the wild type protein, and replacement of both was found to further enhance this resistance (the lysozyme used throughout this thesis does not contain any Cys residues). In a review article, Tsugita (1971) states that when T4 is isolated from bacteriophage-infected *E. coli*, pH below 5 greatly inactivates the isolated enzyme.

In light of these articles it was therefore decided to test the extracts in the buffers used. **Figure 3.7** and **Table 3.1** (key to the buffers in Figure 3.7) give an indication of the effects of all the buffers used, where 100% activity is arbitrarily given to the activity of the enzyme in 20 mM sodium phosphate, 0.2 M sodium chloride, pH 7.2 (buffer

number 2 and the binding buffer in the magnetic supports experiments - see Chapter 5). All buffers were tested on both clarified and crude samples (section **2.4.12**) under conditions similar to those used in Chapter 5 and carried out as follows : 4 µl of extract was added to 746 µl buffer at room temperature. Five minutes after mixing, 100 µl was tested for lysozyme activity. This was repeated with all buffers used. This ratio of extract to buffer gave a linear rate of lysozyme activity over one minute.

In Chapter 5 it will be noted that when buffers of a low pH were used, one tenth of the total volume of 1 M Tris-HCl pH 7.2 was added in order to restore the pH to neutrality. In order to mimic this scenario with the extracts, 75 µl of the Tris buffer was added to the 750 µl in the tube after the 5 minute incubation at room temperature, the tube vortexed, and 110 µl of mixture added to 990 µl cell suspension for activity assays (thus maintaining the proportion of test material to the substrate of suspended cells).

All rates were done in duplicate and an average value taken when calculating activation/deactivation. From **Figure 3.7** it can be seen that there is insignificant difference in the activity of the two extracts tested within the limits of the lysozyme assay used (approximately 15% error limit). However, it should be remembered that these are not absolute values and are only an indication as to whether the buffers used have the ability to activate/deactivate the enzyme. Results given here may be used in Chapter 5 when interpreting activity data.

The enzyme appears to be marginally more active in the 20 mM sodium phosphate (buffer nos. 1-4) than in the 50 mM sodium phosphate buffers (buffer nos. 5-8) over the same pH range (pH 6.8-8.0). There appears to be little difference in the 20 mM phosphate buffers over the pH range tested (also compare buffers 14 and 15), while the activity seems to decrease with an increase in pH value with the 50 mM buffers (this is not quite so obvious with the crude extracts). Buffers 12 and 16 (both 20 mM sodium phosphate, 0.5 M NaCl) indicate that there appears to be little difference in the enzymatic activity at pH 7.2 (no. 12) and pH 3.5 (no. 16). The sodium phosphate buffer used in the lysozyme assays (no. 24) appears to enhance the activity of the enzyme.

When one considers the effect of the concentration of sodium chloride in the binding buffers (20 mM sodium phosphate, pH 7.2) on the lysozyme activity (buffer nos. 9-14), the activity is seen to decrease noticeably in both extracts as the salt concentration of the buffer increases. Buffers 9 (0 M NaCl) and 10 (0.1 M NaCl) appear to have an activation effect on the enzyme when their values are compared to those in buffer 2 (0.2 M NaCl). Becktel and Baase (1985) developed a benchtop plate assay based on the digestion of purified *E. coli* peptidoglycan suspended in an agarose gel matrix. Their studies found that the optimal ionic strength for wild type T4 lysozyme was obtained with 0.1 M NaCl.

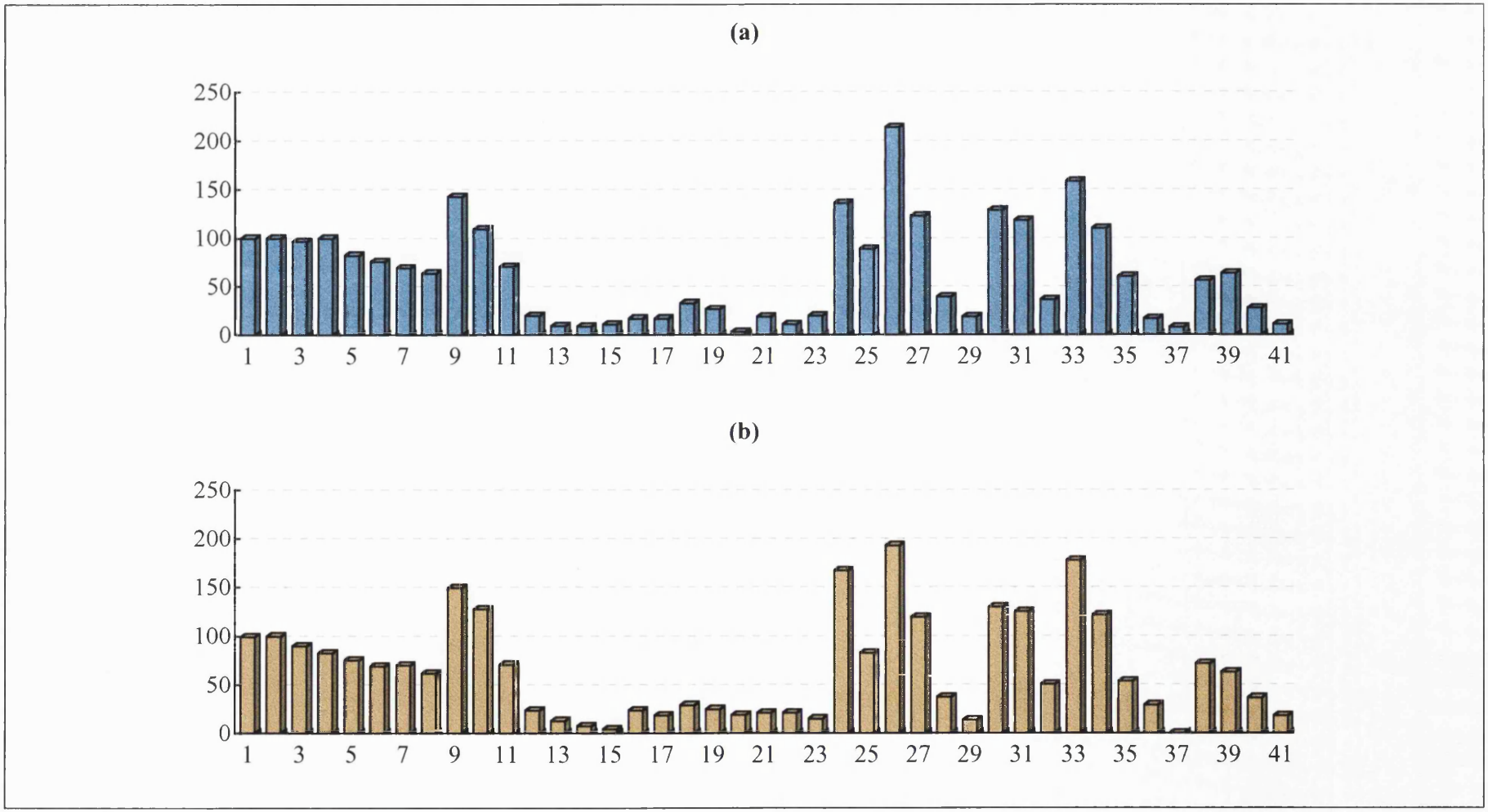


Figure 3.7 The effect of buffers on the activity of T4 lysozyme in: a) clarified; b) crude extracts.

Table 3.1 Buffer identification for Figure 3.7

No.	Buffer	No.	Buffer
1	20 mM sodium phosphate, 200 mM NaCl, pH 6.8	22	200 mM Histidine in Binding buffer, pH 7.2
2	20 mM sodium phosphate, 200 mM NaCl, pH 7.2 (Binding)	23	100 mM EDTA in Binding buffer, pH 8.0
3	20 mM sodium phosphate, 200 mM NaCl, pH 7.6	24	67 mM sodium phosphate, pH 6.2 (Activity assay)
4	20 mM sodium phosphate, 200 mM NaCl, pH 8.0	25	100 mM sodium acetate, pH 3.8
5	50 mM sodium phosphate, 200 mM NaCl, pH 6.8	26	100 mM sodium acetate, 250 mM NaCl, pH 3.8
6	50 mM sodium phosphate, 200 mM NaCl, pH 7.2	27	100 mM sodium acetate, 500 mM NaCl, pH 3.8
7	50 mM sodium phosphate, 200 mM NaCl, pH 7.6	28	100 mM sodium acetate, 750 mM NaCl, pH 3.8
8	50 mM sodium phosphate, 200 mM NaCl, pH 8.0	29	100 mM sodium acetate, 1000 mM NaCl, pH 3.8
9	20 mM sodium phosphate, pH 7.2	30	100 mM sodium acetate, 500 mM NaCl, pH 3.5
10	20 mM sodium phosphate, 100 mM NaCl, pH 7.2	31	100 mM sodium acetate, 500 mM NaCl, pH 3.8
11	20 mM sodium phosphate, 250 mM NaCl, pH 7.2	32	100 mM sodium acetate, 500 mM NaCl, pH 4.8
12	20 mM sodium phosphate, 500 mM NaCl, pH 7.2	33	100 mM sodium acetate, pH 3.8 + Tris
13	20 mM sodium phosphate, 750 mM NaCl, pH 7.2	34	100 mM sodium acetate, 250 mM NaCl, pH 3.8 + Tris
14	20 mM sodium phosphate, 1000 mM NaCl, pH 7.2	35	100 mM sodium acetate, 500 mM NaCl, pH 3.8 + Tris
15	20 mM sodium phosphate, 1000 mM NaCl, pH 6.8	36	100 mM sodium acetate, 750 mM NaCl, pH 3.8 + Tris
16	20 mM sodium phosphate, 500 mM NaCl, pH 3.5	37	100 mM sodium acetate, 1000 mM NaCl, pH 3.8 + Tris
17	100 mM sodium phosphate, 500 mM NaCl, pH 7.0	38	100 mM sodium acetate, 500 mM NaCl, pH 3.5 + Tris
18	10 mM Imidazole in Binding buffer, pH 6.9	39	100 mM sodium acetate, 500 mM NaCl, pH 3.8 + Tris
19	100 mM Imidazole in Binding buffer, pH 6.9	40	100 mM sodium acetate, 500 mM NaCl, pH 4.8 + Tris
20	100 mM Imidazole in Binding buffer, pH 8.0	41	20 mM sodium phosphate, 500 mM NaCl, pH 3.5 + Tris
21	100 mM Histidine in Binding buffer, pH 7.2		

Many different types of buffers were tested for their ability to elute the recombinant protein from the magnetic supports. When imidazole was used in the elution buffer, the activity is marginally greater with the lower molarity (comparing 18 and 19) although variation is within the error limits of the assay. Also activity is marginally greater with the lower pH (19-pH of 6.9, and 20-pH 8.0). All three buffers appeared to have a negative effect on the enzyme activity and since they were all made up in the binding buffer, it can only be concluded that this effect is due to the presence of the imidazole. When histidine (also made up in the binding buffer) was used in competitive elution studies (21 and 22), the activity was greatly effected at pH 7.2 at 100 mM and 200 mM respectively.

On comparing the sodium chloride concentration in the sodium acetate elution buffers (25-29), it can be seen from **Figure 3.7** that there is a maximum activity obtained at 250 mM NaCl, and the following trend is seen in both crude and clarified extracts : 250 mM > 500 mM > 0 mM > 750 mM > 1000 mM. The 250 mM and 500 mM solutions gave activities greater than 100%. When considering the same buffer (100 mM sodium acetate, 500 mM NaCl) at different pH values (30-32), there is marginal difference between pH 3.5 and 3.8, but both have enhanced activity while that at pH 4.8 is reduced.

On comparing the experiments into which Tris buffer was added (34-41), with their counterparts (25-32 and 16 respectively), the activity in the buffers with the Tris were found to be lower than those without Tris. The only difference in the latter set of assays carried out was that 110 µl of sample was assayed in 990 µl resuspended *M. lysodeikticus* cells but this maintained the ratio of enzyme to substrate and therefore it can be concluded that the low pH does not appear to have a damaging effect on enzyme activity.

3.6 Summary

This chapter has described the methods employed to obtain a polyhistidine-tailed clone of bacteriophage T4 lysozyme. In all, only one method was successful and the mutation rate for the desired construct using this method was less than 1%. The mutation rate might have been increased by an alteration of several of the parameters such as extended flanking regions on the mutagenic oligo or annealing conditions. The resulting construct did not contain five His residues as intended, but Chapters 4 and 5 demonstrate that the resulting construct proved very efficient in its binding efficiency to metal ions.

Also described in this chapter is an account of the routine expression (also see Chapter 2 section **2.4.10**) of the recombinant protein, its activity, and distribution within

the culture. The presence of lysozyme in the culture cells can be detected as little as 30 minutes after induction and may even be present in detectable amounts before this time (not determined here). It is interesting to note that lysozyme is released into the culture medium and that this has a lethal effect on the remaining cells in the culture.

The final section of this chapter deals with the effect of the buffers (used throughout this thesis) on the activity of the enzyme. This section shows that different buffers can have the ability to exert different effects (activate or deactivate) on the enzyme and enables the reader to interpret the data in the following two chapters by using it as a rough guide while comparing activities. However, it should be emphasised that the data presented in section **3.5** is only true for the recombinant bT4l and cannot be applied to other proteins which might be purified by elution from chelating matrices.

Tsugita and Inouye (1968) published the complete primary structure of bT4l from *E. coli* T4 and established that the size of this wild type protein has a molecular weight of 18,635. The pseudo wild type protein produced by pHSe5 and used in this thesis differed from the wild type at two residues (Cys54Thr and Cys97Ala) and has a molecular weight of 18,601. Since the polyhistidine was composed of four His residues plus a Gln residue, the overall molecular weight of this recombinant protein is 19,367.

The recombinant protein can therefore be expressed in sufficient quantities to enable purification studies to take place, and these will be discussed in the following two chapters.

CHAPTER FOUR

PURIFICATION OF THE RECOMBINANT PROTEIN BY COLUMN CHROMATOGRAPHY

4.1 Introduction

Bacteriophage T4 lysozyme (bT4l) has previously been purified to homogeneity by several methods which essentially utilise weak acid ion exchange resins for chromatography and Sephadex for molecular sieving. Several groups have used ion exchange columns such as CM-Sephadex (Alber *et al.*, 1988), CM-Sepharose (Alber and Matthews, 1987), or CM-Sepharose followed by SP Sephadex (Griffey *et al.*, 1985; Muchmore *et al.*, 1989; Poteete *et al.*, 1991). DEAE-cellulose (basic columns) and CM-cellulose followed by a gel filtration column were used by Perry and Wetzel (1986). Recently, Düring (1993) described a purification method for single-step isolation of a recombinant bT4l. The method involved the fusion of six His residues to the N-terminus of the protein and passing the lysate through a commercially available Ni-NTA column. However, evidence from SDS-PAGE gels suggests that the size of the eluted proteins is dependent on the elution conditions and the recovery and purification factors obtained were not stated.

The ideal purification procedure would be one which involved a single step from the crude mixture while maintaining biological activity of the protein in as high a yield as possible. The technique of affinity chromatography fulfils these criteria as it can be used to recover small amounts of biological material from large amounts of contaminating substances in a single step.

As seen in Chapter 3, the polyhistidine-containing protein can be expressed in adequate quantities to allow purification studies to take place. Prior to purification studies involving the novel magnetic support material (Chapter 5), it was necessary to establish the ability of this histidine-containing tail to allow selective purification of the recombinant protein on tried and tested affinity columns. Therefore, this chapter deals with the attempted purification of the recombinant and wild type proteins by column chromatography from a crude culture mixture (see section **2.4.11** for sample preparation) using HiTrap™ chelating columns.

4.2 Column chromatography

All the chromatography described in this chapter was carried out on 5 ml HiTrap™ Chelating Sepharose High Performance® columns (Pharmacia LKB Biotechnology, Sweden) and used according to the manufacturer's instructions. These columns were made of medical grade polypropylene which is non-interactive with biomolecules. The Chelating Sepharose High Performance used consisted of highly cross-linked agarose beads to which IDA had been coupled by stable ether groups via a spacer arm. The gel is stable over a large pH range (2-14), tolerates all commonly used aqueous buffers

(although Tris-HCl is not recommended as a binding buffer) and the 5 ml columns have a maximum flow rate of 20 ml/minute.

In general, when the Chelating Sepharose is charged with suitable metal ions, the column selectively retains the desired protein if the complex-forming amino acid residues are exposed on the surface of the protein. At neutral pH the surface His residues are largely unprotonated and free to coordinate with the metal. The histidine-containing recombinant protein used in this study was designed so that the "tail" would protrude from the folded protein and therefore the His residues would be available for complexing with the metal ions on the column. Elution of the desired protein from the column can be achieved by several methods such as pH adjustment (this procedure should only be used when the protein is not effected by low pH values), addition of a competitive ligand such as imidazole, or by stripping the metal ions from the gel by the use of chelating agents such as EDTA.

4.3 The use of HiTrap columns

The columns were used for the attempted isolation of both the recombinant and the wild type lysozyme as outlined in Chapter 2 section **2.4.16**. Three different metal ions were used in the chromatographic runs - Ni, Zn, and Cu.

4.3.1 Isolation of the recombinant protein on a HiTrap Ni column - column 1

In total, 24 ml of sample (8.14 mg of total protein) were loaded onto a HiTrap column which had been charged with nickel ions as outlined in Chapter 2 section **2.4.16**. Graphs of the pH, lysozyme activity, and total protein content for each fraction collected, against the elution volume can be seen in **Figure 4.1**. The numbers on the graph in Figure 4.1 **(c)** indicate the fraction numbers which were the samples used for the SDS-PAGE gel. A photograph of the 12% SDS-PAGE gel can be seen in **Figure 4.2**.

From **Figure 4.1 (a)** and **(b)** it can be seen that there is a peak of lysozyme activity at fractions 23-32 which is equivalent to a pH range of 5.27-4.48 respectively in a total volume of 13 ml. Maximum activity was found in fraction 26 (after 18.2 ml elution buffer had been added) at a pH value of 5.05. The total volume of these ten fractions was 13 ml. The activity recovered in the peak fractions accounted for 72% of the activity recovered. Another estimation as to the efficiency of the column would be protein measurements.

The data shown in **Figure 4.1 (c)** is that of total protein content (mg/ml) of each fraction collected against elution volume. As can be seen from this graph, there were three main peaks of protein distribution. The first peak was small and quite broad (the first 40 ml) and contained the flow-through proteins eluted when the sample was loaded

onto the column and washed through with start buffer - these proteins had not bound to the column. The maximum amount of protein was eluted in fraction 19 (after a total of 48.7 ml of buffer, 9.1 ml of which was the elution buffer, with a fraction pH of 5.89) but it can be seen in **(b)** that there was no activity in this fraction. The third peak in the protein profile had a maximum value at fractions 25 and 26 (after the addition of 16.9 ml of the elution buffer) at a pH of 5.19-5.05 and as noted above, these fractions did possess activity. In total 8.13 mg of protein were loaded onto the column and the total protein collected in all fractions was 7.47 mg. Therefore 92% of the loaded protein was recovered. The amount of protein recovered in the activity peak fractions corresponded to 15% of the total loaded or 17% of the total recovered. On combining the data collected for the second and third protein peaks, these fractions represented 43% of protein loaded and 47% of that recovered.

The photograph of the 12% SDS-PAGE gel run with the samples numbered in Figure 4.1 (c) can be seen in **Figure 4.2**. The maximum amount of protein/ml was found in fraction 19 (lane 5). It can be seen from this figure that a large number of proteins are contained in this fraction and the proteins present exhibit a wide range of sizes. There is a band in this fraction similar in size to that of T4 lysozyme but it can be seen from Figure 4.1 (b) that this fraction did not exhibit any activity. A band of the correct size is present in fractions 23-31 (lanes 7-15 respectively). However, it can be seen from the photograph that there are also several minor contaminants present in these lanes. The fractions containing the largest amount of the recombinant protein are 25-27 (lanes 9-11) which are the fractions that form the third peak in the protein profile in **Figure 4.1 (c)**.

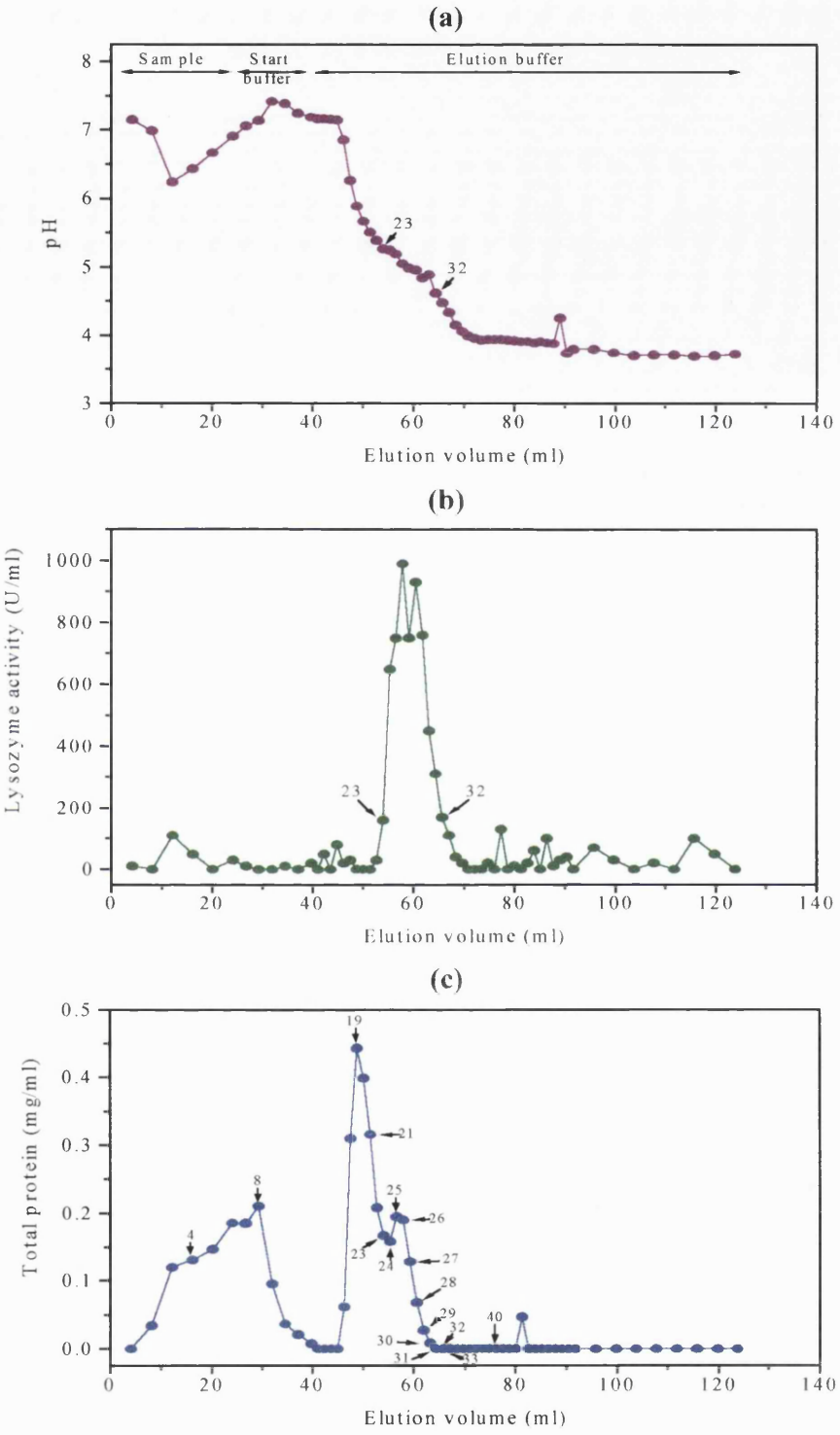


Figure 4.1 Graphs showing (a) pH; (b) lysozyme activity; (c) total protein for each fraction collected against the elution volume when the sample containing the recombinant protein was passed through a HiTrap nickel column - column 1. Samples from the fractions numbered in (c) were run on a SDS-PAGE gel (see **Figure 4.2**).

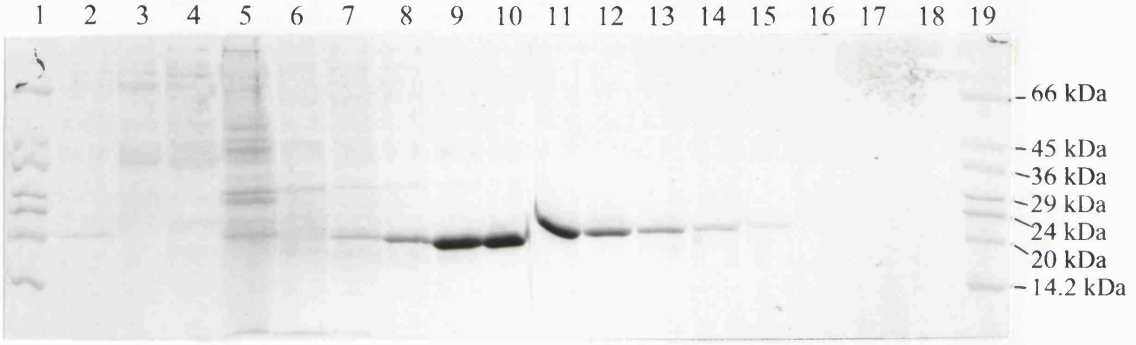


Figure 4.2 Photograph of a 12% SDS-PAGE gel loaded with samples from a HiTrap column charged with Ni showing the purification of the recombinant T4 lysozyme - column 1. The samples in lanes 3-18 inclusive were concentrated 10-fold with TCA precipitation and 20 µl were loaded into the wells. **Lanes 1** and **19**, 5 µl molecular weight markers (Sigma, SDS-7); **lane 2**, 20 µl of the extract loaded onto the column (unconcentrated); **lane 3**, fraction 4; **lane 4**, fraction 8; **lane 5**, fraction 19; **lane 6**, fraction 21; **lane 7**, fraction 23; **lane 8**, fraction 24; **lane 9**, fraction 25; **lane 10**, fraction 26; **lane 11**, fraction 27; **lane 12**, fraction 28; **lane 13**, fraction 29; **lane 14**, fraction 30; **lane 15**, fraction 31; **lane 16**, fraction 32; **lane 17**, fraction 33; **lane 18**, fraction 40.

4.3.2 Isolation of the recombinant protein on a HiTrap Zn column - column 2

In total, 10 ml of sample (2.8 mg total protein) were loaded onto the HiTrap Zn-charged column. Data for the pH, lysozyme activity, and total protein content for each fraction were plotted against elution volume and can be seen in **Figure 4.3**.

On considering the lysozyme activity profile - **Figure 4.3 (b)** - it can be seen that there is one sharp peak which contains most of the eluted activity. The peak is spread over two fractions - numbers 17 and 18 - in a total volume of 2.6 ml. The pH values of these eluted fractions were 5.58 and 5.06 respectively. Maximum activity was in fraction 17 (after 6.5 ml of elution buffer had been added). The activity of the loaded sample was 370 Units/ml and the total activity recovered in all fractions was 2,440 Units, hence 66% of the activity loaded onto the column was recovered. When considering the peak fractions, 86% of the total recovered activity was found in these fractions.

In total 2.81 mg protein was eluted from the Zn column and this amounts to 100% recovery. From **Figure 4.3 (c)** it can be seen that the protein was eluted in two main peaks - fractions 1-8 (pH 7.27-7.12) and 15-18 (6.74-5.06). No lysozyme activity was detected in the first broader peak and the proteins present in these fractions did not bind and were washed straight through. The second peak (initial elution after the addition of 7.8 ml of elution buffer) contained 36% of the total protein eluted/loaded which was present in 5.2 ml. However, although fractions 15 and 16 form a part of the second protein peak there was no activity detected in these samples and it can be concluded that these are other proteins which have bound loosely to the column but were eluted at the higher pH than the T4 lysozyme. As mentioned in the paragraph above, the lysozyme activity was found in fractions 17 and 18 and these fractions also contained a significant amount of protein, 0.44 mg.

Samples from the fractions indicated on the graph of **Figure 4.3 (c)** were used for the loading of a 12% SDS-PAGE gel shown in **Figure 4.4.** Samples in lanes 3-13 inclusive were concentrated 10-fold by TCA precipitation and 20 µl samples were loaded. Lanes 3 and 4 (fractions 3 and 6 respectively) are representative samples from the first protein peak and exhibit a large number of proteins which were washed through the column as they had not bound. Lanes 7 and 8 (fractions 15 and 16) show the array of proteins which were eluted from the column prior to the recombinant protein which can be seen as a strong band in lanes 9 and 10 migrating around the distance of the 20 kDa standard marker. However, it can be seen that these two tracks do not only contain the lysozyme as some contaminants are also present - there is quite a large contaminant in fraction 17 around 45 kDa and this can account for the rather large protein content in this fraction.

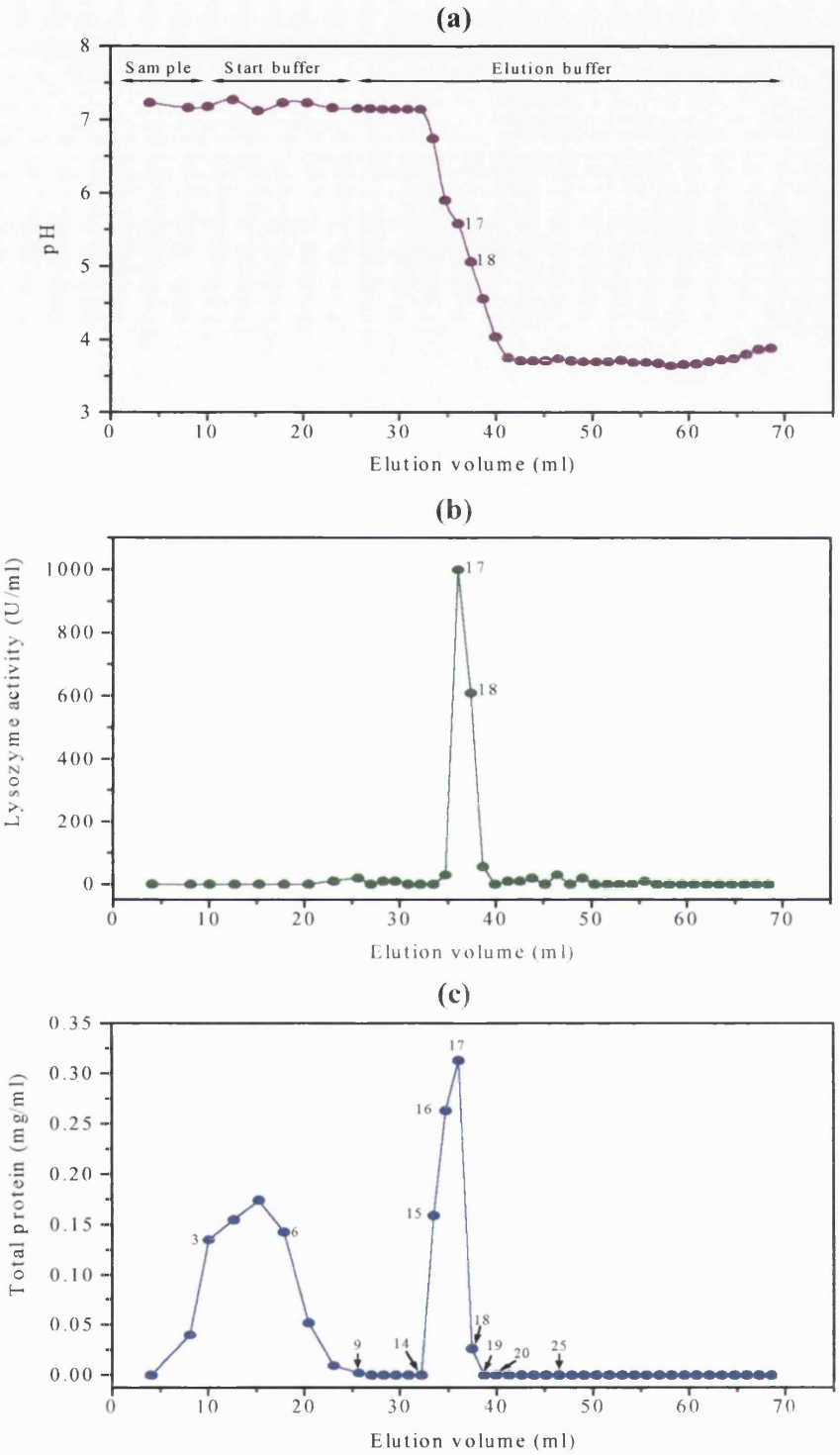


Figure 4.3 Graphs showing (a) pH; (b) lysozyme activity; (c) total protein for each fraction collected against the elution volume when the sample containing the recombinant protein was passed through a HiTrap Zn column - column 2. Samples from the fractions numbered in (c) were run on a SDS-PAGE gel (see **Figure 4.4**).

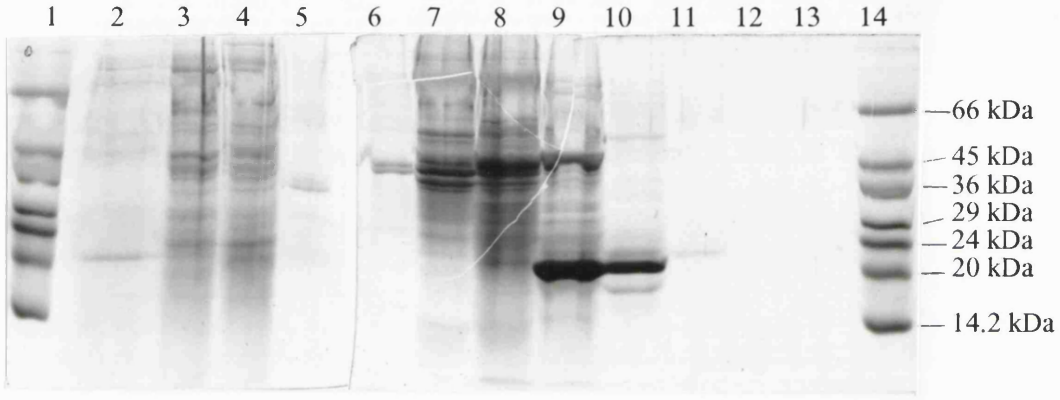


Figure 4.4 Photograph of a 12% SDS-PAGE gel loaded with samples from a HiTrap column charged with Zn showing the purification of the recombinant T4 lysozyme. The samples in lanes 3-13 inclusive were concentrated 10-fold with TCA precipitation and 20 µl were loaded into the wells. **Lanes 1** and **14**, 5 µl molecular weight markers (Sigma, SDS-7); **lane 2**, 20 µl of the extract loaded onto the column (unconcentrated); **lane 3**, fraction 3; **lane 4**, fraction 3; **lane 5**, fraction 9; **lane 6**, fraction 14; **lane 7**, fraction 15; **lane 8**, fraction 16; **lane 9**, fraction 17; **lane 10**, fraction 18; **lane 11**, fraction 19; **lane 12**, fraction 20; **lane 13**, fraction 25.

4.3.3 Isolation of the recombinant protein on a HiTrap Cu column - column 3

A total of 10 ml of sample (2.8 mg of total protein) were loaded onto a HiTrap column which was charged with copper ions. Two different elution buffers were used with this Cu-charged column; the first buffer was that which was routinely used in all previous columns - 20 mM sodium phosphate, 0.5 M NaCl, pH 3.5 - and the second buffer used was 0.1 M imidazole, 1 M NaCl pH 9.35. Data obtained from the collected fractions can be seen in **Figure 4.5** where the pH, lysozyme activity, and total protein for each fraction is plotted against the elution volume.

The graph showing the lysozyme activity for each fraction - see **Figure 4.5 (b)** - indicates that there is a single peak of activity which stretches over 16 fractions (numbers 33-48 inclusive with a total volume of 20.8 ml) and begins after 31.2 ml of elution buffer 1 had been applied to the column. The pH range over these fractions is 3.65-3.59. The activity recovered in fractions 33-48 amounted to 94.5% of the total recovered from the column.

A total of 2.8 mg of protein was applied to this column of which only 1.68 mg was recovered - this is equivalent to a 60% recovery. Fractions 33-48 contained negligible amounts of protein (estimated at 0.01 mg) and hence it can be assumed that the protein which was present in these fractions was quite pure. From **Figure 4.5 (c)** it can be seen that there were three regions where protein was eluted from the column. The first peak (fractions 3-9 inclusive, pH 5.88-7.16) represents the wash-through proteins - the proteins which were not bound to the copper ions. The second peak (fractions 17-33, pH 5.72-3.65) contained the proteins which had bound but were eluted at pH values higher than that of the lysozyme. The third peak (fractions 50-52, pH 7.46-8.77) represents the proteins which required the more stringent elution conditions of buffer 2 - 0.1 M imidazole, 1 M NaCl, pH 9.35, which acted as a competitive ligand.

Equal volumes of the fractions numbered in **Figure 4.5 (c)** were concentrated 10-fold and loaded onto a SDS-PAGE gel, a photograph of which can be seen in **Figure 4.6**. The lysozyme assays indicated that the activity was distributed over fractions 33-48 inclusive and lanes 9-16 contain alternate fractions across this region. There appears to be maximum amount of T4 lysozyme in lanes 12 and 13 corresponding to fractions 40 and 42 which verifies the results of **Figure 4.5 (b)**. As suggested earlier, these fractions appeared to have a low level of contaminants. A large number of proteins can be seen in lane 17 (fraction 51) indicating that a large number of proteins stick to the copper ions but require different elution conditions to the lysozyme. The gel is stained lightly and the faint bands of T4 lysozyme in lanes 9, 15 and 16 do not show in the photograph. As mentioned earlier, 60% of the loaded protein was recovered. This estimate is low as the protein assay not was sensitive enough to be able to quantify the trace amounts present in some fractions. This is evident when comparing **Figure 4.5 (c)** with the photograph

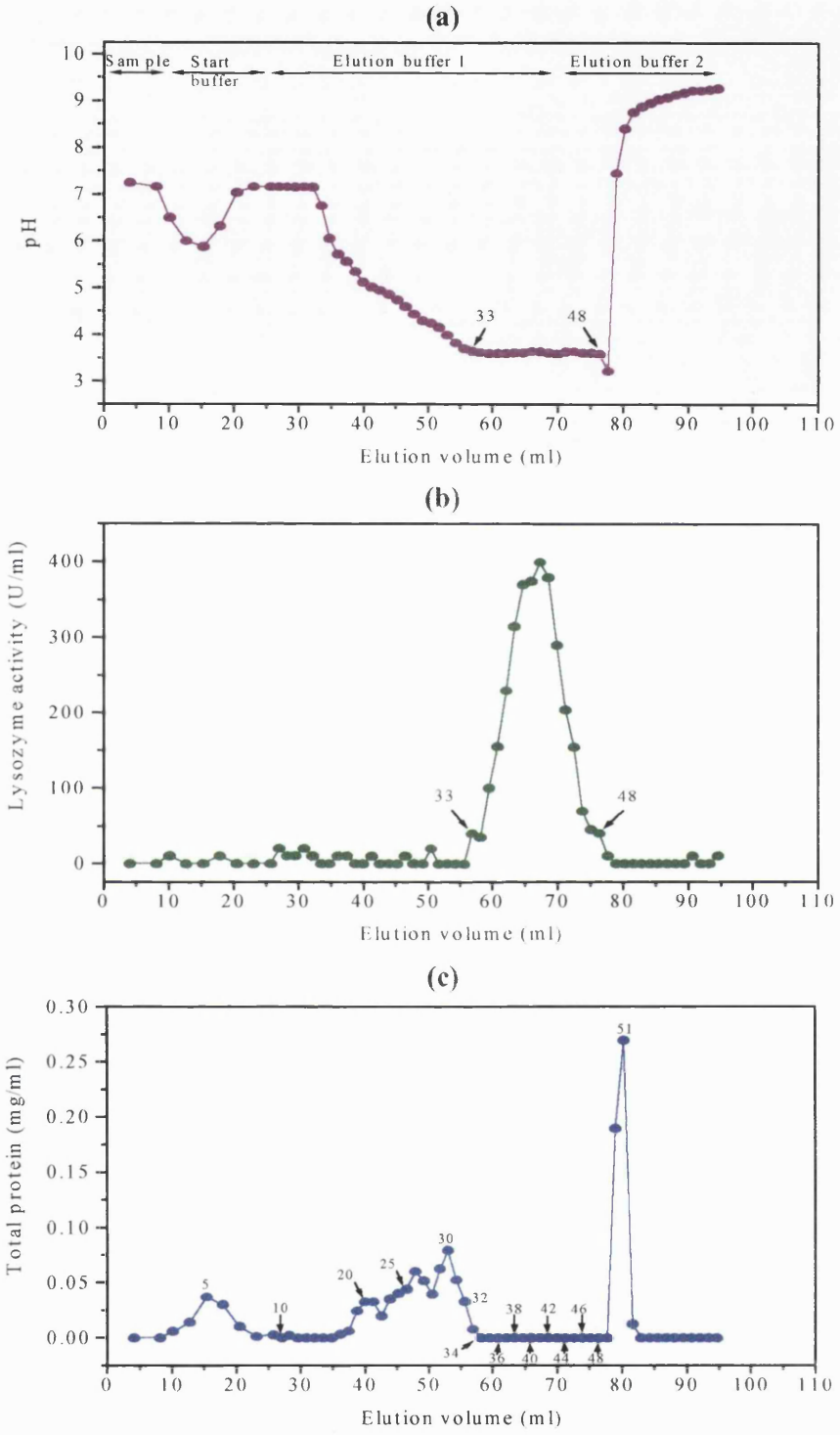


Figure 4.5 Graphs showing (a) pH; (b) lysozyme activity; (c) total protein for each fraction collected against the elution volume when the sample containing the recombinant protein was passed through a HiTrap Cu column - column 3. Samples from the fractions numbered in (c) were run on a SDS-PAGE gel (see **Figure 4.6**).

of **Figure 4.6** - the graph indicates that protein was beyond the limit of detection of the assay for fractions 34-49, yet when some of these fractions were electrophoresed (lanes 10-14 of the SDS-PAGE gel) protein is present.

Figure 4.6 Photograph of a 12% SDS-PAGE gel loaded with samples from a HiTrap column charged with Cu showing the purification of the recombinant T4 lysozyme - column 3. The samples in lanes 2-17 inclusive were concentrated 10-fold with TCA precipitation and 20 µl were loaded into the wells. **Lanes 1** and **18**, 5 µl molecular weight markers (Sigma, SDS-7); **lane 2**, 20 µl of the extract loaded onto the column ; **lane 3**, fraction 5; **lane 4**, fraction 10; **lane 5**, fraction 20; **lane 6**, fraction 25; **lane 7**, fraction 30; **lane 8**, fraction 32; **lane 9**, fraction 34; **lane 10**, fraction 36; **lane 11**, fraction 38; **lane 12**, fraction 40; **lane 13**, fraction 42; **lane 14**, fraction 44; **lane 15**, fraction 46; **lane 16**, fraction 48; **lane 17**, fraction 51.

4.3.4 Isolation of the recombinant protein on an uncharged HiTrap column - column 4

In order to conclude that the recombinant protein is binding to the column as a result of an interaction between the metal ions on the column and surface sites on the protein, an uncharged column was run under identical conditions to the three outlined above. Graphs showing the pH and lysozyme activity for each fraction collected against the elution volume can be seen in **Figure 4.7**.

Approximately 2.8 mg of total protein contained in the 10 ml sample was applied to the top of the column in the usual manner. The graph shown in **Figure 4.7 (b)** indicates that the activity was eluted from the column in one peak which corresponded to fractions 2-6 inclusive over the pH range of 7.21-7.08. During the elution of these fractions the sample and start buffer were applied to the top of the column and one can therefore conclude that all the recombinant protein was being washed straight through and none appears to have bound. Other fractions exhibited minimal lysozyme activity but this was taken to be a background count and insignificant due to the inaccuracy of the assay method. Seventy percent of the activity loaded and 54% of that recovered was located in the five peak fractions.

Since the reason for this column was to ensure that the lysozyme was been retarded in the column by the metal ions present it was not thought necessary to assess the amount of protein in the column as it was predicted that all were eliminated from the matrix in the wash step and not bound to the iminodiacetic acid on the surface of the sepharose. Samples were not run on a SDS-PAGE gel.

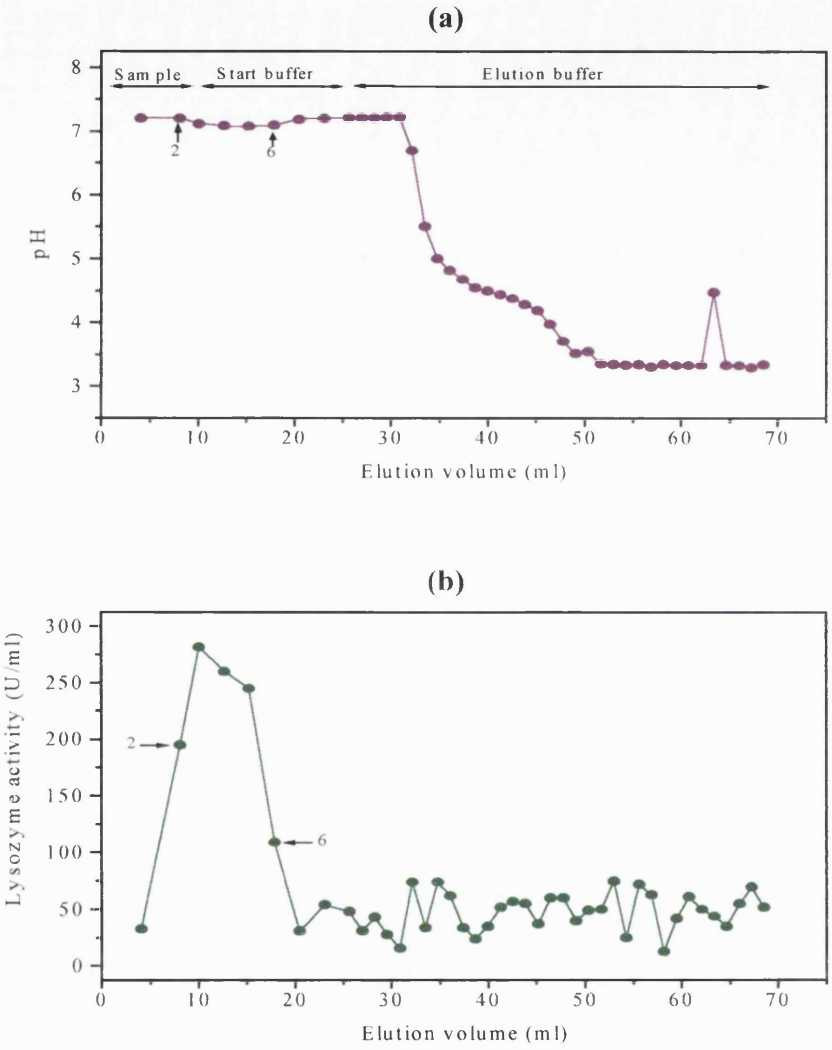


Figure 4.7 Graphs showing (a) pH; (b) lysozyme activity for each fraction collected against the elution volume when the sample containing the recombinant protein was passed through an uncharged HiTrap column - column 4.

4.3.5 Isolation of the wild type protein on a HiTrap Ni column - column 5

In order to state conclusively that the binding of the recombinant protein to the HiTrap column charged with Ni was due solely to the presence of the polyhistidine tail, the wild type protein was applied to the same type of column and run under identical conditions including the sample preparation. **Figure 4.8** shows the data obtained from this column - pH, lysozyme activity, and total protein measurements.

The activity data as seen in **Figure 4.8 (b)** shows that the activity was mainly distributed over seven fractions (2-8 inclusive) which had a pH range of 6.85-7.41. No elution buffer had passed through the column and hence it can be concluded that the lysozyme was not bound to the Ni. The peak region contained 92% of the total activity recovered from the column.

The graph which displays the elution volume against the total protein eluted - **Figure 4.8 (c)** - has two peaks. The first one was over fractions 2 to 7 and these were the unbound proteins. The second peak was fractions 15-20 which were eluted at a pH of 6.12-5.25. These proteins have bound to the Ni on the matrix. From the data obtained for the recombinant protein in column 1 (section **4.3.1**) and shown in **Figure 4.1 (c)**, the second protein peak was seen to be eluted at a similar pH. The amount of protein applied to column 5 was 2.56 mg and the amount recovered in all fractions was 2.45 mg (96%), of which 56% was present in fractions 2-8. It is interesting to compare the protein present in the second peak of this column (which represented 44% of the total recovered) to the second and third peaks with the recombinant protein in section **4.3.1** (which represented 47% of the total recovered). From this comparison it is possible to conclude that only a small percentage of the protein recovered in this region of column 1 (i.e. fraction 19) is comprised of the recombinant protein.

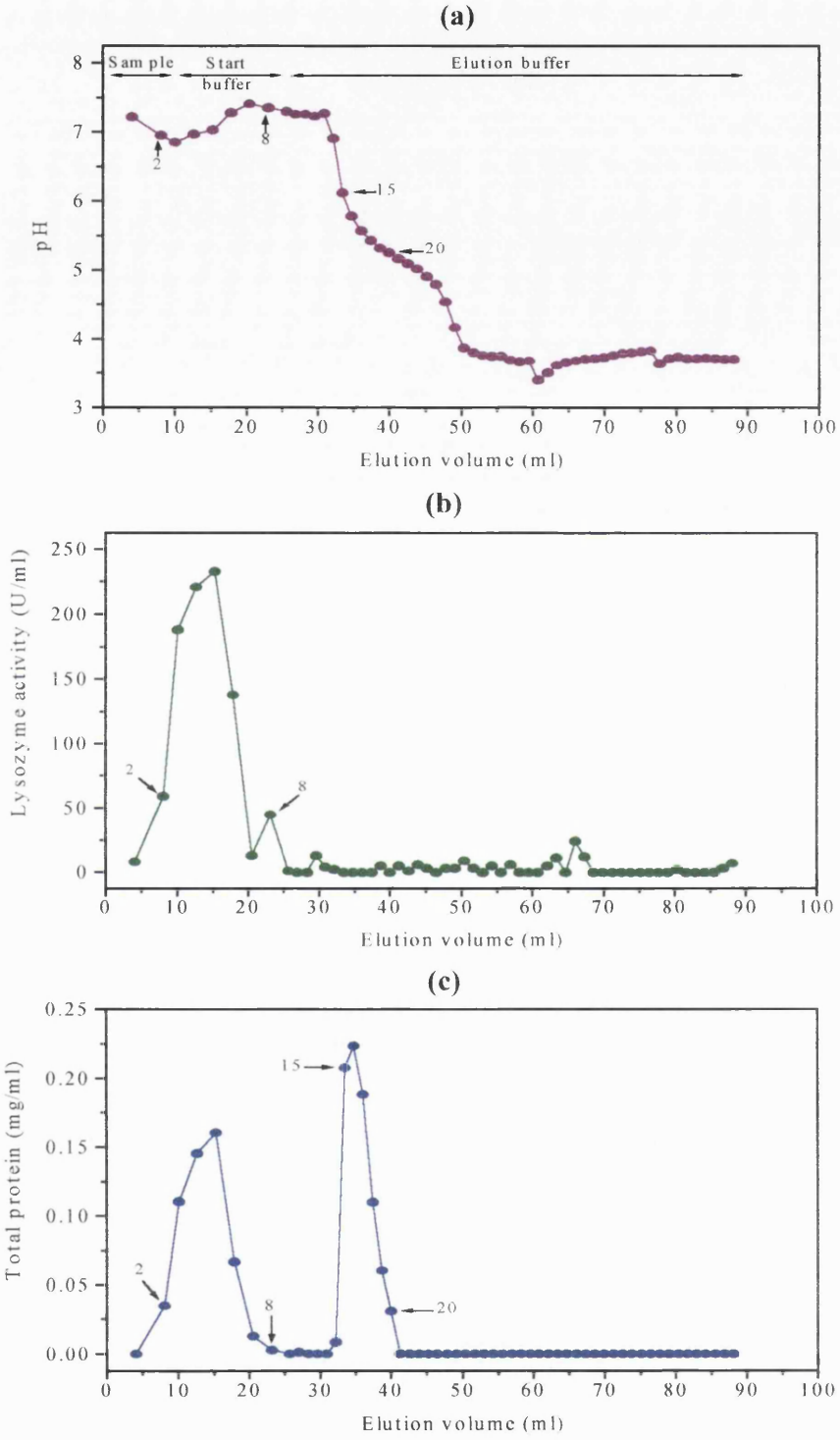


Figure 4.8 Graphs showing (a) pH; (b) lysozyme activity; (c) total protein for each fraction collected against the elution volume when the sample containing the wild type protein was passed through a HiTrap Ni column - column 5.

4.3.6 Isolation of the wild type protein on a HiTrap Zn column - column 6

In order to compare the ability of the Zn to purify of the recombinant and wild type proteins, 2.56 mg of total protein from a culture of pHSe5 was loaded onto a HiTrap column which was charged with Zn by the method described earlier. **Figure 4.9** shows the data obtained from this column - pH, lysozyme activity, and total protein measurements plotted against the elution volume.

From graph **(b)**, the lysozyme assay data, it can be seen that there is one region of the eluted volume where the lysozyme activity was present - fractions 1-8 inclusive at a pH range of 7.06-6.98. Fractions 1-8 accounted for 91% of that recovered. Since this activity came off the column during the loading and washing steps it can be concluded that the lysozyme was washed through the column without binding to the zinc.

Of the 2.56 mg of protein loaded, **Figure 4.9 (c)** shows that the protein was eluted in two regions. The first was contained in fractions 2-6 and are the proteins that were washed through the column between the pH range of 7.02- 6.98 and included the lysozyme. These amounted to 87% of that loaded, or 85% of that recovered. The second and smaller peak at fractions 15-17 contained 0.4 mg protein (15% of that recovered) at the measured pH of 6.64-5.71. The corresponding column on which the recombinant protein was separated (column 2, section **4.3.2**) also had a peak of protein eluted between pH 6.74-5.06 (35% of protein loaded and eluted) and the lysozyme was eluted in this region.

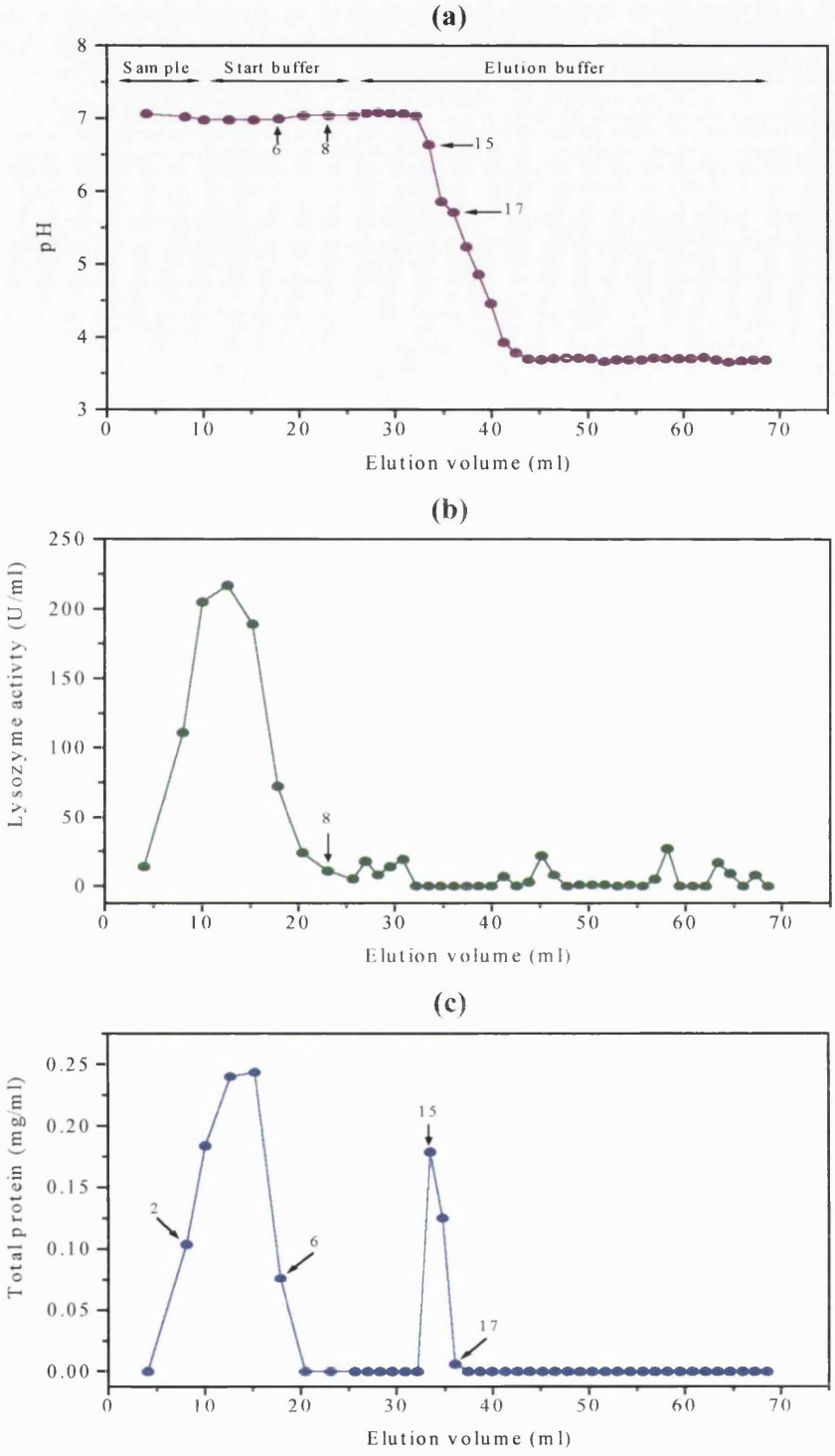


Figure 4.9 Graphs showing (a) pH; (b) lysozyme activity; (c) total protein for each fraction collected against the elution volume when the sample containing the wild type protein was passed through a HiTrap Zn column - column 6.

4.3.7 Isolation of the wild type protein on a HiTrap Cu column - column 7

This column enables comparisons to be drawn with column 3 which was run in section **4.3.3** above to purify the recombinant protein. **Figure 4.10** shows the pH, lysozyme activity, and total protein content of each fraction plotted against the elution volume for the sample which contained the wild type protein. As in the earlier Cu-charged column, two elution buffers were used (the first was 20 mM sodium phosphate, 0.5 M NaCl, pH 3.8, and the second was 0.1 M imidazole, 1 M NaCl, pH 9.35).

A total of 2.56 mg of total protein were applied to the column. From **Figure 4.10 (b)**, it can be seen that there is one region of activity which was found in fractions 5-12, between pH 6.8 and 7.22. Of the 2,115 Units applied to the top of the column, a total of 78% of the recovered activity was located in fractions 5-12. There is some evidence for a very weak interaction of the wild type protein with the Cu-charged column as the activity peak is slightly retarded compared to the Ni and Zn columns. However, the enzyme was easily displaced at neutral pH, and virtually all was eluted before the start of the pH elution gradient.

Of the protein loaded at the start of the experiment, it was calculated that a total of 0.45 mg had been recovered. This protein estimation is on the low side and a further indication of this can be seen for the wash through proteins of fractions 1-12 which was a negligible value. In the column 3 run (section **4.3.3**), the wash-through protein amounted to 15% of that recovered; in this column the equivalent region amounted to 5% of that recovered. It can be seen from **Figures 4.5 (c)** and **4.10 (c)** that there appear to be three regions where protein has been eluted. With the wild type sample they are around fraction(s) 5, 25-27 (pH 4.43-3.88), and 50-51 (pH 7.7-8.5). It is interesting to compare these regions to those of equivalent pH readings in the column of the recombinant protein (section **4.3.3**) - fraction 5 would correspond to the wash through proteins, fractions 25-27 here have a pH similar to those of the second peak of the earlier column (fractions 26-30) where some proteins had bound to the column but were eluted just prior to the lysozyme, and fractions 50-51 in both columns have similar pH values and the proteins which compose these peaks have bound tightly to the Cu and were only stripped from the columns by the stringent conditions of the second elution buffer.

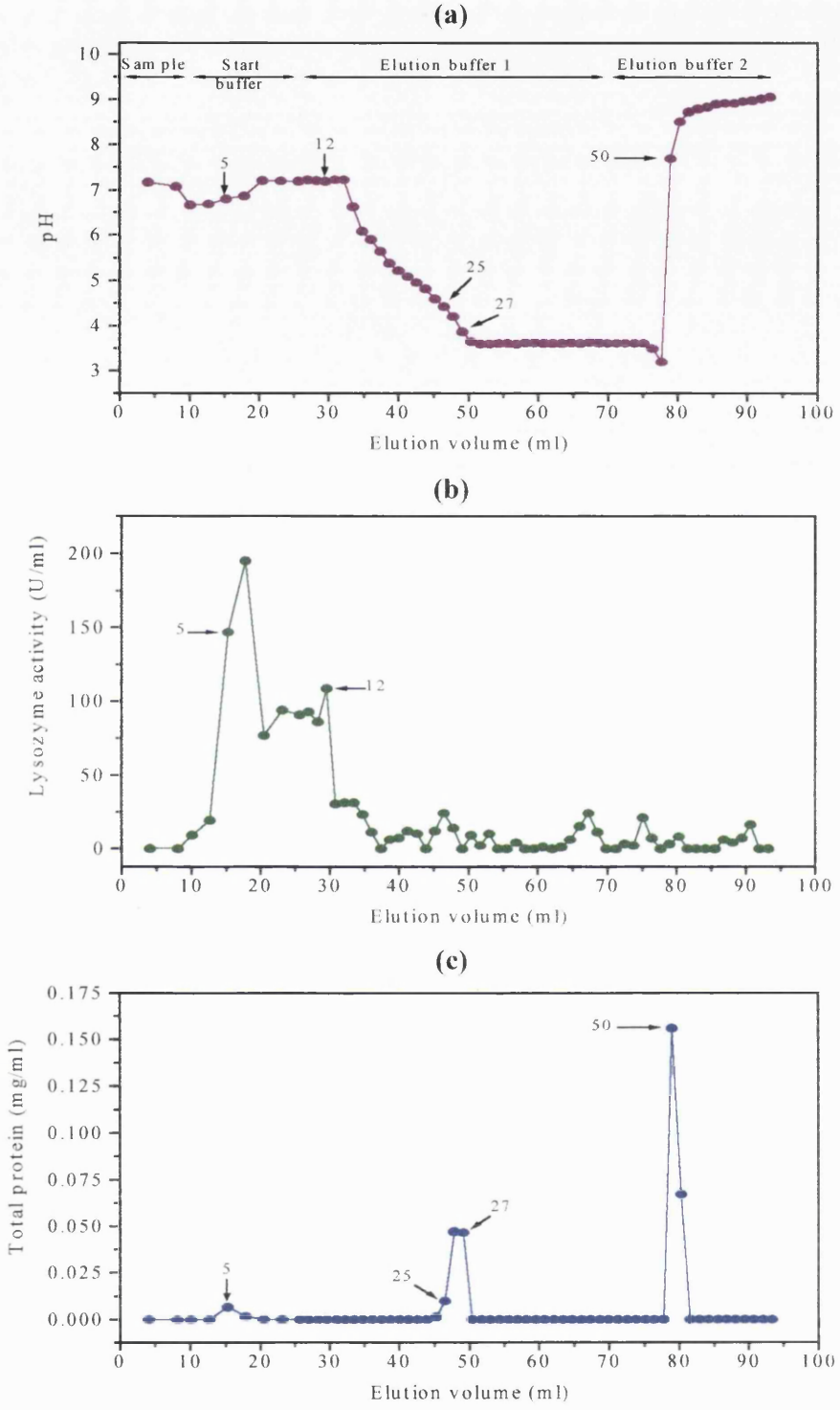


Figure 4.10 Graphs showing (a) pH; (b) lysozyme activity; (c) total protein for each fraction collected against the elution volume when the sample containing the wild type protein was passed through a HiTrap Cu column - column 7.

It should be noted that each of the fractions eluted from all three charged columns which were loaded with the recombinant protein (i.e. columns 1-3) were tested for leaching of the metal ion from the column by atomic absorption spectrometry and results showed that insignificant levels were recorded (data not shown).

4.4 Summary

Chapter 3 described the construction and expression of the recombinant protein. This chapter deals with the attempted purification of this histidine-containing protein by column chromatography on nickel-, zinc-, and copper-charged columns which was necessary before purification attempts were made on the novel magnetic supports (Chapter 5). In order to state that the successful purification was due to an interaction between the protein and the metal ions on all three of the columns, an equivalent sample was passed through an uncharged column and it was seen that the protein was not retained.

To prove that binding resulted due to the presence of the histidine-containing tail and not with any other region of the protein, a sample which contained the wild type tail-free lysozyme was passed through columns charged with Ni, Zn, and Cu ions under identical conditions. The results of these columns indicated that the wild type T4 lysozyme was not retained on the column in any way and was washed straight through being eluted in the first few fractions and prior to the application of any elution conditions. The wild type T4 lysozyme contains a single His residue within the active cleft at position 31. However, it is thought to be unlikely that it is accessible to the immobilized metal ions (B.W. Matthews, personal communication) and a single His residue would not cause retention of the protein.

From these results it can be concluded that the recombinant protein can be purified (to different extents) from a clarified mixture of *E. coli* culture on a HiTrap Chelating Sepharose column charged with Ni, Zn, or Cu ions. The fusion tail enabled rapid selective purification from a heterogeneous mixture.

The strength of binding to the different metal-charged IDA Sepharose varied. The recombinant protein was quickly desorbed from the Zn columns (between pH 5-5.6) in a low volume but some contaminating proteins were visible in the SDS-PAGE gel (**Figure 4.4**). The protein bound more tightly to the Ni column (an elution pH of 4.5-5.3), although not so concentrated, was more pure. The retention of the tailed lysozyme was tightest on the Cu-charged column where it was eluted at an extremely low pH - 3.59-3.65. All bound recombinant protein was efficiently eluted from all the charged columns tested. The binding of protein to the Cu ions was so strong that a more stringent buffer (0.1 M imidazole, 1 M NaCl, pH 9.35) was tested for the ability to elute

other bound proteins. This second elution buffer did indeed elute further proteins but it was not thought necessary to use this second elution buffer on the other types of columns as binding of the recombinant was not as strong. With the Cu-charged column, the elution volume was large (20.8 ml, 10 ml sample loaded) but the protein purity was good. An ideal purification procedure would be to run a Cu-charged column for purity followed by a Zn-charged column to concentrate the sample. The choice of which metal ion to employ is influenced by a balance of purity, yield and speed and hence the column of choice would have to be copper. It should be stressed that this is not necessarily the best for all proteins containing a polyhistidine tail as the harsh elution conditions may not be survived by other proteins. A summary of the data obtained from each column can be seen in **Table 4.1**.

The recombinant protein was purified effectively and efficiently from these chelating columns charged with Ni, Zn, and Cu on the small scale outlined above. However, column chromatography would pose a number of problems for a large-scale purification including flocculation and clogging of the Sepharose. Larger columns would have to be used and there is a limit to the size that are available and practical to handle. Also, the sample required some pre treatment which is time consuming and therefore would add to the processing costs at a large scale. It was in order to overcome these problems that purification of the recombinant protein was tested on magnetic particles which were coated with IDA and had the facility to be charged with metal ions, thereby attempting to mimic the scenario achieved with the columns outlined in this chapter. Experiments with these novel magnetic supports are outlined in Chapter 5.

In the table opposite (**Table 4.1**) the results of the copper columns should be treated with caution as the protein content of many of the peak fractions were below the limit of detection and hence the total protein in the peak fractions has been underestimated. Consequently, the data for the Total protein, Specific Activity, Purification factor, and Yield for these two columns have been affected and are not true values. The actual protein content of the peak fractions is greater than that stated in the table and therefore the Specific activity, Purification factor, and Yield values are smaller than those given.

Table 4.1 Summary of the purification data for each column with recombinant and wild type proteins

	Activity peak fraction numbers	Elution pH	Total activity (Units)	Total protein (mg)	Specific Activity (Units/mg)	Purification factor*	Yield (%)#
Recombinant extract	_	_	27,240	8.14	3,346	1	100
Nickel column	23-32	5.27-4.48	7,839	1.24	6,322	1.89	28.8
Recombinant extract	_	_	3,700	2.8	1,321	1	100
Zinc column	17-18	5.58-5.06	2,093	0.44	4,757	3.6	56.6
Recombinant extract	_	_	3,700	2.8	1,321	1	100
Copper column	33-48	3.65-3.59	4,167	0.01 ξ	416,000	315	113
Recombinant extract	_	_	4,170	2.8	1,489	1	100
Uncharged column	2-6	7.21-7.08	2,940	_	_	_	71
Wild type extract	_	_	2,115	2.56	826	1	100
Nickel column	2-8	6.85-7.41	2,302	1.37	1,680	2.0	109
Wild type extract	_	_	2,115	2.56	826	1	100
Zinc column	1-8	7.06-6.98	1,577	2.24	704	0.85	74.6
Wild type extract	_	_	2,115	2.56	826	1	100
Copper column	5-12	6.8-7.22	1,944	0.02 ξ	97,200	118	92

* Purification factor = $\frac{\text{Specific activity of peak}}{\text{Specific activity loaded}}$

\# Yield = $\frac{\text{Activity in peak fractions}}{\text{Total activity loaded}} \times 100$

ξ These values tended to err on the side of underestimation as the protein content of some fractions were essentially on or below the limits of detection of the assay used (see text section **4.3.3**) and hence the Specific activity and Purification factor values for these copper-charged columns are not true values.

_ indicates that these values were not determined.

CHAPTER FIVE

PURIFICATION OF THE RECOMBINANT PROTEIN ON NOVEL MAGNETIC SUPPORTS

5.1 Introduction

The results of the last chapter showed that the recombinant protein could be selectively isolated and purified from a heterologous mixture of fermentation products by column chromatography using charged chelating sepharose columns. Although the techniques used were successful, they would not be recommended for a large-scale purification procedure for several reasons, such as fouling of the column, a size limitation on the columns available, the dialysis for pre treatment which would be time consuming, and the extensive cleaning procedures which would be required for the matrix.

In order to overcome some of these problems, it was decided to test the ability of the novel magnetic particles to selectively purify the recombinant protein from a crude mixture on a small pilot scale but always keeping in mind that the procedures must have the ability to be scaled up. The theory behind the following procedures again utilises the specific interaction between the polyhistidine tail and divalent metal ions.

A description of the method of production of the beads is given in Chapter 2 section **2.4.17,** and the general method for their use is also outlined in that section. Use of the support can be divided into several sections such as charging of the particles, binding of the recombinant protein to the particles, and elution of the required protein from the particles.

This chapter deals with the purification of the recombinant T4 lysozyme with these magnetic beads and assesses the parameters which should be taken into consideration when using these novel supports so as to provide a basic protocol to optimise the quality and quantity of the recovered recombinant T4 lysozyme.

Since Cu-charged sepharose columns yielded the strongest binding in the column chromatography as seen in the last chapter, it was decided to carry out most of the experiments with the magnetic supports charged with this metal unless stated otherwise in the text. In all experiments both "clarified" and "crude" samples were tested (see Chapter 2, section **2.4.12** for nomenclature). In each experiment the general usage of the magnetic support was employed as described in Chapter 2 section **2.4.17.3,** unless otherwise stated.

Lysozyme assays and protein estimations were carried out for each supernatant sample collected. Samples were stored on ice prior to the lysozyme assays which were carried out as soon as possible after all samples had been collected. The samples were then frozen at -20°C and assayed for protein content when time allowed using the Pierce Protein Plus assay which is more sensitive than the Bio-Rad Bradford assay.

5.2 Sample preparation

Initial studies which were carried out attempted to mimic the conditions used in the column purifications described in the previous chapter and hence the samples were prepared by the same method (section **2.4.11**). However, binding could not be achieved under these conditions and a change of fermentation broth from Nutrient No.2 to Luria-Bertani broth had no apparent effect. It was then decided to pellet the cells by centrifugation, wash them in buffer, prior to sonication to release the lysozyme, after which binding to the beads was successful. A general method of sample preparation for use with the magnetic supports is outlined in section **2.4.12**.

It is, at present, unknown why binding could only be achieved after washing the cells before releasing the intracellular proteins, but it is perhaps due to the presence of some component(s) in the broth which may interfere with the binding mechanism. The reason why the method of direct sonication into the media worked with the column chromatography was perhaps that this component(s) was relatively small and was eliminated from the loaded sample by the dialysis and/or filtering steps which were carried out prior to the use of the chelating columns. An indication may be given by the composition of the nutrient broth used which can be seen in **Appendix 4**. It is noted that many divalent ions are present in the media and one possibility is that some of these formed a complex with the histidine tails on the recombinant protein hence there were no vacant sites available to bind to the charged magnetic supports.

In this chapter, "eluted fractions" refers to those fractions collected after the addition of the chosen elution buffer had been applied to the tubes and prior to stripping the beads of the metal ion, "total activity (or protein) recovered" refers to the activity or protein recovered in all fractions for each tube (**i.e.** Unbound, Wash 1 and 2, Elute 1 and 2, Strip), and "eluted as a % recovered" refers to the activity (or protein) present in the eluted fractions expressed as a percentage of the total activity (or protein) recovered.

5.3 Capacity of the novel magnetic supports

In order to work at the most efficient level, it was necessary to find the capacity of the beads for the recombinant protein. To try to accomplish this task, and since the magnetic support was not freely available, the volume of the beads and the conditions were kept constant while the amount of total protein added was varied.

For this experiment, beads from batch 5 were used (see **Table 2.3**) and each tube contained 50 µl of bead suspension which was equivalent to 1 mg support. An estimation of the protein content of each of the two extracts was measured with the Bio-Rad Bradford reagent - the clarified sample contained 2.5 mg/ml total protein while the crude extract contained 3.25 mg/ml total protein. The protein and binding buffer (20 mM sodium acetate, 0.5 M NaCl, pH 7.2) were added to the Cu-charged beads and allowed to interact at 4°C for 13 hours with mixing. The protein was eluted from the support with 350 µl 0.1 M sodium acetate, 0.5 M NaCl, pH 3.8.

A summary of the lysozyme activity data for each collected fraction can be seen in **Table 5.1**. The amount of protein added to the tubes increased from tube numbers 1-7 (clarified sample) and 8-13 (crude sample). It appears from the data obtained for the Unbound fractions that the samples collected in tubes 6, 7, 12, and 13 exhibited lysozyme activity indicating that the beads in these tubes had reached their capacity of binding as excess lysozyme was present in these collected fractions - the activity of the Unbound fraction from tube 7 accounted for 14% of the total activity recovered in all six fractions for this tube, and similarly the activity of the Unbound fraction for tube 13 accounted for 13% of the total activity recovered from this tube. Low amounts of activity were present in the Wash 1 and 2 fractions when the beads were washed with binding buffer, but slightly higher levels were seen in the tubes which were presented with maximum amount of protein (7 and 13). As expected, the total activity eluted with the elution buffer (0.1 M sodium acetate, 0.5 M NaCl, pH 3.8) in the two fractions (labelled Elute 1 and Elute 2) from the beads also increased as the amount of protein loaded increased. The activity recovered in these two Elute fractions for each tube represented anything from 77-97% of the total activity recovered from the six steps. Insignificant activity was present in any of the Strip fractions.

Table 5.1 To find the capacity of 1 mg of beads - data shows the amount of lysozyme activity (in Units) of each collected fraction.

Sample	Tube No.	Volume of extract added (µl)	Unbound fraction	Wash 1	Wash 2	Elute 1	Elute 2	Strip	Total activity recovered
Clarified	1	18.75	2	14	22	77	52	0	167
	2	37.5	12	10	5	186	121	1	335
	3	56.25	0	12	7	279	169	6	473
	4	75	22	21	0	330	202	0	575
	5	93.75	2	0	14	369	234	0	619
	6	140.6	60	25	4	410	273	0	772
	7	187.5	130	38	39	435	259	0	901
Crude	8	37.5	0	4	0	191	99	4	298
	9	56.25	0	0	4	290	146	3	443
	10	75	0	9	0	320	198	10	537
	11	93.75	11	0	0	375	223	0	609
	12	140.6	36	0	10	408	265	13	732
	13	187.5	121	29	49	442	276	8	925

The data presented in **Table 5.2** shows the amount of total protein present in each fraction. Results for both extracts showed that the protein present in the Unbound fraction accounted for 36-70% of the total protein recovered from all six steps, and that this value increased proportionally to the amount of protein added initially (i.e. 36% was found in tube 1 and 70% was found in tube 7). Small amounts of protein were recovered in the two Wash fractions as was the case with the activity data. Of the protein eluted with the sodium acetate buffer (Elute 1 and 2), on average 13% (clarified) or 12% (crude) of the total activity recovered from all six steps was located in these two fractions and a smaller proportion was eluted in the second fraction. It should be noted that the recombinant T4 lysozyme was found to represent approximately 15% of the total protein in the extracts (as seen on SDS-PAGE gels). After elution of the recombinant protein, up to 60 μg of protein was found to be still bound to the beads, thus indicating that other proteins bound to the copper - it was not within the scope of this project to investigate these further.

Table 5.2 To find the capacity of 1 mg beads - data shows the total protein content (in μg) of each fraction collected.

Sample	Tube No.	Volume of extract added (μl)	Unbound fraction	Wash 1	Wash 2	Elute 1	Elute 2	Strip	Total protein recovered
Clarified	1	18.75	24	0	7	5	5	24	65
	2	37.5	36	7	0	8	0	46	97
	3	56.25	55	0	7	14	5	49	130
	4	75	83	7	8	19	6	52	175
	5	93.75	102	7	8	23	6	55	201
	6	140.6	185	9	8	34	8	60	304
	7	187.5	266	12	7	42	6	46	379
Crude	8	37.5	36	7	8	7	0	32	90
	9	56.25	56	8	7	11	0	45	127
	10	75	81	7	7	18	7	59	179
	11	93.75	106	9	8	24	5	61	213
	12	140.6	189	11	8	35	9	61	313
	13	187.5	294	13	8	45	9	58	427

The Unbound fractions for both crude and clarified extracts (tubes 1-13 inclusive) were examined visually for the presence of T4 lysozyme by electrophoresing unconcentrated samples on SDS-PAGE gels. Similar results were obtained for both fractions and a photograph of the silver-stained gel for the crude samples is shown in **Figure 5.1**. It can be seen that there is a band which corresponds to a protein the size of the recombinant lysozyme in lane 7 (tube 13) and it may also be present in lane 6 (tube 12) - this can also be inferred from the activity data for these fractions which indicate that the capacity of the magnetic particles for the recombinant protein in these tubes has been exceeded. Lanes 3 and 4 contain trace quantities of a protein whose size is slightly larger than that of the recombinant T4 lysozyme.

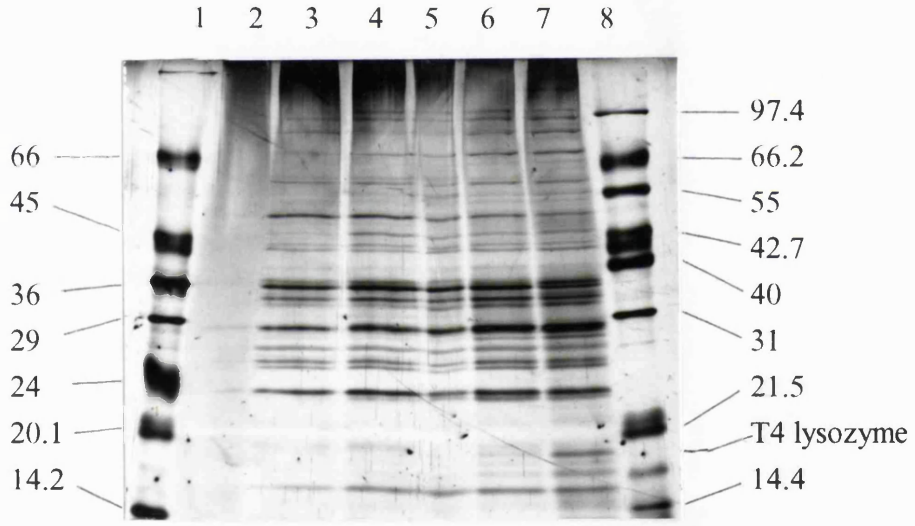


Figure 5.1 A photograph of the 15% SDS-PAGE silver-stained gel loaded with unconcentrated samples from the Unbound fractions of the crude extract (tubes 8-13 inclusive) in order to find the capacity of 1 mg of the magnetic support particles. A protein band corresponding in size to that of the T4 lysozyme (~19 kDa) can be seen in lane 7 indicating that not all the recombinant protein has bound to the support as the capacity of the beads has been exceeded. **Lane 1**, 0.1 μl molecular weight markers (Sigma, SDS-7); **lane 2**, 30 μl of sample from tube 8; **lane 3**, 15 μl from tube 9; **lane 4**, 10 μl from tube 10; **lane 5**, 7.5 μl from tube 11; **lane 6**, 5 μl from tube 12; **lane 7**, 3.75 μl from tube 13; **lane 8**, 0.1 μl molecular weight markers (Promega, Mid Range). The sizes of the molecular weight markers are in kDa.

The graph shown in **Figure 5.2** is a plot of the recovered activity in the two eluted fractions (Elute 1 and 2) against the amount of total protein presented to the beads (as measured by the Bio-Rad Bradford protein assay) for both clarified and crude extracts. It can be seen that the performance of the magnetic supports was not adversely affected by the presence of suspended solids (of the crude extract) in this first cycle of operation.

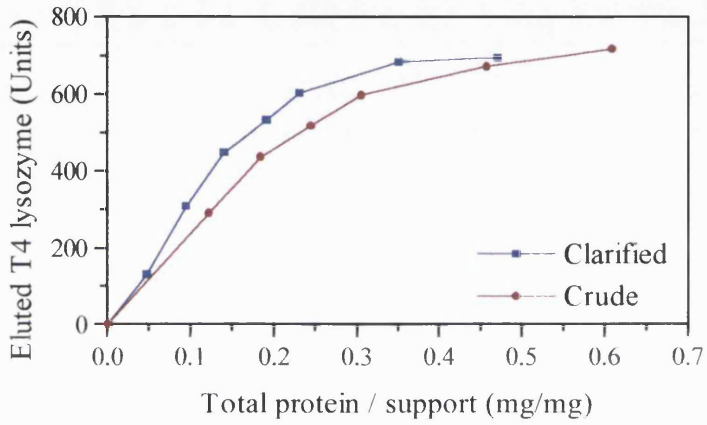


Figure 5.2 A graph of the eluted T4 lysozyme activity recovered (Elute 1 and Elute 2 fractions) plotted against the total amount of protein presented to 1 mg support for both clarified and crude extracts.

On the basis of these results, it was decided to use 0.3-0.35 mg total protein of clarified extract, and 0.4-0.45 mg total protein of crude extract per mg magnetic support particles for future experiments.

5.4 The number of wash steps required after binding

In the column chromatography of Chapter 4 the matrix was flushed through with a minimum of three column volumes of start buffer to ensure that all unbound and loosely bound proteins were eliminated and did not contaminate the purified product. This step is also required when the magnetic supports are employed as even careful aspiration of the Unbound fraction tended to leave a small amount of liquid behind which was entrapped between the beads. Of course, if the capacity of the beads for the recombinant protein was exceeded, part of this liquid would also contain some unbound lysozyme. Time and materials are important factors in a purification procedure and therefore it is necessary to find the minimum amount of washes required in order to eliminate any undesired proteins.

All Wash steps were carried out at room temperature thus keeping the cost low as refrigeration was not required. Not only should the number of washes be considered but also the volume of buffer required per Wash step. All experiments were carried out in microcentrifuge tubes and therefore there was a limit to the maximum volume of liquid permitted. However, it was observed that too much liquid did not allow optimum mixing of the tube contents. It was therefore decided to use 1 ml volumes of the Wash (binding) buffer.

In all the experiments discussed above, two Wash steps were carried out. **Table 5.1** suggests that even when the capacity of the beads is exceeded, two Washes were adequate to eliminate excess protein as indicated by the activity data for both extracts but small amounts of lysozyme activity were found to be present in the second Wash step. However, in order to ensure that no other proteins are present, the protein estimations for the collected fractions should be consulted. Results shown in **Table 5.2** and **Table 5.6** indicate that protein is detected in both Wash fractions but those of **Table 5.4** indicate that no protein was detected in the second Wash fractions in all but one tube (out of 12). In general, twice as much protein is present in the Wash 1 fraction compared to the Wash 2.

From these results, it was decided to continue to Wash 1 mg of the beads twice with 1 ml of binding buffer at room temperature by vortexing for twenty seconds followed by magnetic separation and aspiration of the resulting liquid phase.

5.5 The effect of sodium chloride concentration on binding

The presence of sodium chloride in the binding buffer is thought to minimise non specific ionic interactions (Dean *et al.*, 1985). In Chapter 4, 0.5 M NaCl was employed in both the start and elution buffers. In order to optimise binding, a buffer containing 20 mM sodium phosphate, pH 7.2, with a range of six different sodium chloride concentrations (0-1 M) was tested in the following experiment.

A single culture was induced, aliquoted into six tubes, and centrifuged. Each of the six bacterial pellets was resuspended in a different binding buffer to enable comparisons to be made. In order to standardise the amount of protein presented to the beads, protein estimations were carried out on the samples with the Bio-Rad Bradford assay - 0.35 mg total protein of clarified extract and 0.45 mg total protein of crude extract were used. The protein and the respective binding buffers were added to the designated tubes which were then shaken on a vibrax at 4°C for 13 hours. The elution

buffer used was 0.1 M sodium acetate, 0.5 M NaCl, pH 3.8 and each tube contained 50 µl of bead suspension from batch 6 - equivalent to 1 mg beads.

Results of the activity estimations for each fraction can be seen in **Table 5.3**. It should be repeated here that lysozyme activity can be effected by the buffer composition and section **3.5** should be consulted as a rough guide to aid interpretation of the results. A reason for not directly comparing the actual activities obtained for each tube is that although tubes 1-6 and 7-12 contained approximately equal amounts of protein, the activities were not necessarily comparable (as different buffers were used). From **Figure 3.7 (a)** it can be seen that (compared to the activity in the standard of 20 mM sodium phosphate, 200 mM NaCl, pH 6.8) the enzyme appears to be activated in the buffers containing 0 M and 0.1 M NaCl and deactivated in the other four buffers. On considering this fact, it would appear that the higher the concentration of NaCl present in the buffer, the greater the "true" activity recovered for the clarified extract. However, **Figure 3.7 (a)** should only be taken as an indication of the activity and perhaps information obtained from protein estimations and SDS-PAGE gels can clarify which would be the best binding buffer, not only in terms of amount of protein bound but also the purity.

Taking the results for the clarified extract as they stand, little variation can be seen in the total activities recovered in all the steps (tubes 1-6, ranging from 593-671 Units). The activity recovered in the Unbound fractions are low as are those recovered in the two Wash steps and the Strip fractions. Of the total activity recovered from the clarified sample, not less than 87% was located in the Elute 1 and 2 fractions for any binding buffer.

The total activities recovered in the six steps for each of the crude samples (tubes 7-12) varied more than those obtained for the clarified samples, ranging from 644-814 Units. In general, the Unbound fractions of the crude extracts/samples tended to contain higher activities than the Unbound samples of the clarified extract, and this is also true of the Wash samples. The binding buffer which contained 0.25 M NaCl (tube 9) exhibited the highest activity values obtained for the Unbound, the two Wash steps and the Strip, and the eluted activities (Elute 1 and 2) represented 75.6% of the total recovered for this buffer. With all other buffers tested there was a minimum of 82% of the total activity present in the two eluted fractions.

The data found in **Table 5.4** shows the protein content of each fraction (in µg as estimated by the Pierce Protein Plus assay). The first conclusion that should be noted is the low recovery of total protein compared to that loaded - this indicates that not all proteins bound to the beads are stripped off with 0.1 M EDTA in 20 mM sodium phosphate, 0.5 M NaCl, pH 8 (this will be discussed later in section **5.14**). A large proportion of the protein did not bind to the support and came out in the Unbound fraction. On considering the protein present in the two eluted fractions, expressed as a

percentage of the total recovered in all fractions for each tube, 9.7% was recovered for tube 1, 10.3% for tube 2, 9.9% for tube 3, 8.3% for tube 4, 7.2% for tube 5, and 7.6% for tube 6, and a similar trend is seen when the eluted fractions are expressed as a percentage of the protein loaded. **Figure 5.3** is a plot of the eluted protein (as a percentage of the total protein recovered) as a function of the concentration of the sodium chloride in the binding buffer from the data obtained.

It can be noted that a maximum of 10 μg was present in Wash 1 and those of Wash 2 were below the limit of detection of the assay (with the exception of tube 1). Maximum amount of protein was eluted from tube 1 with similar amounts present for tubes 7 and 8 (where the binding buffer contained 0 M and 0.1 M salt respectively).

This protein data, together with the lysozyme activity data, would indicate that a binding buffer which contained NaCl in the range 0-0.25 M would permit the best binding for the clarified extract.

Table 5.3 Activity (in Units) of each fraction collected in order to assess the effect of the concentration of sodium chloride (in the 20 mM sodium phosphate, pH 7.2 binding buffer) on binding.

Sample	[NaCl] (M)	Tube No.	Unbound fraction	Wash 1	Wash 2	Elute 1	Elute 2	Strip	Total activity recovered
Clarified	0	1	16	17	10	364	225	11	643
	0.1	2	23	14	16	363	208	12	636
	0.25	3	36	0	23	361	239	10	669
	0.5	4	16	13	8	342	233	6	618
	0.75	5	35	17	17	333	251	18	671
	1.0	6	0	5	22	323	228	15	593
Crude	0	7	15	23	11	393	238	10	690
	0.1	8	78	13	7	386	216	3	703
	0.25	9	113	29	43	361	255	13	814
	0.5	10	56	41	24	336	251	7	715
	0.75	11	47	21	16	361	250	11	706
	1.0	12	20	6	0	358	252	8	644

Table 5.4 Protein (in μg) of each fraction collected in order to assess the effect of the concentration of sodium chloride (in the 20 mM sodium phosphate, pH 7.2 binding buffer) on binding.

Sample	[NaCl] (M)	Tube No.	Unbound fraction	Wash 1	Wash 2	Elute 1	Elute 2	Strip	Total protein recovered	Protein loaded
Clarified	0	1	194	8	5	21	4	24	256	287
	0.1	2	137	9	0	19	0	20	185	320
	0.25	3	147	8	0	19	0	18	192	351
	0.5	4	158	6	0	17	0	25	206	348
	0.75	5	197	5	0	13	4	18	237	380
	1.0	6	149	0	0	14	0	22	185	333
Crude	0	7	191	8	0	19	2	19	239	315
	0.1	8	134	10	0	21	0	22	187	336
	0.25	9	159	10	0	13	0	19	201	354
	0.5	10	150	8	0	8	0	20	186	330
	0.75	11	180	7	0	8	2	16	213	305
	1.0	12	152	6	0	13	3	21	195	260

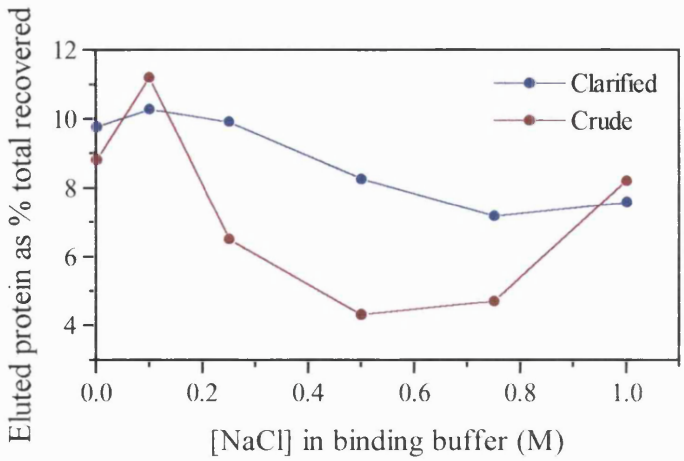


Figure 5.3 The protein eluted from the magnetic bead support with 0.1 M sodium acetate, 0.5 M NaCl, pH 3.8 (expressed as a percentage of the total protein eluted in all six steps) for each concentration of sodium chloride in the binding buffer (20 mM sodium phosphate, pH 7.2).

A photograph of a silver-stained 15% SDS-PAGE gel with samples of equal volume from the Elute 1 fraction for each binding buffer used can be seen in **Figure 5.4**. The samples from tubes 3 (clarified, 0.25 M NaCl) and 8 (crude, 0.1 M NaCl) show contaminating proteins were present. Since the desired purification procedure required a combination of maximum amount of protein recovered with the minimal amount of contamination, and without permanent inactivation of the enzyme, it was decided to use a binding buffer that contained 0.2 M NaCl.

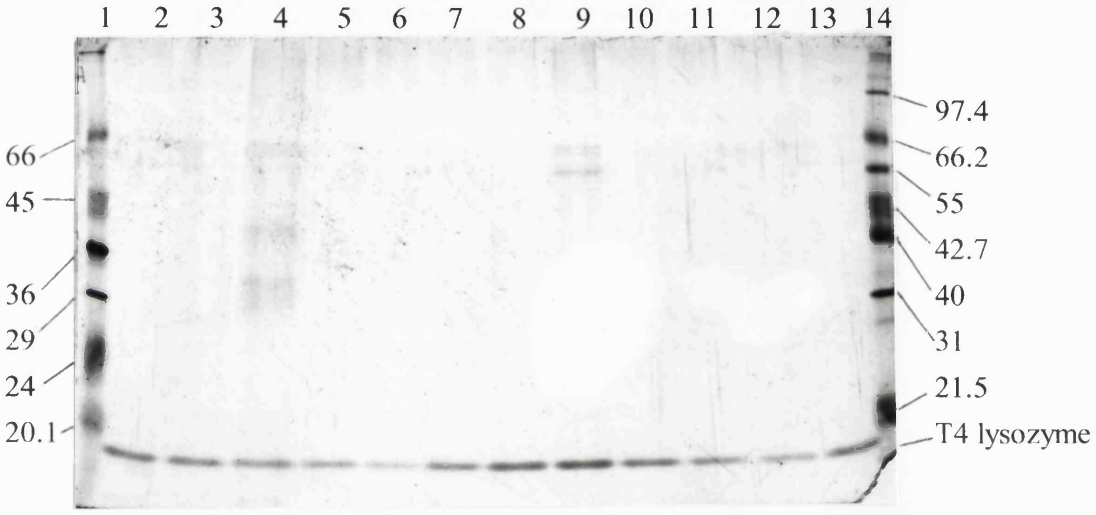


Figure 5.4 A photograph of the 15% SDS-PAGE silver-stained gel loaded with unconcentrated samples from the Elute 1 fractions to determine the optimum concentration of sodium chloride in the binding buffer. Lanes 2-13 inclusive were loaded with 5 µl samples. **Lane 1**, 0.1 µl molecular weight markers (Sigma, SDS-7); **lane 2**, tube 1 (0 M, clarified); **lane 3**, tube 2 (0.1 M, clarified); **lane 4**, tube 3 (0.25 M, clarified); **lane 5**, tube 4 (0.5 M, clarified); **lane 6**, tube 5 (0.75 M, clarified); **lane 7**, tube 6 (1 M, clarified); **lane 8**, tube 7 (0 M, crude); **lane 9**, tube 8 (0.1 M, crude); **lane 10**, tube 9 (0.25 M, crude); **lane 11**, tube 10 (0.5 M, crude); **lane 12**, tube 11 (0.75 M, crude); **lane 13**, tube 12 (1 M, crude); **lane 14**, 0.1 µl molecular weight markers (Promega, Mid Range). The sizes of the molecular weight markers are in kDa.

5.6 20 mM versus 50 mM sodium phosphate as a binding buffer

As mentioned in Chapter 4, Pharmacia did not recommend the use of a Tris-HCl buffer as the binding buffer on the HiTrap columns as "it tends to reduce binding and should only be used when the metal-protein affinity is high" (manufacturer's guideline booklet for HiTrap™ affinity columns, Pharmacia LKB Biotechnology, Sweden) and it is known that Tris can act as a weak chelating agent in such instances. The binding buffer used in all columns in Chapter 4 was 20 mM sodium phosphate, 0.5 M NaCl, pH 7.2. Porath (1987) has suggested the following order of salts for the relative contribution to protein precipitation from aqueous solutions : phosphates > sulphates > acetates > chlorides > nitrates > thiocyanates. Hochuli *et al.* (1987) used columns containing the resin nitrilotriacetic acid (NTA) coated with nickel ions to bind peptides and proteins which contained neighbouring histidine residues. The proteins were loaded onto such columns and the columns were equilibrated with either 0.1 M sodium phosphate, 0.5 M NaCl, pH 8 or 0.01 M sodium phosphate, 0.2 M NaCl, pH 7. Hochuli *et al.* (1988) purified a modified mouse dihydrofolate reductase containing two histidine residues with the use of a Ni-NTA column as previous, and used a 0.05 M sodium phosphate buffer, pH 8 for binding. M(II)-IDA columns were used by Hemdan *et al.* (1989) to isolate histidine-containing "model proteins" and used 0.02 M sodium phosphate, 1 M NaCl, pH 7 as the binding buffer. Earlier experiments with these novel magnetic support particles used a sodium phosphate buffer (S. O'Brien, personal communication). Since the binding of the recombinant protein to the sepharose columns in the last chapter was successful, it was decided to investigate the possibility of using a sodium phosphate buffer of higher molarity - 50 mM.

The samples were prepared as outlined in section **2.4.12**. Eight bacterial pellets from the same culture were resuspended in one of eight different buffers - 20 mM sodium phosphate, 0.2 M NaCl, pH 6.8 / 7.2 / 7.6 / 8.0 and 50 mM sodium phosphate, 0.2 M NaCl, pH 6.8 / 7.2 / 7.6 / 8.0 - and then sonicated. The protein concentration for each of the 16 samples was estimated with the Bio-Rad Bradford assay reagent and equivalent amounts of total protein were loaded onto the beads. Fifty microlitres of bead suspension from batch 7 (~1 mg) were charged with copper, protein was diluted to the required concentration with the same buffer as that used for resuspension of the cells, and the protein was allowed to bind at 4°C for 13 hours. As previously described, 0.1 M sodium acetate, 0.5 M NaCl, pH 3.8 was used as the Elution buffer in all cases.

The activity of each collected fraction was estimated and results are shown in **Table 5.5**. Although approximately equal amounts of protein were added to each tube, the activities were not necessarily the same and hence direct activity unit comparisons were not drawn. However, comparisons were made on the basis of a percentage of the total activity recovered. Using the data of the eluted activity (combined activity for

Elution steps 1 and 2) expressed as a percentage of the total activity recovered for each tube, it can be seen that the values obtained (for each pH, e.g. comparing tubes 1 and 5, 2 and 6, 3 and 7) were greater when the 20 mM sodium phosphate buffer was employed in all but one case (crude extract, pH 7.2) for both extracts. A graph displaying these results can be seen in **Figure 5.5**.

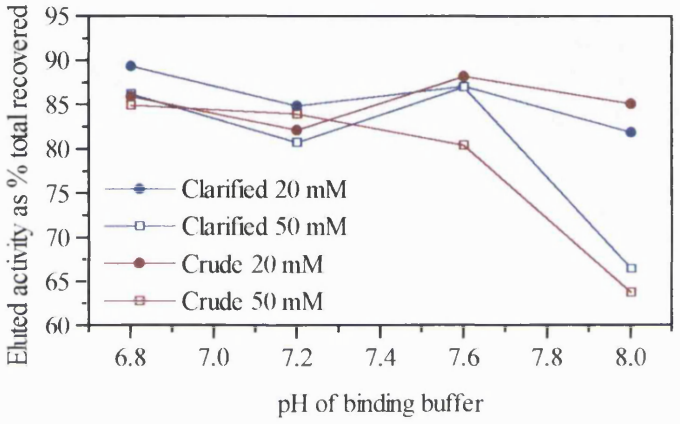


Figure 5.5 Graph of the eluted activity (combined values for the Elute 1 and 2 fractions) expressed as a percentage of the total activity recovered in all six fractions against the pH of the binding buffers (20 mM or 50 mM sodium phosphate, 0.5 M NaCl).

A similar trend can be found in the results obtained with protein estimations for each collected fraction which are to be found in **Table 5.6**. For each pH value investigated, the percentage of eluted protein (expressed as a percentage of the total protein recovered in the six steps) is greater when the 20 mM sodium phosphate buffer was used for binding and this is also true if the eluted protein is expressed as a percentage of total protein loaded.

Not only would the lower molarity of the buffer be cheaper for scale-up purposes, but it can be seen from the results obtained that the proportion of activity and protein eluted (compared to the total recovered) is greater when the 20 mM sodium phosphate is used rather than the 50 mM buffer. Perhaps even a lower molarity of sodium phosphate would yield similar results and this would be favourable for larger scale systems, but this was not investigated here.

5.7 pH of the binding buffer

This section was carried out in conjunction with section **5.6**. Having chosen the molarity of the sodium phosphate binding buffer in the previous section, the pH of the binding buffer was investigated. Adsorption of the protein should be carried out at neutral to alkaline pH so that the electron donor groups on the protein are unprotonated and to minimise damage to recombinant proteins which may be fragile. Preliminary work suggested that binding was achieved around neutral pH (O. Thomas, personal communication) and hence a range of 6.8-8.0 was investigated. Results of the activity and protein estimations are shown in **Tables 5.5** and **5.6** respectively.

It can also be seen from **Figure 5.5** that, in general, the higher the pH of the binding buffer, the lower the eluted activity when expressed as a percentage of the total activity recovered (this held true for both extracts and for both 20 mM and 50 mM phosphate buffers) over the pH range 6.8-8.0.

Although the samples all originated from the same induced bacterial culture they were all treated with different phosphate binding buffers and hence direct comparisons of activity are not recommended. Hence a more realistic comparison would be to base all calculations as percentages of total activity recovered in all fractions. Considering the data in **Table 5.5** for the 20 mM binding buffer, 82-89% of the total activity recovered was present in the Elute 1 and 2 fractions for the clarified extract, while for the tubes that contained the crude extract 82-88% of the total recovered activity was eluted in the two fractions. However, a more marked range of eluted activity (when expressed as a percentage of total activity recovered in the six steps) was found with the 50 mM phosphate binding buffer where values ranged from 66-86% for the clarified sample and 64-85% for the crude sample (see right-most column on **Table 5.5**). Information from **Figure 3.7** (which indicates the activities of the extracts in the different buffers) indicates that there is little difference with the clarified extract and only a small decrease in the activities of the crude sample as the pH of the buffers increased.

On the basis of the activity data presented here it would not be recommended to use a phosphate binding buffer with a pH of 8.0 or above.

Footnote to **Table 5.5** (next page):
* Total activity recovered in all six steps (per row of table)
# Activity present in the two eluted fractions expressed as a percentage of the total activity recovered in all fractions

Table 5.5 The activities (in Units) of all fractions collected to allow a comparison in binding between 20 mM and 50 mM sodium phosphate, 0.5 M NaCl, buffers of different pH.

Sample	Sodium phosphate Molarity	pH	Tube	Unbound fraction	Wash 1	Wash 2	Elute 1	Elute 2	Strip	*Total activity recovered	#Eluted as % recovered
Clarified	20 mM	6.8	1	49	18	22	431	315	0	835	89
		7.2	2	57	20	38	409	302	12	838	85
		7.6	3	38	32	20	410	314	17	831	87
		8.0	4	67	39	40	422	302	14	884	82
	50 mM	6.8	5	54	24	20	421	319	21	858	86
		7.2	6	77	40	40	393	324	14	888	81
		7.6	7	44	22	13	368	306	22	775	87
		8.0	8	102	90	86	311	274	18	881	66
Crude	20 mM	6.8	9	26	50	32	422	312	13	855	86
		7.2	10	65	29	29	392	282	24	821	82
		7.6	11	59	13	12	389	276	5	754	88
		8.0	12	81	32	0	399	288	7	807	85
	50 mM	6.8	13	83	26	0	390	320	18	837	85
		7.2	14	78	29	25	389	325	6	851	84
		7.6	15	53	34	45	354	284	25	794	80
		8.0	16	133	91	90	317	277	25	932	64

Activity data (**Table 5.5**) would indicate that there does not seem to be a trend between the pH and the eluted activity. Results from the protein estimations of each fraction are shown in **Table 5.6** and indicate that approximately equal amounts of protein are recovered from all six fractions when expressed as a percentage of the protein loaded for each tube (approximately 70% in each case - see ξ column in **Table 5.6**). However, when the eluted protein (from the Elute 1 and 2 fractions) is expressed either as a percentage of total protein presented to the magnetic particles (* column), or as a percentage of the total protein recovered from all six fractions (# column), it can be seen that in general the value decreased as the pH of the solution increased - this is true for both extracts. The range of values is wider for the binding buffer containing 50 mM sodium phosphate.

Results from this section would indicate that the pH of the binding buffer should be within the range of 6.8-7.2. Since binding was known to occur at a neutral pH (O. Thomas, personal communication) values below pH 6.8 were not included in this study.

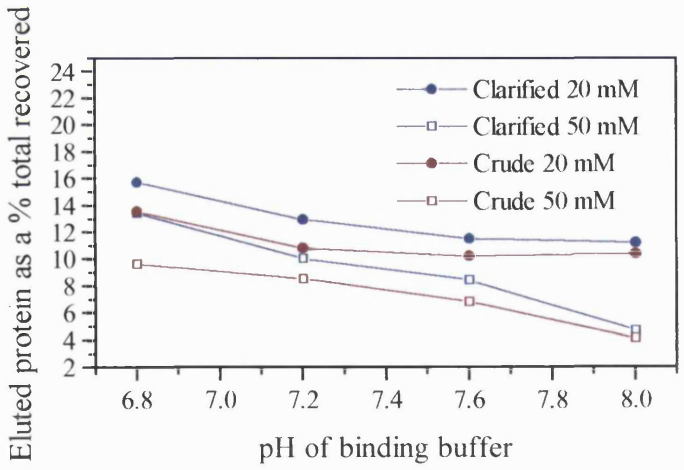


Figure 5.6 Graph of the eluted protein (Elute 1 and 2) expressed as a percentage of the total protein recovered in all six fractions against the pH of the binding buffers (20 mM or 50 mM sodium phosphate, 0.5 M NaCl).

Footnote for **Table 5.6** (next page):
* Protein present in the two eluted fractions expressed as a percentage of the total protein loaded
# Protein present in the two eluted fractions expressed as a percentage of the total protein recovered
ξ Total protein recovered in all six fractions expressed as a percentage of the protein loaded

Table 5.6 The protein content (in μg) of all fractions collected to allow a comparison in binding between 20 mM and 50 mM sodium phosphate, 0.5 M NaCl, buffers of different pH.

Sample	Sodium phosphate Molarity	pH	Tube	Unbound fraction	Wash 1	Wash 2	Elute 1	Elute 2	Strip	Total protein loaded	*Eluted as % total loaded	Total protein recovered	#Eluted as % total recovered	ξ Recovered as % loaded
Clarified	20 mM	6.8	1	152	11	5	29	8	31	343	10.7	236	15.7	68.8
		7.2	2	167	13	6	27	6	37	364	9	256	12.9	70.3
		7.6	3	185	12	6	23	7	28	363	8.3	261	11.5	71.9
		8.0	4	191	14	6	25	5	28	365	8.2	269	11.2	73.6
	50 mM	6.8	5	160	14	6	25	8	33	343	9.6	246	13.4	71.7
		7.2	6	198	18	7	20	8	28	371	7.5	279	10	75.2
		7.6	7	173	14	8	15	6	34	351	6	250	8.4	71.2
		8.0	8	219	16	7	8	5	22	349	3.7	277	4.7	79.4
Crude	20 mM	6.8	9	178	11	6	28	8	36	381	9.4	267	13.5	70.1
		7.2	10	206	12	6	25	7	39	413	7.7	295	10.8	71.4
		7.6	11	206	12	7	23	7	40	413	7.3	295	10.2	71.4
		8.0	12	221	12	0	23	7	25	422	7.1	288	10.4	68.2
	50 mM	6.8	13	221	17	8	22	9	46	431	7.2	323	9.6	74.9
		7.2	14	236	16	7	19	8	31	418	6.5	317	8.5	75.8
		7.6	15	216	16	8	14	6	35	424	4.7	295	6.8	69.6
		8.0	16	300	16	9	10	5	25	450	3.3	365	4.1	81.1

5.8 Time for binding

In order for a purification to be successful as a viable procedure, the costings should be taken into account. Not only should the cost of the materials be considered but also the equipment used, the purity (and biological activity if required) of the desired end product, time taken to carry out the whole purification procedure in terms of man hours, and the number of processes that can be completed within the working schedule. As mentioned earlier, this chapter deals with assessing that these novel magnetic supports work and in determining the essential parameters for their use on a small pilot scale so as to provide information which may be used in a scale-up purification.

For convenience, in all the experiments mentioned above, binding was allowed to take place at 4°C overnight / 13 hours after which all the recombinant protein appeared to have bound (negligible activity was found to be present in the Unbound fractions). Since time is an important factor in any process, it is desirable to establish the minimum time required for binding the maximum amount of the recombinant protein. (Section **5.3** dealt with finding the capacity of the beads)

Initially binding times (at 4°C) over the range of 30 minutes to 13 hours were tested. Forty-five microlitres of bead suspension (0.67 mg) from batch 12 were charged with copper and loaded with 0.3 mg of total protein of both crude and clarified extracts (as measured by the Bio-Rad Bradford assay). The binding buffer used was 20 mM sodium phosphate, 0.5 M NaCl, pH 7.2, and the Elution buffer was 0.1 M sodium acetate, 0.5 M NaCl, pH 3.8. When the activities of all the collected fractions were measured, it was found that in all the test cases, the Unbound fractions contained virtually no activity (less than 1% of the total activity recovered, data not shown) and similarly for the two Wash fractions, and the Strip fraction. It was therefore concluded that maximum binding of the lysozyme could be achieved in 30 minutes or less (based on the activity data).

To determine whether maximum binding could be accomplished in under 30 minutes, binding was allowed to take place for 5, 10, 20, and 30 minutes, again at 4°C. The binding and elution buffers used were the same as above. Fifty microlitres of bead suspension (approximately 0.76 mg) from batch 13 were used and again 0.3 mg of total protein (as measured by the Bio-Rad Bradford assay) of both crude and clarified extracts were applied to the beads. Samples were diluted in assay buffer (67 mM sodium phosphate, pH 6.2) in order to obtain a linear reading, carried out in duplicate, and the average value was used to calculate the activity of each collected fraction - the data is shown in **Table 5.7**. A minimum of 97.6% of the total activity recovered was eluted in the three fractions in a total volume of 1.65 ml of 0.1 M sodium acetate, 0.5 M NaCl, pH 3.8. A photograph of a SDS-PAGE gel in which TCA concentrated samples of the Unbound fractions were loaded is shown in **Figure 5.7.** The volumes of all the samples

in lanes 2-11 in the gel were equal. Lanes 2 and 7 contain the clarified and crude extracts (respectively) which were presented to the charged magnetic supports. From this photograph it is clear that all the recombinant protein appears to have bound within 5 minutes (as no protein band present in the Unbound fractions corresponds in size to that of the T4 lysozyme) and this can be seen to be true for both extracts.

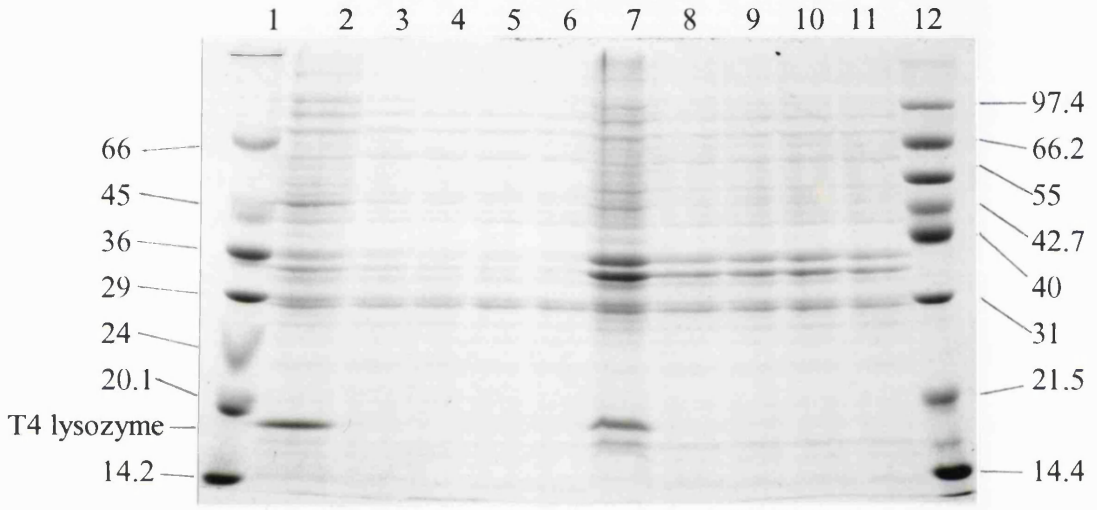


Figure 5.7 A photograph of the Coomassie-stained 15% SDS-PAGE gel loaded with samples from the Unbound fractions to determine the minimum time required for binding. Samples in lanes 2-11 inclusive were prepared by TCA precipitating 150 µl of sample 10-fold. Lanes 2-6 were loaded with clarified extract, and lanes 8-11 with crude extract. **Lane 1**, 5 µl molecular weight markers (Sigma, SDS-7); **lane 2**, loaded clarified extract; **lane 3**, 5 minutes binding; **lane 4**, 10 minutes binding; **lane 5**, 20 minutes binding; **lane 6**, 30 minutes binding; **lane 7**, loaded crude extract; **lane 8**, 5 minutes binding; **lane 9**, 10 minutes binding; **lane 10**, 20 minutes binding; **lane 11**, 30 minutes binding; **lane 12**, 5 µl molecular weight markers (Promega, Mid Range). The band of the recombinant T4 lysozyme is indicated in the loaded clarified sample (lane 2). The sizes of the molecular weight markers are in kDa.

Table 5.7 Minimum binding time study - the activity (in Units) for each fraction collected.

Sample	Tube No.	Time (in mins)	Unbound fraction	Wash 1	Wash 2	Elute 1	Elute 2	Elute 3	Total activity recovered	Eluted activity as a % total recovered
Clarified	1	5	107	21	21	6,903	2,431	177	9,660	98.5
	2	10	93	25	20	7,769	2,305	220	10,432	98.7
	3	20	95	30	12	7,274	2,343	189	9,943	98.6
	4	30	59	29	17	7,728	2,277	196	10,306	99
Crude	5	5	166	37	23	6,738	2,316	196	9,476	97.6
	6	10	177	34	29	7,370	2,393	199	10,202	97.6
	7	20	125	31	23	8,236	2,426	218	11,059	98.4
	8	30	111	42	22	8,126	2,420	218	10,939	98.4

Results here indicate that the minimum time for binding (with 20 mM sodium phosphate, 0.5 M NaCl, pH 7.2) 0.3 mg of total protein for both clarified and crude extracts to 0.76 mg of copper-charged support at 4°C is 5 minutes.

5.9 Elution buffers

Having discussed the capacity of the beads, the type of binding buffer, the time required for binding, and the number of Wash steps required, it was then decided to investigate the elution buffers. Initial studies with these novel magnetic particles (S. O'Brien, personal communication) used 0.1 M sodium acetate, 0.5 M NaCl, pH 3.8 to elute the bound protein from the beads and in all experiments mentioned above this buffer was employed.

Results obtained in Chapter 4 indicated that the recombinant protein was strongly bound onto the copper-charged columns as the protein was eluted at a low pH (around 3.6) which would have altered the degree of ionization of charged groups on the protein at the binding sites. At low pH values the electron donor groups are protonated. It is noted that while not every matrix, ligand and adsorbed substances can withstand such severe conditions, it did not appear to have any deleterious effect on either the HiTrap columns or the novel magnetic supports. It is also appreciated that this low pH would not be suitable for elution in a general purification protocol for proteins, and therefore other agents were investigated for suitability.

Many different chemicals have been used to elute purified products in these chelating systems by such methods as an increase in ionic strength, competitive binding, the use of reducing agents, and hence it would be a difficult task to test each and every possible candidate. Therefore, a selection of buffers were chosen and tested for their ability and efficiency to elute the recombinant lysozyme from the copper-charged magnetic particles. Hochuli *et al.* (1988) noted that the use of reducing agents (such as mercaptoethanol and dithiothreitol) also had the ability to form metal complexes by the thiol groups and therefore the affinity of polyhistidine peptides for Ni-NTA decreased.

5.9.1 Sodium acetate and Imidazole

Initial studies centred on eluting the bound protein with imidazole (Aldrich Chemical Co., Gillingham, Dorset, UK) in the binding buffer which acted as a competitor for binding to the metal chelate, and also comparing the effects of the sodium acetate buffer at different pH. An advantage with the use of imidazole is that a neutral pH can be used. Bead suspension from batch 8 was used (0.965 mg) in each tube and the beads were washed and charged with copper. The pelleted cells were resuspended in 20 mM sodium phosphate, 0.2 M NaCl, pH 7.2 (the binding buffer) and sonicated to release the

intracellular proteins. The beads were challenged with 0.35 mg of total protein of the clarified and 0.45 mg of total protein of the crude extracts (as measured by the Bio-Rad Bradford assay). The tubes were placed in a vibrax shaker overnight at 4°C.

For tubes 1-5 inclusive, the same quantity of total protein was added and likewise for tubes 6-10. Therefore an equivalent amount of activity and protein should have been present in the Unbound, Wash 1 and Wash 2 steps. The lysozyme activities of each collected fraction is shown in **Table 5.8**. Results obtained with both extracts showed that a general trend was apparent on considering the total activity eluted : 0.1 M sodium acetate, 0.5 M NaCl, pH 3.8 > sodium acetate pH 3.5 > 0.01 M imidazole > sodium acetate pH 4.8 > 0.1 M imidazole. However, when the eluted activity was expressed as a percentage of the total recovered, 0.1 M imidazole > sodium acetate pH 3.5 > sodium acetate pH 3.8 > sodium acetate pH 4.8 > 0.01 M imidazole for the clarified extract, and sodium acetate pH 3.5 > sodium acetate pH 3.8 > 0.1 M imidazole > 0.01 M imidazole > sodium acetate pH 4.8 for the crude sample. The main reason for these conflicting results is probably due to activation / deactivation of the enzyme by the elution buffers.

The protein content of each fraction is shown in **Table 5.9**. From the column which shows the total protein eluted, it would appear that 0.1 M imidazole > 0.01 M imidazole > sodium acetate pH 3.5 > sodium acetate pH 3.8 > sodium acetate pH 4.8 for both extracts. When the eluted protein was expressed as a percentage of the total recovered, the same trend was evident with a maximum value of 39%. It has been mentioned elsewhere in this thesis that the T4 lysozyme represented approximately 15% of the total protein in the extracts and hence it should be concluded that the eluted protein is not pure and/or the composition of the buffers has interfered with the protein estimation assay. To assess the purity of the eluted protein, 15% SDS-PAGE gels were loaded with precipitated samples from the Elute 1 steps. A photograph of the gel run for tubes 6-10 is shown in **Figure 5.8**. As unequal volumes of protein were applied to the gel (see figure legend for quantities) direct comparison of all lane contents is not possible. Minor contaminants can be seen when the recombinant protein is eluted with 0.01 M and 0.1 M imidazole in binding buffer, pH 6.9 and therefore purification with these solutions is not recommended (the presence of these contaminants explains the relatively high protein estimations obtained with these buffers when the eluted protein was expressed as a percentage of the total recovered). The lysozyme was recovered in a more pure form with the sodium acetate buffers although quantities recovered depended on the solution pH. Large quantities and highly pure lysozyme were obtained with 0.1 M sodium acetate, 0.5 M NaCl, pH 3.5 as the elution buffer. A similar result was obtained with a gel of the corresponding fractions for tubes 1-5 (not shown).

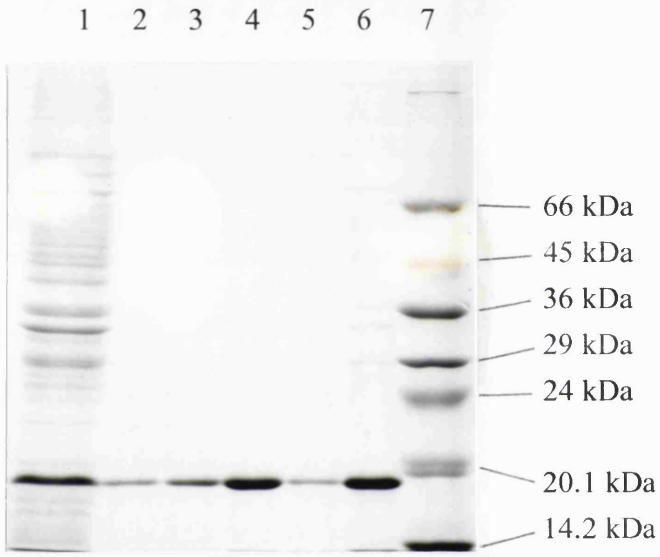


Figure 5.8 Photograph of a 15% Coomassie-stained SDS-PAGE gel loaded with concentrated samples from the Elute 1 fractions of the beads loaded with crude extract - see section **5.9.1**. **Lane 1**, 150 µl neat crude extract; **lane 2**, 82.5 µl, 0.1 M sodium acetate, 0.5 M NaCl, pH 4.8; **lane 3**, 82.5 µl, 0.1 M sodium acetate, 0.5 M NaCl, pH 3.8; **lane 4**, 82.5 µl, 0.1 M sodium acetate, 0.5 M NaCl, pH 3.5; **lane 5**, 75 µl, 0.01 M imidazole in binding buffer, pH 6.9; **lane 6**, 75 µl, 0.1 M imidazole in binding buffer, pH 6.9; **lane 7**, 5 µl molecular weight markers (Sigma, SDS-7).

Table 5.8 Choice of elution buffer (see section **5.9.1**) - activity data (in Units) for each fraction.

Sample	Tube No.	Unbound fraction	Wash 1	Wash 2	Elute 1	Elute 2	Strip	Total activity recovered	Total activity eluted	Eluted as a % total recovered	Elution buffer
Clarified	1	52	3	37	318	223	56	689	541	78.5	0.1 M sodium acetate, 0.5 M NaCl, pH 4.8
	2	55	37	42	450	404	16	1,004	854	85.1	0.1 M sodium acetate, 0.5 M NaCl, pH 3.8
	3	54	34	35	436	412	22	992	848	85.5	0.1 M sodium acetate, 0.5 M NaCl, pH 3.5
	4	44	37	40	319	257	42	739	576	77.9	0.01 M imidazole in binding buffer, pH 6.9
	5	44	2	15	353	131	6	551	484	87.8	0.1 M imidazole in binding buffer, pH 6.9
Crude	6	41	40	48	308	236	49	722	545	75.3	0.1 M sodium acetate, 0.5 M NaCl, pH 4.8
	7	32	42	35	471	411	24	1,015	882	86.9	0.1 M sodium acetate, 0.5 M NaCl, pH 3.8
	8	31	35	34	451	404	11	966	855	88.5	0.1 M sodium acetate, 0.5 M NaCl, pH 3.5
	9	36	37	31	329	255	28	716	584	81.6	0.01 M imidazole in binding buffer, pH 6.9
	10	31	38	27	367	147	3	613	514	83.8	0.1 M imidazole in binding buffer, pH 6.9

Table 5.9 Choice of elution buffer (see section **5.9.1**) - protein data (in μg) for each fraction.

Sample	Tube No.	Unbound fraction	Wash 1	Wash 2	Elute 1	Elute 2	Strip	Total protein recovered	Total protein eluted	Eluted as a % total recovered	Elution buffer
Clarified	1	156	18	0	19	14	97	304	33	10.9	0.1 M sodium acetate, 0.5 M NaCl, pH 4.8
	2	156	18	0	42	15	36	267	57	21.3	0.1 M sodium acetate, 0.5 M NaCl, pH 3.8
	3	156	23	0	48	14	40	281	62	22	0.1 M sodium acetate, 0.5 M NaCl, pH 3.5
	4	152	19	0	67	21	60	319	88	27.6	0.01 M imidazole in binding buffer, pH 6.9
	5	150	19	0	93	17	0	279	110	39.4	0.1 M imidazole in binding buffer, pH 6.9
Crude	6	191	19	16	19	16	35	296	35	11.8	0.1 M sodium acetate, 0.5 M NaCl, pH 4.8
	7	191	18	0	34	18	37	298	52	17.4	0.1 M sodium acetate, 0.5 M NaCl, pH 3.8
	8	186	21	0	49	14	54	324	63	19.4	0.1 M sodium acetate, 0.5 M NaCl, pH 3.5
	9	186	21	0	53	24	0	284	77	27.1	0.01 M imidazole in binding buffer, pH 6.9
	10	193	18	0	84	23	0	318	107	33.6	0.1 M imidazole in binding buffer, pH 6.9

5.9.2 Sodium acetate, Imidazole, and Histidine

Several other elution buffers were then investigated for their efficiency to elute the bound protein from the beads. Qiagen Inc. (California, USA) market the QIA*express*™ Ni-NTA Protein Purification System for proteins carrying a tag consisting of six consecutive histidine residues, where the recombinant protein can be eluted by 0.1-0.2 M imidazole at pH 8.0. In section **5.9.1** above, the pH of the imidazole solution was 6.9 and therefore a change in pH of the solution was tested. Also in this section are results gathered when histidine (Sigma Chemical Co.) in binding buffer was used as the elution buffer. In order to compare the results obtained here with the results of **5.9.1**, 0.1 M sodium acetate, 0.5 M NaCl, pH 3.8 was also tested, as different extracts and beads were used here to those previously.

The protein samples were prepared as outlined in section **2.4.12** and the cells were resuspended in 20 mM sodium phosphate, 0.5 M NaCl, pH 7.2 which was also used as the binding buffer. Sixty microlitres of bead suspension from batch 10 (0.94 mg) were placed into each tube and charged with copper. Binding buffer and protein samples (0.35 mg of total protein as determined by the Bio-Rad Bradford assay) were allowed 13 hours to bind at 4°C and were shaken on a vibrax shaker as usual.

The lysozyme activity of each collected fraction is displayed in **Table 5.10**. The samples were diluted in lysozyme assay buffer to obtain a linear decrease in absorption and an average of the duplicate results was used in the calculations. Since an equal amount of protein was added to tubes 1-4 and 5-8, and all tubes treated identically up to the elution steps, the Unbound and Wash steps will not be discussed. Considering tubes 1-4, maximum activity was eluted with the sodium acetate buffer. The second highest activity was found with the 0.1 M imidazole at pH 8. Little activity was present when histidine acted as the elution buffer. When the eluted activity was expressed as a percentage of the total activity recovered, the sodium acetate and imidazole were highest at 98.9% and 98.8% respectively. Values for the two buffers which contained the histidine were much lower - 80.1% and 69.8%. Similar results were obtained with the crude extract except the imidazole buffer yielded marginally larger activities and percentages than the sodium acetate buffer.

Activity values are not an accurate measurement of the quantity and quality of the eluted product and protein estimations are shown in **Table 5.11**. Maximum amount of total protein was recovered with the histidine-containing buffers and the minimum with the sodium acetate buffer. In addition this is also true for the total protein eluted and also when the eluted protein was expressed as a percentage of the total protein recovered. Similar quantities of protein were eluted with the two histidine-containing buffers. As mentioned above in section **5.9.1**, the recombinant T4 lysozyme represented approximately 15% of the total protein in the extracts, and therefore the results obtained

(when the eluted protein is expressed as a percentage of the total recovered) indicate that the protein was not as pure as is desired. A photograph of samples from the Elute 1 fractions of all the tubes can be seen in **Figure 5.9**. Equal volumes of each sample were TCA precipitated and applied to the gel, and therefore direct comparison is possible. Lanes 3-6 represent tubes 1-4, and lanes 8-11 represent tubes 5-8 respectively. It can be seen that although more of the recombinant protein is eluted with the histidine- and imidazole-containing buffers, the 0.1 M sodium acetate buffer provided the most pure product.

Of the elution buffers tested in this section, the buffers which produced the recombinant T4 lysozyme in as pure a form as possible were the 0.1 M sodium acetate, 0.5 M NaCl buffers and of these three buffers, maximum yield was achieved at pH 3.5. However, it is appreciated that this low pH may be too severe for many proteins.

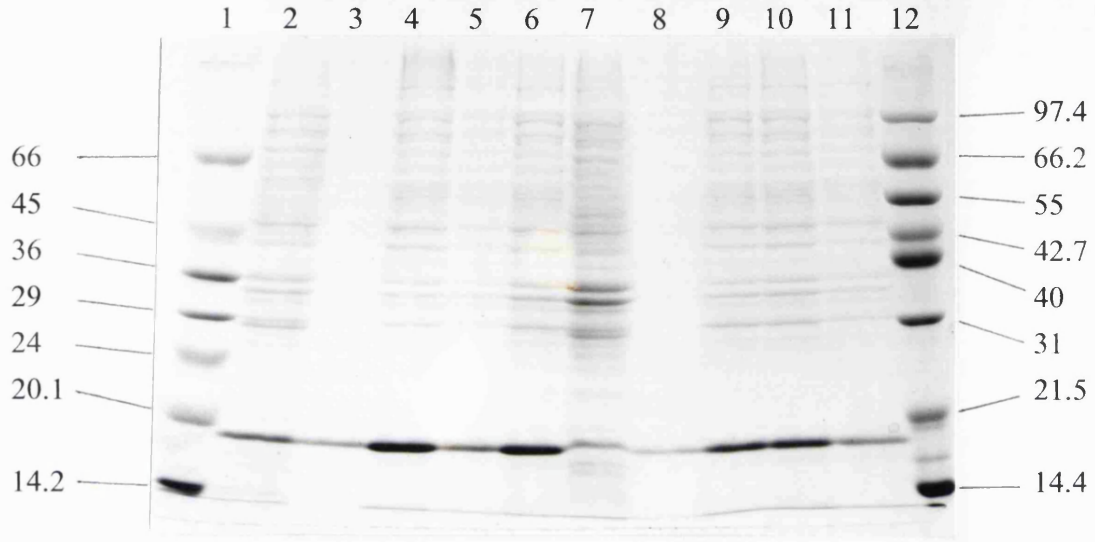


Figure 5.9 Photograph of a 15% Coomassie-stained SDS-PAGE gel loaded with concentrated samples from the Elute 1 fractions of the beads loaded with clarified (lanes 3-6) and crude (lanes 8-11) extract - see section **5.9.2**. **Lane 1**, 5 µl molecular weight markers (Sigma, SDS-7); **lane 2**, 75 µl, clarified extract; **lane 3**, 75 µl tube 1 (0.1 M sodium acetate, 0.5 M NaCl, pH 3.8); **lane 4**, 75 µl tube 2 (0.1 M histidine in binding buffer, pH 7.2); **lane 5**, 75 µl tube 3 (0.2 M histidine in binding buffer, pH 7.2); **lane 6**, 75 µl tube 4 (0.1 M imidazole in binding buffer, pH 8.0); **lane 7**, 75 µl, crude extract; **lane 8**, 75 µl tube 5 (0.1 M sodium acetate, 0.5 M NaCl, pH 3.8); **lane 9**, 75 µl tube 6 (0.1 M histidine in binding buffer, pH 7.2); **lane 10**, 75 µl tube 7 (0.2 M histidine in binding buffer, pH 7.2); **lane 11**, 75 µl tube 8 (0.1 M imidazole in binding buffer, pH 8.0); **lane 12**, 5 µl molecular weight markers (Promega, Mid-Range). The sizes of the molecular weight markers are in kDa.

Table 5.10 Choice of elution buffer (see section **5.9.2**) - the activity (in Units) for each fraction.

Sample	Tube No.	Unbound fraction	Wash 1	Wash 2	Elute 1	Elute 2	Strip	Total activity recovered	Total activity eluted	Eluted as a % total recovered	Elution buffer
Clarified	1	34	24	33	6,440	3,255	12	9,798	9,695	98.9	0.1 M sodium acetate, 0.5 M NaCl, pH 3.8
	2	38	9	17	252	34	7	357	286	80.1	0.1 M histidine in binding buffer, pH 7.2
	3	36	25	31	188	34	4	318	222	69.8	0.2 M histidine in binding buffer, pH 7.2
	4	38	17	20	6,755	65	6	6,901	6,820	98.8	0.1 M imidazole in binding buffer, pH 8.0
Crude	5	24	26	10	4,095	2,660	7	6,822	6,755	99	0.1 M sodium acetate, 0.5 M NaCl, pH 3.8
	6	34	19	17	229	20	2	321	249	77.6	0.1 M histidine in binding buffer, pH 7.2
	7	20	18	14	172	26	13	263	198	75.3	0.2 M histidine in binding buffer, pH 7.2
	8	29	1	32	9,870	54	6	9,992	9,924	99.3	0.1 M imidazole in binding buffer, pH 8.0

Table 5.11 Choice of elution buffer (see section **5.9.2**) - the protein estimations (in μg) for each fraction.

Sample	Tube No.	Unbound fraction	Wash 1	Wash 2	Elute 1	Elute 2	Strip	Total protein recovered	Total protein eluted	Eluted as a % total recovered	Elution buffer
Clarified	1	183	17	3	20	12	16	251	32	12.7	0.1 M sodium acetate, 0.5 M NaCl, pH 3.8
	2	176	18	2	125	29	0	350	154	44	0.1 M histidine in binding buffer, pH 7.2
	3	176	18	0	124	37	0	355	161	45.3	0.2 M histidine in binding buffer, pH 7.2
	4	179	16	0	102	20	0	317	122	38.5	0.1 M imidazole in binding buffer, pH 8.0
Crude	5	186	18	2	15	16	14	251	31	12.4	0.1 M sodium acetate, 0.5 M NaCl, pH 3.8
	6	180	22	1	127	31	0	361	158	43.8	0.1 M histidine in binding buffer, pH 7.2
	7	180	18	4	122	33	0	357	155	43.4	0.2 M histidine in binding buffer, pH 7.2
	8	180	19	0	98	21	25	343	119	34.7	0.1 M imidazole in binding buffer, pH 8.0

5.10 The effect of the concentration of sodium chloride in the elution buffer

Using 0.1 M sodium acetate, pH 3.8 as the elution buffer, it was not known what effect the concentration of sodium chloride had on the efficiency of elution of the bound protein. Keeping all other parameters constant, five different concentrations of sodium chloride were tested. Cells from an induced culture were resuspended in 20 mM sodium phosphate, 0.5 M NaCl, pH 7.2 which also served as the binding buffer. Fifty microlitres of suspended beads from batch 11 (0.8 mg) were charged with copper as in all previous experiments and loaded with equal amounts of protein (0.35 mg of total protein for both extracts as determined by the Bio-Rad Bradford assay). Binding was allowed to take place overnight at 4°C and fractions were collected as above using a different elution buffer for each tube. **Figure 3.7** (buffer number 25-29) is an indication of the effect of these buffers on the activity of the lysozyme in the extracts and not how the buffers can effect the purified protein.

Each fraction was diluted in lysozyme assay buffer to obtain a linear decrease in absorbance with time. Results of these assays are shown in **Table 5.12**. For the tubes which contained the clarified extract (tubes 1-5), not less than 86% of the total activity recovered was present in the three eluted fractions, and not less than 79.3% in the tubes that contained the crude extract (tubes 6-10). Maximum activity was recovered when 0.25 or 0.5 M NaCl was present in the elution buffer. Very low activities were obtained when 1 M NaCl was present and this held true for both types of sample. Therefore, based on the activity data of each fraction collected, it could be concluded that a concentration of 0.25-0.5 M NaCl in this elution buffer would yield the highest activity recoverable.

Since the type of buffer used can effect the enzyme activity, protein assays were conducted on all the fractions and results can be seen in **Table 5.13**. It should be remembered that the amount of protein added to tubes 1-5 was the same, and that added to tubes 6-10 was equal. It appears from this set of data that the higher the salt concentration, the lower the amount of protein is stripped from the beads with the solution of EDTA. The higher the salt concentration the greater the proportion of the eluted protein present in the first eluted fraction (as at 0 M NaCl three elution steps are required, while at 1 M NaCl only one step contained detectable protein). However, in comparing tubes 1-5, although tube 5 eluted the most protein in the first elution step, this was the only protein detected in all of the three eluted fractions. In total, for tubes 1-5 inclusive, the total protein eluted was maximum when 0.25 M NaCl was employed, and for tubes 6-10, a maximum recovery of 23 μg was achieved with the buffers containing 0 M NaCl and 0.25 M NaCl.

Table 5.12 To test the effect of the [NaCl] in 0.1 M sodium acetate, pH 3.8 as elution buffer - the activity (in Units) recovered for each fraction collected.

Sample	[NaCl] (M)	Tube No.	Unbound fraction	Wash 1	Wash 2	Elute 1	Elute 2	Elute 3	Strip	Total activity recovered	Eluted as a % of total recovered
Clarified	0	1	91	11	24	362	1,367	989	21	2,865	94.9
	0.25	2	70	55	31	3,273	1,344	874	6	5,653	97.1
	0.5	3	29	16	21	3,754	1,040	732	17	5,609	98.5
	0.75	4	32	27	39	3,176	138	67	1	3,480	97.2
	1	5	29	13	44	504	44	29	8	671	86
Crude	0	6	74	43	31	39	693	789	0	1,669	91.1
	0.25	7	51	22	10	2,041	1,455	939	0	4,518	98.2
	0.5	8	65	19	40	2,618	1,005	720	4	4,471	97.1
	0.75	9	40	25	27	211	96	49	1	449	79.3
	1	10	35	31	47	110	39	14	3	279	58.4

Table 5.13 To test the effect of the [NaCl] in 0.1 M sodium acetate, pH 3.8 as elution buffer - the protein content (in μg) recovered for each fraction collected.

Sample	[NaCl] (M)	Tube No.	Unbound fraction	Wash 1	Wash 2	Elute 1	Elute 2	Elute 3	Strip	Total protein recovered	Total protein eluted	Eluted as a % of total recovered
Clarified	0	1	169	15	0	7	10	10	101	312	27	8.7
	0.25	2	184	13	0	18	11	7	98	331	36	10.9
	0.5	3	177	13	0	20	7	0	97	314	27	8.6
	0.75	4	180	11	0	20	6	0	96	313	26	8.3
	1.0	5	171	11	7	23	0	0	89	301	23	7.6
Crude	0	6	173	12	9	8	8	7	137	354	23	6.5
	0.25	7	173	13	8	13	10	0	130	347	23	6.6
	0.5	8	161	11	8	14	8	0	123	325	22	6.8
	0.75	9	173	13	7	16	6	0	121	336	22	6.5
	1.0	10	159	11	0	15	0	0	119	304	15	4.9

For an enzyme purification, the procedure requires maximising the amount of protein recovered combined with maintenance of biological activity. On considering the results shown in **Tables 5.12** and **5.13** these criteria could be achieved for the recombinant T4 lysozyme by the use of 0.25 M NaCl if the elution buffer used was 0.1 M sodium acetate, pH 3.8.

5.11 The number of elution steps required

A purification process requires the end product to be as pure as possible but also in as high a concentration as possible. There comes a point where no more protein can be eluted from the support and since each elution step takes extra time, it is desirable to know at what stage this optimum is reached.

In total 0.76 mg of support from batch 13 were charged with copper, loaded with 0.33 mg of total protein in 20 mM sodium phosphate, 0.5 M NaCl, pH 7.2, and shaken for 10 minutes at 4°C. Five hundred microlitres of 0.1 M sodium acetate, 0.5 M NaCl, pH 3.8 was used as the elution buffer for each fraction. Activities of the eluted fractions are shown in **Table 5.14**. **Figure 5.10** shows the eluted activity for each fraction expressed as a percentage of the total activity recovered. It can be seen from the data with the clarified extract that in terms of total activity eluted, 67% is present in the first fraction, 96% is present in the first two fractions, and 99% is present in the first three fractions. From the data for the crude extract, a similar scenario was found (54%, 93%, 98%). A decision should therefore be made as to whether it would be worthwhile to collect the third elution fraction or whether the cost of this procedure would outweigh the extra protein collected.

Table 5.14 Optimum number of elution steps - the activity (in Units).

Sample	Elute 1	Elute 2	Elute 3	Elute 4	Elute 5	Total activity eluted
Clarified	908	402	39	7	3	1,359
Crude	413	294	40	12	5	764

Therefore, in an experiment where 0.76 mg of support would be presented with 0.33 mg of total protein, a minimum of two and preferably three elution steps should be used.

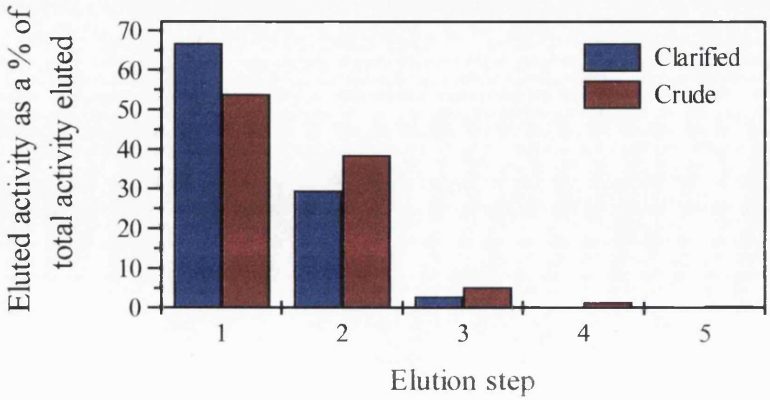


Figure 5.10 The activity eluted in consecutive steps (with 0.1 M sodium acetate, 0.5 M NaCl, pH 3.8) expressed as a percentage of the total activity eluted.

5.12 The volume of elution

All these small-scale experiments were carried out in microfuge tubes, and due to the nature of the magnetic separation rack used, separation in any volume less than 300 µl proved difficult and resulted in aspiration of support. As a result, if two elution steps are employed, the recombinant protein was purified but not concentrated. However, when working at a larger scale it can be envisaged that the volume of elution buffer could be altered so that the purified protein can be concentrated while remembering that the volume should not be too low so that adequate mixing can still occur.

5.13 The ability of other metal ions on the magnetic support to bind the recombinant protein

In the HiTrap columns (Chapter 4), zinc and nickel were also tested for their ability to retain the recombinant protein. It was decided to test the ability of these and other divalent ions to bind the recombinant proteins when attached to the magnetic support particles.

5.13.1 A selection of metal ions made in Elution buffer

In all the above experiments, the beads were charged with copper (copper chloride, 5 mg/ml solution in 0.1 M sodium acetate, 0.5 M NaCl, pH 3.8). Solutions of the same concentration and in the same buffer were made for the following: $CuCl_2$, $ZnCl_2$, $NiCl_2.6H_2O$, $MgCl_2.6H_2O$ (Fisons, Scientific Apparatus, Loughborough, UK), $CaCl_2.2H_2O$, $FeCl_2.4H_2O$ (Sigma Chemical Co.), $FeCl_3.6H_2O$. As a control, for each extract one tube containing uncharged beads (IDA on the surface) was set up.

Sixteen tubes were set up each containing bead suspension from batch 9 (45 µl, approximately 0.88 mg) and charged with the above solutions by shaking the tubes on a vibrax shaker (setting 1800) at room temperature for 2 hours as usual. Equal volumes of protein were added to each tube (0.35 mg of extract) in 20 mM sodium phosphate, 0.5 M NaCl, pH 7.2 (the binding buffer) and the elution buffer used was 0.1 M sodium acetate, 0.5 M NaCl, pH 3.8. All tubes were treated identically and hence any differences were due to the different metal ion.

Results obtained with the lysozyme assay for all collected fractions can be seen in **Table 5.15**. It can be noted that in comparing tubes 1-8 (contained clarified extract) that the total activity recovered was least when the beads charged with copper (tube 1), and this was also true for the tubes containing the crude extract. The reason for this may be that the lysozyme was activated to some extent with the other metal ions tested although this also was evident when no metal was present (the beads in tubes 8 and 16 had no metal ion attached to the IDA on their surface). Another, and more likely, explanation for this result is that the majority of the recovered activity in these other tubes (those not charged with copper) was located in the Unbound and Wash fractions and therefore not exposed to the stringent low (and potentially denaturing) pH of the sodium acetate elution buffer. With the exception of the copper, it appeared that insignificant binding occurred with the other metals tested based on the activity data as most of the activity recovered was present in the Unbound and the two Wash fractions. Slightly higher binding was achieved with the crude extract but again only significant binding was obtained with the copper-charged support. A three-dimensional graph of this activity data for the tubes loaded with the clarified extract can be seen in **Figure 5.11**.

Protein estimations for the fractions are in **Table 5.16**. When the eluted protein was expressed as a percentage of the total protein recovered, the result obtained with the clarified extract and copper was almost four times that of any of the others at 19%, which is approximately the content of the expressed lysozyme in the extracts. For the crude extract, only copper showed any significant binding of protein. Results indicate that 3.7% of the total protein recovered was located in the three elution steps when IDA / no metal was present on the surface of the beads. The Strip fractions indicate that, of the metal ions tested, only copper retained protein although this was not the T4 lysozyme.

SDS-PAGE gels were loaded with samples from the Elute 1 fraction for each tube. Similar results were obtained for both the clarified and crude extracts and a photograph of the gel for tubes 1-8 is shown in **Figure 5.12**. It can be seen from the Coomassie-stained gel that purified recombinant protein is only evident in lane 3, when the magnetic supports were charged with copper.

Table 5.15 The ability of selected metal ions (in elution buffer) to bind the recombinant protein - the activity (in Units) for each fraction.

Sample	Tube No.	Unbound fraction	Wash 1	Wash 2	Elute 1	Elute 2	Elute 3	Strip	Total activity recovered	Eluted as a % of total recovered	Metal ion
Clarified	1	67	31	29	349	302	193	29	1,000	84.4	Cu
	2	621	439	231	65	93	50	13	1,512	13.7	Zn
	3	625	455	238	62	82	47	0	1,509	12.6	Ni
	4	623	425	169	58	67	63	9	1,414	13.3	Mg
	5	620	451	199	68	90	48	6	1,482	13.9	Ca
	6	628	479	225	70	90	57	11	1,560	13.9	Fe^{2+}
	7	611	503	266	59	94	49	14	1,596	12.6	Fe^{3+}
	8	615	421	176	51	60	34	18	1,375	10.5	-
Crude	9	46	14	10	341	285	186	15	897	90.5	Cu
	10	412	402	205	86	141	93	16	1,355	23.6	Zn
	11	438	391	257	78	150	104	0	1,418	23.4	Ni
	12	419	393	236	91	153	113	17	1,422	25.1	Mg
	13	599	371	153	57	98	59	0	1,337	23.4	Ca
	14	517	394	179	88	129	88	0	1,395	21.8	Fe^{2+}
	15	230	356	215	61	135	137	14	1,148	29	Fe^{3+}
	16	519	391	196	72	133	89	7	1,407	20.8	-

Table 5.16 The ability of selected metal ions (in elution buffer) to bind the recombinant protein - the protein (in μg) for each fraction.

Sample	Tube No.	Unbound fraction	Wash 1	Wash 2	Elute 1	Elute 2	Elute 3	Strip	Total protein recovered	Eluted as a % of total recovered	Metal ion
Clarified	1	212	19	6	35	16	8	15	311	19	Cu
	2	319	20	7	8	7	6	0	366	5.7	Zn
	3	334	24	9	6	8	6	0	387	5.2	Ni
	4	332	19	7	6	7	7	0	378	5.3	Mg
	5	319	24	10	8	6	8	0	375	5.9	Ca
	6	315	27	7	6	6	8	0	369	5.4	Fe^{2+}
	7	310	23	5	6	4	5	0	353	4.2	Fe^{3+}
	8	315	18	4	1	5	7	0	350	3.7	-
Crude	9	141	17	0	24	6	2	8	198	16.2	Cu
	10	272	21	0	0	0	2	0	295	0.7	Zn
	11	274	24	0	2	0	0	0	300	0.7	Ni
	12	272	22	0	0	0	2	0	296	0.7	Mg
	13	272	22	0	0	0	0	0	294	0	Ca
	14	272	19	0	0	0	0	0	291	0	Fe^{2+}
	15	249	24	0	0	0	0	0	273	0	Fe^{3+}
	16	263	19	0	0	0	0	0	282	0	-

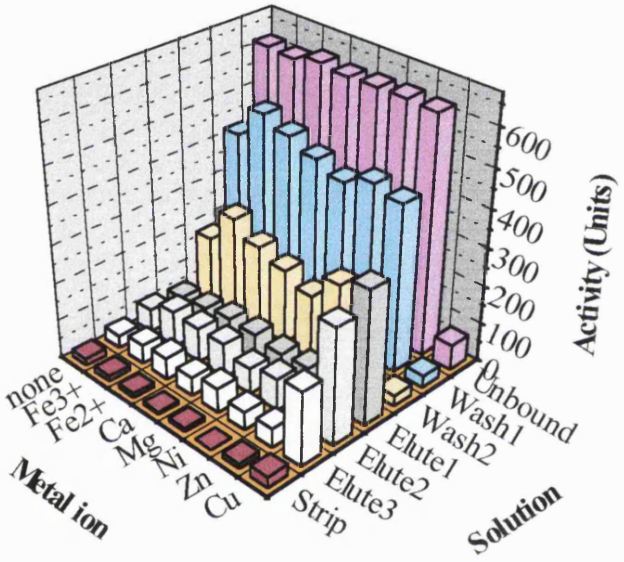


Figure 5.11 A three-dimension graph representing the activity data for each fraction collected (**Table 5.15)** for the beads presented with the clarified extract - the ability of selected metal ions (made in elution buffer) to bind the recombinant protein.

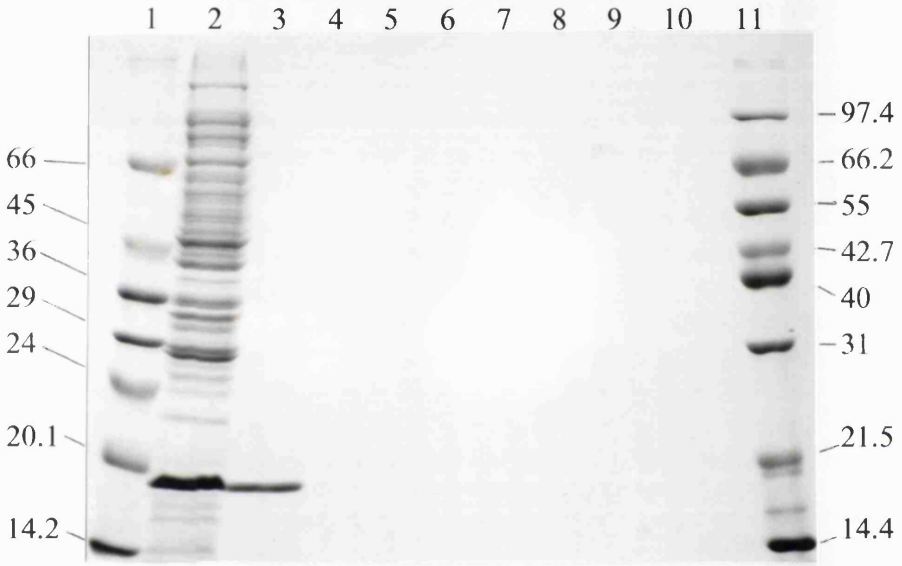


Figure 5.12 Photograph of a 15% Coomassie-stained SDS-PAGE gel with concentrated samples from the Elute 1 fractions of the beads charged with selected metal ions (in 0.1 M sodium acetate, 0.5 M NaCl, pH 3.8) and loaded with clarified extract - see section **5.13.1**. **Lane 1**, 5 µl molecular weight markers (Sigma, SDS-7); **lane 2**, 161 µl, loaded clarified extract; **lane 3**, 82.5 µl tube 1 ($CuCl_2$); **lane 4**, 82.5 µl tube 2 ($ZnCl_2$); **lane 5**, 82.5 µl tube 3 ($NiCl_2.6H_2O$); **lane 6**, 82.5 µl tube 4 ($MgCl_2.6H_2O$); **lane 7**, 82.5 µl, tube 5 ($CaCl_2.2H_2O$); **lane 8**, 82.5 µl tube 6 ($FeCl_2.4H_2O$); **lane 9**, 82.5 µl tube 7 ($FeCl_3.6H_2O$); **lane 10**, 82.5 µl tube 8 (uncharged/IDA); **lane 11**, 5 µl molecular weight markers (Promega, Mid-Range). The sizes of the molecular weight markers are in kDa.

5.13.2 A selection of metals in distilled water

In Chapter 4 it was concluded that zinc and nickel were able to bind the recombinant protein though at lower levels than copper. A possible reason why these two metal ions were not successful in the above section (**5.13.1**) may lie in the fact that these solutions were made with the elution buffer which had a low pH (3.8) and therefore the metals may have been prevented from binding to the IDA coat on the beads. It was therefore decided to test the ability of copper, zinc, and nickel made in distilled water to bind the recombinant protein.

Solutions (5 mg/ml) of $CuCl_2$, $ZnCl_2$, and $NiCl_2.6H_2O$ were made in distilled water, and for comparison, a copper solution in the elution buffer was also used. Fifty microlitres of bead suspension from batch 11 (0.8 mg) were placed in each tube, washed in distilled water and charged accordingly - see **Table 5.17**. As usual, 20 mM sodium phosphate, 0.5 M NaCl, pH 7.2 and 0.1 M sodium acetate, 0.5 M NaCl, pH 3.8 were the binding and elution buffers respectively. In total 0.35 mg of protein were presented to each set of the charged beads and all aspirated fractions were assayed for lysozyme activity (**Table 5.17**) and protein content (**Table 5.18**).

Samples were diluted to give a linear decrease in absorption with time in the lysozyme assay and carried out in duplicate. An average of these duplicates was used in the calculations. Results from **Table 5.17** indicate that there is little difference between the two copper solutions. By making the nickel solution in distilled water and not the elution buffer, binding was achieved while much lower activities were found when zinc was used. Therefore, according to the activities of the fractions recovered, the strength of binding of the recombinant lysozyme was in the following order : Cu > Ni >> Zn. This result follows the pattern found with the HiTrap columns in Chapter 4. For both extracts, approximately 33% of the total activity recovered was eluted from the zinc-charged beads while the nickel-charged supports yielded a value close to that of copper. Since the modified T4 lysozyme was eluted from the nickel columns under less stringent conditions (at a pH of approximately 4.5-5.3), there is a possibility that this and other histidine-containing proteins could be eluted likewise from nickel-charged magnetic supports and thus reducing the risk of disruption of the protein. A 3-D graph displaying the activity results for the beads presented with clarified extract can be seen in **Figure 5.13**.

Protein estimations (**Table 5.18**) for tubes 4 and 8 were carried out on a different day and hence direct comparison of results were not used. When the eluted protein was expressed as a percentage of the total protein recovered, copper > nickel >> zinc as was also found with the lysozyme activities. Protein was not detectable in any of the Elute 3 fractions. It is interesting to note that not only does copper elute the highest percentage of total protein recovered, but also the greatest amount of protein present in the Strip fractions.

To obtain an insight into the purity of the eluted protein(s), samples from the Unbound and Elute 1 fractions for tubes 1-3 and 5-8 were TCA precipitated and

electrophoresed on SDS-PAGE gels, and the 15% gels were stained with Coomassie. Similar results were obtained with both extracts and a photograph of these fractions for the clarified samples is shown in **Figure 5.14**. The Unbound fraction samples are in the lanes preceding the samples from the Elute 1 factions for each metal used. The Unbound sample in lane 7 (zinc-charged support) contained a band which corresponds in size to the recombinant protein but no band of equivalent size is present in the Elute 1 fraction (lane 8). As expected from the data in **Tables 5.17** and **5.18**, the single protein band in lane 4 (Elute 1 for copper) is more intense than that of lane 6 (Elute 1 for nickel). It appears that the nickel-charged support has the ability to selectively bind the recombinant protein as the presence of contaminating proteins is not evident in this gel stained with Coomassie.

Table 5.17 The ability of selected divalent metal ions (in distilled water) to bind the recombinant protein - the activity (in Units) located in each fraction.

Sample	Tube No.	Unbound fraction	Wash 1	Wash 2	Elute 1	Elute 2	Elute 3	Strip	Total activity recovered	Eluted as a % of total recovered	Metal ion solution
Clarified	1	47	0	0	7,565	1,194	837	4	9,647	99.5	Cu in distilled water
	2	160	224	141	6,795	1,176	749	3	9,248	94.3	Ni in distilled water
	3	1,823	1,480	414	801	583	425	9	5,535	32.7	Zn in distilled water
	4	29	16	21	3,754	1,040	732	17	5,609	98.5	Cu in Elution buffer
Crude	5	49	18	38	4,197	945	768	11	6,026	98.1	Cu in distilled water
	6	141	104	97	5,236	832	624	28	7,062	94.8	Ni in distilled water
	7	1,403	973	321	443	542	352	12	4,046	33	Zn in distilled water
	8	65	19	40	2,618	1,005	720	4	4,471	97.1	Cu in Elution buffer

Table 5.18 The ability of selected divalent metal ions (in distilled water) to bind the recombinant protein - the protein (in μg) located in each fraction.

Sample	Tube No.	Unbound fraction	Wash 1	Wash 2	Elute 1	Elute 2	Elute 3	Strip	Total protein recovered	Eluted as a % of total recovered	Metal ion solution
Clarified	1	192	18	3	38	12	0	169	432	11.6	Cu in distilled water
	2	281	22	3	25	6	0	75	412	7.5	Ni in distilled water
	3	295	24	8	2	0	0	56	385	0.5	Zn in distilled water
	4	236	13	0	52	18	0	194	513	13.6	Cu in Elution buffer
Crude	5	141	17	4	20	9	0	111	302	9.6	Cu in distilled water
	6	160	21	8	14	0	0	84	287	4.9	Ni in distilled water
	7	221	18	5	0	0	0	75	319	0	Zn in distilled water
	8	158	11	8	37	21	0	244	479	12.1	Cu in Elution buffer

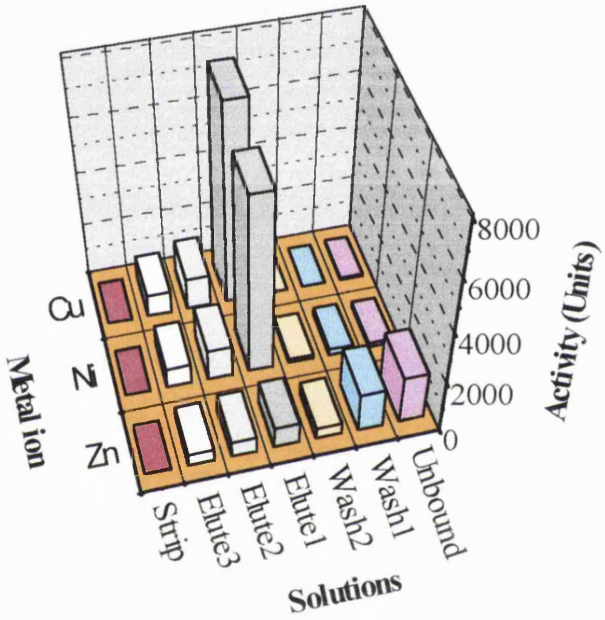


Figure 5.13 A 3-D graph of the activity data of each fraction for the beads charged with the metal ion in water and presented with clarified extract (data from **Table 5.17**).

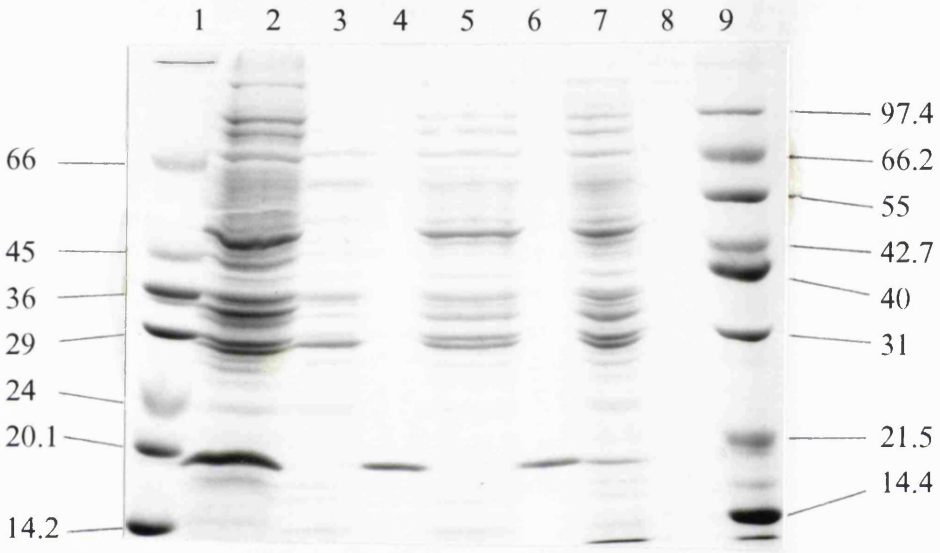


Figure 5.14 Photograph of a 15% Coomassie-stained SDS-PAGE gel with concentrated samples from the Unbound and Elute 1 fractions of the beads charged with selected metal ions (5 mg/ml in distilled water) and loaded with clarified extract - see section **5.13.2**. **Lane 1**, 5 µl molecular weight markers (Sigma, SDS-7); **lane 2**, 161 µl, loaded clarified extract; **lane 3**, 161 µl $CuCl_2$ Unbound fraction (tube 1); **lane 4**, 82.5 µl $CuCl_2$ Elute 1 fraction (tube 1); **lane 5**, 161 µl $NiCl_2.6H_2O$ Unbound fraction (tube 2); **lane 6**, 82.5 µl, $NiCl_2.6H_2O$ Elute 1 fraction (tube 2); **lane 7**, 161 µl, $ZnCl_2$ Unbound fraction (tube 3); **lane 8**, 82.5 µl $ZnCl_2$ Elute 1 fraction (tube 3); **lane 9**, 5 µl molecular weight markers (Promega, Mid-Range). The sizes of the molecular weight markers are in kDa.

From the activity results obtained in this section, there appears to be insignificant difference in the binding ability between the copper solutions (5 mg/ml in distilled water or elution buffer) used to charge the magnetic supports. All the other tested metals failed to specifically bind the recombinant protein when the beads were charged at pH 3.8 in elution buffer. However, binding was achieved when the nickel solution used to charge the beads was made in distilled water. Although the activity data for the beads charged with zinc exhibited activity in these fractions, no protein was detected with either the protein assay or by SDS-PAGE analysis. As with the column chromatography, the strength of binding was as follows: Cu > Ni >> Zn. If the histidine-containing protein to be purified was denatured at the low pH used in the elution buffer, a less stringent approach could be the use of nickel-charged particles and elution at a pH in the region of 4.5-5.3 as with the columns, although a lower yield would be obtained compared to isolation with copper.

5.14 Reuse of the magnetic supports

As mentioned earlier, in order for a purification process to be commercially viable, careful consideration must be taken to keep the costing to a minimum. The core magnetic supports used are expensive to purchase and it then takes time to coat them and test each batch for performance. (It should be noted that an initial investigation into large-scale production of the core particles is ongoing in this department.) It would therefore be suitable for the beads to be reusable and to find out what steps are necessary to achieve this. In some instances, the purified protein may be required for therapeutic use (e.g. antibodies) and therefore the purity must pass certain criteria as specified by bodies like the FDA (Food and Drug Administration) such as sterility and lack of contaminating proteins.

Several factors should be considered such as the need for stripping the support after each use, the possibility of having to recharge after each cycle, the stringency of the cleaning process of the support required to fulfil the FDA requirements, and how all these would effect the structure and composition of these novel supports.

Pharmacia recommend that the HiTrap columns may be reused if re-charged after each separation. However, if the metal ion was to be changed the column should firstly be stripped of the existing metal with a solution of EDTA and then charged as usual - this was not found to reduce the capability of the column. Common processes approved by the FDA are the use of sodium hydroxide solution to clean columns and sterilization by autoclaving. However, none of these steps are recommended for these magnetic supports as it is thought that irreversible damage would occur after several cycles (O. Thomas, personal communication).

Not only were these common procedures not recommended, but all these processes would take time which in itself is costly, therefore investigations into the reusability of the charged magnetic supports without stripping or cleaning were carried out. The beads were charged with copper in the normal fashion and recycled five times. The only form of cleaning process employed was the use of excessive washing steps (five) with elution buffer after which it was deemed that all the bound lysozyme had been collected.

The aliquoted bead suspension from Batch 13 (50 µl, 0.76 mg) was charged and challenged with either the clarified or crude extracts (0.35 mg of total protein as determined by the Bio-Rad Bradford assay) in the presence of the binding buffer (20 mM sodium phosphate, 0.5 M NaCl, pH 7.2) and allowed to bind at 4°C for 10 minutes. After aspirating away the unbound protein, the support was washed twice in binding buffer as usual, and five times with elution buffer (0.1 M sodium acetate, 0.5 M NaCl, pH 3.8) to ensure that all the bound recombinant protein had been recovered (see section **5.11**). The pH of the beads was then restored to 7.2 by rinsing three times in the binding buffer and the support was rechallenged. In all five cycles were carried out on both sets of beads and the activity and protein data for each fraction collected can be seen in **Tables 5.19** and **5.20** respectively.

Each elution step was carried out in a volume of 500 µl. All samples were diluted in assay buffer (67 mM sodium phosphate, pH 6.2) to obtain a linear decrease in absorption with time and tested in duplicate. The activity data of **Table 5.19** indicates that the eluted activity decreased as the activity in the Unbound fraction increased. When the eluted activity was expressed as a percentage of the total recovered for each cycle, 96% (clarified) and 91.5% (crude) were found in the first cycle, and this value steadily decreased over the remaining four cycles when 0.7% (clarified) and 1.1% (crude) were eluted in the fifth cycle. Correspondingly, when the activity located in the Unbound fractions was expressed as a percentage of the total activity recovered for each cycle, 1.7% (clarified) and 2.8% (crude) were obtained for the first cycle, and for the fifth cycle 82% (clarified) and 89% (crude) was found. A diagrammatic representation of the activity results comparing the activity in the Unbound fractions against the total activity eluted for each cycle can be seen in **Figure 5.15**. Insignificant amounts of activity were located in the Elute 4 and 5 fractions in any cycle indicating that all bound activity had been eluted. From the activity data it would be recommended that only two cycles would prove productive if the magnetic support was used in this manner as the total activity eluted on the third cycle was dramatically reduced.

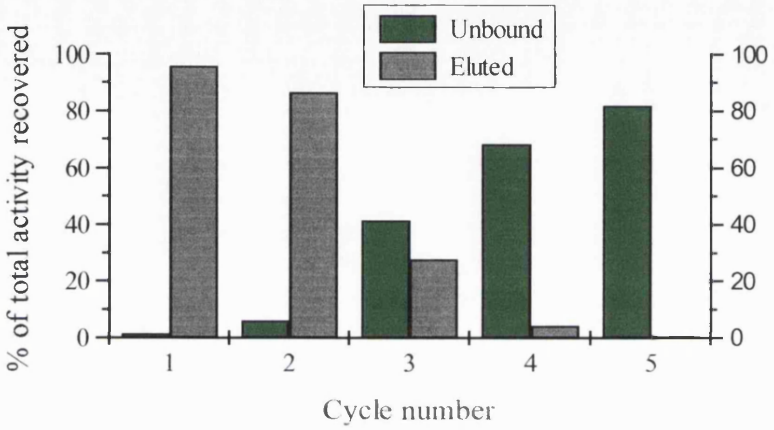
Protein estimations for each fraction are tabulated in **Table 5.20**. Interpretation of these results is more difficult as the identity of the protein(s) present cannot be realised (the detected lysozyme activity can be solely attributed to the presence of the

recombinant protein). A more accurate account was seen when samples were electrophoresed on SDS-PAGE gels. Precipitated samples from the Unbound fractions of each cycle (with both extracts) were run on a 15% gel and a photograph can be seen in **Figure 5.16.** Likewise samples from the Elute 1 fractions can be seen in **Figure 5.17.**

Table 5.19 Recycling the magnetic support - the activity data (in Units) for each fraction.

Sample	Cycle No.	Unbound fraction	Wash 1	Wash 2	Elute 1	Elute 2	Elute 3	Elute 4	Elute 5	Total activity recovered	Unbound as a % of total recovered	Eluted as a % of total recovered
Clarified	1	24	20	13	905	402	39	6	2	1,411	1.7	96
	2	93	66	44	1,144	131	19	6	4	1,507	6.2	86.5
	3	780	445	138	446	60	9	1	6	1,885	41.4	27.7
	4	1,433	438	128	67	13	6	3	4	2,092	68.5	4.4
	5	2,325	420	69	15	2	2	1	0	2,834	82	0.7
Crude	1	16	15	18	413	59	40	12	5	578	2.8	91.5
	2	84	52	35	886	64	35	8	7	1,171	7.2	85.4
	3	521	171	108	418	66	9	4	0	1,297	40.2	38.3
	4	1,403	220	104	57	13	5	2	4	1,808	77.6	4.5
	5	2,730	221	83	24	5	1	3	1	3,068	89	1.1

(a)



(b)

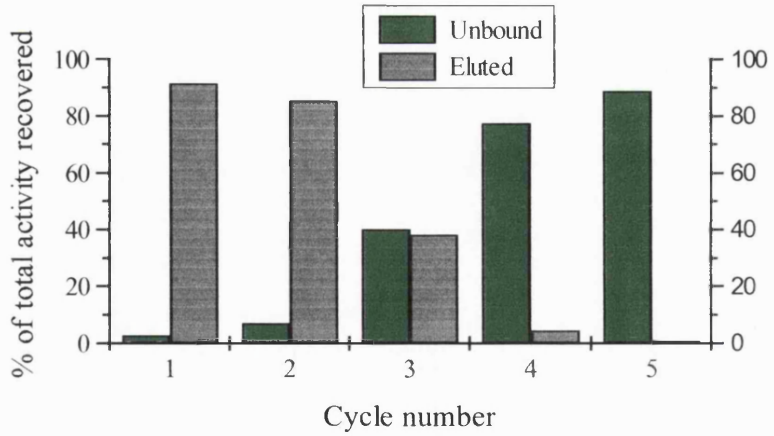


Figure 5.15 To compare the unbound and total eluted activity (expressed as a % of the total activity recovered) for each cycle with **(a)** clarified extract and **(b)** crude extract.

Table 5.20 Recycling the magnetic support - the protein content data (in μg) for each fraction.

Sample	Cycle No.	Unbound fraction	Wash 1	Wash 2	Elute 1	Elute 2	Elute 3	Elute 4	Elute 5	Total protein recovered	Unbound as a % of total recovered	Eluted as a % of total recovered
Clarified	1	180	23	6	21	14	12	14	9	279	64.5	25.1
	2	308	22	11	12	6	9	8	4	380	81.1	10.3
	3	344	22	11	8	9	7	6	0	407	84.5	7.4
	4	334	19	9	13	10	9	8	6	408	81.9	11.3
	5	360	20	7	11	11	12	6	1	428	84.1	9.6
Crude	1	150	12	0	12	9	7	4	6	200	75.0	19.0
	2	255	15	0	1	0	2	7	6	286	89.2	5.6
	3	272	18	1	8	0	7	0	5	311	87.5	6.4
	4	255	12	0	0	5	5	5	9	291	87.6	8.2
	5	353	17	0	1	5	2	5	4	387	91.2	4.4

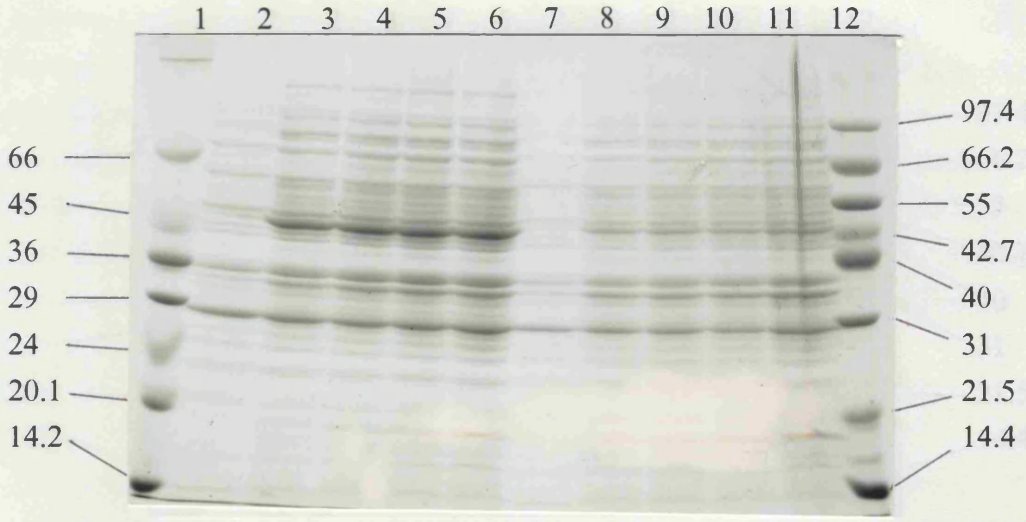


Figure 5.16 Photograph of a 15% Coomassie-stained SDS-PAGE gel with concentrated samples from the Unbound fractions of the copper-charged beads which were recycled. Each lane contained the equivalent of 150 µl of unconcentrated sample. **Lane 1**, 5 µl molecular weight markers (Sigma, SDS-7); **lane 2**, clarified cycle 1; **lane 3**, clarified cycle 2; **lane 4**, clarified cycle 3; **lane 5**, clarified cycle 4; **lane 6**, clarified cycle 5; **lane 7**, crude cycle 1; **lane 8**, crude cycle 2; **lane 9**, crude cycle 3; **lane 10**, crude cycle 4; **lane 11**, crude cycle 5; **lane 12**, 5 µl molecular weight markers (Promega, Mid-Range). The sizes of the molecular weight markers are in kDa.

The photograph showing samples of the Unbound fractions is shown in **Figure 5.16**. Since equal volumes of the samples were applied to the gel direct comparison is possible. A similar scenario is exhibited by both extracts (lanes 2-6 and 7-11). It can be seen that much less protein is present in the Unbound fraction of the first cycle compared to the other four cycles and this correlates with the data in **Table 5.20**. Lanes 4-6 and 9-11 inclusive also show the presence of a protein the expected size of the recombinant T4 lysozyme, and the concentration of this band increased as the number of recycles increased. This fact supports the activity data of **Table 5.19** which indicated that the amount of activity which bound to the beads decreased significantly after the second cycle and was mainly present in the Unbound and the two Wash fractions.

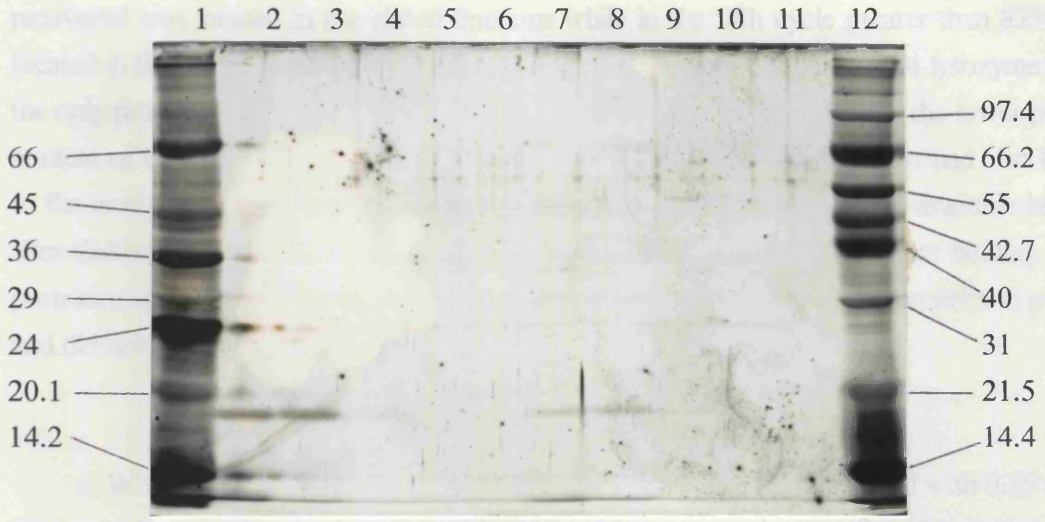


Figure 5.17 Photograph of a 15% Coomassie- and silver-stained SDS-PAGE gel with concentrated samples from the Elute 1 fractions of the copper-charged beads which were recycled. Each lane contained the equivalent of 150 µl of unconcentrated sample. **Lane 1**, 5 µl molecular weight markers (Sigma, SDS-7); **lane 2**, clarified cycle 1; **lane 3**, clarified cycle 2; **lane 4**, clarified cycle 3; **lane 5**, clarified cycle 4; **lane 6**, clarified cycle 5; **lane 7**, crude cycle 1; **lane 8**, crude cycle 2; **lane 9**, crude cycle 3; **lane 10**, crude cycle 4; **lane 11**, crude cycle 5; **lane 12**, 5 µl molecular weight markers (Promega, Mid-Range). The sizes of the molecular weight markers are in kDa.

Contents of the Elute 1 fractions can be visualised from the photograph in **Figure 5.17**. As in Figure 5.16, a similar result is seen with both extracts. Direct comparison of lane contents is possible as equal volumes of the samples were applied to the gel. It can be seen from lanes 2-3 and 7-8 inclusive that the recombinant protein was significantly eluted in the first two cycles only and not in the following cycles. This information compares favourably to the activity results seen in **Table 5.19**.

From **Figures 5.16** and **5.17** and the activity data present in **Table 5.19**, it is possible to trace the location of the recombinant protein. As the cycle number increased the activity was recovered in earlier fractions. In the first cycle greater than 91% of the total activity recovered was located in the eluted fractions while in the fifth cycle greater than 82% was located in the Unbound fraction. As suggested earlier in this chapter, the T4 lysozyme is not the only protein which binds to the copper-charged support. The reason for the lower protein content of the Unbound fraction in the first cycle is that these other proteins had also bound to the available metal sites. Hence in the following rounds the number of available binding sites decreased as the beads were not stripped of these proteins and further binding of all proteins was restricted. This led to an increase in the amount of unbound proteins in general and did not only apply to the recombinant lysozyme.

When 0.76 mg of copper-charged support was repeatedly challenged with 0.35 mg of total protein and the only cleansing of the support between cycles was five elution steps with 0.1 M sodium acetate, 0.5 M NaCl, pH 3.8, - followed by a restoration of the pH to 7.2 with the binding buffer (20 mM sodium phosphate, 0.5 M NaCl, pH 7.2) - the amount of the recombinant protein which bound decreased dramatically after the second cycle. From all the information in this section it would not be recommended to recycle the support in this manner beyond two cycles although if a stripping step were to be incorporated after the elution steps this may resolve the problem. However, it should be noted that repeated stripping of the beads in a high concentration of EDTA may result in irreversible damage to the support by the loss of core iron content (O. Thomas, personal communication). The manufacturers of the core amine-terminated BioMag supports (Advanced Magnetics Inc,. Massachusetts, USA) suggest that antimicrobials such as sodium azide or chlorinated phenol may be used to prevent growth of contaminating microbes, but any solutions used should be checked for the potential to damage the modified coatings as used in all these experiments.

5.15 Method of mixing

On the small-scale pilot experiments in this chapter, mixing was achieved with a vibrax shaker. It is important in all steps that the support remains in suspension and that there is maximal interaction between the protein and the support. Obviously the type of vibrax shakers used in these experiments would not be feasible for large-scale mixing and perhaps a paddle stirrer system in a contained vessel would be sufficient. However, it should be noted that excessive speed may result in degradation of the protein by shear forces or by an excessive temperature increase and the contact time of the protein to the metal ion may not be

sufficient. A different system of mixing could possibly alter the minimum time required for binding.

5.16 Summary

The results of the column chromatography in Chapter 4 proved that the polyhistidine-tagged protein could be purified utilising a specific reaction between the histidine residues and the copper, nickel, and zinc ions which were present on the matrix. The overall conclusion from this chapter is that it is possible to purify the histidine-containing recombinant T4 lysozyme from a heterogeneous mixture of culture proteins with the use of the charged novel magnetic supports. Isolation of the protein was repeatedly accomplished in a highly purified form and many parameters which optimise the isolation of the protein have been investigated. Also concluded from this section is that the recombinant protein can be purified to a similar level from both crude and clarified extracts.

Initial binding problems were overcome by pelleting the cells from the fermentation mixture and resuspending them in buffer before sonication to release the intracellular proteins. Once binding had been achieved it was important to find the capacity of the beads for the recombinant protein in order to purify the maximum amount possible in one step. Optimisation of the binding conditions then followed which involved finding the type of buffer to be used (comparison of different sodium chloride concentrations, the molarity of the components, the pH) and also the minimum time for binding. It was important to establish the number of Wash steps required after binding to eliminate all unbound and non-specifically bound proteins which could otherwise contaminate the purified product. The ability of many potential elution buffers to provide an active, uniform product in a concentrated form was investigated using competitive elution, increasing the ionic strength, and pH gradients. Once the method of elution had been chosen, the number of elution steps required to recover maximal activity was determined. The majority of these parameters were investigated using copper-charged supports and it was unknown whether other metal ions had the ability to purify the protein. Therefore, several other solutions were used to charge the magnetic beads and their success was found to be dependent on the nature of the solutions. As with the HiTrap columns, copper was still found to provide the best results although nickel in distilled water would be a good alternative especially if the protein would require less harsh elution conditions (higher pH). It is not recommended that the support is recycled for more than two consecutive cycles unless stripped of all bound proteins after each cycle and the stripping process must not be too stringent so as to irreversibly damage the support. From the results obtained in this chapter, there appears to be insignificant difference in the isolation of the recombinant protein from the two extracts tested.

A summary of the optimised conditions, as devised in this chapter, for purifying the recombinant T4 lysozyme on a small scale can be seen in **Table 5.21**.

Table 5.21 A summary of the optimised conditions for the purification of the histidine-containing T4 lysozyme with the novel magnetic beads on a small scale.

Capacity of 1 mg magnetic supports	Clarified extract - 0.35 mg/ml of total protein Crude extract - 0.45 mg/ml of total protein
Binding buffer	20 mM sodium phosphate, 0.5 M NaCl, pH 7.2
Minimum time of binding	5 minutes
Number of Wash steps after binding	minimum of 2 elutions
Elution buffer	* 0.1 M sodium acetate, 0.5 M NaCl, pH 3.5 * For less stringent conditions 0.1 M sodium acetate, 0.5 M NaCl, pH 3.8 *or if nickel is the metal of choice 0.1 M sodium acetate, 0.5 M NaCl, pH 4.5
Number of elution steps	> 90% recovered in 2
Metal ions recommended	Copper or nickel
Consecutive recycling	maximum of two cycles

CHAPTER SIX

DISCUSSION

6.1 Introduction

Protein purification procedures strive to economically maximise the yield of the desired protein in as pure and active a form as possible. Although high yields may be achieved for individual steps, the cumulative loss for the overall purification procedure can result in vastly reduced yields (Lagerlöf *et al.*, 1976; Wang *et al.*, 1979) and a reduction in the number of steps required would be desirable. Also, this would usually reduce both the process time and the need for extensive equipment. An ideal purification protocol would be a one-step downstream processing procedure to isolate the desired protein from unrefined fermentation liquor. Since immobilized metal affinity chromatography was introduced by Porath *et al.* (1975) to fractionate human serum proteins on chromatography columns, it has become a widely used technique for protein purification and is based on the affinity of certain amino acid residues (histidine, cysteine and tryptophan), which are present on the protein surface, for first row transition metal ions and zinc. However, most IMAC procedures are only capable of small-scale purification due to limitations imposed by the commonly used matrices.

6.2 Summary of thesis

The aim of this thesis was to devise a novel purification system (based on IMAC) for use as a generic one-step purification system for the recovery of proteins containing surface-exposed histidine residues from *E. coli* fermentation liquors. This would reduce the number of purification steps, time taken to achieve this, and the amount of processing equipment necessary. It was decided to test the effect of the addition of a polyhistidine tail onto a chosen protein to enable its facile purification. The system was to be initially tested on traditional column supports and if successful, isolation by novel magnetic particles was to be investigated at bench scale but with a view to scale-up of the isolation procedure.

Bacteriophage T4 lysozyme was the chosen protein for several reasons. It is known to be a small protein whose 3-D structure (Matthews and Remington, 1974; Remington *et al.*, 1978; Weaver and Matthews, 1987), amino acid (Tsugita and Inouye, 1968) and DNA sequences (Owen *et al.*, 1983) have been determined. The enzyme has previously been purified over several steps using various combinations of column chromatography and filtration procedures (Tsugita and Inouye, 1968; Szewczyk *et al.*, 1982; Griffey *et al.*, 1985; Perry and Wetzel 1986; Alber and Matthews 1987; Alber *et al.*, 1988; Muchmore *et al.*, 1989; Poteete *et al.*, 1991), and more recently Düring (1993) has described the use of a single column for the isolation of a histidine-containing recombinant lysozyme variant. As an enzyme its activity can be assayed (Parry *et al.*, 1965; Locquet *et al.*, 1968; Mark, 1970; Mörsky, 1983; Becktel and Baase, 1985).

From the data obtained on the amino acid composition and the 3-D structure of the protein it is known to contain one hidden histidine residue and the three tryptophan residues are sequestered into the centre of the molecule. A cysteine-free variant was available on a clone (kindly donated by B.W.Matthews) and hence any binding to transition metal ions could be attributed to the additional tail. The protein is produced intracellularly which was desirable as it is possible to control its release from the cells thereby reducing the volume to be processed if required. Studies have also shown that both the N- and C-terminals are known to be away from the active site and that up to two residues can be removed from the C-terminus without significant change to the properties of the enzyme (B.W.Matthews, personal communication). Since lysozyme is an endoacetylmuramidase it cleaves bonds in the cell wall of many bacteria and the hen egg white lysozyme is an important enzyme used in the isolation of many other intracellular proteins. However, it has restricted use mainly due to the commercial cost of the lysozyme but also as it can act as an additional contaminant to the final product. It could potentially become more widely used if these problems could be overcome by selective retrieval of the lysozyme and reuse in further fermentation broths.

Having decided on a polyhistidine tail attached to the C-terminus of the protein, the exact type of tail was looked into. To a first approximation, proteins were reported to be retained on metal affinity columns according to the number of accessible histidine residues (Yip *et al.*, 1989; Arnold, 1991; Todd *et al.*, 1991). However, it is important not to have too many interactions occurring as noted by Skerra *et al.* (1991) when the fusion of a series of nine histidine residues resulted in binding that was too strong to allow significant elution. Several successful isolations of proteins have been accomplished with 2-6 adjacent histidine residues (Hochuli *et al.*, 1988; Düring, 1993; Raupach *et al.*, 1994).

Activity studies indicated that the presence of the tail did not effect the activity of the recombinant enzyme. Once the polyhistidine tail had been successfully added by site-directed mutagenesis (which was confirmed by sequencing), it was necessary to determine whether the fusion tail did in fact enable the selective purification of the recombinant protein on chelating columns charged with either nickel, zinc or copper. Minor sample treatment involving direct sonication of the cells into the fermentation broth, dialysis against the column wash buffer and sample filtration had to be carried out prior to the loading of the sample onto the columns. The protein was selectively retained and eluted from these charged columns and not retained to any extent on an uncharged column. In order to prove that the binding was as a result of the presence of the polyhistidine region, pseudo wild type lysozyme (containing no fusion tail) was passed through similar columns under identical conditions with the result that no protein bound.

Having established that the polyhistidine tail enabled selective, high yield purification of the recombinant lysozyme on standard chelating columns, purification was attempted using novel magnetic particle supports (developed elsewhere in this department). It was envisaged that if this methodology was successful, scale-up of the purification process could be accomplished with ease. Initial attempts used samples which were sonicated whole fermentation broths and these were unsuccessful. However, when the culture was centrifuged and the cells washed in buffer prior to sonication, successful isolation of the recombinant protein was achieved (see section **6.8**). In order to maximise the yield of the isolated protein, many aspects of the processing strategy involving the magnetic particles were investigated such as the capacity of the novel particles, buffer composition, minimal time for optimal binding, and recycling of the beads - these aspects were discussed individually in Chapter 5.

It is therefore possible to state that high level purification of the recombinant bacteriophage T4 lysozyme was achieved on both chelated columns and also with the use of the novel magnetic particles. Parameters for the use of the novel particles have been refined for the isolation of this protein but it is felt that little alteration would be required to the purification protocol for use as a generic method of one-step purification of other proteins containing surface-exposed histidine residues.

6.3 Current purification methods for bacteriophage T4 lysozyme isolation

As mentioned earlier, the use of polyhistidine tails to enable protein purification is becoming a popular technique for column chromatography mainly due to the commercial manufacture of several kits. The question now raised is how does the purification of the bacteriophage T4 lysozyme by this method compare to the other methods of its purification?

As previously mentioned in Chapter 1, most groups have concentrated on the use of several steps to achieve a homogeneous product. The first group to isolate the enzyme was Tsugita and Inouye in 1968 using ion exchange chromatography followed by molecular sieving and this general trend was followed by the majority of other workers. However, due to the number of steps involved, on average only approximately 40% yield was achieved. Recently, Düring (1993) described a unique method utilising the advances in purification technology, namely that of immobilized metal affinity chromatography. A commercially available kit (from Qiagen) was used to attach a polyhistidine tail (six consecutive histidine residues) to the N-terminus of the lysozyme gene and the supernatant lysate was passed through a Ni-NTA agarose column for a one-step purification procedure. However, the size of the eluted recombinant lysozyme differed depending on the method of elution and it was thought that a premature

cleavage reaction had occurred during the affinity chromatography at low pH incubations. Although the article indicates that as high as 50 mg of lysozyme fusion protein could be obtained per litre of culture, no clear indication of the purity of the recovered product is stated.

The recombinant lysozyme used in this thesis was similar to that of Düring in that it contained a polyhistidine tail but on the C-terminus and it was purified on IDA chelated columns. The work presented here has extended the available technology by applying the existing technology in the attempted (and successful) purification on novel magnetic particles in place of the columns.

In the light of the work in this thesis being used as a general purification strategy, whether it be for small- or large-scale purification, several aspects must be discussed.

6.4 Site-directed mutagenesis

The reader will perhaps have noted that the addition of the polyhistidine tail to the C-terminus of the bacteriophage T4 lysozyme was difficult to achieve by the methods of site-directed mutagenesis used here (indicated in Chapter 3). The frequency of mutation could possibly have been increased by several modifications to the procedure, such as the presence of larger overlapping regions (from the proposed site of mutagenesis) in the mutagenic oligonucleotide which would form a stronger complex with the single-stranded template DNA. Further alterations such as reassessing the annealing conditions could also be investigated. However, it must be agreed that the technique used in this thesis would need to be improved if it was to be used as a generic protocol for protein manipulation as used here. A further method of mutagenesis, untested in this thesis, would perhaps be the use of the polymerase chain reaction but this would be a costly exercise as several oligonucleotides would be required, misincorporation of bases is not rare, and again conditions of annealing would have to be determined.

6.5 Design of the polyhistidine tail

A second point is that the polyhistidine tail was designed to contain five consecutive histidine residues. However, on sequencing the recombinant protein containing the desired antibiotic resistance profile, it was discovered that the tail was in fact composed of His-Gln-His-His-His. This may have arisen for several reasons, notably mutagenesis in the third base of the second codon where instead of CAT there

was CAG, or through experimental error in the production of the mutagenic oligonucleotide. Despite this fact it was decided to go ahead and test this recombinant protein for the ability to bind to chelated metal ions on several IDA-Sepharose columns. Binding was successful and specific and it was decided to use this protein in further studies.

It can therefore be concluded that adequate binding was achieved with this alternative tail and it should be noted that the His-X_3-His α-helical conformation still held. The presence of five consecutive histidine residues may result in binding which would require more stringent elution conditions from the metal ions although several groups have successfully used up to six consecutive histidine residues for protein isolation so this may not be an issue.

6.6 Cleavage of the fusion tail

Tails with a variety of characteristics, sizes and specificities, ranging from entire enzymes to antigenic epitopes, have been designed for fusion to virtually any target protein which can be expressed in bacterial, cell line or yeast systems. Fusion tails have been added to either the N- or the C-terminus, and can be added to both.

Whether or not the fusion tail should be removed is dependent on the end-use of the target protein. Ford *et al.* (1991) recommend that for lab-scale characterization of a protein that has previously been difficult to obtain in sufficient quantities, it may be possible to leave the tail present providing initial studies indicate that the biological function of the target protein is not effected. This article also indicates that the fusion tail on antibodies could promote antigenicity. On the other hand, if interference is experienced, the tail should be removed. For pharmaceutical or therapeutic applications, precise removal of the fusion tail is usually desired in order to achieve absolute product validity.

If it is necessary for the tail to be removed, two methods exist for specific excision of the fusion tail from the target protein - enzymatic and chemical. Some of the fusion proteins which have been isolated to date have had the tail cleaved enzymatically (e.g. carboxypeptidase A, Factor Xa) and the peptide separated from the desired protein (Sassenfeld and Brewer, 1984; Brewer and Sassenfeld, 1985; Moks *et al.*, 1987; Hopp *et al.*, 1988; Smith and Johnson, 1988; Hochuli *et al.*, 1988; Düring, 1993). These enzymatic cleavage sites can be incorporated into the design of the fusion tail but it should be pointed out that it is essential to ensure that the cleavage site is not found within the target protein! However, cleavage in this manner is not very efficient and also requires further column purification to separate the tail from the target protein. Chemical cleavage methods, although in general highly efficient, are usually too harsh for

the majority of isolated proteins. An added problem with the use of chemicals is that the cleavage sites are specified by only one or two residues which are usually also found within the protein.

Düring (1993) used the enzyme Factor Xa to cleave the histidine-containing tail from a target protein. This involved a buffer exchange step followed by digestion with the Factor Xa for up to 24 hours after which correct cleavage could not be detected on a Coomassie-stained SDS-PAGE gel even after several days of incubation with the enzyme. Cleavage of the tail can be time consuming, inefficient and also possibly result in a loss of yield. Since there is no ideal method for tail removal, the need to eliminate the fusion tail at large scale is problematic.

Therefore, a general rule of thumb would be that the fusion tail should only be removed if it is a necessity as it can be time consuming, potentially denaturing, inefficient, and lead to reduced yields. However, if it is necessary, it is possible as tails can be designed to incorporate known cleavage sites. Specific proteolytic cleavage sites can be genetically engineered into the fusion tail systems for the removal of the tail from the target protein after purification.

Since the polyhistidine tail used in this study did not appear to effect the activity of the enzyme, it is thought to be non-antigenic, and the purified protein was not to be used therapeutically or pharmaceutically, it was not thought necessary to remove the tag in this instance. This is obviously less time consuming as a purification procedure. An added advantage to leaving the tail on the bT4l is that if the enzyme is used to disrupt cells in other fermentations, it can be retrieved from the crude liquor and reused thereby reducing the number of contaminating proteins and reducing costs.

6.7 Growth media

In chapter 3 it was mentioned that when the bacterial culture was grown in nutrient broth media, lysozyme was found to be present in both the cells and the medium even as little as 30 minutes post-induction of the expression plasmid. The enzyme thus present was able to lyse other intact cells in the culture leading to further culture lysis. The four strong promoters upstream of the lysozyme gene are responsible for this rapid production.

In the isolation of the recombinant enzyme by column chromatography, the samples loaded onto the columns were in effect cultures which had been directly sonicated and then dialysed and filtered in order to maximise the recovery of the protein. However, sample preparation in this manner was not sufficient for the isolation of the recombinant protein on the magnetic particles where the cells had to be collected and

washed and the culture medium discarded, thereby disposing of a considerable amount of the required protein - this will be discussed in section **6.8**.

With respect to the leakage of the enzyme into the media, changing the broth to Luria-Bertani had little effect on the distribution of activity in the liquor. Studies elsewhere in this department (O'Brien *et al.*, 1996[b]) have indicated that leakage of the enzyme into the medium can be dramatically reduced by the use of a highly nutrient medium (rich in yeast extract - 40 g/l) and a growth temperature of 28°C. It was proposed that the high concentration of yeast extract present in the medium is thought to lead to the formation of stronger cell walls resulting in reduced leakage into the medium and hence reduced cell lysis. A reduction in growth temperature may also have aided the containment of the enzyme within the cells.

Use of this high biomass medium would therefore enable the intracellular concentration of the recombinant protein to be maximised, so discarding the large volume of culture supernatant would not result in any significant loss of yield (and smaller volumes are easier to handle). Obviously for other proteins, leakage into the media would not be so detrimental as further culture lysis would not arise; therefore for many expression systems it would not be a problem and the technique could be used as a generic system.

6.8 Sample preparation

Another question to be raised, and related to that above (section **6.7**), is why it is necessary for the samples presented to the magnetic particles to be treated differently from those loaded onto the columns?

It is thought that a component(s) may be present in the culture medium which interferes with the binding event of the protein to the beads. Similar results were obtained with the use of both nutrient and Luria-Bertani broths. Prevention of the recombinant protein binding to the beads could be possible for example if the component(s) has the ability to bind to the surface of the beads, or has the ability to bind to the recombinant protein (hence further binding of the protein to the beads is not permitted). **Appendix 4** shows a list of components of Nutrient broth No.2 which contains several divalent ions such as magnesium (492 parts per million/202 μM), zinc (27 parts per million/4 μM), copper (7 parts per million/1 μM), iron (107 parts per million/19 μM) which could potentially bind to the surface of the protein molecules and thereby block further binding of the protein to the chelated metal ions on the surface of the beads. Another possible component(s) of the media could be the presence of amino acids such as His, Cys or Trp binding to the metal ion before interaction with the fused protein. (It was not within the scope of this project to investigate further)

If this is the case, why does this scenario not occur with the columns? The sonicated culture samples presented to the columns were dialysed and filtered prior to loading and it is therefore thought that a small molecular weight component(s) is responsible for the lack of binding of the recombinant protein to the novel particles. Supporting evidence for this theory was seen by Martin (1994) where small molecular weight supernatant components were found to interfere with the metal affinity purification of single-chain antibodies but conditions improved on dialysis of the sample.

It should be mentioned that although the cells are collected and washed prior to sonication and the supernatant discarded, it is much more desirable to work with smaller volumes. Therefore, an ideal situation for the production of bT4l would be the growth of the *E. coli* in the high biomass medium (where loss due to leakage of the lysozyme into the medium is kept to a minimum), collection of the cells, and wash and sonicate the cells to maximise the amount of recombinant protein available for binding to the magnetic particles. Considering the time scale it would probably be less time consuming to centrifuge the cells prior to sonication than to dialyse larger volumes after sonication as was the case with the column samples. For large scale the cells could be concentrated by a cross-flow filtration system which would allow for buffer changes. Cell disruption on a large scale would be carried out by homogenisation rather than by sonication (sonication is not suitable for large-scale processing because of difficulties in providing adequate cooling at high power input).

6.9 Columns versus magnetic particles

In comparing the results of the purification on the chelated columns to that of the magnetic particles (Chapters 4 and 5), similar quantities were recovered and similar purities obtained. The sample preparation was slightly different but dialysis and filtration would not be recommended for large volumes. The times taken for each is approximately equal but passage of larger volumes through larger columns would increase the time required to obtain the end product while this would not necessarily occur in scale-up of the bead technology. There is a physical limit as to the maximum size of columns which can be used while batch isolation with the beads is dependent upon the efficient production of a magnetic field for retention of the particles.

6.10 Leaching of the metal ions on elution

Atomic absorption spectrophotometric studies of the eluted fractions from all the columns indicate that there was no leakage of any of the metal ions (Zn, Ni or Cu) along with the recombinant protein as their presence was not detected in these samples by this instrument (the limits of detection with these lamps are in the order of 1 part per million/ less than 1 μM). Studies elsewhere in this department (O. Thomas, personal communication) have indicated that metal leaching from these novel magnetic particles does not occur under the experimental conditions used in this thesis. However, if metal ions were stripped from the beads or columns, they could be collected and retrieved with a chelating solution (EDTA is perhaps the most common) but this would add several other steps to the purification protocol (the EDTA would then have to be eliminated) as a whole which is not desirable.

6.11 Metal ions

As indicated in Chapter 5, although the best results for the purification of the recombinant lysozyme was obtained when copper was the chosen metal ion, this may not be desirable for a general technique for a couple of reasons - the low pH of the elution buffer may be too stringent for some proteins, and the use of copper may not gain approval from such bodies as the FDA especially if the purified product is to be used in diagnostics. A safer alternative would possibly be the use of nickel ions where a higher elution pH (and therefore less stringent) can be used successfully (similar recoveries obtained with copper and nickel - see section **5.13.2**) although nickel is also toxic and potentially carcinogenic. Of the other divalent ions tested (section **5.13.1**) no significant binding of the recombinant lysozyme was achieved.

6.12 Scale-up of bead production

In order to scale-up the isolation of a target protein, the availability of the coated magnetic particles would have to improve. Throughout this thesis the core beads were processed in small batches (Chapter 2 section **2.4.17**). These core beads were also relatively expensive to purchase from the manufacturer. Current work in this department is involved with the large-scale production of these novel components thereby the increasing demand can be met at a fraction of the commercial cost. The IDA-coated particles have the advantage of a long shelf life (at least six months) when stored in buffer at 4°C. Technology for the production of different types of beads are also being investigated in this department especially hematite particles coated with dye ligands for the isolation of nucleotide dependent enzymes such as dehydrogenases from unclarified

yeast homogenates (P. Pannu), and perfluorocarbon supports coated with IDA and charged with zinc for the isolation of monoclonal antibodies from CHO culture supernatants (P.E. Morgan). However, the purification protocols as used in this thesis would have to reassessed for the use of other bead particles.

6.13 Scale-up of the magnetic bead purification

In this thesis the successful use of the novel magnetic particles as a one-step purification strategy have only been used on a small scale. However, there is potential that this methodology can be scaled up in a cost effective manner. Results from Chapter 5 show the strength and versatility of this developed technology, as similar results were obtained for each experiment irrespective of the presence or absence of suspended solids in the culture medium.

Reproduction of the batch affinity adsorption process at large scale should be relatively easy to accomplish and the real challenge will be the efficient collection of the magnetic particles. Due to the limitations on the strengths of available magnetic fields, it is difficult to collect magnetic materials from large volumes of liquid using a simple externally applied magnetic field. High gradient magnetic separators (HGMS) overcome the problem. The first large-scale industrial application of HGMS was by the J.M.Huber Company in 1969 for the removal of impurities from Kaolin clay used in the paper industry (Hirschbein *et al.*, 1982). The mined clay is often discoloured by approximately 1 nm particles which are weakly magnetic. The contribution of the magnetic separation step to the final cost of the clay was small and it is now an established technology used in this industry. High processing rates are possible with this system.

HGMS contain high-gradient magnetic filters which consist of a ferromagnetic matrix packed into a canister. The canister is then held between the poles of a powerful electromagnet. The matrix distort the externally applied magnetic field and create local gradients of high strength which attract and retain the magnetic particles when the external field is switched on. When the magnetic field is turned off, the particles can be captured from the canister by flushing with liquid. Large-scale separation of magnetic particles (bound to target proteins) from complex biological liquors using HGMS is currently being investigated in this department.

Separation in this manner would involve adding the supports to the crude culture homogenate in a stirred batch mode. The feed containing the particles and homogenate mix would then be pumped into the small canister and the magnetic field applied. The entrapped support would then be washed to remove any unbound and loosely bound material, followed by the application of the elution buffer to elute the target protein. The supports can be cleaned *in situ* or after their removal from the canister (achieved by

switching off the magnetic field and flushing the canister with water). To put this into a practical perspective, results from section **5.3** indicate that a capacity of 0.45 mg total protein can be applied to 1 mg support. Assuming that the bT4l expression level is approximately 15%, 0.0675 mg of the recombinant protein can be bound by 1 mg of the charged support (or 67.5 mg of target protein/g support). If 1 litre of sonicated *E. coli* culture produces approximately 320 mg total protein (as loaded onto the columns - Chapter 4), then approximately 50 mg of the target protein is present. Therefore either a 2 ml canister with a capacity of 0.1-1 g support processing up to 0.5 l fermentation broth, or a 10 ml canister with a capacity of 0.5-5 g support processing up to 2.2 l fermentation broth (S. O'Brien, personal communication) would suffice.

6.14 Difficulties in scale-up

Although the major challenge in the scale-up of the system refined in Chapter 5 is the collection of the magnetic particles, several other areas are of major importance. For example, in order to maximise the interaction of the beads and the target protein, the beads should be kept in suspension prior to their collection and retrieval. This can be achieved in tubes by vortexing or rotation of the tubes and it is possible that a paddle-like system/in-line mixer could achieve this with larger volumes. The longer the contact time required for binding of the protein to the particles, the more important the mixing of the beads in suspension becomes. In section **5.8** the minimum time for optimal binding of the recombinant protein to the charged magnetic particles was investigated. Assuming that the whole process is scaled up from the bench, since the binding capacities of the beads remain the same, it can be envisaged that the optimal binding time should be similar providing that the mixing is adequate.

A problem encountered in the use of the particles at such a small scale as that seen in Chapter 5, was that potential aspiration (and hence loss) of the beads was possible if one tried to maximise the amount of liquid recovered from the tubes. This was mainly due to the position of the permanent magnets in the small scale retrieval rack. However, this could be overcome with the use of a HGMS system.

Ideally, the beads would be more cost efficient if they could be reused. Methods for cleaning the particles would have to be investigated and should, preferentially, be carried out *in situ*. Effective cleaning is not only important from the aspect of efficient binding but is essential if the isolated product is to be used diagnostically or therapeutically and would have to meet specific guidelines for sterility. Any cleaning procedures tested would have to be verified to ascertain that they did not have a detrimental effect on the particle coatings and would not cause phenomena such as metal

leaching or damage to the IDA. Acceptable cleaning criteria (passed by the FDA) such as cleaning with sodium hydroxide could be investigated.

6.15 Commercial aspects

As mentioned previously in this chapter, one reason for choosing the bacteriophage T4 lysozyme as the protein to test the proposed system, was for its commercial potential - economically it would be highly advantageous if it could be specifically isolated from bacterial cultures and recycled.

At the time of writing this thesis, molecular-grade hen egg white lysozyme available from Sigma (catalogue reference L 7651) cost £727.70 per 100 g. If we take as an example the periplasmic isolation of plasmid-encoded α-amylase from an *E. coli* host (C. French, 1993). The cells from a 5 litre culture were resuspended in 1 litre of a solution containing 500 μg hen egg white lysozyme/ml, thus requiring 0.5 g of lysozyme (which at the cost quoted above, amounts to £3.64). One can therefore imagine the vast expense for a 1000 litre fermentation culture if the same protocol is followed. In general, cell disruption is a high-cost, energy-intensive unit operation (Engler, 1985) and tend to contribute to a significant part of the total production costs.

Recent work carried out elsewhere in this department (O'Brien *et al.*, 1996[b]) has extended the work presented here and investigated the possibility of recycling bT4l. In summary, the recombinant lysozyme has been selectively isolated from an *E. coli* culture by the use of a Cu-IDA Sepharose column (as described in Chapter 4), concentrated, dialysed, and used to release periplasmic α-amylase from other *E. coli* cultures. The histidine-tailed lysozyme was subsequently isolated from this amylase-containing liquor by IMAC for reuse. The enzyme was found to retain its activity throughout the process.

Bacteriophage T4 and hen egg white lysozyme have the same mode of lytic action. However, Tsugita (1971) reported that the T4 lysozyme is approximately two hundred and fifty times more active than the hen egg white lysozyme when *E. coli* cells are the substrate, thus making it an attractive prospect for periplasmic and intracellular protein isolation procedures. Since purified bT4l is not commercially available from any of the major chemical suppliers its use as a substitute for hen egg white lysozyme has not been an option. Not only have we devised a system for production and specific isolation of a recombinant active bT4l, but it has already proved its usefulness in other isolation protocols where *E. coli* is the host with the extremely attractive prospect of recycling.

6.16 Conclusion

Protein purification involves differentiating the unique physiochemical properties of a desired protein from other proteins with similar properties in a crude mixture. A purification protocol should meet several criteria; have a minimal effect on the tertiary structure, have no permanent effect on the biological activity of the enzyme, and be applicable to a number of different proteins. The recovery process of a recombinant protein from a host organism needs to be simple, efficient and economical if it is to translate into industrial use. Traditional affinity supports and ligands require highly clarified liquors and are usually employed during the final steps of purification. Proteinaceous affinity ligands such as antibodies and lectins are not an attractive choice for use in harsh environments due to their high cost, low binding capacity and susceptibility to stringent cleaning conditions.

A magnetic approach offers a number of advantages which could be useful at large scale. The target protein can be recovered from crude cultures containing suspended solids (and nucleic acids) thereby eliminating the need for centrifugation, dialysis and filtration which can be expensive and time consuming. Centrifugation can lead to aerosol production which does not occur with IMAC, therefore reducing the quantity of contaminated liquid and equipment. The non porous supports used here have a large surface area, are easier to handle than traditional chromatographic matrices and are more amenable to scaling up. These particles are also robust and able to withstand low pH conditions (elution of the recombinant lysozyme at pH 3.5 without leaching of the metal ions). Also, HGMS devices are low cost and efficient pieces of equipment.

The availability of several commercially-available kits which combine the advances in recombinant DNA technology and IMAC has enabled many proteins to be isolated. This thesis is another example of the successfulness of the technology and also shows an extension to the application of IMAC using novel non porous magnetic particles for the rapid one-step, high recovery, of highly pure target protein from a complex culture liquor which could be adapted for isolation of many soluble proteins. The methodology for the repeated recovery of a recombinant T4 lysozyme from *E. coli* cultures has been presented which may lead to the wider use of this enzyme in the isolation of intracellular proteins.

APPENDICES

Appendix 1

The complete amino acid sequence of the bacteriophage T4 lysozyme (Tsugita and Inouye (1968) *Journal of Molecular Biology* **37**, 201-212).

1 5 10 15
Met-Asn-Ile-Phe-Glu-Met-Leu-Arg-Ile-Asp-Glu-Gly-Leu-Arg-Leu-Lys-Ile-Tyr-Lys-

20 25 30 35
Asp-Thr-Glu-Gly-Tyr-Tyr-Thr-Ile-Gly-Ile-Gly-His-Leu-Leu-Thr-Lys-Ser-Pro-Ser-Leu-

40 45 50 55
Asn-Ala-Ala-Lys-Ser-Glu-Leu-Asp-Lys-Ala-Ile-Gly-Arg-Asn-Cys-Asn-Gly-Val-Ile-Thr-

60 65 70 75
Lys-Asp-Glu-Ala-Glu-Lys-Leu-Phe-Asn-Gln-Asp-Val-Asp-Ala-Ala-Val-Arg-Gly-Ile-Leu-

80 85 90 95
Arg-Asn-Ala-Lys-Leu-Lys-Pro-Val-Tyr-Asp-Ser-Leu-Asp-Ala-Val-Arg-Arg-Cys-Ala-Leu-

100 105 110 115
Ile-Asn-Met-Val-Phe-Gln-Met-Gly-Glu-Thr-Gly-Val-Ala-Gly-Phe-Thr-Asn-Ser-Leu-Arg-

120 125 130 135
Met-Leu-Gln-Gln-Lys-Arg-Trp-Asp-Glu-Ala-Ala-Val-Asn-Leu-Ala-Lys-Ser-Arg-Trp-Tyr-

140 145 150 155
Asn-Gln-Thr-Pro-Asn-Arg-Ala-Lys-Arg-Val-Ile-Thr-Thr-Phe-Arg-Thr-Gly-Thr-Trp-Asp-

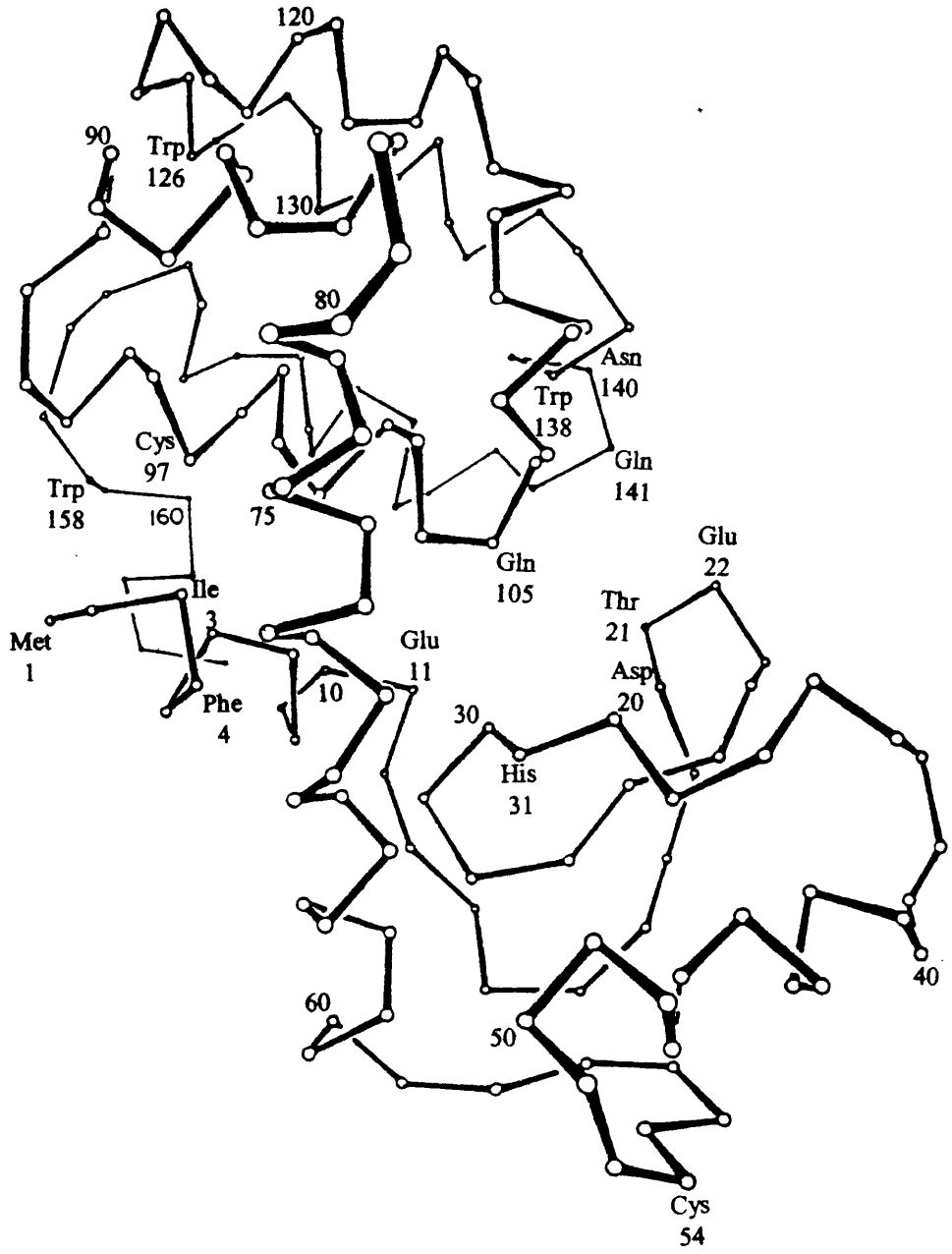
160
Ala-Tyr-Lys-Asn-Leu

Appendix 2

The complete nucleotide sequence of the bacteriophage T4 lysozyme gene (Owen *et al.* (1983) *Journal of Molecular Biology* **165**, 229-248).

```
1               10              20              30              40
ATGAATATATTTGAAATGTTACGTATAGATGAACGTCTTAGACTTAAAA

50              60              70              80              90
TCTATAAAGACACAGAAGGCTATTACACTATTGGCATCGGTCATTTGCTT

100             110             120             130             140
ACAAAAAGTCCATCACTTAATGCTGCTAAATCTGAATTAGATAAAGCTAT

150             160             170             180             190
TGGGCGTAATTGCAATGGTGTAATTACAAAAGATGAGGCTGAAAAACTCT

200             210             220             230             240
TTAATCAGGATGTTGATGCTGCTGTTCGCGGAATTCTGAGAAATGCTAAA

250             260             270             280             290
TTAAAACCGGTTTATGATTCTCTTGATGCGGTTCGTCGCTGTGCATTGAT

300             310             320             330             340
TAATATGGTTTTCCAAATGGGAGAAACCGGTGTGGCAGGATTTACTAACT

350             360             370             380             390
CTTTACGTATGCTTCAACAAAAACGCTGGGATGAAGCAGCAGTTAACTTA

400             410             420             430             440
GCTAAAAGTAGATGGTATAATCAAACACCTAATCGCGCAAAACGAGTCAT

450             460             470             480             490
TACAACGTTTAGAACTGGCACTTGGGACGCGTATAAAAATCTA
```

Appendix 3



A perspective drawing illustrating the polypeptide backbone of wild type bacteriophage T4 lysozyme. The approximate position of each alpha carbon atom is indicated by an open circle. Also shown are the cysteine residues, N- and C-termini, and the active site cleft is indicated. (Modified from Matthews and Remington, 1974)

Appendix 4

Composition of Nutrient Broth No.2 (Oxoid Ltd., Basingstoke, Hampshire)

10 g/l Lab Lemco Powder, 10 g/l Peptone, 5 g/l NaCl

Typical analysis (w/w):

	Peptone	Lab Lemco	
% moisture	4.5	3.9	
% ASH	7.6	14.1	
% Amino nitrogen	2.6	2.5	
% Total nitrogen	14	12.4	
pH	8.3	7.3	
Buffer capacity B1	6.6	10	
Buffer capacity B2	7.3	4.5	
% NaCl	1.6	5.7	
% K	3.6	1.9	
Parts per million:			Total (μM)
Total Ca	690 (172 μM)	238 (59 μM)	231
Free Ca	383 (96 μM)	163 (41 μM)	137
Mg	355 (146 μM)	137 (56 μM)	202
Fe	88 (16 μM)	19 (3 μM)	19
Cu	5 (0.8 μM)	2 (0.3 μM)	1.1
Pb	0.4 (0.02 μM)	0.3 (0.01 μM)	0.03
Mn	3.4 (0.6 μM)	0.4 (0.07 μM)	0.7
Sn	1.0 (0.08 μM)	9.8 (0.8 μM)	0.9
Zn	9.2 (1.4 μM)	18 (2.8 μM)	4
Co	0.1 (0.02 μM)	0.5 (0.08 μM)	0.1
Total P	0.8 (0.3 μM)	0.7 (0.2 μM)	0.5
% Total lipids	0.31	0.37	

Appendix 5

Publications:

Sloane, R.P., Ward, J.M., O'Brien, S.M., Thomas, O.R.T. and Dunnill, P. (1996) Expression and purification of a recombinant metal-binding T4 lysozyme fusion protein useful for repeated cell lysis. *Journal of Biotechnology*, accepted.

O'Brien, S.M., Sloane, R.P., Thomas, O.R.T. and Dunnill, P. (1996) Non-porous magnetic chelator supports. Characterisation and selective recovery of a recombinant T4 lysozyme from crude *E. coli* extract. *Journal of Chromatography*, submitted.

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