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CHEMICAL EXTRACTION OF RECOMBINANT PROTEIN FROM THE CYTOPLASM OF *ESCHERICHIA COLI*

BY

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AMENDMENTS

The following amendments should be made to the text of the thesis.

p 11	line 12	Millar should read Miller
p 62	line 6	total should read recombinant
p 62	line 7	recombinant should read total
p 66	Figure 3.3	x axis title, M should read mM
p 67	Figure 3.4	x axis title, M should read mM
p 118	line 25	4.4 should read 4.3
p 119	line 6	4.5 should read 4.4
p 130	line 10	51% should read 60%
p 130	line 12	79% should read 75%
p 130	line 13	69% should read 61%
p 141	line 3	5.3 should read 5.4
p 146	line 2	5.4 and 5.5 should read 5.6 and 5.7
p 146	line 3	5.5 should read 5.1
p 148	line 11	Costa and Cabral should read Santos *et al.*
p154	lines 13-14	5.10, 5.11 and 5.12 should read 5.12, 5.13 and 5.14
p 166	line 1	permeabilisation should read diafiltration
p 176	line 3	reference Santos *et al.* 1991 should be moved to p 184

The following recommendations are made for future work on chemical extraction of recombinant proteins from inclusion bodies located in the cytoplasm of *E. coli.*

1. Characterise the extract achieved by the new selective extraction procedure.

    a) Measure the levels of key co-extracts such as nucleic acids, lipopolysaccharide, and proteases such as OmpT.

    b) Determine the advantages and disadvantages of the procedure with respect to co-extraction of contaminants.

2. Undertake the procedure with a range of other recombinant proteins.

   a) Determine the role of the cysteine residues on the effectiveness of the selective-extraction procedure.

   b) Determine the procedures effectiveness at the selective-extraction of protease sensitive proteins such as IGF-2.

3. Improve and optimise the prototype pilot-scale selective-extraction process.

   a) Determine the role of the cell concentration on the operation of the permeabilisation step with the aim of optimizing this step.

   b) Determine the role of recombinant protein concentration on the operation of the solubilisation step with the aim of optimizing this step.

   c) Test various membranes for their reduced adsorption of the recombinant protein.

   d) Optimise the diafiltration steps to maximize separation of the contaminants while retaining most of the recombinant product.

   e) Carry out the pilot-scale selective-extraction process and define the processes capabilities.

4. Carry out economic analysis of the pilot-scale selective-extraction process and compare it to conventional extraction.

5. Determine whether additional process improvement such as reagent recycle is required to make this process economically attractive to manufacturers of recombinant protein products.

This work contains no work that has been submitted for the award of any other degree or diploma in any university or any other tertiary institution and to the best of my knowledge and belief, contains no material previously published or written by another person, except were due reference is made in the text.

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15 Sept 1997

Signature (Robert John Falconer) Date

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Lastly I would like to thank Susie, Rachael, and Ben for enriching my life outside of University and adding happiness to a previously mundane existence.

SUMMARY

The majority of processes published for the recovery of recombinant proteins from inclusion bodies located in the cytoplasm of *Escherichia coli* follow a conserved set of process steps. This process (conventional extraction) comprises mechanical disruption of the bacteria, centrifugation to isolate the insoluble fraction, dissolution of inclusion bodies, followed by refolding and purification of the protein. This approach is usually effective but can be cumbersome and inefficient for some proteins.

Alternative extraction procedures developed for the recombinant protein Long-R^3-IGF-I are detailed in this thesis. They include nonselective and selective extraction procedures.

A permeabilisation procedure using a combination of 6 M urea, 3 mM EDTA, and 0.1 M Tris buffer at pH 9.0 proved to be as effective at releasing intracellular protein from *E. coli* as mechanical disruption. This combination (referred to as basic permeabilisation solution) formed the basis for subsequent extraction protocols. Permeabilisation was independant of the bacterial growth-phase.

Treatment with basic permeabilisation solution plus the reducing agent dithiothreitol (DTT) resulted in the complete extraction of Long-R^3-IGF-I protein from inclusion bodies located in the cytoplasm of *E. coli*. This nonselective extraction procedure also released the host-cell contents. The procedure recovered approximately 100% (w/w) of the Long-R^3-IGF-I protein at 16% (w/w) purity (compared to total protein). The low purity can constitute a problem during the purification of recombinant protein and the procedure may need to be combined with a selective process step to overcome this limitation. The kinetics of both recombinant and total protein release were first order with time constants less than 5 min. The nonselective extraction procedure has immediate application for the extraction of recombinant protein from bacterial cells prior to analysis, for example by HPLC.

Addition of the disulphide reagent 2-hydroxyethyldisulphide (2-HEDS) to the basic permeabilisation solution minimised solubilisation of the Long-R^3-IGF-I inclusion bodies while allowing permeabilisation to proceed. A two-stage laboratory procedure was developed that used basic permeabilisation solution plus 2-HEDS during the first

permeabilisation step, centrifugation to separate the insoluble from the soluble waste, and basic permeabilisation solution plus DTT during the inclusion body solubilisation step. The extraction recovered 83% (w/w) of Long-R^3-IGF-I with a purity of 46% (w/w). The results were comparable to those obtained by conventional extraction. This procedure, termed selective extraction, could be directly applied to the purification of milligram quantities of Long-R^3-IGF-I.

A scaleable selective extraction procedure was developed that used tangential flow filtration in place of centrifugation, and used a stirred reactor for the permeabilisation and solubilisation reactions. Results for the extraction of Long-R^3-IGF-I using a 100 kilodalton nominal cut-off filter demonstrated that the procedure was able to extract Long-R^3-IGF-I. A recovery of 51% (w/w) of product at a purity of 32% (w/w) was obtained without any optimisation studies. On optimisation, it was estimated that this procedure has the potential to recover 79% (w/w) Long-R^3-IGF-I at a purity of 69% (w/w). The improved purity relative to the small-scale procedure is due to the selectivity provided by the final filtration step. The optimised pilot-scale selective extraction process would be capable of processing large quantities of Long-R^3-IGF-I at high efficiency

Application of the nonselective and selective extraction procedures to recombinant proteins other than Long-R^3-IGF-I will be dependant on a series of factors. In some cases nonselective extraction could require higher urea concentrations or other chaotropic agents to solubilise the recombinant inclusion bodies. Selective extraction relies on the presence of cysteine residues in the recombinant protein for the reversible formation of disulphide bonds that stabilise the inclusion body during the first stage (cell permeabilisation). Recombinant proteins with few or no cysteine residues may not be suited to this approach. The procedures need to be tested with a variety of recombinant proteins before their generic applicability can be demonstrated.

The selective extraction procedure has some key advantages including simplicity, low equipment requirement, flexibility, and speed. Further development, such as increasing the cell concentration and introducing reagent recycle, may be necessary to reduce operational costs and make the procedure commercially competitive.

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CHAPTER 1

INTRODUCTION

Summary

This introduction describes the procedures used for the recovery of recombinant proteins from inclusion bodies in *Escherichia coli* that have been published. The majority of recovery procedures follow a highly conserved set of steps. These comprise mechanical disruption, centrifugation, and inclusion body solubilisation. This basic procedure is often enlarged to remove insoluble contaminants (including the outer-membrane protease OmpT) from the preparation. The additions can include multiple centrifugation steps or inclusion-body washing procedures.

A few alternative approaches exist including secretion of the protein into the periplasm where it is easier to recover using methods based on chemical extraction. These are described and their potential for replacing the conventional techniques is discussed.

The aim of this project is to develop a superior chemical extraction procedure for the recovery of recombinant protein from inclusion bodies located in the cytoplasm of intact *E. coli* that is competitive with the conventional approach.

1.1 Manufacture of recombinant proteins in *Escherichia coli*

Advances in genetic engineering over the last twenty years have enabled a new industry manufacturing pharmaceutical agents, diagnostic products, veterinary agents, and industrial enzymes to evolve. Ability to transfer genetic material from diverse origins to prokaryote (and later to eukaryote) cells led to technology for the large scale manufacture of recombinant proteins. This technology allows the manufacture of proteins previously available only in low quantities or available only from undesirable sources such as human (or animal) blood and tissue. The implications of the new technology were recognised (Emtage, 1985) and it has led to the commercial production of several potent biopharmaceuticals. In 1982 the only recombinant biotechnology product was human insulin. By 1993 twenty one products had reached the market place with combined sales of $US 4000 million (Barnacal, 1995). Biopharmaceutical products produced using genetically engineered organisms include interferon, tissue plasminogen activator (tPA), hepatitis B vaccine, erythropoietin (EPO), somatotropin, insulin, antihaemophiliac factor, granulocyte colony stimulating factor (G-CSF), granulocyte macrophage colony stimulating factor (GM-CSF), interleukin-2, and monoclonal antibody products (Price, 1993). The manufacture of industrial catalysts such as recombinant renin and penicillin acylase are examples of non-medical applications of this technology that will become increasingly important in the future.

The host organisms used in the production of the majority of recombinant proteins are the bacterium *E. coli*, the yeast *Saccharomyces cerevisiae,* and the mammalian cell line CHO (chinese hamster ovary). More recently the range of host cell lines has expanded to include insect cells, the methylotrophic yeast *Pichea pastoris*, plant cells, and transgenic animals. *E. coli* remains, however, the most widely used host for the manufacture of non-glycosylated recombinant proteins. The wealth of knowledge of *E. coli* genetics and biochemistry is a great asset that has made it possible to construct vectors for the high expression of foreign genes in a reproducible, highly productive and economic manner. Difficulties in genetic manipulation, low productivity or high cost of cultivation of many alternative host lines has limited their commercial application (Rudolph and Lilie, 1996).

Recombinant proteins that require glycosylation, or that are difficult to refold, can be expressed in eukaryotic cells where these difficulties can be overcome.

Recombinant proteins expressed in genetically engineered *E. coli* usually accumulate in the cell's cytoplasm. The protein can be in either a soluble or insoluble form, or as mixture of the two. Accumulated insoluble protein is often visible under a phase contrast microscope as "phase bright bodies", referred to as inclusion bodies. Many recombinant proteins are manufactured using genetically modified *E. coli* where the recombinant protein accumulates as inclusion bodies.

The formation of recombinant inclusion bodies simplifies the initial recovery steps from the bacterial host. The physical properties of inclusion bodies (their size and density) allow their separation from the bulk of the host organism's soluble components. This initial purification is invaluable as the reduction in purification steps can provide substantial savings in terms of time and money for the total process. Inclusion bodies, however, require dissolution to release the recombinant proteins. Strong denaturing agents are usually required which not only solubilise the inclusion body but denature the protein. The protein requires refolding to regain its native conformation. The refolding step can be simple for some of the smaller simple proteins like insulin-like growth factor 1 (Hejnaes *et al.*, 1992), but can be a costly low-yielding process for larger complex proteins like tPA (Datar *et al.*, 1993). Production of recombinant protein as inclusion bodies in *E. coli* remains the most widely used method for production and dominates the published methodologies.

1.2 Traditional recovery strategies

Since the late 1970's when recombinant proteins were first expressed in *E. coli*, a large number of purification procedures have been published. While the majority of the processes were for the purification of small quantities of protein in the laboratory, the strategies closely resemble those used in subsequent industrial-scale processes. Analysis of published purification processes demonstrated that the strategies used were quite conservative for the initial process steps, while the later process steps were highly variable (Fischer *et al.*, 1993).

Table 1.1 Strategy for isolation of bioactive proteins from inclusion bodies (modified figure from Fischer *et al.*, 1993).

1. Expression of product in *E. coli* cells
2. Extraction from *E. coli* cells
3. Removal of soluble contaminants
4. Reduction of insoluble contaminants
5. Dissolution of insoluble material
6. Initial fractionation
7. Renaturation of the product
8. Final purification

Initial recovery steps (steps 2 to 5 in Table 1.1) are dictated by the physical properties of the inclusion body and its location within the *E. coli* cell. Extraction of the inclusion bodies from the cell (step 2) is usually achieved using mechanical disruption. The resulting mixture includes inclusion bodies, fragments of cell wall, and soluble host cell components. The size and density of inclusion bodies allows their separation from the soluble contaminants by either centrifugation or diafiltration (step 3). Further removal of

cell debris is possible with the incorporation of washing steps (step 4). Recombinant protein is then recovered from the inclusion body by dissolution using strong denaturing agents (step 5). Subsequent purification and renaturation steps can be made up of a range of different process steps, and there is great variety between the strategies used for different proteins. The range of purification steps is shown in Table 1.3. Protein renaturation is not conservative. A wide range of conditions have been applied for the refolding of proteins, many of which are likely to be protein specific.

1.2.1 Extraction

Mechanical disruption is the preferred technique for the extraction of recombinant protein inclusion bodies from the confines of the bacterial cell wall. The units most suited to the large-scale disruption of micro-organisms are the high-pressure homogeniser and the bead mill. The sonicator and French press are usually limited to laboratory-scale applications (Engler, 1985).

High-pressure homogenisers consist of a positive-displacement pump (operating at about 75-100 MPa) that forces the suspension through the centre of the valve seat, radially across the seat face, and into the impact ring (Middelberg, 1995). The mechanism of disruption is uncertain, though recent evidence suggests that disruption occurs in regions of high pressure gradient near the valve inlet and impact ring (Kleinig and Middelberg, 1996). Mechanical disruption causes the organism's cell wall to be torn apart non-specifically. The homogenate of *E. coli* containing inclusion bodies is made up of inclusion bodies, cell wall fragments, and soluble host cell components. Repeated passes though a homogeniser are required for complete cell disruption. Repeated passes have no significant effect on inclusion body size but do result in a decrease in cell-wall fragment size (Keshavarz-Moore *et al.*, 1991; Wong, 1996).

An alternative to the high-pressure homogeniser is the bead mill. Bead mills contain glass beads (usually less than 1.5 mm) that are agitated by a rotary shaft of various designs. Disruption is thought to occur in the active volume between colliding beads (Middelberg,

1995). While this technique is effective for bacteria such as *E. coli,* it is better suited for the disruption of larger organisms such as yeast and fungi.

1.2.2 Removal of soluble contaminants

Centrifugation.

Centrifugation is widely used for the separation of small particles of biological origin (Hoare and Dunnill, 1989). At the industrial scale disc-stack centrifuges are used that allow a continuous flow of the process suspension to pass though the machine. Particulates sediment to the edge of the disc-stack, and the clarified liquor passes back out of the top of the disc-stack.

The basis for separation by centrifugation is explained by Stokes law,

$$\upsilon_g = \Delta\rho\, d^2 g / 18\mu \qquad - (1.1)$$

where $\Delta\rho$ is the difference between the density of the particle and the density of the surrounding medium, d is the diameter of the particle, g is the gravitational acceleration and μ is the viscosity of the solution. This law can be modified to apply to the operation of centrifuges by replacing the g with $\omega^2 r$ where ω is the angular velocity and r is the radial position of the particle. The centrifuge can be characterised by equation 1.2,

$$K = \omega^2 r / g \qquad - (1.2)$$

where K is the relative centrifugal force.

Particle size and buoyant density are critical for particle separation using this technology. Examples of inclusion bodies where the size and density have been studied include the recombinant proteins γ-interferon and prochymosin. The sizes were calculated to be 0.81 ± 0.17 µm and 1.28 ± 0.46 µm, respectively (Taylor *et al.*, 1986). Determination of the

buoyant density is more difficult. The voidage in the inclusion bodies (estimated to be 70 and 85% respectively for the two examples) allows entry of the suspending liquid into the inclusion bodies altering the buoyant density. Observation of inclusion bodies using electron microscopy reveals some variation in the morphology of the bodies. Inclusion bodies located in the cytoplasm can be regular cylindrical particles differing significantly from those located in the periplasm that are smaller, irregular and semi-spherical in shape (Bowden *et al.*, 1991). The size, shape and density of inclusion bodies is not always uniform even within an individual bacterium. Recombinant *Vitreoscilla* haemoglobin (Vhb) protein formed two discrete inclusion body morphologies that could be separated by differential centrifugation and had different levels of contamination by host cell components (Hart *et al.*, 1990).

Cross-flow filtration

Cross-flow filtration involves the flow of the process material parallel to the filtration membrane surface in order to minimise the build up of solids on the membrane surface. Maintenance of a relatively clean membrane surface improves the performance of the system reducing the level of flux decline with time. Many factors affect the performance of cross-flow filtration systems. These include flow-rate, transmembrane pressure, pore size, membrane charge and hyrophobicity, the ionic composition of the liquid phase, the concentration of the soluble macromolecules, and the nature of the insoluble components.

Cross-flow filtration in diafiltration mode (where fresh buffer is supplied at the same rate as the permeate is removed) can be used to separate soluble from insoluble components. Cross-flow filtration was successfully applied to the separation of soluble proteins from *E. coli* cell lysate containing recombinant protein inclusion bodies (Forman *et al.*, 1990). Removal of the soluble protein was effective with 87% of the soluble protein removed after three volume exchanges using constant volume diafiltration. Retention of the soluble protein was found to be dependant on the flux rate across the membrane. High transmembrane pressure gave increased flux rates which also correlated with higher protein retention, limiting the operation of the process. The nature of the cell debris also

affects the performance of cross-flow filtration of disrupted bacterial cells (Quirk and Woodrow, 1984).

Cross-flow filtration presents significant economic advantages over centrifugation for specific applications such as cell debris processing (Datar and Rosen, 1993) and is worth serious consideration for the separation of soluble components away from insoluble inclusion bodies.

1.2.3 Reduction of insoluble contaminants

Inclusion body preparations usually contain contaminating molecules that are either integral components of the inclusion body, molecules adhering to the surface of the inclusion body, or material that copurifies with the inclusion body (the insoluble fraction of *E. coli*). Bovine somatotropin inclusion bodies prepared from cell homogenate by centrifugation with one wash step (using water) was found to contain a range of contaminating molecules including bacterial proteins, other proteins encoded on the plasmid (e.g. β-lactamase), four subunits of RNA polymerase, outer-membrane proteins (Omp A, C and F), rRNA, plasmid DNA, and lipopolysaccharide (LPS) (Hartley and Kane, 1987). The outer membrane contaminants include the outer-membrane serine protease OmpT. It is specific to dibasic sites (i.e. two consecutive basic amino acid residues). This protease has been implicated in the degradation of a range of normal bacterial and foreign proteins (including IGF-2) that come into contact with the outer-membrane during cell lysis or subsequent purification (Miller, 1996).

Homogenisation of *E. coli* cells creates cell debris made up of irregularly shapes fragments of cell wall. Cell debris can be separated from inclusion bodies using differential centrifugation relying on the difference in size and density of the two components. Differential centrifugation has varying levels of success due to adhesion of cell wall components to the surface of the inclusion bodies. Sucrose gradient centrifugation was unable to remove outer membrane contamination during the purification of β-lactamase

inclusion bodies (Valax and Georgiou, 1993). The hydrophobic nature of some inclusion body surfaces and cell debris is a likely cause of adhesion.

At a commercial scale disc-stack centrifugation is used to recover inclusion bodies from homogenate. This technique can be used to remove the bulk of the cell debris from inclusion body preparations using the difference in size and density of the two types of particles. The size of cell debris resulting from mechanical disruption vary according to the disruption regime used. Debris size was shown to reduce after 5 successive passes though a high pressure homogeniser (to approximately 0.3 μm) (Wong, 1996). The differences in size between the cell debris and the inclusion body allows some separation of the two by centrifugation. Simulated recovery of prochymosin inclusion bodies in a disc centrifuge (Westfalia, BSB7) using PVA grade efficiency curves demonstrates that it is theoretically possible to recover about 95% (w/w) of the inclusion bodies while reducing the level of cell debris by about 75% (w/w) at a specific flow rate (200 L/h) (Keshavarz-Moore *et al.*, 1991). In practice inclusion body separation from cell debris can be difficult. Where cell debris and inclusion body size distributions overlap centrifugation will not perfectly separate the two fraction. The recovery of Long-IGF-2 inclusion bodies (where inclusion bodies only reached 0.35 to 0.45 μm diameter) required multiple centrifuge passes to achieve a significant reduction in outer-membrane contamination (Wong, 1996).

Washing strategies

To reduce insoluble contaminants from the inclusion body preparation a range of different types of washes have been developed. The aim of the washing buffer components is to solubilise the contaminating material or to reduce its adhesion to the inclusion body, without significantly solubilising the recombinant protein. Washing buffers often include either detergents such as Triton X-100 or deoxycholate, or a chaotropic agent such as urea (Fischer *et al.*, 1993).

An example of a recovery process that incorporates a wash step is the manufacture of recombinant bovine growth hormone (Langley *et al.*, 1987). The inclusion body preparation was washed using a buffer comprising 20 mM Tris-HCl, 5 mM EDTA, and

0.02% lysozyme, followed by 2% deoxycholate. The quantities of the product (r-bGH), total protein, phospholipid, nucleic acid, and endotoxin were determined at key stages of the process. The results (shown in Table 1.2) demonstrated successful removal of the bulk of cell-wall associated phospholipid and endotoxin, nucleic acid and soluble host-cell protein from the preparation. Note that after inclusion body washing the protein was released by solubilisation with 6 M guanidine HCl followed by gel filtration through a Sephacryl S-200 column. The profile of the gel filtration chromatograph showed that a considerable level of high and some low molecular weight contaminants still remained in the inclusion body preparation.

Table 1.2 Reduction in the quantities of host cell components during recovery of r-bGH (Langley *et al.*, 1987).

Purification step	*Product r-bGH (g)*	*Total protein (g)*	*Phospholipid (nmol)*	*Nucleic acid (g)*	*Endotoxin (mg)*
1. Broken cells	15.4	80.4	8.87	19.72	160-1600
2. Broken cell pellet washed with water	15.35	29.1	2.30	2.07	120-1200
3. Pellet fraction after extraction with the washing buffer	14.1	17.9	0.10	0.09	1-10
4. Pooled material from Sephacryl S-200 column	6.6	6.6	nr	nr	nr

nr Not recorded

A similar washing protocol was used for the removal of contaminants from recombinant *Vitreoscilla* haemoglobin inclusion bodies (Hart *et al.*, 1990). In this study the detergent Triton X-100 was used in parallel with deoxycholate, both at 2% (w/v). Triton X-100 proved to be a less powerful at solubilising host cell membranes than deoxycholate though it could be suitable for use with inclusion bodies which are soluble in a deoxycholate solution.

Removal of protease activity associated with the outer membrane fraction was successfully achieved by the incorporation of a wash step using 2.5% (w/v) octyl-glycoside during the recovery of recombinant creatine kinase (Babbitt *et al.*, 1990). This protease activity is probably due to OmpT a protease that is responsible for the degradation of a range of native *E. coli* proteins and recombinant proteins in *E. coli* that come into contact with the outer-membrane (Millar, 1996).

Characterisation of prochymosin inclusion bodies (both cytoplasmic and periplasmic) separated from cell debris by sucrose density centrifugation followed by a wash with 1.5% (w/v) octyl glucoside, showed that the remaining polypeptide contaminants ranged from 5 to 50% (w/w) of the total protein content, that phosopholipids composed 0.5 to 13% (w/w) of the inclusion body, and that nucleic acid was a minor contaminant. Periplasmic inclusion bodies contained higher levels of contaminants than those of cytoplasmic origin (Valax and Georgiou, 1993).

Cross-flow filtration in diafiltration mode can be used to separate soluble from insoluble components. The flexibility of cross-flow filtration makes the addition of a washing protocol quite simple. The washing solution can be added directly to the process solution to solubilise the contaminating material or by diafiltration using the washing solution to replace the permeate. Diafiltration with 1.75 M guanidine-HCl proved effective for the extraction of contaminants from recombinant interleukin-2 inclusion bodies providing a higher purity and recovery than washing in conjunction with batch centrifugation (Meagher *et al*, 1994).

1.2.4 Solubilisation of the inclusion bodies

Inclusion bodies usually require strong denaturing conditions to dissolve the aggregated protein into a monomeric form. The formation of inclusion bodies was thought to be due to precipitation of the protein as its concentration was higher than the solubility limit. The requirement for strong denaturing conditions demonstrates that the intermolecular bonds are not those observed in "salting out" precipitation (which can redissolve on dilution). Studies of aggregation during refolding *in-vitro* demonstrate that aggregation can be due to the interaction of partially folded intermediates (Mitraki and King, 1989). Exposure of hydrophobic zones on the partially folded intermediates was found to be the mechanism for aggregation during refolding of the enzyme rhodanase (Tandon and Horowitz, 1987). Detergent inhibited aggregate formation. Further stabilisation of the inclusion body can be caused by intermolecular covalent disulphide bonding, which probably results from air oxidation after cell lysis. Disulphide bond formation within the cytoplasm can be inhibited by the reducing environment inside the bacterial cytoplasm (Tuggle and Fuch, 1985).

The chaotropic agents urea and guanidine HCl are the most common solubilising agents used to process a range of recombinant protein inclusions (Fischer *et al.*, 1993). The mechanisms for urea and guanidine HCl denaturation of monomeric proteins have been studied. Urea is believed to be able to denature protein due to its ability to weaken hydrogen bonds that provide much of the protein's structural strength (Kamoun, 1988). The mechanism for protein denaturation by chaotropic agents such as urea and guanidine HCl has been studied using X-ray crystallographic methods (Hibbard and Tulinsky, 1978). Electron density maps for the protein chymotrypsin in the presence of dilute urea and guanidine HCl demonstrated that urea bound to sites in the hydrophobic interior and protein surface, while guanidine HCl bound only to the protein surface (Hibbard and Tulinsky, 1978). The study also showed that urea binding was not always accompanied by structural changes in the protein. Denaturation of proteins by urea and guanidine HCl have also been studied using techniques such as UV differential spectroscopy, circular dichromism, fluorescence and NMR (Pace, 1986). Protein denaturation can be explained in terms of a denaturant binding model.

Solubilisation of inclusion bodies is more complex than denaturation of monomeric protein. The bonding within an inclusion body includes intermolecular and intramolecular bonds with hydrogen, hydrophobic forces, and often covalent disulphide bonds are present. Solubilisation is further complicated by the ability of some proteins to phase partition at high concentrations (Thomson *et al.*, 1987). This would interfere with mixing in the vicinity of the inclusion body.

Few detailed studies on the solubilisation of inclusion bodies are available in the literature. Most investigators use highly-concentrated solutions of chaotrope without testing the suitability of lower concentrations (Marston and Hartley, 1990). Dissolution with low concentrations of urea was demonstrated for the recombinant fusion protein Long-R^3-IGF-I (Greenwood *et al.,* 1994). Studies need to be carried out to determine the role of environmental conditions such as pH, temperature, and chaotropic agent concentration on solubilisation.

Alternative methods that have been used for solubilising specific inclusion bodies include extremes of pH (Fischer *et al.*, 1993) and the cationic surfactant n-cetlytrimethylammonium chloride (Puri *et al.*, 1992). The reducing agents dithiothreitol (DDT) and β-mercaptoethanol are often required for the solubilisation of proteins containing cysteine residues. The reducing agents prevent the formation of disulphide bonds that could cause aggregation and prevent complete denaturation.

1.2.5 Purification and renaturation.

The soluble recombinant protein that results from the primary separation steps (homogenisation, centrifugation and dissolution) is usually in a denatured form and is contaminated with components of the bacterial host cell. This material usually requires refolding back to its native configuration prior to recovery by a series of purification steps.

Prior to renaturation, an initial purification step is usually incorporated to remove low molecular weight contaminants that can interfere with the renaturation step. Size exclusion chromatography or ultrafiltration are often applied.

Renaturation is then carried out to obtain the correct native conformation. Conditions used for renaturation vary greatly and are dependant on the specific protein (excellent reviews of protein refolding techniques include those by De Bernardez-Clark and Georgiou (1991), and Rudolph and Lilie (1996).

The final purification strategy tends to vary greatly due to the wide variety of separation techniques available to the biochemical engineer. The choice and order of the purification and renaturation methods applied to the protein is dictated by the required level of purity and the conformation of the final protein product, the nature of the protein, the nature of the key contaminants, and the final product formulation. While the range of available separation processes is large, there are several heuristics that aid in selection a suitable of purification strategy. Typical heuristics given by Asenjo and Patrick (1990) are:

1. Choose separation methods based on different properties of the protein.
2. Choose conditions that exploit the greatest differences between the protein and the key contaminants.
3. Separate the most plentiful impurities first (usually achieved during the primary separation steps).
4. Use a highly selective step as soon as possible.
5. Carry out the most arduous or expensive step last.

The separation methods and principles for their action are outlined in Table 1.3. Selection of the purification steps also needs to take into account the amenability of the methods to scale-up. Purification steps have to be gentle to the protein product. Proteins can be susceptible to deterioration by denaturation, aggregation, enzymatic or chemical modification, and adhesion to surfaces. Final selection of a purification method is often heavily influenced by the preference of the individual purification scientist and the method's availability. As the biotechnology industry matures, economic and engineering considerations need to be taken seriously to assure the processes suitability in a increasingly competitive environment.

Table 1.3 Separation principles and techniques in biotechnology (modified from Janson and Ryden, 1993).

Separation Principle	Separation Technique
Temperature stability	Heat denaturation
Solubility	Salt precipitation Solvent precipitation Polymer precipitation Isoelectric precipitation Partitioning in aqueous two-phase systems Partition chromatography
Size and shape	Size exclusion chromatography Ultrafiltration
Net charge	Ion exchange chromatography
Hydrophobicity	Hydrophobic interaction chromatography Reverse phase chromatography
Biological function	Biospecific affinity chromatography
Antigenicity	Immunosorption
Carbohydrate content	Lectin affinity chromatography
Content of free -SH	Covalent chromatography
Metal binding	Immobilised metal chromatography
Miscellaneous	Hydroxylapatite chromatography Dye ligand affinity chromatography

1.3 Alternative recovery strategies

1.3.1 Expression

Genetic engineering has been used to aid the recovery of recombinant proteins from host bacteria. One strategy involves cloning the protein-encoding sequence onto an endogenous secretory signal sequence, facilitating secretion of the protein into the bacterial periplasmic space. Recovery strategies for periplasmic material are significantly different from those used in the extraction of cytoplasmic material as discussed in section 1.3.2.

Export into the periplasmic space has advantages and disadvantages (Simmons and Yansura, 1996). Advantages include a natural N-terminal amino acid sequence, enzymatic catalysis of disulphide bond formation, and physical separation from the bulk of the endogenous molecules. Disadvantages include low levels of secretion, incomplete processing of the precursor, and aggregation of proteins secreted at high levels in the periplasm.

Secretion into the periplasm is accompanied by proteolytic cleavage of the leader sequence. Once inside the periplasm (an oxidising environment) the protein can refold. The endogenous foldases (Dsb A, B, C, and D) catalyse disulphide bond formation, and the rearrangement or isomerisation of incorrect disulphide bonds. Correct disulphide bond formation can be enhanced by coexpression of the eukaryotic enzyme DPI that catalyses cysteine oxidation and hence disulphide bond isomerisation. It also acts as a chaperone. This strategy has been used to correctly fold proteins like pectate lyase C and tPA (Georgiou and Valax, 1996).

Secretion levels can be enhanced by the optimisation of translational levels to match secretion levels (Simmons and Yansura, 1996).

Insulin-like growth factor has been expressed in a construct that secretes the growth factor into the periplasmic space (Hart *et al*, 1994). The recombinant protein accumulates as a

mixture of soluble protein and aggregates within the periplasm The recovery protocol used chemical permeabilisation (urea and cysteine) and an aqueous two-phase extraction step (PEG/phosphate) to recover a relatively pure product.

Another strategy is to produce soluble correctly-folded protein in the bacterial cytoplasm. Over-expression of endogenous chaperone proteins has been used to prevent aggregation of unfolded recombinant products. This strategy has been used to assemble complex multimeric as well as monomeric proteins (Georgiou and Valax, 1996). Recovery protocols for correctly folded cytoplasmic proteins need to be designed so the correct conformation of the protein is maintained. Soluble cytoplasmic proteins will be contaminated by a wide array of endogenous molecules on extraction from the bacterial cell. The purification of such a product would benefit from a highly specific purification step to minimise process complexity.

1.3.2 Chemical permeabilisation

An alternative to mechanical disruption for product extraction is chemical permeabilisation. A wide variety of chemical treatments have been used to permeabilise the cell walls of bacteria with mixed success. Chemical permeabilisation has been used for the extraction of a range of different proteins. These include native *E. coli* proteins, correctly refolded recombinant protein located in the periplasm, recombinant inclusion bodies located in the periplasm, and recombinant protein located in the cytoplasm. Native and correctly refolded recombinant proteins require extraction without alteration of their structure, while the recombinant proteins that are not in their native conformation often require denaturation to be released into the aqueous phase. These constraints often influence the choice and success of the chemical extraction procedure chosen. Many chemical extraction procedures also attempt to achieve a degree of selectivity during extraction and do not aim to maximise total protein release (i.e. they do not aim to maximise cell wall permeabilisation). These factors need to be taken into account when evaluating chemical permeabilisation techniques.

Cell wall barrier

For a chemical permeabilisation technique to be successful, the barrier presented by the cell wall needs to be overcome. The cell wall of *E. coli* comprises three structural components, the outer membrane, the peptidoglycan layer, and the inner (or cytoplasmic) membrane (see Figure 1.1).

The outer membrane envelopes the bacterium covering the structural peptidoglycan layer. It presents the first barrier between the environment and the *E. coli* cytoplasm. The major components of the outer membrane are shown in the Table 1.4

Table 1.4 Major components of the outer membrane of *Escherichia coli* (modified table from Nikaido, 1996).

Component	Molecules per cell	Surface area (μm^2)
Lipopolysaccharide	34.6×10^5	4.9 (outer surface)
Proteins (Porins & OmpA)	2×10^5	1.8 (outer & inner surface)
Lipoprotein	7×10^5	0.5 (inner surface)
Phosopholipid	87×10^5	4.1 (inner surface)

Lipopolysaccharide (LPS) plays a strong role in the barrier function of the outer membrane and protects the bacterium against surface-active agents such as bile salts. Wild-type outer membranes demonstrate low permeability to hydrophobic molecules, while some mutations affecting the structure of the LPS result in dramatic increases in permeability to

hydrophobic molecules. Phospholipid bilayers alone present a poor barrier to these molecules (Nikaido, 1996).

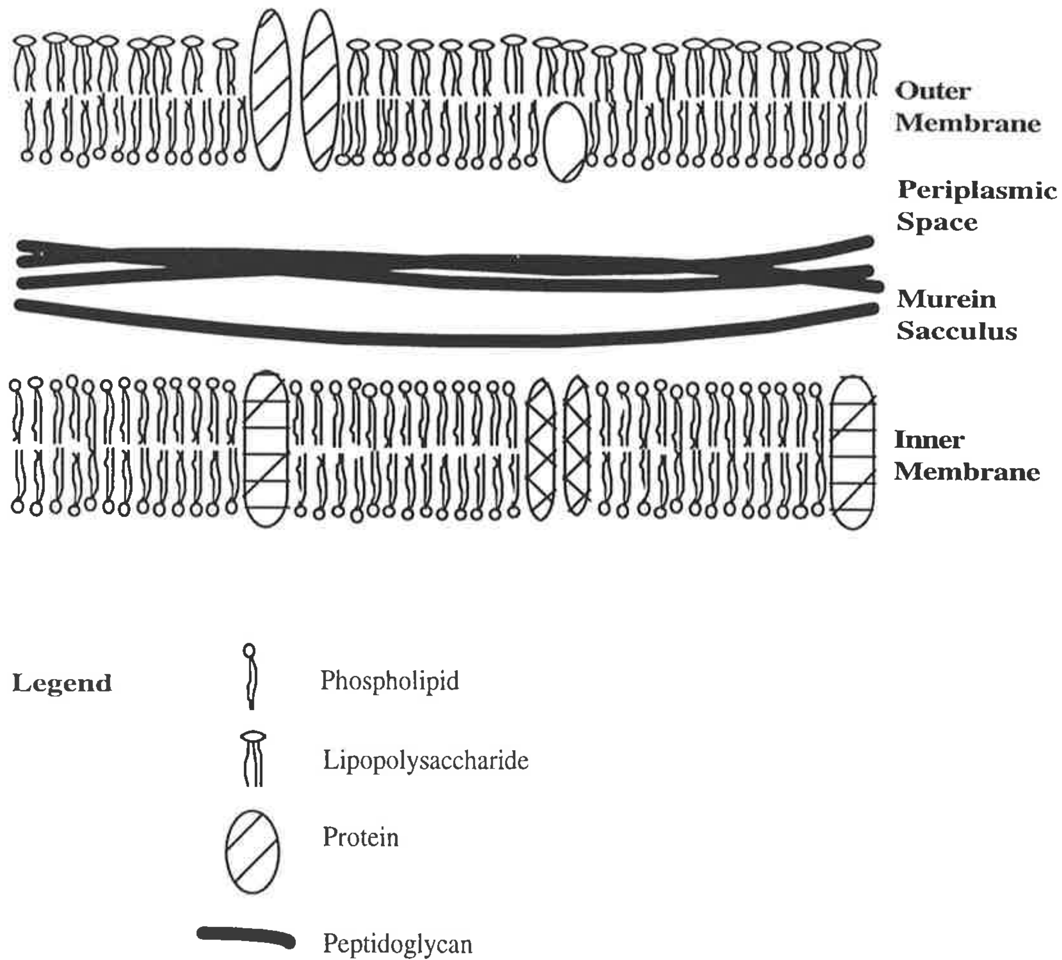


Figure 1.1 Simplified schematic representation of the structure of the *Escherichia coli* cell wall viewed as a cross section.

The peptidoglycan layer (or murein sacculus) is the rigid shape-determining structure in the cell wall and is located in the periplasmic space between the outer and inner membranes. The peptidoglycan polymer is made up of two amino sugars and four amino acids (Park, 1987). The thickness of the peptidoglycan layer varies depending on the growth phase of the culture. The thickness measurements for stationary and logarithmic cells correspond to four to five layers and two to three layers of peptidoglycan, respectively (Leduc *et al.,* 1989).

The inner (or cytoplasmic) membrane is predominantly a phospholipid bilayer containing 75% of the cell's phospholipid and 6 to 9% of total cell protein. The inner membrane is only permeable to water and small hydrophobic molecules. Many of the proteins involved in the cell's bioenergetic and biosynthetic reactions are located in this membrane. This membrane is relatively weak, relying on the outer membrane for defence against molecules such as surface active agents like bile salts (Cronan *et al.*, 1987).

A range of chemical agents have been used to modify, permeabilise or disrupt *E. coli* cell walls. These methods can be broken down into two main groups; those that release periplasmic protein by permeabilising the outer membrane only, and those that release all protein by permeabilising both the inner and outer membranes.

Periplasmic protein release

To release protein from the periplasm of *E. coli,* only the outer membrane of the bacterium has to be permeabilised. The bulk of the host cell protein can be contained in the intact protoplast thus providing a useful degree of selectivity. This simplifies the subsequent purification requirements.

The chelating agent ethylenediaminetetraacetate (EDTA) has been used to permeabilise the outer membrane of *E. coli.* EDTA removes divalent cations that stabilise the LPS matrix in the outer-membrane of *E. coli.* Quantities of LPS shed from the outer membrane (Marvin *et al.*, 1989). The outer membrane nevertheless maintains its structure, possibly

by the filling gaps with phospholipid. The EDTA-treated outer membrane is permeable to a range of hydrophobic molecules unable to penetrate the untreated membrane (Leive, 1974). EDTA has been used in conjunction with other chemical agents for the release of protein located in the periplasm. The chaotropic agent guanidine HCl plus EDTA has been shown to successfully release the periplasmic protein penicillin acylase from *E. coli* (Novella *et al.*, 1994). Lysozyme combined with EDTA was used for the extraction of RNase associated with the inner membrane of *E. coli* (Neu and Heppel, 1964).

Polymyxin B is a polycationic agent that disrupts or disorganises the outer membrane. This antibiotic has been used to selectively release protein from the periplasm of actively growing *E. coli* (Cerny and Teuber, 1971). Polymyxin B, however, is an expensive option for the release of periplasmic protein and is limited to laboratory applications.

The chaotropic agent urea has been used in combination with the reducing agent dithiothreitol (DTT) to extract recombinant IGF-I from inclusion bodies located in the periplasmic space of *E. coli* (Hart *et al.*, 1994). The reducing agent DTT has a role in solubilising inclusion bodies. It weakens intermolecular covalent disulphide bonds formed between IGF-I molecules. The role of DTT, if any, in permeabilising the outer membrane is not known.

The solvents toluene and chloroform have also been used to release periplasmic proteins (Teuber, 1970, Ames *et al.*, 1984). Toluene treatment, however, also releases a proportion of the cytoplasmic contents due to damage caused to the inner membrane (Jackson and Demoss, 1965).

Total protein release

To release both periplasmic and cytoplasmic proteins, both the inner and outer membrane have to be permeabilised. Several methods for the release of protein from the cytoplasm of *E. coli* have been examined. These include treatment with chaotropic agents (urea, guanidine HCl, and ethanol), solvents (toluene), and detergents (such as the anionic

detergent sodium dodecyl sulphate (SDS), the non-ionic detergent Triton-X100, and the cationic detergent cetyltrimethylammonium bromide (CETAB)).

Chaotropic agents have the ability to denature proteins and disrupt membrane structures (Hatefi and Hanstein, 1974). Low concentrations of chaotropic agents have been used to lyse log-phase *E. coli* (Ingram, 1981). However, urea proved to be relatively ineffective. The mechanism causing lysis in this case is probably weakening of the growing peptidoglycan structure. The chaotropic agent guanidine-HCl used in combination with the non-ionic detergent Triton X-100 released approximately 50% of total cell protein from chilled logarithmic-phase *E. coli* (Hettwer and Wang, 1989, Naglak and Wang, 1991). The mechanism for this combination is unclear.

A wide range of detergents compromise the cell wall of *E. coli* with limited success (Vaara, 1992). The detergents can be classified into the following categories: non ionic, anionic, cationic, and type B surfactants. Detergents alone usually show little effect on *E. coli* cells but when combined with EDTA and lysozyme can result in considerable lysis. The effectiveness of the detergents at lysing *E. coli* spheroplasts is as follows (Birdsell and Cota-Robles, 1968):

Brij 58 (non-ionic) > Triton X-100 (non-ionic) > Duponal (anionic) > Deoxycholate (type B)

The following detergents are effective at solubilising the inner membrane but ineffective against the outer membrane: sodium-lauryl sarcosinate (anionic) (Filip *et al.*, 1973), sodium dodecyl sulphate (anionic) (Woldringh, 1970), Triton-X100 (non-ionic) (Schnaitmann, 1971a), and Brij 58 (non-ionic) (Birdsell and Cota-Robles, 1968). The cationic detergent cetyltrimethylammonium bromide (CETAB) has been used to release transaminase B from *E. coli* for analytical purposes, and must therefore have some effect on the outer membrane (Whittaker and Jackson, 1980). The outer membranes of gram-negative enteric bacteria can be permeabilised by monocationic detergents such as benzalkonium chloride (BAC) and CETAB although the mechanism is not known (Vaara, 1992).

Toluene is the solvent that has been most investigated for the permeabilisation of *E. coli*. It is a well known antimicrobial agent and has been used in a variety of research protocols. Treatment with toluene damages the inner membrane of the bacteria (DeSmet *et al.*, 1978). Observation of freeze fractured cells by electron microscopy shows considerable damage to the inner membrane while the outer membrane remains intact. Treatment with 5% (v/v) toluene releases up to 25% of total cell protein and 85% of RNA (Jackson and Demoss, 1965). Treatment with 1% (v/v) toluene is enhanced by the addition of EDTA with 28% of the total cell protein released (DeSmet *et al.*, 1978). EDTA permeabilisation of the outer membrane complements toluene's action on the inner membrane. The application of solvents for the permeabilisation of *E. coli* have generally been for the release of periplasmic proteins, which is difficult to justify as the solvents are better suited to permeabilisation of the inner membrane (Teuber, 1970, Ames *et al.*, 1984).

The enzyme lysozyme can be utilised to digest the peptidoglycan layer of the cell wall. The outer membrane must be weakened (EDTA is often used) to provide the enzyme with access to the peptidoglycan layer (Schnaitmann, 1971b). This treatment, however, does not compromise the inner membrane and requires further augmentation to release cytoplasmic material.

Chemical permeabilisation of bacterial cells has found very few large-scale applications. An exception is the recovery of cholesterol oxidase from *Nocardia* using 0.5% Triton X-100 (Asenjo and Patrick, 1990). Use of chemical permeabilisation in the laboratory is wide-spread and several processes have been suggested for industrial use. The full potential of this technique is yet to be realised.

Chemical pretreatments can be used to weaken the cell wall prior to mechanical disruption. A pre-treatment using a combination of EDTA and lysozyme on *E. coli* cells prior to mechanical disruption results in marginally greater levels of disruption (Lutzer *et al.*, 1994). The lysozyme digests the peptidoglycan which is an important structural component of the cell wall. Peptidoglycan has been shown to be an important factor affecting homogenisation efficiency (Middelberg *et al.*, 1992).

Extraction of Recombinant Protein from the Cytoplasm of *E. coli.*

High concentrations of the chaotropic agent guanidine-HCl (7M) have been used to release recombinant interferon (INF-γ) from the cytoplasm of *E. coli* (Kung, 1984). The product was presumably in the liquid phase as sonication (with no additional dissolution step) was able to release active protein. The effectiveness of the chemical treatment was compared with sonication. Protein extraction by sonication resulted in proteolytic breakdown of the interferon, while chemical extraction resulted in release of correct-length protein. The fractional product release was not determined, so the effectiveness of this treatment is not known. A combination of the chaotropic agent urea and the reducing agent cysteine (7M and 50 mM respectively), has been used to extract recombinant AE-IGF-I from *E. coli* (Hejnaes *et al.*, 1992). No details were given for this procedure.

1.4 Project aims and thesis structure

1.4.1 Project aims

Processes for the recovery of recombinant proteins from inclusion bodies located in the cytoplasm of *Escherichia coli* tend to follow a highly conservative set of process steps (Fischer *et al.*, 1993). The scarcity of feasible alternatives can be of concern when technical, legal, or economic difficulties arise that render the selected manufacturing route non-viable for a given protein. This project attempts to develop a novel alternative technology that will help redresses this shortcoming.

The specific project aims are:

1. discover an alternative extraction technology for the recovery of recombinant protein from inclusion bodies located in the cytoplasm of genetically engineered *Escherichia coli*;

2. investigate the role of the components that make up the novel extraction procedure;

3. model the kinetics of the novel extraction procedure;

4. characterise the end product of the novel extraction procedure and compare it with that produced by traditional extraction procedures;

5. assess the suitability of the novel extraction procedure for application to small and large scale biotechnology processes.

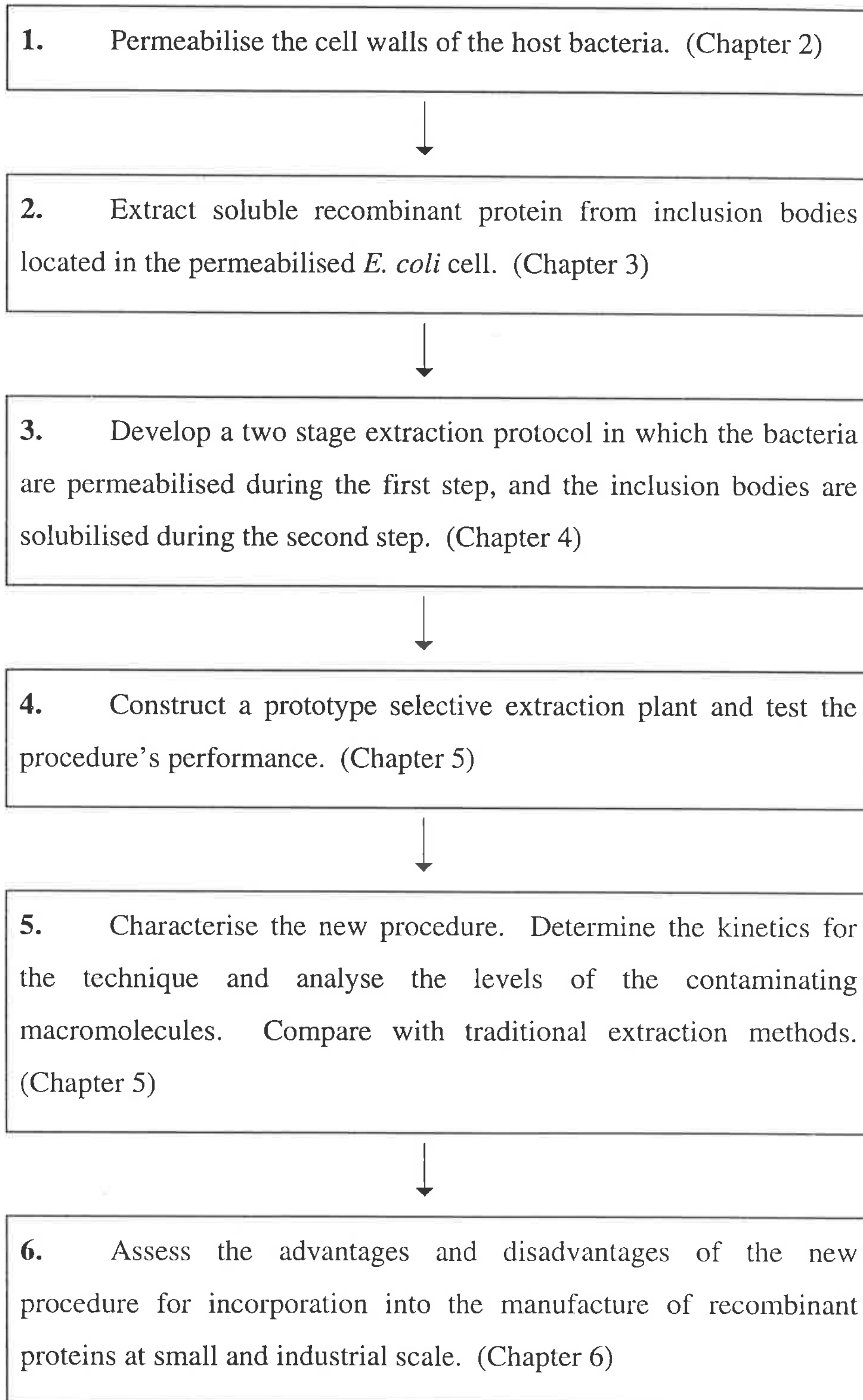
1.4.2 Bacterial stain and encoded protein

The genetically engineered *E. coli* strain used in this project is used for the commercial production of an insulin-like growth factor-I analogue. The details of the strain and its encoded recombinant protein are as follows:

Bacterial strain:	*E. coli* strain JM101 (lacI^{q}) containing the plasmid p[Met^{1}]-pGH(11)-Val-Asn-[Arg^{3}]-IGF-I with the IPTG inducable lac promotor.
Recombinant protein:	Met^{1}-pGH(11)-Val-Asn-[Arg^{3}]-IGF-I subsequently referred to as Long-R^{3}-IGF-I, is an 83 amino acid analog of human insulin-like growth factor-I (IGF-I) (King *et al.,* 1992). It is comprised of 13 amino acids from the N-terminus of porcine (met) growth hormone followed by the complete human IGF-I sequence with a glutamate substitution for the asparagine at position 3. The protein contains 3 intramolecular disulphide bonds in its native state and has a molecular weight of 9110 Daltons. On expression, the protein should accumulate in the bacterial cytoplasm as no secretion signal sequence is present.
Protein Expression.	Long-R^{3}-IGF-I is only detectable in the insoluble phase after homogenisation and separation by centrifugation as demonstrated by HPLC and PAGE analysis (PAGE analysis is shown in this thesis in Figure 4.12). Inclusion bodies are visible using phase-contrast microscopy and are consistent with cytoplasmic accumulation (personal observation).

1.4.2 Thesis structure

To achieve the aims detailed in section 1.4.1 the research followed the steps shown below.



CHAPTER 2

PERMEABILISATION OF *ESCHERICHIA COLI*

Summary

Permeabilisation of the cell wall of *Escherichia coli* was determined by measuring the protein levels released from treated bacteria into the extracellular-phase. Treatment with a combination of the chelating agent ethylenediaminetetraacetate (EDTA) and the chaotropic agent urea is highly effective at releasing protein from *Escherichia coli*. 6 M urea in the presence of 3 mM EDTA can release cytoplasmic protein from both logarithmic-phase and stationary-phase *E. coli* cells at levels equivalent to mechanical disruption. The concentrations of the two chemical agents were the major variables affecting protein release. Several minor variables and interactions were also identified. The kinetics of protein release was first order. The time constant was approximately 2.5 min and was independent of the urea concentration.

2.1 Introduction

The first stage in the recovery of recombinant proteins that have accumulated in the cytoplasm of the host bacterium is to overcome the barrier presented by the bacterial cell wall. The bacterial cell wall is comprised of an outer membrane, layers of peptidoglycan (the murein sacculus), and an inner membrane. Traditionally the cell wall is broken using mechanical disruption, a procedure that fragments the cell wall and releases the cellular contents.

Chemical permeabilisation of the cell wall of *E. coli* is a widely studied alternative to mechanical disruption. A wide range of chemical agents have been used including detergents, solvents, chaotropic agents, and chelating agents (see section 1.3.2 of this thesis). These chemical agents are effective as they either compromise the membrane structures or weaken the peptidoglycan layer. Chemicals such as EDTA and Polymyxin B specifically weaken the outer membrane. Other permeabilising agents such as bile salts are effective against the inner membrane but are ineffective against the outer membrane. The peptidoglycan layer can be enzymically degraded by lysozyme or its construction compromised by the presence of the chaotropic agent ethanol. The chemical permeabilisation protocols presented in the literature, however, do not match the effectiveness of mechanical disruption at releasing intracellular protein. The chemical permeabilisation techniques release only a fraction of the cellular constituents and are often effective against particular growth phases of the bacteria (Naglak *et al.,* 1990). To match mechanical disruption as an extraction procedure a method has to give total release of the cellular components. It would also be advantageous if it were independent of the growth-phase of the bacteria, was rapid, used cheap components, and was amenable to scale-up.

The research presented in this chapter attempts to establish a chemical permeabilisation procedure that can replace mechanical disruption for the extraction of molecules located in the cytoplasm of *E. coli.*

2.1.1 Research Goals

The research presented in this chapter aims to:

- select an analytical technique suitable for monitoring permeabilisation of *E. coli* cells;
- select a combination of chemical agents that permeabilise *E. coli* cells releasing levels of intracellular components into the extracellular phase at levels similar to mechanical disruption;
- determine the significance and role of the chemical and environmental parameters on permeabilisation;
- determine the kinetics of the permeabilisation process.

2.2 Selection of analytical techniques and initial screen

The analytical techniques used for monitoring chemical treatment of *E. coli* cells need to provide qualitative and quantitative information on the operation of the process. Three assays were selected for assessment. Particle size analysis using a Joyce-Loebl disc centrifuge has been applied to the analysis of mechanical cell disruption (Middelberg, 1992) and can provide useful information on the particle sizes created by disruption. This is particularly informative when assessing the disruption of cells containing inclusion bodies where the cells and the inclusion bodies can be quantitated during the same assay. Optical density is a simple analytical tool that has the advantage that it can be easily incorporated into continuous monitoring of the process at laboratory and industrial scale (used by Ingram, (1981) for the monitoring of bacterial lysis). Intracellular protein release is an effective method for monitoring bacterial cell permeabilisation (Hettwer and Wang, 1989; Naglak and Wang, 1991). It provides directly-useful data as protein release is the ultimate aim of this process.

The chemical agents trialed in this section are the chelating agent ethylenediaminetetraacetate (EDTA) and the chaotropic agent urea. EDTA is a well known agent for permeabilising the outer membrane of *E. coli* and allows a range of different molecules access into the bacterial cell (Leive, 1974). Urea is a commonly used denaturant of proteins (Kamoun, 1988) and could help destabilise the membrane structure like other chaotropic agents (Hatefi and Hanstein, 1974). The combination of the two chemical agents was thought to have a high probability of success due to EDTA's capacity to compromise the integrity of the outer membrane and urea's potential to destabilise the inner membrane. The chemicals are compatible with subsequent processing of recombinant proteins where product denaturation is not of concern.

2.2.1 Method

A glycerol stock of *E. coli* strain JM101 (lacI^q) containing the plasmid p[Met^1]-pGH(11)-Val-Asn-[Arg^3]-IGF-I was streaked onto a C1 minimal media agar plate (Appendix B1)

and incubated at 37°C for 24 h. A shake flask containing 10 mL of sterile modified C1 media (Appendix B1) was inoculated with a colony from the plate and then incubated at 37°C for 6 h. 0.1 mL of broth was used to inoculate 3 x 2 L flasks each containing 660 mL of sterile modified C1 media. The flasks were incubated at 37°C and agitated for 17 h. The *E. coli* samples were harvested (10000 xg, 4°C, 30 min) and resuspended in phosphate-buffered saline (Appendix B3) to give an A_{600} of 80.

The test solutions containing 10 mM EDTA, 0.1 M Tris and various concentrations of urea were adjusted to pH 9.0 using conc. HCl. The test solutions (4.75 mL) (see Table 2.1 for composition) were placed in a 25 mL MacCartney bottle. The *E. coli* sample (0.25 mL) (prepared following the protocol detailed above) was added to each test solutions and agitated in a shaking water bath, set at 37°C. Test solutions were sampled at 30 min. The samples were analysed using particle size analysis (Appendix A1), optical density monitored (Appendix A2), and protein estimated (Appendix A3).

Table 2.1 Composition of the chemical treatment groups used in the initial screen.

	Treatment Group	Composition
1	PBS	see Appendix B3.
2	0M Urea	0M Urea, 10mM EDTA, 0.1M Tris, pH 9.0
3	1M Urea	1M Urea, 10mM EDTA, 0.1M Tris, pH 9.0
4	2M Urea	2M Urea, 10mM EDTA, 0.1M Tris, pH 9.0
5	3M Urea	3M Urea, 10mM EDTA, 0.1M Tris, pH 9.0
6	6M Urea	6M Urea, 10mM EDTA, 0.1M Tris, pH 9.0

2.2.3 Results and Discussion

2.2.3.1 Particle size analysis

Particle size analysis using the Joyce-Loebl analytical disc centrifuge has been applied to the analysis of cell disruption by mechanical methods such as high-pressure homogenisation (Middelberg, 1992) and to the estimation of inclusion body size (Taylor, *et al.*, 1986). The method separates the particles by centrifugation. Sedimentation of the particles is monitored spectrophotometrically. The time for the particle to reach the detector, t, is described by a modification of Stokes' law (Taylor *et al.*, 1986),

$$t = 18\mu \ln(r_o/r_i) / \Delta\rho \, d^2 \, \omega^2 \qquad - (2.1)$$

where μ is the spin fluid viscosity, r_o is the radius at $t = 0$, r_i is the radius at the detector, $\Delta\rho$ is the difference between the density of the particle and the density of the surrounding medium, d is the diameter of the particle, and ω is the disc's angular velocity. This method is able to resolve particles such as cells, inclusion bodies, and cell debris. The absorbance measured by the detector is used to quantify the levels of the different sized particles.

Results for the analysis of chemically-treated cells by particle-size analysis are shown in Figures 2.1. The results show that a significant change is made to the cells by the chemical treatment. The quantity of cells observed as a peak of 1.2 µm is diminished by the treatment with urea. The analysis does not, however, provide a clear indication of what is actually happening. If the chemical treatment of the cells did increase cell permeability the buoyant density of the cells would be expected to change. The $\Delta\rho$ value could approach zero as the intracellular component diffuse out of the cells. The analysis is sensitive to buoyant density as well as particle size (equation 2.1). Interpretation of the result is subsequently difficult.

Particle size analysis does demonstrate that chemical treatment is having an effect on the bacterial cells. However, as the technique does not provide much information as to the changes occurring to the cell it was not used in subsequent experiments.

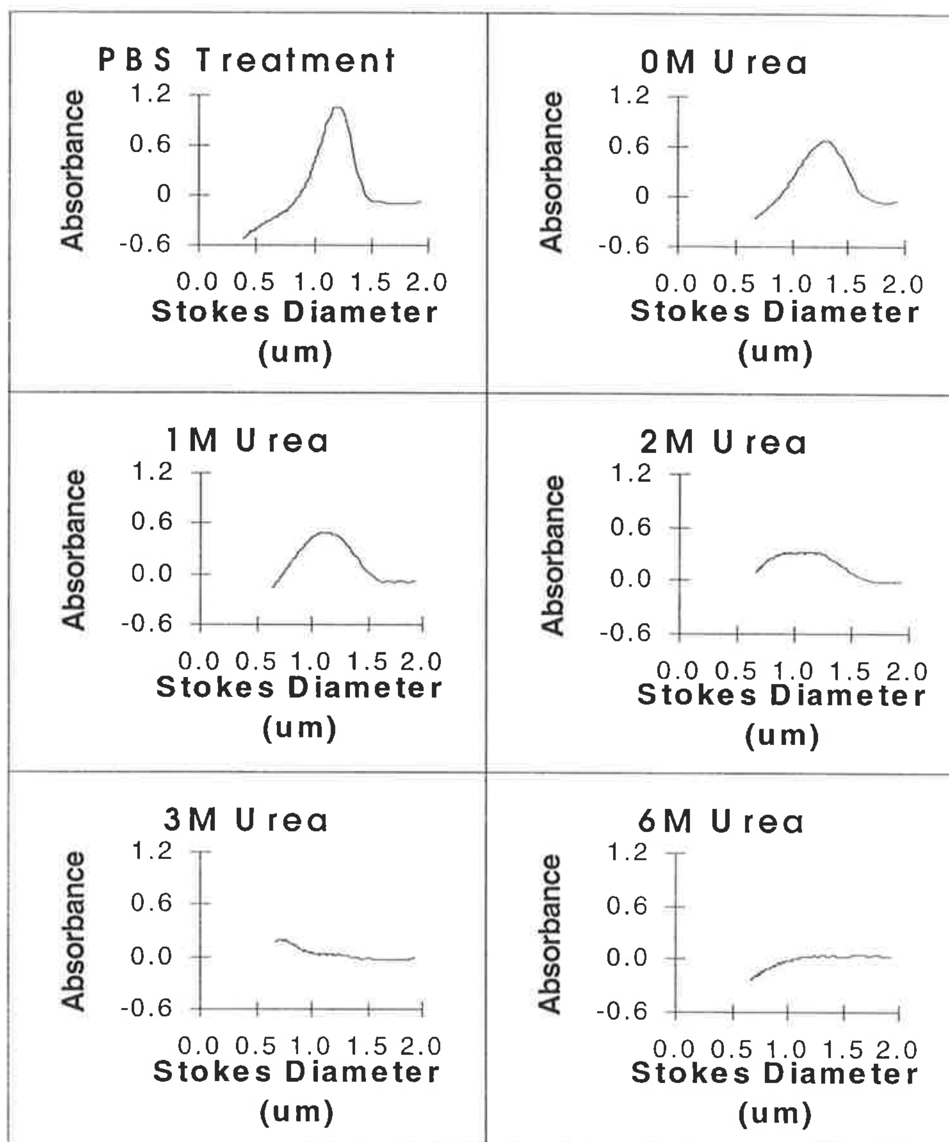


Figure 2.1 Particle size analysis of chemically treated *E. coli* cells. The treatment groups comprise PBS or various concentrations of urea plus 10 mM EDTA and 0.1 M Tris at pH 9.0 (as detailed in Table 2.1).

2.2.3.2 Absorbance at 600 nm

The results of the analysis of chemically permeabilised cells using absorbance measurement at 600 nm (A_{600}) are shown in Figure 2.2. These results show that the chemical treatment did have an effect on the A_{600} value. However, the assay does not provide any real insight into what is happening at the cellular level.

Measurement of optical density is a commonly used assay for the quantitation of bacterial cells in solution. Changes in optical density can be due to changes in the cell size and in the density of their intracellular constituents. The absorbance of a culture has a complex functional relationship to particle size, depending on the extinction coefficient at low particle sizes. Absorbance may therefore increase or decrease depending on optical properties and the absolute particle size. Interpretation of turbidity readings is also complicated by the number of factors that can alter the optical density reading (Russell *et al.*, 1973). Aggregation of intracellular components can increase the suspensions absorbance. Cell permeabilisation or lysis can result in a decrease (Ingram, 1981), while cell fragmentation can increase or decrease the optical density. Changes in the extracellular phase, salt concentration, refractive index and temperature can also effect the results (Russell *et al.*, 1973).

Absorbance measurement remains a particularly useful method for monitoring a bacterial suspension as it is very simple, rapid, and is easily adapted for on-line process monitoring.

The absorbance measurement provides evidence that the chemical treatment does have an impact on *E. coli* cells. The limitation is that the assay does not directly determine the nature of the changes taking place. The assay is inappropriate for the investigation of the effectiveness and nature of chemical permeabilisation treatments as it is unable to distinguish between the range of effects that could be occurring.

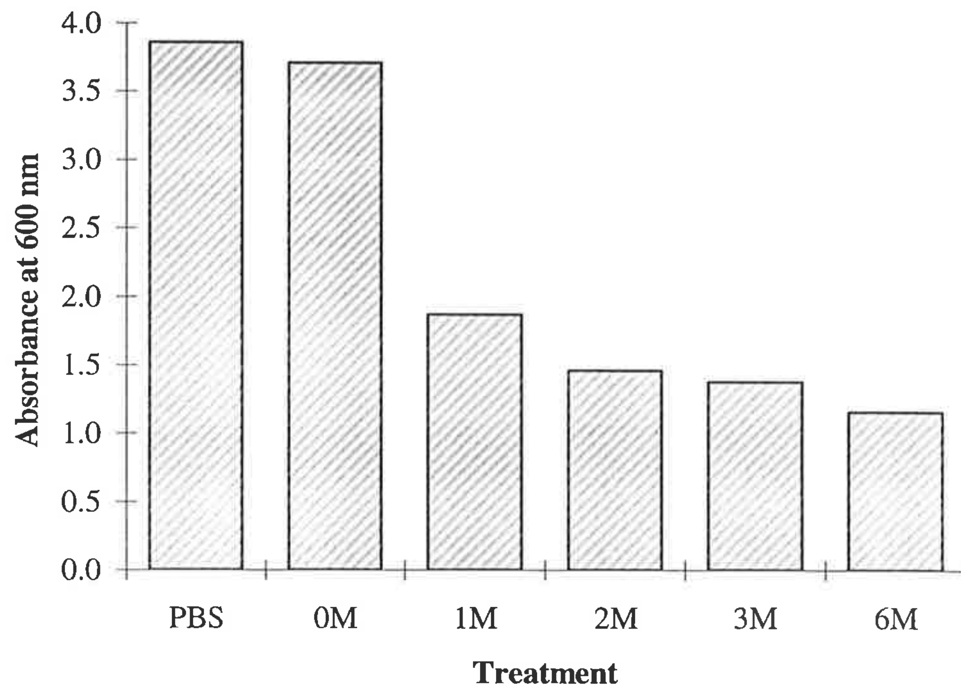


Figure 2.2 Absorbance at 600 nm of chemically treated *E. coli* cells. The treatment groups comprise PBS or various concentrations of urea plus 10 mM EDTA and 0.1 M Tris at pH 9.0 (as detailed in Table 2.1).

2.2.3.3 Protein estimation

The total protein concentration in solution can be measured by the colourimetric change that occurs when protein comes into contact with the dye Commassie Blue (Bradford, 1976). This assay is referred to as the Bradford assay and is available as a commercial kit from BioRad (Sydney, Australia). During disruption or permeabilisation of a bacterial cell, intracellular protein is released into the surrounding solution. Measurement of the intracellular protein that diffuses into the extracellular phase should provide a direct measurement of the effectiveness of the chemical treatment at compromising the integrity of the bacterial cell wall. This analytical technique has been used for monitoring permeabilisation studies (Hettwer and Wang, 1989; Naglak and Wang, 1991).

The results for the analysis of chemically-treated cells are shown in Figure 2.3. This analytical method was selected for quantifying permeabilisation during chemical treatments for the remainder of this chapter. It has the advantage that total protein release into the extracellular phase is a parameter that must be measured to characterise process effectiveness.

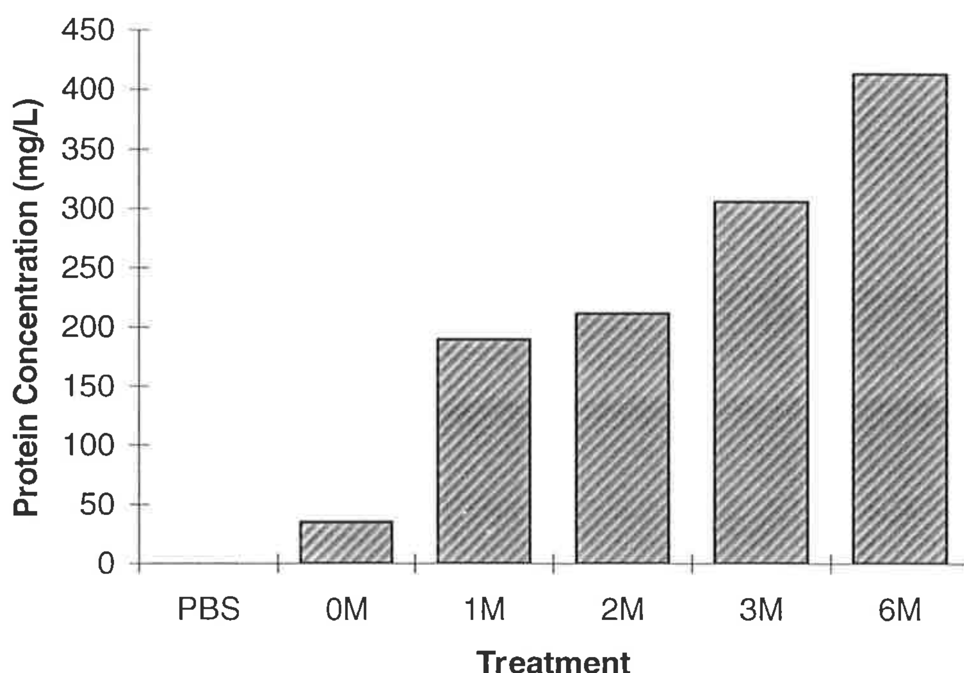


Figure 2.3 Protein release into the extracellular phase from chemically treated *E. coli* cells. The treatment groups comprise PBS, or various concentrations of urea plus 10 mM EDTA and 0.1 M Tris at pH 9.0 (as detailed in Table 2.1).

2.2.3.4 Protein estimation on-line

Further experimentation was conducted to see if the suspension absorbance could be directly related to protein release from the cells (measured using the method reviewed in section 2.1.3). Data were analysed using the Jandel Scientific Table Curve™ 2D program (AISN Software) to fit a curve to the data, calculate the constants for the equation, and conduct the statistical analysis.

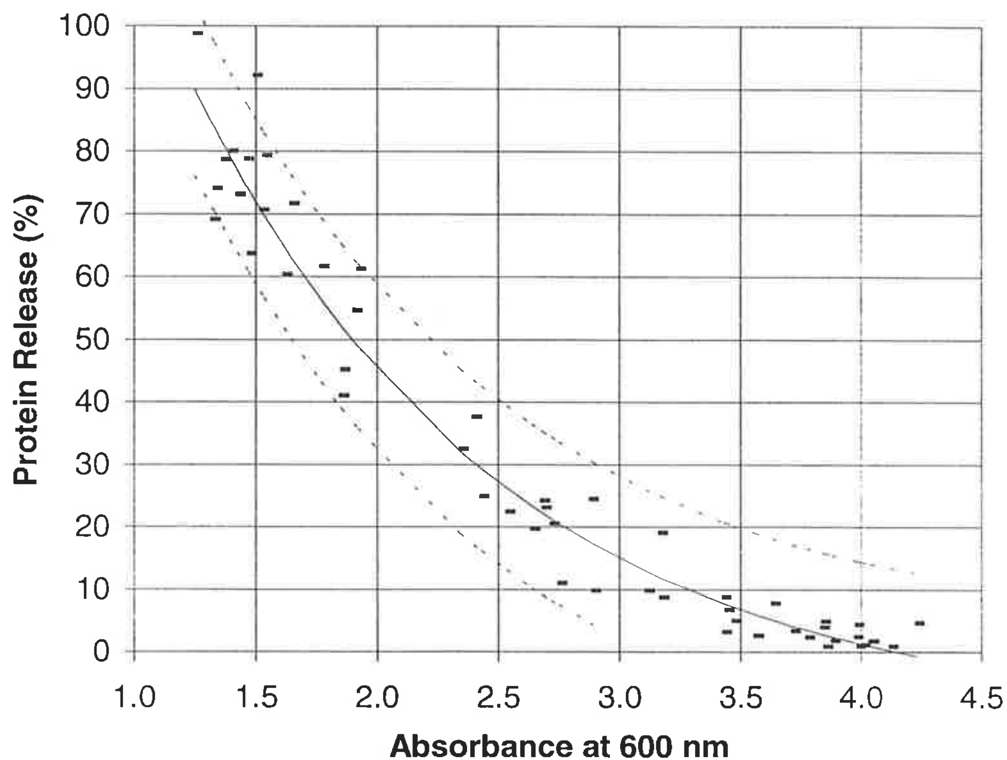


Figure 2.4 Protein release from chemically treated *E. coli* against the absorbance at 600nm of the bacterial suspension. The solid line is the fitted curve (equation 2.2) and the broken lines are the 95% confidence limits.

An equation was selected as it satisfactorily described the relationship between the protein release and the optical density of the *E. coli* solution with various chemical treatments. The correlation between A_{600} and protein release is described by equation (2.2),

$$Y = a + b \exp(-cx) \qquad - (2.2)$$

where x is the A_{600} value and Y is the percentage protein released. a, b, and c are constants. The results, the fitted curve, and the 95% confidence limits are shown in Figure 2.4. The R^2 value for this curve is 0.96.

The relationship between A_{600} and the protein release from the cells treated with the chemical agents does indicate that the measurement of optical density could be utilised for the on-line measurement of the permeabilisation process.

2.2.3.5 Initial screen

The results of this experiment show that treatment with a combination of urea and EDTA has a significant impact on *E. coli*. Analysis by each of the analytical techniques used detected some change in the cells following treatment.

Particle size analysis detected a decrease in the amount of material between 0.5 and 1.5 μm size on addition of urea to the test solutions (Figure 2.1). Increasing the concentration of urea resulted in less material detected between 0.5 and 1.5 μm. This change could be due to a decrease in cell size, or a decrease in buoyant density due to an increase in cellular permeability.

Chemical treatments resulted in a decrease in optical density of the bacterial solutions (Figure 2.2). Treatment with 10 mM EDTA and 0.1M Tris had very little effect on the bacterial solution's optical density. The presence of urea, however, caused a marked decrease in optical density. The drop in absorbance has been used as a measure of cellular lysis (Ingram, 1981) but can be due to several changes in the cells. Optical density of a

particulate solution has a complex dependence on particle size (see section 2.2.3.2), so a decrease in absorbance does not guarantee that a change in cell size has taken place. A change in the composition of the cell could also change its optical density. Permeabilisation could reduce a suspension's optical density due to the removal of the cellular contents into the extracellular phase. This assay illustrates that changes are occurring in the bacterial solution but, like particle size analysis, it is not particularly informative as to the nature of these changes.

Measurement of the protein levels released into the extracellular-phase provides a clear indication that the treatment of the cell suspension with urea and EDTA is compromising the integrity of the bacterial cell wall (Figures 2.3). The protein comes from the interior of the bacterial cells and requires the loss of integrity of the cell wall to be released into the solution. The results for protein release after the chemical treatment observed in this experiment indicate that these treatments are worth further investigation. The levels of protein release from the cell need to be directly compared to that obtained using mechanical disruption to determine the effectiveness of the treatments.

2.3 Multifactorial experiment

The composition of the extraction buffer used in section 2.2 included the chaotropic agent urea, the chelating agent EDTA, and the buffer Tris. The role of these different components needs to be determined. Other variables that could effect permeabilisation include the bacterial growth phase and reaction time. A multifactorial experiment was used to determine the significance of the variables on protein release. Such an experimental design allows statistical analysis of the results to be carried out to prove the significance of a given variable. Protein release can be directly compared to that achieved by mechanical disruption.

2.3.1 Method

Two multifactorial experiments were conducted using cells produced in two separate fermentation runs. The fermentation protocol is described in Appendix C1. The *E. coli* cells were stationary phase or logarithmic phase, and contained no measurable levels of recombinant protein. Reaction temperatures for the first and second experiment were 20°C and 37°C, respectively. The following variables were tested for each experiment:

1. bacterial growth phase (logarithmic or stationary)
2. buffer (0.1 M Tris or 0.1 M borate)
3. EDTA concentration (0 or 3 mM)
4. urea concentration (0, 2, 4, or 6 M)
5. time (30 or 90 min)

Each of the potential combinations were tested; a total of 64 for each experiment. Each test solution was adjusted to pH 9.0 using conc. HCl. In all cases, 4.75 mL of the test solution was placed in a 25 mL MacCartney bottle. The negative control was PBS (Appendix B3). The *E. coli* sample (0.25 mL) was added to each test solution which was

then agitated in a shaking water bath. Reactions were sampled at 30 and 90 min. Protein release was determined as described in Appendix A3.

The 100% protein release value was determined using untreated cells passed three times through an APV-Gaulin homogeniser at 56 MPa with debris removed by centrifugation at 10,000 xg for 15 min prior to protein estimation. All data for chemical release experiments are expressed as a percentage of the protein released by mechanical disruption.

Initial analysis demonstrated that a linear scale was inappropriate. Experimental data were therefore converted to the logit scale and subjected to statistical analysis. A value of 109.3% was used for the maximum protein release. This value was derived from the analysis and may be greater than 100% due to the solubilisation of insoluble membrane-associated protein during the chemical treatment. For each urea and EDTA concentration an average was calculated over several factors: the reaction temperature, bacterial growth phase, buffer system, and reaction time. The difference between the mean values was calculated for the factors that the statistical analysis showed to be important: bacterial growth phase, reaction time, the interaction between urea and EDTA, and the interaction between urea concentration and time. Averages for the urea and EDTA concentrations were adjusted by the difference between the mean values for above factors. Results were then converted back to the original (linear) scale. The analysis gave the fitted results for protein release for the four urea concentrations in the presence or absence of EDTA for logarithmic and stationary phases bacteria and the 30 and 90 min reaction times.

2.3.2 Results and Discussion

The most important observation from this experiment is that virtually all of the total cellular protein from the *E. coli* suspension is released at 6 M urea concentration in the presence of 3 mM EDTA. The method of determining total available protein for release is mechanical disruption using three passes through an APV-Gaulin homogeniser set at 56 MPa with debris removed by centrifugation prior to protein estimation. The total available protein was 481 ± 48 mg/L for the stationary-phase cells and 570 ± 22 mg/L for

logarithmic-phase cells. Chemical treatment with 6 M urea plus 3 mM EDTA for 90 min released 499 ± 29 mg/L for the stationary-phase cells and 559 ± 42 mg/L for logarithmic-phase cells. This indicates that the chemical treatment method can match the level of protein release achieved by mechanical disruption. The recovery of protein by this chemical permeabilisation technique is superior to those previously published (Naglak *et al.*, 1990).

Figures 2.5 and 2.6 show experimental results for a typical treatment group from the multifactorial experiment. Urea and EDTA concentrations have a clear impact on total protein release from the treated cells.

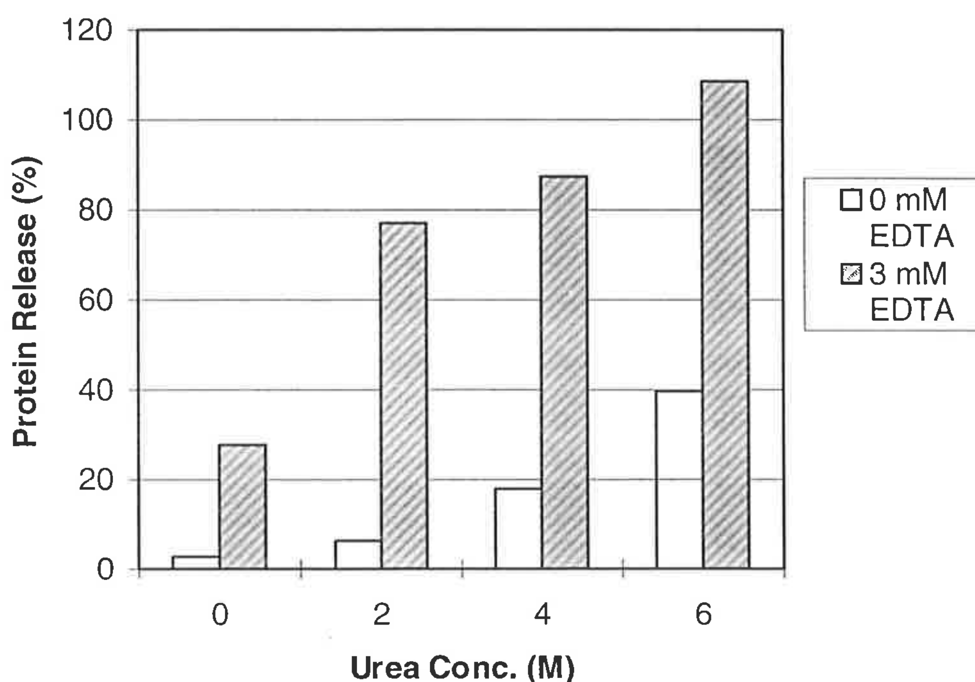


Figure 2.5 Protein release from chemically treated logarithmic-phase *E. coli*. The treatment groups contained urea (0, 2, 4, or 6 M), EDTA (0 or 3 mM), 0.1 M Tris, pH 9.0, and operated at 37°C for 90 min.

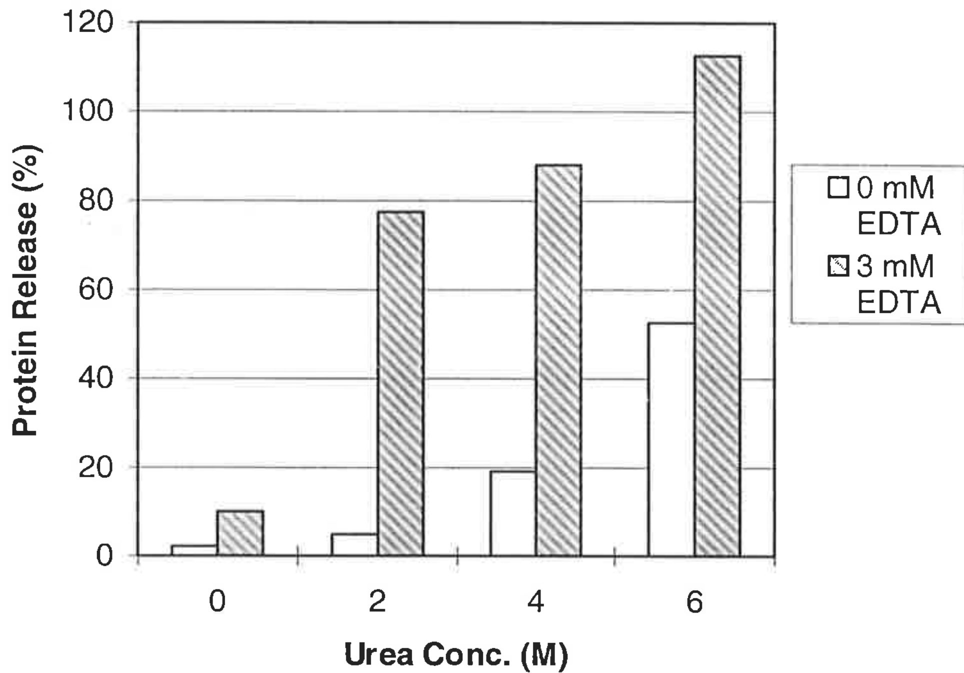


Figure 2.6 Protein release from chemically treated stationary-phase *E. coli*. The treatment groups contained urea (0, 2, 4, or 6 M), EDTA (0 or 3 mM), 0.1 M Tris, pH 9.0, and operated at 37°C for 90 min.

Results from the statistical analysis of the multifactorial experiment are shown in Tables 2.2 and 2.3. Minor interactions that were not significant are not included in the tables. Varying the urea and EDTA concentrations has by far the largest effect on the efficiency of bacterial permeabilisation (accounting for 530 of the 610 total sum of squares). Tables 2.2 and 2.3 show that several other variables and interactions are important. Clearly, reaction time, the interaction between EDTA and urea, and the interaction between urea and time are important. Growth phase of the bacteria appears to be an important term (accounting

for 19 sum of squares) but the design of the experiment did not allow the significance of this to be assessed. Interactions between the other variables did not prove to be significant. Interestingly, the choice of buffer did not have any significant effect. The significance of the different reaction temperatures could not determined by this experiment due to the physical constraints on the experiment (each reaction temperature was tested using different fermentation batches). The remaining sum of squares (after the above factors are taken into account) represents 7.2% variation. This could be due to random variation in the system. The fitted means using the results from this experiment are summarised in Table 2.4 (logarithmic-phase bacteria) and Table 2.5 (stationary-phase bacteria).

The chelating agent EDTA is a well known agent for the permeabilisation of the *E. coli* outer-membrane and it is not surprising that its role in permeabilising *E. coli* is significant under the conditions of this experiment. EDTA allows access into the bacterial cell of a range of different molecules that would not otherwise penetrate the outer membrane (Leive, 1974). Urea is a commonly-used denaturant of proteins (Kamoun, 1988) and could help destabilise the membrane structure like other chaotropic agents (Hatefi and Hanstein, 1974). Presumably, having gained access to the inner (or cytoplasmic) membrane due to the permeabilisation of the outer membrane by EDTA, the urea was able the permeabilise this barrier and allow the release of the cytoplasmic contents. The combination of the two agents is clearly complementary and very effective.

Chemical treatment with 6 M urea and 3 mM EDTA results in approximately 100% release of the bacterial protein from both stationary and logarithmic-phase cells. This differs from many permeabilisation techniques which are sensitive to the bacterial growth-phase. Treatment of *E. coli* with the antibiotic Polymyxin-B can cause autolysis resulting in the release of 68% (w/w) of the total protein in logarithmic cells. Stationary-phase cells were quite stable (Cerny and Teuber, 1971). The use of low concentrations of chaotropic agents alone can cause cell lysis but is dependant on the growth-phase; antibiotics that inhibited cell growth also prevented lysis (Ingram, 1981). The reason for the dependence of these treatments on the growth-phase of the bacteria is unlikely to be due to the composition of the cell wall. The cell wall does change with the growth-phase of the

bacteria (the structural component, peptidoglycan has been shown to increase from a two to three layer structure to a four to five layer structure when passing from exponential to stationary-phase growth (Leduc *et al.*, 1989)), and does effect mechanical disruption (Middelberg *et al.*, 1992). Autolysis, however, usually requires active synthesis of the cell wall. It is the weakening of the growing cell wall structure that causes the lysis. Treatment with urea and EDTA is not so subtle. Urea and EDTA directly compromise the integrity of the inner and outer membrane components of the cell wall. This process is independant of the bacterial growth rate.

The choice of buffer had no significant effect on protein release in this experiment. Tris buffer has been shown to increase the outer membrane permeability of *E. coli* although this phenomenon was minimal at a Tris concentration of 0.1 M (Irvine *et al.*, 1981). This suggests that buffer selection can be made on economic grounds.

Variables that were not tested in this experiment such as cell concentration, pH, and redox potential will need to be tested and optimised prior to this technique's commercial application.

Table 2.2 Statistical analysis of the multifactorial experiment. Abbreviations are Df Degrees of freedom, Pr (F) Probability of similarity.

	Df	Sum of Squares	Pr (F) **-highly significant
Urea	3	266.6	< 0.001**
EDTA	1	262.9	< 0.001**
Buffer	1	0.9	0.186
Time	1	6.2	< 0.001**
Growth Phase	1	19.0	0.086
Total		610	

Table 2.3 Minor, significant interactions in the multifactorial experiment. Abbreviations are Df Degrees of freedom, Pr (F) Probability of similarity.

	Df	Sum of Squares	Pr (F)
Urea : EDTA	3	8.3	0.003
Urea : Time	3	2.8	0.006
EDTA : Time	1	0.9	0.049

Table 2.4 Fitted mean values of percentage protein release from logarithmic-phase cells at the tested urea and EDTA concentrations.

	Time (min)	0 M Urea	2 M Urea	4 M Urea	6 M Urea
0 mM EDTA	30	2.0	5.0	15.5	44.8
	90	3.0	7.6	22.3	56.8
3 mM EDTA	30	14.8	68.9	83.0	100.7
	90	21.3	79.3	90.8	103.6

Table 2.5 Fitted mean values of percentage protein release from stationary-phase cells at the tested urea and EDTA concentrations.

	Time (min)	0 M Urea	2 M Urea	4 M Urea	6 M Urea
0 mM EDTA	30	1.0	2.4	7.8	26.6
	90	1.4	3.7	11.6	36.4
3 mM EDTA	30	7.4	48.1	64.9	92.3
	90	11.0	60.1	75.8	97.7

2.4 Interaction between EDTA and urea

Both urea and EDTA concentration were shown to be the most important factors affecting protein release from treated cells in section 2.3. The interaction between urea and EDTA was significant. This section presents a more detailed study of the effect of EDTA and urea on protein release. EDTA concentrations between 0.001 and 3 mM, and urea concentrations between 0 and 6 M, were used to permeabilise the cells. The amounts of the chemical reagents required to permeabilise the cells directly affects the cost of the procedure.

2.4.1 Method

Test solutions were prepared for every combination of the following urea and EDTA concentrations: 0, 0.5, 1.0, 2.0, 4.0, and 6.0 M urea; and 0.01, 0.03, 0.1, 0.3, 1.0, and 3.0 mM EDTA (36 test solutions total). All test solutions were buffered with 0.1 M Tris and adjusted to pH 9.0 using conc. HCl. The negative control was PBS (Appendix B3). Each test solution (4.75 mL) was held in a 25 mL MacCartney bottle. *E. coli* used in this experiment were stationary phase and originated from a single fermentation run (Appendix C1). The *E. coli* sample (0.25 mL) was added to each test solution that was then agitated in a shaking water bath at 37°C. Reactions were sampled at 30 min.

Samples were analysed for total protein release into the extracellular phase (Appendix A3). The 100% protein release value was determined using untreated cells passed three times through an APV-Gaulin homogeniser at 56 MPa with debris removed by centrifugation at 10,000 xg for 15 min prior to protein estimation. All data for chemical release experiments are expressed as a percentage of the protein released by mechanical disruption.

2.4.2 Results and Discussion

Results for protein release from *E. coli* cells treated with various urea and EDTA concentrations are shown in Figure 2.7.

As indicated by the multifactorial experiment, the interaction between urea and EDTA is not simple. The curve for protein release against urea concentration is non-linear for both low (0 to 0.03 mM) and high (greater than 0.1 mM) EDTA concentrations. The relationship between protein release and urea concentration is also different for low and high EDTA concentrations. At low EDTA concentrations the protein release is low until high urea concentrations (4 and 6 M) are used; however at high EDTA concentrations the lower urea concentrations (0.5 to 2 M) do stimulate protein release.

Maximum levels of protein release were achieved at a urea concentration of 6 M in the presence of greater than 0.1 mM EDTA. This demonstrates that the EDTA concentration in the previous sections was in excess by 30 fold and that excess EDTA does not play any further role in permeabilisation.

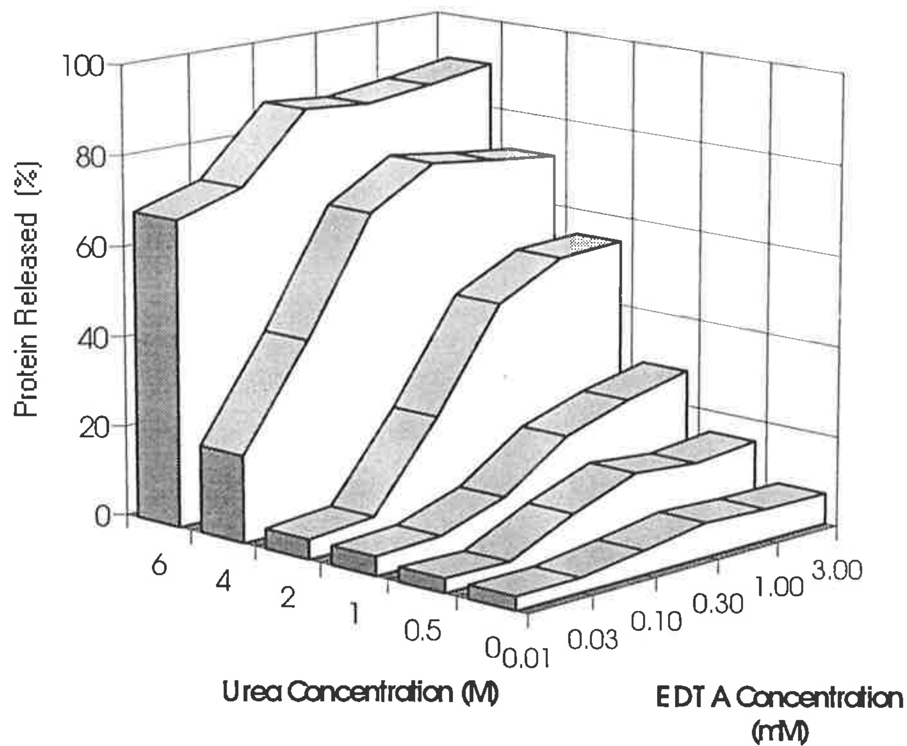


Figure 2.7 Protein release from chemically treated stationary-phase *E. coli* at a range of urea and EDTA concentrations. The treatment groups contained urea (0, 0.5, 1, 2, 4, or 6 M), EDTA (0, 0.01, 0.03, 0.1, 0.3, 1, or 3 mM), 0.1 M Tris, pH 9.0, and operated at 37°C for 90 min.

2.5 Kinetics of protein release

Reaction time is an important consideration when assessing a technique's suitability for application to commercial processes, especially where a product is unstable or where process delay causes an economic penalty. In the multifactorial experiment, reaction times of 30 and 90 min were used (see section 2.3). Analysis showed that these times constituted a significant but small variable. In this study sample times as short as 1 min were used to study this variable in greater depth.

2.5.1 Method

Test solutions were prepared containing 3 mM EDTA, 0.1 M Tris buffer, and 0, 2, 4, or 6 M urea. The pH was adjusted to 9.0 using conc. HCl. The negative control was PBS (Appendix B3). Each test solution (4.75 mL) was held in a 25 mL MacCartney bottle. *E. coli* used in this experiment were stationary phase and originated from a single fermentation run (Appendix C1). The *E. coli* sample (0.25 mL) was added to each test solution that was then agitated in a shaking water bath at 37°C. Reactions were sampled at 1, 2, 5, 15, and 60 min. The PBS negative control sample provided the time zero value.

Samples were added to PBS (to give a 1:10 dilution) as described in Appendix B3. The dilution effectively quenches the reaction. Urea and EDTA are diluted, isotonic conditions are provided that reduce further lysis, and the salts constitute antichaotropic agents that further inhibit the action of urea.

Samples were analysed for total protein release into the extracellular phase (Appendix A3). The 100% protein release value was determined using untreated cells passed three times through an APV-Gaulin homogeniser at 56 MPa with debris removed by centrifugation at 10,000 xg for 15 min prior to protein estimation. All data for chemical release experiments are expressed as a percentage of the protein released by mechanical disruption.

Data were analysed using the Jandel Scientific Table Curve™ 2D program, (AISN Software) to fit a curve to the data, calculate the constants for the kinetic equation, and conducted the statistical analysis.

2.5.2 Results and Discussion

The results for protein release against time are shown in Figure 2.8 along with the fitted curve. The kinetics of protein release during chemical treatment are assumed to be first-order. The fractional protein release at time t is given by equation (2.3),

$$Y = (Y_0 - Y_{max}) \exp (-t / \tau) + Y_{max} \qquad - (2.3)$$

where Y_0 and Y_{max} are the initial and maximum fractional releases, respectively, and τ is the time constant.

Table 2.6 Parameters for the equation describing protein release from stationary phase *E. coli* cells treated with urea and EDTA.

Urea Conc. (M)	Y_{max} (%)	Y_0 (%)	τ (minutes)
0	15.4 ± 0.6	8.2 ± 0.4	12.3 ± 3.0
2	55 ± 1	8.0 ± 1.7	2.3 ± 0.3
4	72 ± 1	6.6 ± 2.6	2.6 ± 0.3
6	102 ± 2	6.4 ± 3.2	2.7 ± 0.3

Regression results are shown in Table 2.6. The time constants (τ) for the different urea concentrations are very close, approximately 2.5 min. However, when no urea was present the time constant increases to 12.3 min. Increasing the urea concentration clearly has a

definite effect on the maximum protein release (as demonstrated in sections 2.3 and 2.4), but did not have a major influence on the speed of reaction.

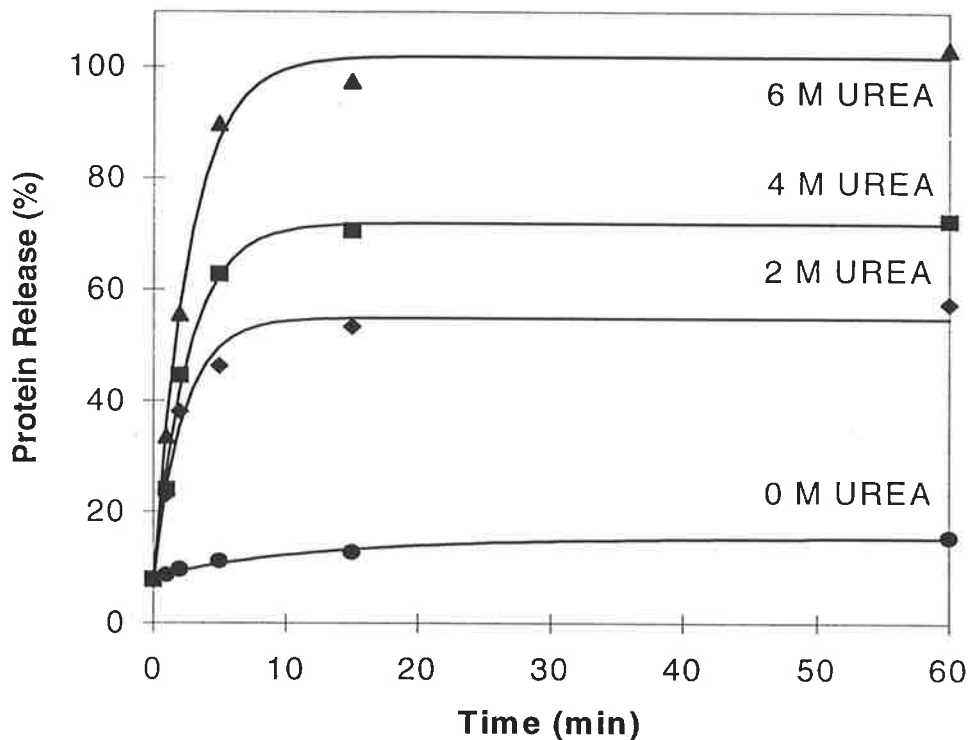


Figure 2.8 Protein release from chemically treated stationary-phase *E. coli* against time. The treatment groups contained urea (0, 2, 4, or 6 M), 3 mM EDTA, 0.1 M Tris, pH 9.0, and operated at 37°C for 90 min.

The reaction rate does not constitute a limitation on the use of this permeabilisation technique in commercial applications. A treatment time (of approximately 30 min) to release cellular protein compares favourably with mechanical disruption, where the flow rate through the homogeniser causes a bottle-neck when large process volumes are being treated and multiple passes are required.

2.6 Conclusion

Chemical treatment of *E.* coli with a combination of urea (a chaotropic agent) and EDTA (a chelating agent) is a potent method for the release of intracellular protein from both logarithmic-phase and stationary-phase cells. The level of protein release achieved with chemical treatment using 6 M urea and greater than 0.1 mM EDTA is comparable to that achieved by high-pressure homogenisation. An important feature of this permeabilisation technique is that it is applicable for both logarithmic and stationary cells. Many chemical permeabilisation techniques have been limited in their application due to their dependence on the growth phase of the bacteria.

The success of this chemical permeabilisation technique will be very dependent on the type of product being extracted from the bacteria. The presence of urea can be detrimental when the native structure of macromolecule needs to be maintained. Urea's action as a protein denaturant is well documented (Kamoun, 1988). Denaturation, however, is not of concern during the extraction of most recombinant proteins from the cytoplasm of *E. coli.* Recombinant proteins are usually not in their native configuration inside the *E. coli* cell, and require denaturation and subsequent renaturation to attain a biologically-active conformation. The presence of urea is often desirable during the processing of recombinant proteins as it can maintain protein solubility especially at high protein concentrations.

The purity of an extracted product will be effected by the technique used to extract it from the *E. coli* cell. Extraction of soluble proteins from the cytoplasm of *E. coli* using a mechanical or chemical technique is unlikely to result in radically different levels of product purity. The chemical method could, however, result in the solubilisation of higher levels of macromolecules associated with bacterial membrane. Extraction of insoluble proteins (including recombinant protein inclusion bodies) from the cytoplasm of *E. coli* is likely to a more complex process than that developed for soluble proteins. The role of the chemical agents on cell permeabilisation and protein solubilisation needs to be

experimentally determined in each case before the suitability of the chemical extraction technique is known.

The potential advantages of this chemical treatment are its simplicity, potential economic benefits, and relative speed. The replacement of the high-pressure homogeniser or ball mill with a stirred tank or plug-flow reactor can present economic advantages. The chemical agents (urea and EDTA) are reasonably cheap and should not constitute a major component in the overall costs associated with product recovery. The speed of the reaction is also advantageous. In practice, chemical treatment should take a fraction of the time required for mechanical disruption and is more amenable to continuous process development. This will be of benefit where the disruption step constitutes a process bottleneck.

CHAPTER 3

NONSELECTIVE EXTRACTION OF RECOMBINANT PROTEIN

Summary

Chemical permeabilisation was applied to the extraction of the recombinant protein Long-R^3-IGF-I from intact *E. coli* cells. Treatment with the basic permeabilisation solution (6 M urea, 3 mM EDTA, 0.1 M Tris at pH 9.0 developed in chapter 2) plus 20 mM of the reducing agent dithiothreitol (DTT) proved effective at extracting the recombinant protein. The kinetics of nonselective extraction of the recombinant protein was first-order with a time constant of 3 min. Urea plays an important role in both permeabilisation of the cell wall and dissolution of the inclusion body. Conversely, EDTA was only involved in cell wall permeabilisation while DTT enhanced recombinant protein extraction. pH proved to be important with lower levels of protein release achieved at low pH values (<9). An increase in cell concentration had a minor effect on recombinant protein release and caused an observable increase in viscosity.

Nonselective extraction recovered comparable levels of recombinant protein to that achieved by conventional extraction (mechanical disruption followed by centrifugation). However, the relative concentration of directly-extracted recombinant protein was low (16% (w/w) of the total protein) due to contamination by bacterial cell components.

3.1 Introduction

Recovery of recombinant proteins from the cytoplasmic inclusion bodies usually requires a series of steps including cell disruption, separation of the insoluble inclusion bodies from the soluble fraction, inclusion body washing, solubilisation of the inclusion bodies, refolding, and purification of the protein (Fischer *et al.*, 1993). Release of inclusion bodies from the confines of the cell is usually achieved by mechanical disruption, using equipment such as high-pressure homogenisers, bead mills, or sonicators (Middelberg, 1995). Inclusion bodies are then separated from the soluble host-cell components by centrifugation or diafiltration. Further removal of the insoluble host-cell components can be achieved using washing steps that can include the addition of various chemical agents to solubilise any contaminating material. The inclusion body is then dissolved using denaturants such as the chaotropic agents urea or guanidine hydrogen chloride. Reducing agents such as dithiothreitol (DTT) or β-mercaptoethanol are often used to eliminate intermolecular disulphide bonds when cysteine residues are present in the protein. The resulting product is in a denatured state and requires refolding back to its native configuration. Further purification steps are often necessary to meet final product specifications.

Direct extraction of recombinant protein from intact cells is rarely reported. An exception is the solubilisation of interferon-γ inclusion bodies located in the host bacteria described in US Patent 4,476,049. Guanidine hydrogen chloride was used to release the protein into the extracellular phase (Kung, 1984). Details of the process such as the level of contamination, the kinetics of release, and the role of the chemical agents are not given.

The research presented in this section adapts the permeabilisation technology (developed in chapter 2) for the direct extraction of the recombinant protein Long-R^3-IGF-I from inclusion bodies located in the cytoplasm of *E. coli*. Long-R^3-IGF-I is only detected in the insoluble phase after homogenisation and separation by centrifugation as demonstrated by HPLC and PAGE analysis (see Figure 4.12 for PAGE analysis), inclusion bodies are visible using phase-contrast microscopy. The chemicals tested were the chaotropic agent

urea, the chelating agent EDTA, and the reducing agent DTT. Urea is known to solubilise Long-R^3-IGF-I inclusion bodies *in-vitro* (Greenwood *et al.*, 1994) and is involved in cell-wall permeabilisation. EDTA aids permeabilisation of bacterial cell walls. DTT is known to effect the dissolution of inclusion bodies that contain cysteine residues.

3.1.1 Research goals

The research presented in this chapter aims to:

- discover a combination of chemical agents that can extract insoluble recombinant protein from the cytoplasm of *E. coli* at levels similar to traditional procedures (mechanical disruption, centrifugation, followed by *in-vitro* dissolution);

- determine the role of the chemical and environmental parameters on *in-situ* dissolution;

- establish the kinetics of *in-situ* dissolution;

- determine the process yield and product purity.

3.2 Role of the chemical agents

For *in-situ* dissolution to succeed, a series of reactions need to take place. The cell wall barrier has to be permeabilised for the denaturant to diffuse to the inclusion body, the inclusion body has to dissolve, and the solubilised protein has to diffuse into the extracellular phase.

This section investigates the role of chemical agents in the extraction of the recombinant protein Long-R^3-IGF-I from inclusion bodies located in the cytoplasm of intact *E. coli*. The chemical agents are the chaotropic agent urea, the chelating agent EDTA, and the reducing agent DTT. Urea plus DTT has been shown to be effective at dissolving Long-R^3-IGF-I inclusion bodies already extracted from *E. coli* cells (Greenwood *et al.,* 1994). Urea is also effective at permeabilising *E. coli* cell walls, a phenomenon that is enhanced by the addition of EDTA (chapter 2). Combinations of these three chemical agents are therefore tested for the extraction of Long-R^3-IGF-I from inclusion bodies located in the cytoplasm of intact *E. coli.*

Direct comparison of *in-situ* and *in-vitro* dissolution demonstrates the role of the chemical agents in inclusion body solubilisation, as well as the permeabilisation- solubilisation process. This procedure was used to study the roles of urea, EDTA, the reducing agent DTT, and the protease inhibitor $ZnCl_2$ on *in-situ* inclusion body dissolution.

3.2.1 Method

3.2.1.1 Part A

Test solutions were prepared containing each combination of the following: urea (0, 2, 4, or 6 M), EDTA (0 or 3 mM), DTT (0 or 20 mM), and 0.1 M Tris buffer. The pH was adjusted to 9.0 using conc. HCl. A total of 16 combinations were examined. The test solution (4.75 mL) was held in a 25 mL MacCartney bottle. *E. coli* used in this experiment were induced for the expression of Long-R^3-IGF-I which accumulated as inclusion bodies in the host cytoplasm (Appendix C2). *E. coli* sample (0.25 mL) was added to each test solution which was then agitated in a shaking incubator set at 37°C. Test solutions were sampled at 30 min.

Samples were analysed for total protein release into the extracellular phase (Appendix A3) and recombinant protein release into the extracellular phase (Appendix A4).

3.2.1.2 Part B

Test solutions were prepared containing 6 M urea, 20 mM DTT, and 0.1 M Tris buffer, and the following additions: no addition, 3 mM EDTA, and 5 mM $ZnCl_2$. The pH was adjusted to 9 using conc. HCl. The test solution (4.75 mL) was held in a 25 mL MacCartney bottle. *E. coli* and extracted inclusion bodies were used in this experiment. Both were from *E. coli* induced for the expression of Long-R^3-IGF-I which accumulated as inclusion bodies in the host cytoplasm (Appendix C2 and C3). *E. coli* or inclusion body extract (0.25 mL) were added to each test solution which was then agitated in a shaking incubator set at 37°C. Test solutions were sampled at 30 min.

Samples were analysed for total protein release into the extracellular phase (Appendix A3) and recombinant protein release into the extracellular phase (Appendix A4).

3.2.2 Results

3.2.2.1 Part A

Combinations of the chemical agents urea, EDTA, and DTT were tested on *E. coli* containing inclusion bodies of the recombinant protein Long-R^3-IGF-I (as described in section 3.2.1.1) and the total and recombinant protein levels in the extracellular phase quantified. The results for total protein release are shown in Figure 3.1, and for recombinant protein release in Figure 3.2.

Measurable quantities of the recombinant protein (Long-R^3-IGF-I) were released into the extracellular phase by chemical treatment. The presence of urea, EDTA, and DTT had a positive effect on the levels of both recombinant and total protein released. Maximal dissolution occurred in the presence of 6 M urea, 3 mM EDTA, and 20 mM DTT. This treatment released 73 mg/L Long-R^3-IGF-I and 464 mg/L total protein into the extracellular phase. Recombinant protein constituted 16% (w/w) of the total protein in solution.

The urea concentration directly affected the level of recombinant protein and total cellular protein released into the extracellular phase (this phenomenon was consistent for all urea treatments whether EDTA or EDTA and DTT were present). The presence of both DTT and EDTA was important for maximising release of recombinant protein from the cell. Removal of either EDTA and/or DTT resulted in a drop in the release of recombinant protein and total cellular protein to the extracellular phase.

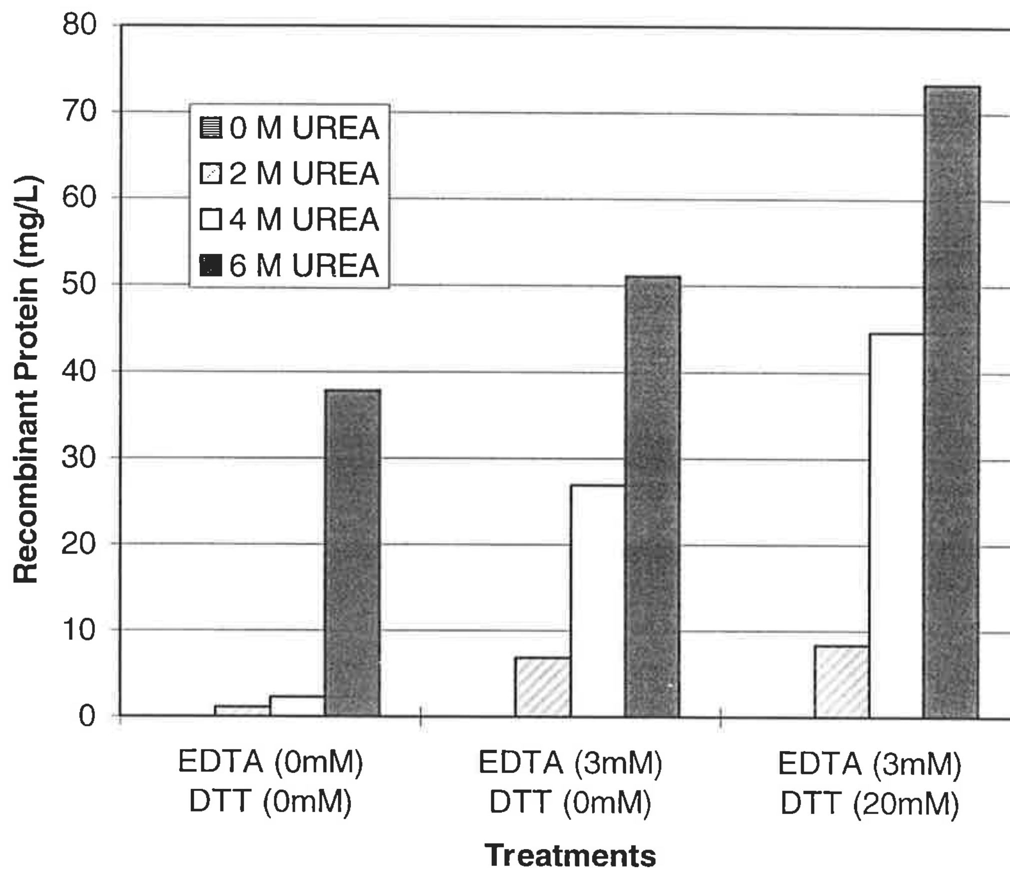


Figure 3.1 Recombinant protein (Long-R^3-IGF-I) release from *E. coli* containing inclusion bodies following treatment with a combination of urea, DTT and EDTA in 0.1 M Tris (pH 9.0) for 30 min at 37°C. (Note 0 M urea released no Long-R^3-IGF-I).

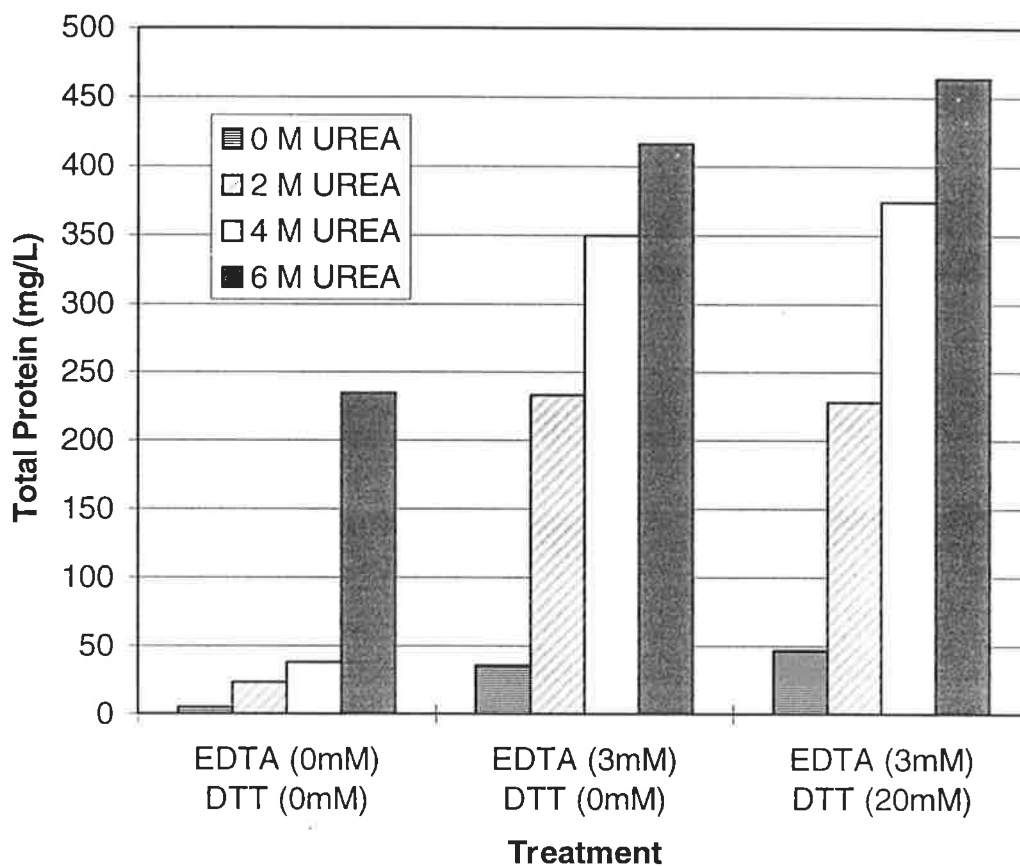


Figure 3.2 Total protein release from *E. coli* containing recombinant protein inclusion bodies following treatment with a combination of urea, DTT and EDTA in 0.1 M Tris (pH 9.0), for 30 min at 37°C.

3.2.2.2 Part B

A comparison of the chemical treatment of inclusion bodies extracted from *E. coli* cells (*in-vitro* dissolution) and inclusion bodies still located inside intact bacterial cells (*in-situ* dissolution) is shown in Figure 3.3 (recombinant protein release) and Figure 3.4 (total protein release).

The results demonstrate that treatment with the combination of 6 M urea, 3 mM EDTA, and 20 mM DTT was as effective at solubilising Long-R^3-IGF-I inclusion bodies while still located inside the cell as it was at solubilising extracted inclusion bodies (Figure 3.3). This indicates that direct extraction of recombinant protein from *E. coli* cells can be used as a replacement for *in-vitro* inclusion body dissolution (after extraction using mechanical disruption and centrifugation) without significant loss in the amount of product recovered. The major difference between direct extraction from cells and *in-vitro* inclusion body solubilisation was the purity of the end product (Table 3.1). The total protein released from treated whole cells was significantly higher than that released from extracted inclusion bodies (Figure 3.4). This is due to the separation of the inclusion bodies from the soluble phase by centrifugation for the *in-vitro* method.

Chemical treatment of intact cells without EDTA present released a significantly lower level of both recombinant protein and total protein into the extracellular phase (Figures 3.3 and 3.4, respectively). Removal of EDTA, however, had no effect on the solubilisation of extracted inclusion bodies. The presence of the protease inhibitor $ZnCl_2$ had no significant effect on inclusion body solubilisation whether *in-vitro* or *in-situ* (Figure 3.3). The recombinant protein used in this trial Long-R^3-IGF-I is therefore not observably susceptible to degradation by $ZnCl_2$-inhibited proteases (such as OmpT) under the conditions used in this study. The level of OmpT coextraction can be critical to the success or failure of many processes for the recovery of active recombinant proteins (Grodberg and Dunn, 1988). Testing with a protease-susceptible protein is required to evaluate differences between the two processes regarding the level of protease coextraction.

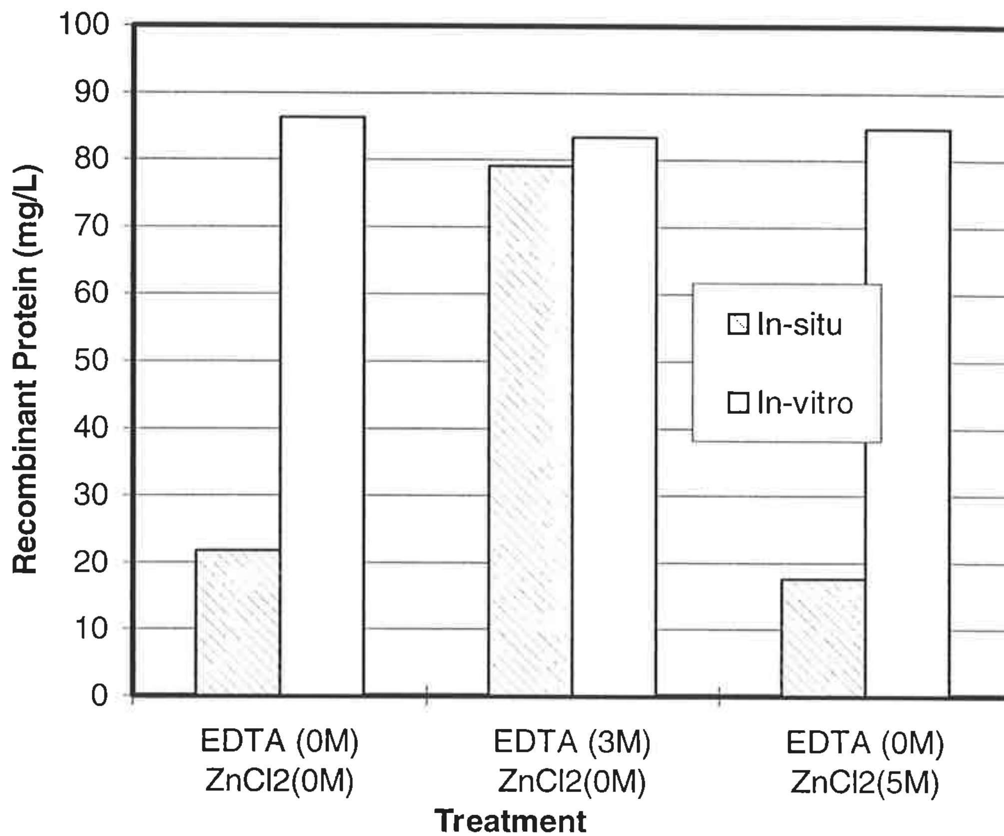


Figure 3.3 Comparison of the recombinant protein release (Long-R^3-IGF-I) from chemically-treated *E. coli* containing inclusion bodies (*in-situ* solubilisation) and from extracted Long-R^3-IGF-I inclusion bodies (*in-vitro* solubilisation). All treatments contained 6 M urea, 20 mM DTT, 0.1 M Tris (pH 9.0) and operated at 37°C for 30 min.

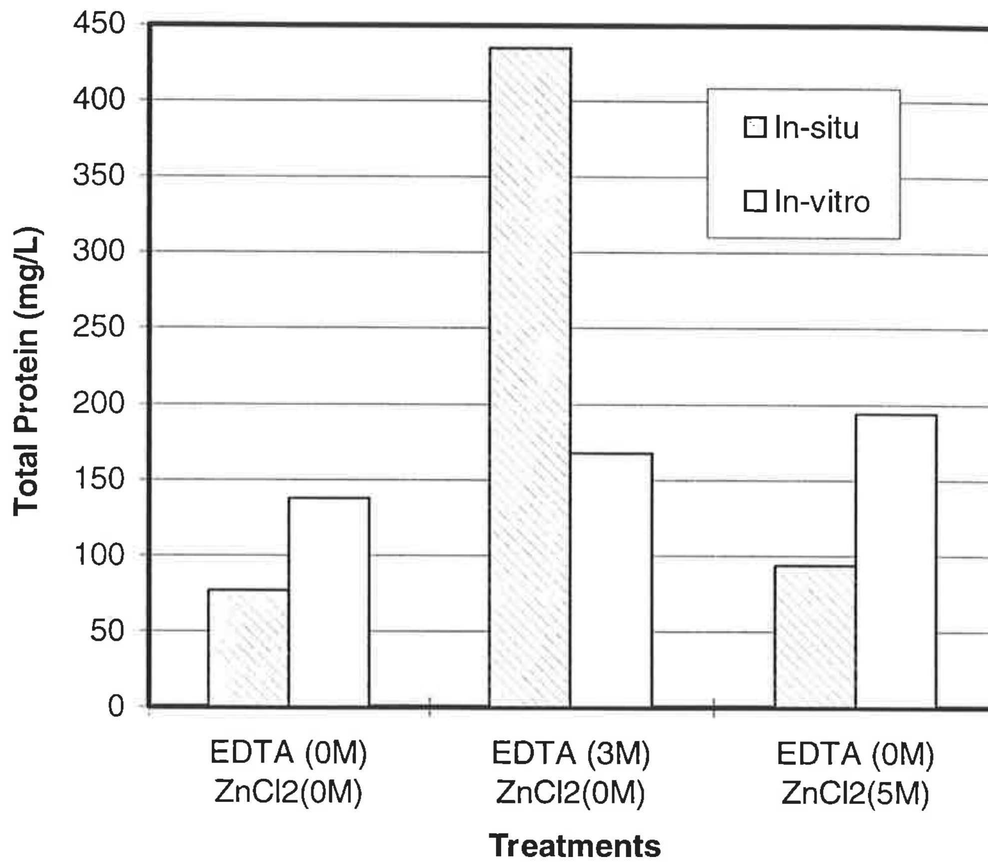


Figure 3.4 Comparison of the total protein release from chemically-treated *E. coli* containing inclusion bodies (*in-situ* solubilisation) and from extracted Long-R^3-IGF-I inclusion bodies (*in-vitro* solubilisation). All treatments contained 6 M urea, 20 mM DTT, 0.1 M Tris (pH 9.0) and operated at 37°C for 30 min.

Table 3.1 Comparison of *in-situ* dissolution with mechanical disruption followed by *in-vitro* dissolution for the release of the recombinant protein (Long-R^3-IGF-I) from inclusion bodies.

	Total Protein (mg/L)	Recombinant Protein (mg/L)	% Recovery (w/w)	% Relative Concentration (w/w)
In-situ dissolution	435	79 ± 3	95	18
In-vitro dissolution	167	83 ± 3	100	50

3.2.3 Discussion

The role of the chemical agents urea, EDTA, and DTT in extracting Long-R^3-IGF-I from intact *E. coli* cells can be discussed in light of the experimental results shown in Figures 3.1, 3.2, 3.3, and 3.4, and from the known properties of the chemicals.

EDTA is known to permeabilise the outer membrane of *E. coli* (Leive, 1974), and enhances the action of urea in permeabilising both stationary and logarithmic phase cells (chapter 2). The role EDTA plays in permeabilising the cell wall of intact bacteria is again demonstrated by the enhanced levels of total protein release in the presence of EDTA. However, it has little or no effect on protein release from extracted inclusion bodies (treatments 1 and 2 in Figure 3.4). The presence of EDTA does allow access for denaturant into the cell and enables solubilised protein to leave, but played no direct role in solubilising the inclusion body.

DTT is a reducing agent and is known to enhance the solubilisation of many inclusion bodies after extraction from their bacterial hosts (Chang and Swartz, 1993). This is due to

its ability to weaken disulphide bonds that arise due to air oxidation of the exposed inclusion bodies. The experimental results shown in Figure 3.1 indicate that DTT plays a significant role in the extraction of Long-R^3-IGF-I from *E. coli* cells. DTT enhances the level of Long-R^3-IGF-I released into the extracellular phase by approximately 30% (it has a lesser effect on total protein release from the cell, see Figure 3.2). The cytoplasm of an *E. coli* cell is a reducing environment (Tuggle and Fuch, 1985) where disulphide bond formation is unlikely. Disulphide bond formation could, however, take place due to air oxidation of the protein on lysis of the cells. The presence of DTT prevents air oxidation from taking place. While intramolecular disulphide bond formation would be expected to have little impact on Long-R^3-IGF-I release, the formation of intermolecular bonds could give rise to insoluble aggregate formation or stabilise already-present inclusion body structures, and would directly reduce the levels of Long-R^3-IGF-I in the extracellular phase.

The chaotropic agent urea plays a dual role in the release of Long-R^3-IGF-I from *E. coli* cells. Urea concentration plays an important role in the solubilisation of Long-R^3-IGF-I inclusion bodies *in-vitro* (Greenwood *et al.*, 1994). Urea has also been shown to play an important role in the permeabilisation of the *E. coli* cell wall (chapter 2). The levels of recombinant protein and total protein release shown in Figures 3.1 and 3.2, respectively, show that the proportion recombinant protein to total protein differs for the different treatment combinations. Intermediate urea concentrations (2M) are moderately effective at releasing total protein but are ineffective at releasing recombinant protein. This could be due to urea's ability to permeabilise the cell wall at low concentrations, but it's inability to dissolve the inclusion body. 2 M urea and 3 mM EDTA is capable of releasing 233 mg/L total protein (50% of that released by 6 M urea, 3 mM EDTA, and 20 mM DTT), while only releasing 8 mg/L recombinant protein (11% of that released by 6 M urea, 3 mM EDTA and 20 mM DTT). This indicates that urea's role is more complex than simply permeabilising the cell wall, and that its function in the solubilisation of the inclusion bodies *in-situ* is also important.

3.3 Role of pH

This experiment aims to determine the effect of pH on cell permeabilisation and recombinant protein extraction from intact *E. coli* cells. Solution pH is a factor that has to be taken into account when working with biological systems. The charge on macromolecules comprising the bacterial cell wall and the inclusion bodies is dependant on pH. This is likely to effect the cohesiveness of these structures.

3.3.1 Method

Test solutions were prepared containing 6 M urea, 3 mM EDTA, 0 or 20 mM DTT, with 0.1 M Tris buffer for pH 9, 8, and 7; and 0.1 M Acetic acid for pH 6, 5, and 4. The pH was adjusted to 9, 8, and 7 using conc. HCl, and to 6, 5, and 4 using 2 M NaOH. The test solution (4.75 mL) was held in a 25 mL MacCartney bottles. The *E. coli* used in this experiment were induced for the expression of Long-R^3-IGF-I which accumulated as inclusion bodies in the host cytoplasm (Appendix C2). *E. coli* sample (0.25 mL) was added to each test solution which was then agitated in a shaking incubator set at 37°C. Reactions were sampled at 30 min.

Samples were analysed for total protein release into the extracellular phase (Appendix A3) and recombinant protein release into the extracellular phase (Appendix A4).

3.3.2 Results and discussion

pH of the chemical treatment has a dramatic effect on both recombinant and total protein release from treated cells (Figures 3.5 and 3.6, respectively). Reduction in the treatment solution pH resulted in a decline in both recombinant and total protein release. At acidic pH the treatment solution (6 M urea and 3 mM EDTA) has virtually no effect on the permeability of the bacterial cell wall. Above pH 6 an increase in pH results in an increase in the protein released. This release is further enhanced by the presence of the reducing agent DTT.

Detailed elucidation of the role pH on permeabilisation and inclusion body solubilisation is limited in this study due to the experimental design. pH obviously plays a role in cell wall permeabilisation, as total protein release is dramatically affected by the solution pH. Why this effect happens is not demonstrated. The role of the charge of components of the bacterial wall, or the effect of pH on the chemical permeabilising agents, could result in the alteration of cell-wall permeabilisation.

The enhancement of both recombinant and total protein release from treated cells by DTT is dependant on solution pH. While DTT shows little enhancement of total protein release at pH 9.0 it does have a sizeable impact at pH 7.0 and 8.0. This increase in permeability could be due to an increase in cell-wall permeability (possibly due to denaturation of the protein component of the cell wall) or due to enhanced mobility of the cellular proteins when in a reduced state.

Adjustment of the treatment pH below 9, did not enhance either the release of recombinant protein nor the purity of the recombinant protein released into the extracellular phase.

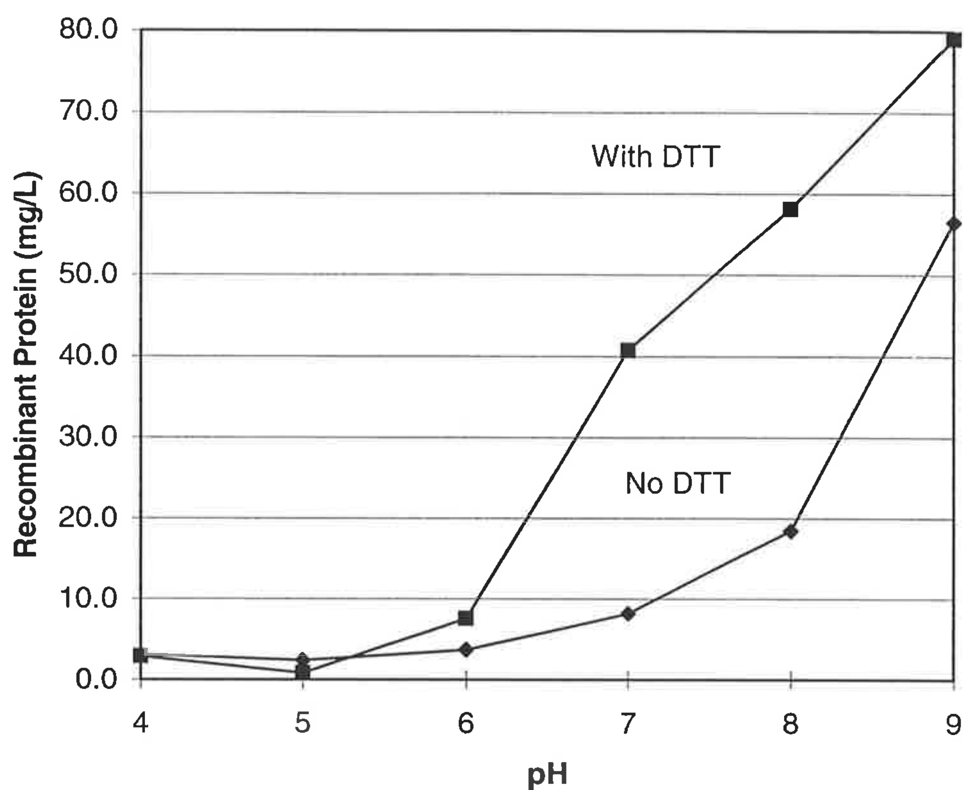


Figure 3.5 Recombinant protein (Long-R^3-IGF-I) release from *E. coli* containing inclusion bodies as a function of pH. Treatment groups contained 6 M urea, 3 mM EDTA, 0 or 20 mM DTT, 0.1 M Tris (for pH between 7 and 9) or 0.1 M sodium acetate (for pH between 4 and 6) buffer, and operated at 37°C for 30 min.

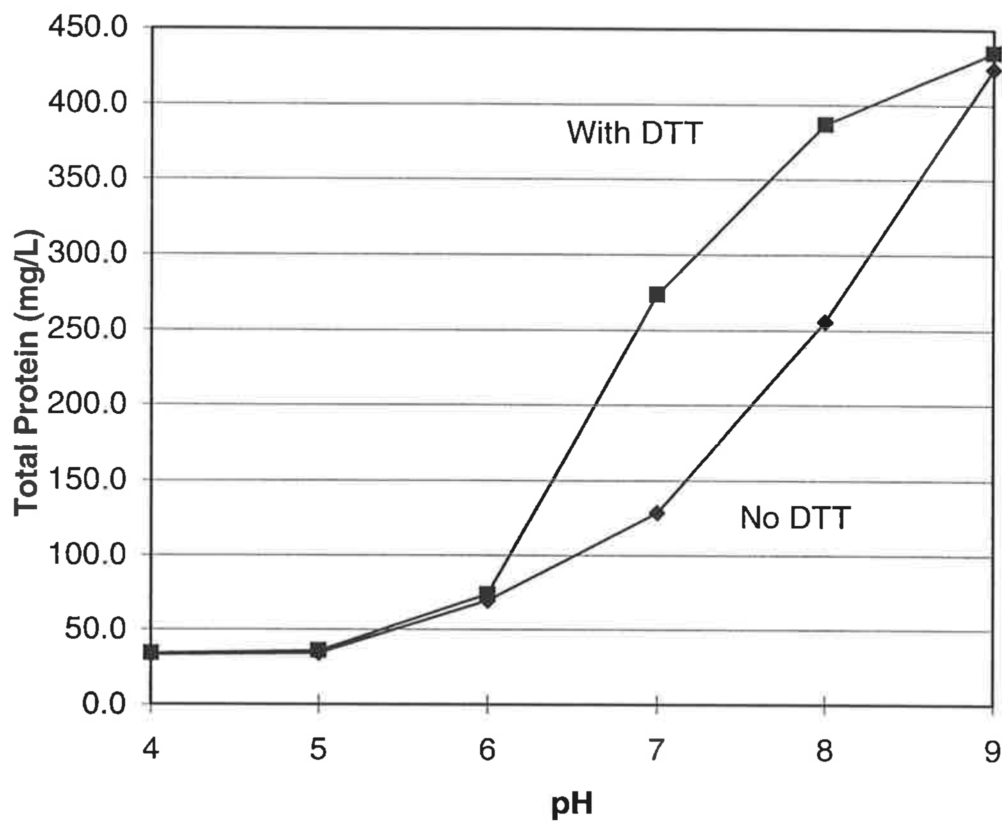


Figure 3.6 Total protein release from *E. coli* containing inclusion bodies as a function of pH. Treatment groups contained 6 M urea, 3 mM EDTA, 0 or 20 mM DTT, 0.1 M Tris (for pH between 7 and 9) or 0.1 M sodium acetate (for pH between 4 and 6) buffer, and operated at 37°C for 30 min.

3.4 Role of cell concentration

Experimental data shown in preceding sections were obtained using a cell concentration with an absorbance reading at 600nm (A_{600})) of 4. The cell concentration used during the chemical treatment has a direct impact on process economics. It dictates the size of vessel that can be used for the reaction, and the amount of the chemical reagents needed to extract a given amount of product.

This experiment aims to study the role of cell concentration on release of recombinant protein and total protein release from *E. coli* containing recombinant inclusion bodies, using cell concentrations from A_{600} of 4 to 20.

3.4.1 Method

Test solutions were prepared containing 6 M urea, 3 mM EDTA, 20 mM DTT, and 0.1 M Tris buffer. The pH value was adjusted to 9 using conc. HCl. The test solution (4.75 mL) was held in a 25 mL MacCartney bottle. *E. coli* used in this experiment were induced for the expression of Long-R^3-IGF-I which accumulated as inclusion bodies in the host cytoplasm (Appendix C2). The cell pellet was resuspended in 0.1 M Tris buffer (pH 9.0). The following volumes of cell concentrate were added to test solutions: 0.25, 0.375, 0.5, 0.75, 1.0, 1.25 mL (with the total volume made up to 5 mL using 0.1 M Tris buffer). The samples were then agitated in a shaking incubator set at 37°C. Reactions were sampled at 30 min.

Samples were analysed for total protein release into the extracellular phase (Appendix A3) and for recombinant protein release into the extracellular phase (Appendix A4).

3.4.2 Results and discussion

In this study the chemical extraction of Long-R^3-IGF-I was carried out using cell concentrations from A_{600} of 4 to 20. The increase in cell concentration had no measurable impact on the proportion of total cell protein released per unit of cells (Figure 3.8). Increased cell concentration did, however, have an impact on the release of recombinant protein from the treated cells (Figure 3.7).

The reason for the decrease in the proportion of recombinant protein released into the extracellular phase with increased cell concentration is not apparent. It is unlikely to be due to EDTA limitation as previous studies have shown that this is in excess by about 30 fold (section 2.5 of this thesis) and the fact that permeabilisation, as demonstrated by total protein release, was unaffected. The level of urea available to enter the cells could be reduced due to binding to the abundant protein in solution. This is possible though it has not interfered with urea's ability to permeabilise the cell walls, as evidenced by the release of total protein from the cells. A reduction of urea available for dissolution of the inclusion bodies would decrease the levels of recombinant protein released into the extracellular phase.

The increase in cell concentration caused a visually-detectable rise in viscosity of the test solution. The increase in viscosity is probably due to a release of nucleic acid. Increases in viscosity of the process solution can directly affect the operation of the chemical extraction step through its effect on mixing and on subsequent steps such as centrifugation, pumping, and tangential flow filtration. This problem could be resolved by mechanically or enzymically breaking the causative polymers.

The results from this study indicate that increasing the cell concentration during chemical extraction without compromising performance is not a simple procedure. The quantity of the recombinant product will not always remain proportional to the amount of cells added. The viscosity also has to be considered. The viscosity increase due to released DNA can interfere with mixing during the extraction process and interfere with subsequent process steps. Both these problems need to be resolved at higher cell concentrations.

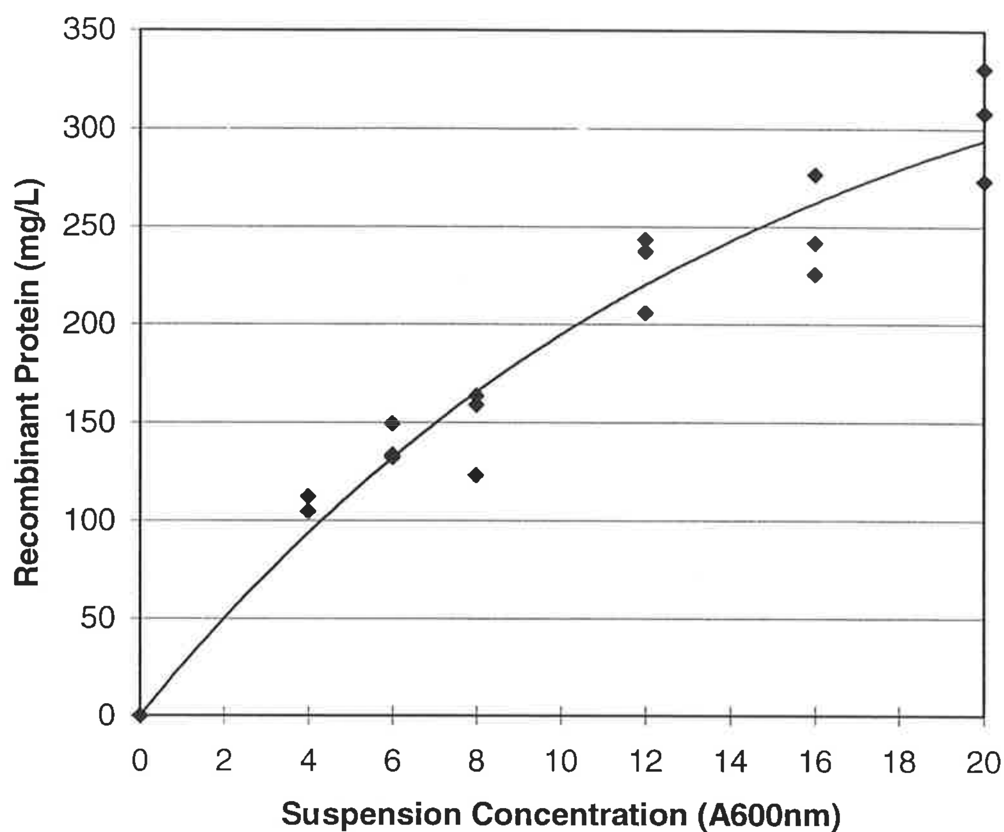


Figure 3.7 Recombinant protein (Long-R^3-IGF-I) release from *E. coli* containing inclusion bodies at various cell concentrations (A600nm). Treatment groups contained 6 M urea, 3 mM EDTA, 0 or 20 mM DTT, 0.1 M Tris buffer (pH 9.0), and operated at 37°C for 30 min.

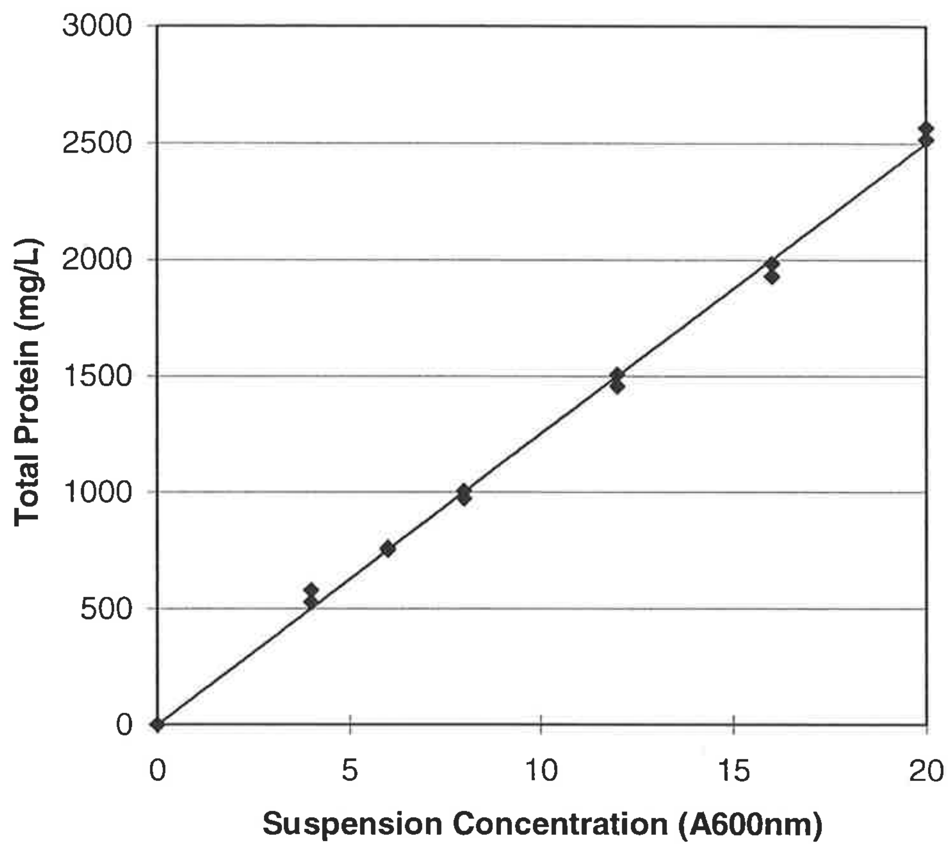


Figure 3.8 Total protein release from *E. coli* containing inclusion bodies at various cell concentrations (A600nm). Treatment groups contained 6 M urea, 3 mM EDTA, 0 or 20 mM DTT, 0.1 M Tris buffer (pH 9.0), and operated at 37°C for 30 min.

3.5 Kinetics of total protein release and *in-situ* dissolution

Reaction kinetics are an important consideration when assessing a technique's suitability for application to commercial processes. The kinetics of cell permeabilisation containing no recombinant inclusion bodies has already been investigated in section 2.6. The reaction was described by a first-order equation (Table 2.6). Kinetics of *in-situ* dissolution, however, is potentially more complex. For recombinant protein to be released from the *E. coli* cell the following events must occur:

1. cell-wall permeabilisation;
2. urea penetration of the cytoplasm;
3. inclusion body dissolution;
4. release of solubilised recombinant protein to the extracellular phase.

The presence of the inclusion body could interfere with the metabolic function of the cell. This in turn could effect the composition and strength of the bacterial cell wall and thus effect the kinetics of *in-situ* dissolution. Many chemical lysis methods do rely on cell growth to be effective as they interfere with cell wall construction. This is unlikely to be a major factor for this procedure as growth-phase is a minor factor affecting permeabilisation of the cell wall (section 2.4 of this thesis).

3.5.1 Method

Test solutions were prepared containing 2, 4, or 6 M urea, 3 mM EDTA, 20 mM DTT, and 0.1 M Tris buffer. The pH was adjusted to 9 using conc. HCl. The test solution (4.75 mL) was held in a 25 mL MacCartney bottle. *E. coli* used in this experiment were induced for the expression of Long-R^3-IGF-I, which accumulated as inclusion bodies in the host cytoplasm (Appendix C2). *E. coli* sample (0.25 mL) was added to each test solution that was then agitated in a shaking incubator set at 37°C. Reactions were sampled at 3, 6, 9, 12, 15, 40, 80, and 120 min.

Samples were analysed for total protein release into the extracellular phase (Appendix A3) and the recombinant protein release into the extracellular phase (Appendix A4).

3.5.2 Results and discussion

The release of recombinant protein and total protein against time during treatment with urea, 3 mM EDTA, and 20 mM DTT are shown in Figures 3.9 and 3.10, respectively.

The kinetics of protein release during chemical treatment can be described using equation 3.1, which is a simplified version of equation 2.1 used to describe permeabilisation (see section 2.6):

$$C = C_{max} (1 - \exp(-t / \tau)) \qquad - (3.1)$$

where C is the protein concentration in the extracellular phase, C_{max} is the maximum protein concentration into the extracellular phase, t is time, and τ is the time constant. The curves shown in Figures 3.9 and 3.10 were determined by regression to equation 3.1. Parameters are shown in Table 3.2. Measurement of the concentrations of recombinant and total protein in the extracellular phase of cell suspensions treated with urea, EDTA, and DTT, indicates that this formula is satisfactory for describing the kinetics of recombinant and total protein release. Treatment with 6 M urea, 3 mM EDTA, and 20 mM DTT quickly released recombinant and total protein into the extracellular phase, with time constants (τ) of 4.1 and 2.9 min, respectively. The time constants for the 2 and 4 M urea treatments are significantly higher than the 6 M urea treatment. This phenomenon differs from that observed for protein release from uninduced cells (section 2.6 of this thesis), where the time constants were not significantly different. The presence of the inclusion body (or the addition of DTT) could play a role in determining the kinetics of the process.

The short period of time taken for chemical extraction of recombinant protein from *E. coli* cells is clearly advantageous as it minimises overall process time and/or unit size. This

procedure has the potential to be significantly quicker to operate than conventional homogenisation.

Table 3.2 Parameters for equation 3.1 describing protein release from *E. coli* cells containing Long-R^3-IGF-I inclusion bodies treated with urea, 3 mM EDTA, and 20 mM DTT (standard error is in brackets).

	Recombinant Protein		Total Protein	
	C_{max} (mg/L)	τ (min)	C_{max} (mg/L)	τ (min)
6 M UREA	76.8 (1.1)	2.9 (0.3)	365 (9)	4.1 (0.5)
4 M UREA	43.0 (1.4)	9.1 (1.0)	299 (7)	15.9 (1.1)
2 M UREA	22.8 (0.6)	27.1 (2.1)	183 (5)	18.4 (1.5)

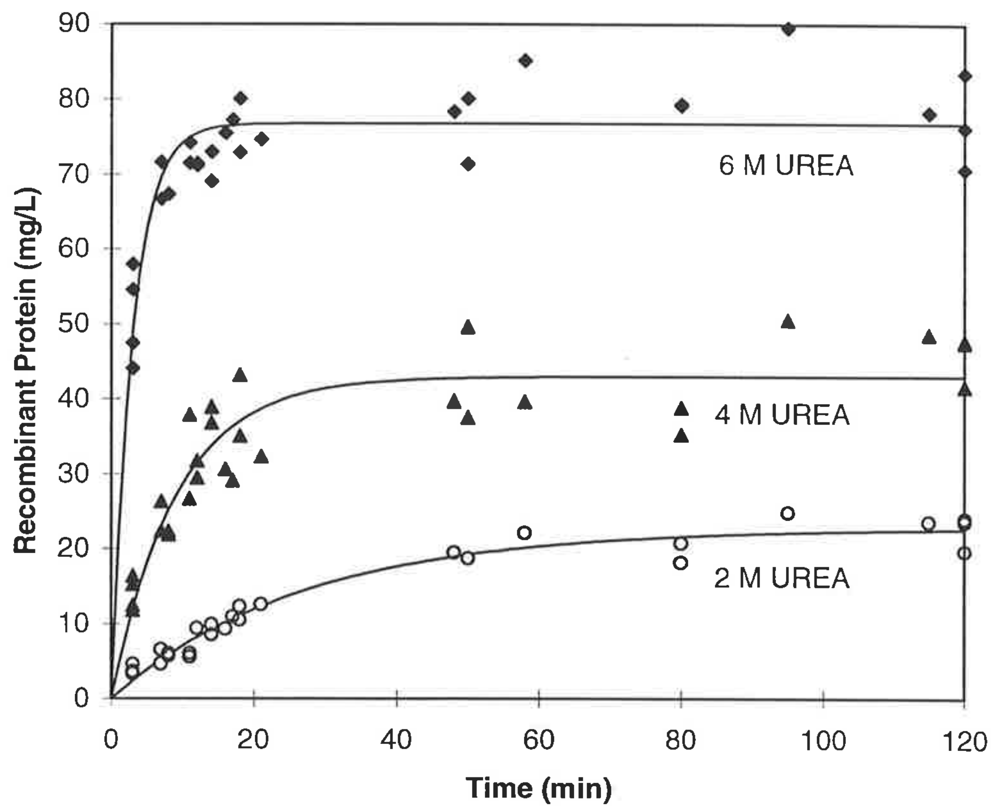


Figure 3.9 Recombinant protein (Long-R^3-IGF-I) release from *E. coli* containing inclusion bodies as a function of time. The treatment groups contained urea (2, 4, or 6 M), 3 mM EDTA, 20 mM DTT, 0.1 M Tris buffer (pH 9.0), and operated at 37°C.

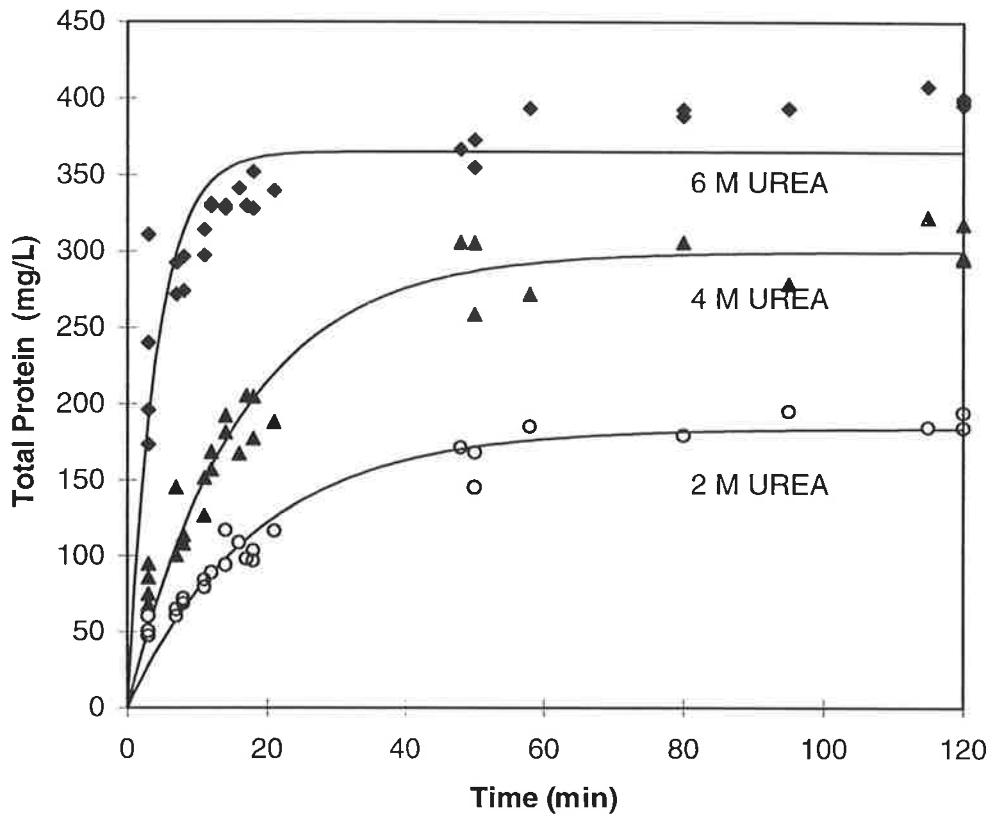


Figure 3.10 Total protein release from *E. coli* containing inclusion bodies as a function of time. The treatment groups contained urea (2, 4, or 6 M), 3 mM EDTA, 20 mM DTT, 0.1 M Tris buffer (pH 9.0), and operated at 37°C.

3.6 Completion of extraction

This section aims to determine the proportions of recombinant protein (Long-R^3-IGF-I) and total protein that were released into the extracellular phase by nonselective extraction. The kinetics of nonselective extraction showed that equilibrium was reached quite rapidly and that after approximately 15 min no significant further protein release occurred. At equilibrium, however, some protein probably remained associated with the bacteria either as insoluble material or as soluble material still located within the cells.

In this experiment, cells were treated using the nonselective extraction procedure developed in section 3.2 of this chapter. Nonselective extraction was then repeated on the insoluble fraction remaining after the first application of the nonselective extraction procedure. The insoluble fraction was resuspended by either vortex mixing or sonication prior to retreatment. Sonication is likely to disrupt the already compromised bacterial cell wall and thus facilitate the release of any entrapped material. An intermediate wash step was used to ascertain the efficiency of separating the soluble and insoluble fractions by centrifugation.

The first nonselective extraction step was conducted with and without the reducing agent DTT present. This further tests the requirement for reducing agent to achieve maximal recovery of recombinant protein.

3.6.1 Method

E. coli used in this experiment were induced for the expression of Long-R^3-IGF-I which accumulated as inclusion bodies in the host cytoplasm (Appendix C2). The reactions were carried out in 20 mL centrifuge tubes agitated in a shaking incubator set at 37°C. The standard solution used to treat the bacteria contained the following: 8 M urea, 4 mM EDTA, 0.1 M Tris buffer, with or without 27 mM DTT. pH was adjusted to 9.0 using conc. HCl.

The treatments were divided into two groups depending on the presence of DTT in the first stage of the treatment (groups A having 0 mM and group B 20 mM DTT).

The following treatments were conducted, with samples taken for analysis at the steps indicated.

Treatment 1.

1. Commence nonselective extraction with the addition of 4.75 mL of standard solution (+/- DTT) for 30 min.
2. Centrifuge at 10,000 xg for 15 min.
3. Remove supernatant (sample collected).
4. Resuspend in 1.25 mL of 0.1 M Tris.
5. Repeat nonselective extraction with the addition of 4.75 mL of standard solution (+DTT) for 30 min.
6. Centrifuge at 10,000 xg for 15 min.
7. Remove supernatant (sample collected).

Treatment 2.

1. Commence nonselective extraction with the addition of 4.75 mL of standard solution (+/- DTT) for 30 min.
2. Centrifuge at 10,000 xg for 15 min.
3. Remove supernatant (sample collected).
4. Resuspend in 2 mL of 0.1 M Tris and mix for 5 min.
5. Centrifuge at 10,000 xg for 15 min.
6. Remove supernatant (sample collected).
7. Resuspend in 1.25 mL of 0.1 M Tris.
8. Repeat nonselective extraction with the addition of 4.75 mL of standard solution (+DTT) for 30 min.
9. Centrifuge at 10,000 xg for 15 min.
10. Remove supernatant (sample collected).

Treatment 3.

1. Commence nonselective extraction with the addition of 4.75 mL of standard solution (+/- DTT) for 30 min.
2. Centrifuge at 10,000 xg for 15 min.
3. Remove supernatant (sample collected).
4. Resuspend in 1.25 mL of 0.1 M Tris.
5. Homogenise with sonicator for 2 seconds.
6. Commence nonselective extraction with the addition of 4.75 mL of standard solution (+DTT) for 30 min.
7. Centrifuge at 10,000 xg for 15 min.
8. Remove supernatant (sample collected).

Table 3.3 Strategies for the recovery of the Long-R^3-IGF-I from *E. coli* cells (where BPS is the basic permeabilisation solution consisting of 6 M urea, 3 mM EDTA, and 0.1 M Tris at pH 9.0).

	First Stage	Additional Treatment	Second Stage
A1	BPS	-	BPS+20 mM DTT
A2	BPS	additional wash step	BPS+20 mM DTT
A3	BPS	pellet resuspension by sonication	BPS+20 mM DTT
B1	BPS+20 mM DTT	-	BPS+20 mM DTT
B2	BPS+20 mM DTT	additional wash step	BPS+20 mM DTT
B3	BPS+20 mM DTT	pellet resuspension by sonication	BPS+20 mM DTT

Samples were analysed for total protein and recombinant protein release into the extracellular phase (Appendices A3 and A4, respectively).

3.6.2 Results and Discussion

Figures 3.11 and 3.12 present the results for repeated nonselective extraction. Nonselective extraction with DTT present (treatments B1 to B3) was effective at releasing 95±4% (w/w) of the recombinant protein from cells. This means that at equilibrium only a small percentage of the protein was still associated with the insoluble fraction, either as insoluble material or as soluble material located within the bacterial cell structure.

The presence of DTT in the extraction buffer enhanced the extraction of recombinant protein from the bacteria, confirming the observations in section 3.2. It raised the recombinant protein levels in the extracellular phase from 371±14 μg to 433±13 μg (equivalent to 74 and 87 mg/L, respectively). The effect on total protein release was much smaller, raising levels from 2170±16 μg to 2280±13 μg (equivalent to 434 and 456 mg/L, respectively).

The addition of a wash step after nonselective extraction both with and without DTT present (treatments A2 and B2) recovered little recombinant protein. Less than 1% (w/w) of the recombinant protein and approximately 5% (w/w) of the total protein was recovered by this step. It can be concluded that the separation of the soluble and insoluble fractions by centrifugation was an effective procedure,

The use of sonication to disperse the pelleted insoluble fraction after the initial nonselective extraction (treatments A3 and B3) had a significant effect on the release of recombinant and total protein during the repeat nonselective extraction. Sonication of the insoluble fraction resulting from nonselective extraction without DTT present (treatment A3) increased recombinant protein release from 42 to 75 μg and total protein from 272 to 575 μg during the repeat extraction. The effect was less pronounced when DTT was present in the initial nonselective extraction. This is expected as nonselective extraction with DTT present achieves a high extraction on the first stage. Sonication probably disrupts any remaining cell wall structure and liberates the entrapped material.

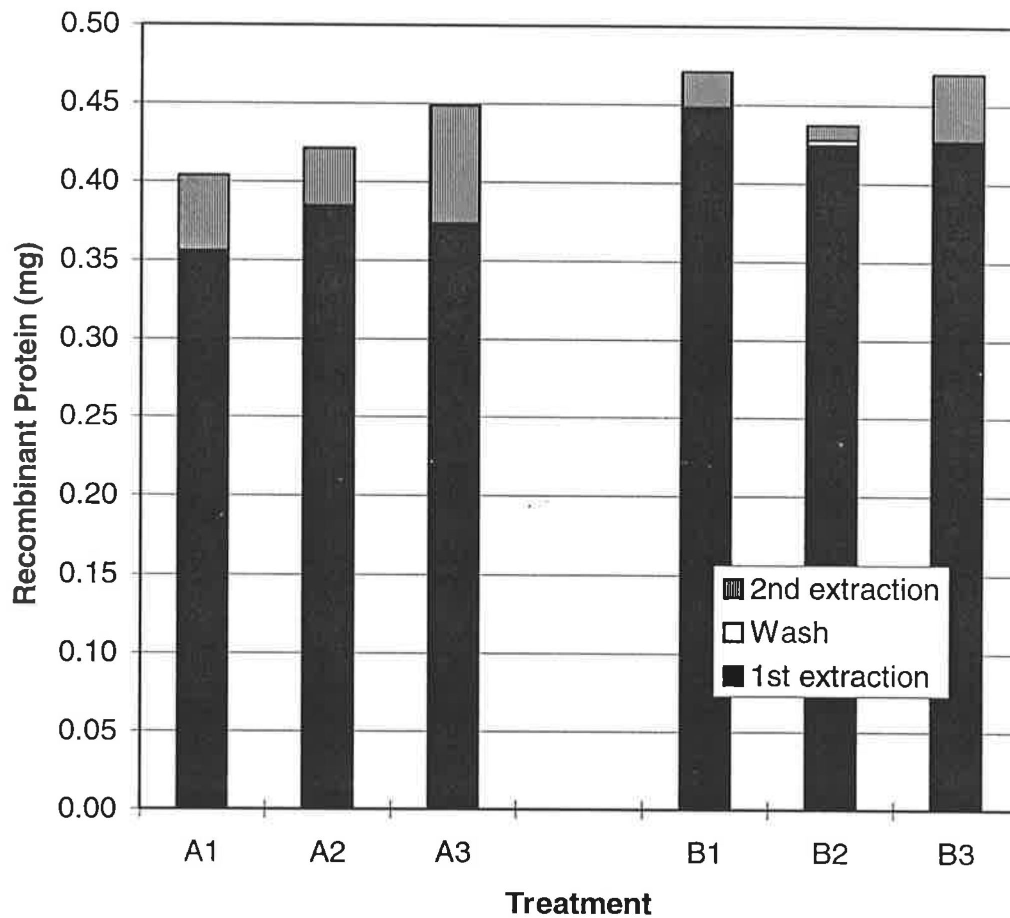


Figure 3.11 Recombinant protein release from *E. coli* during repeated nonselective extraction procedures. The treatment contained 6 M urea, 3 mM EDTA, 0.1 M Tris (pH 9.0), with 0 or 20 mM DTT (groups A and B, respectively), and operated at 37°C. Treatment subgroups A2 and B2 included a wash step and treatment subgroups A3 and B3 included pellet resuspension by sonication, prior to the repeat nonselective extraction step.

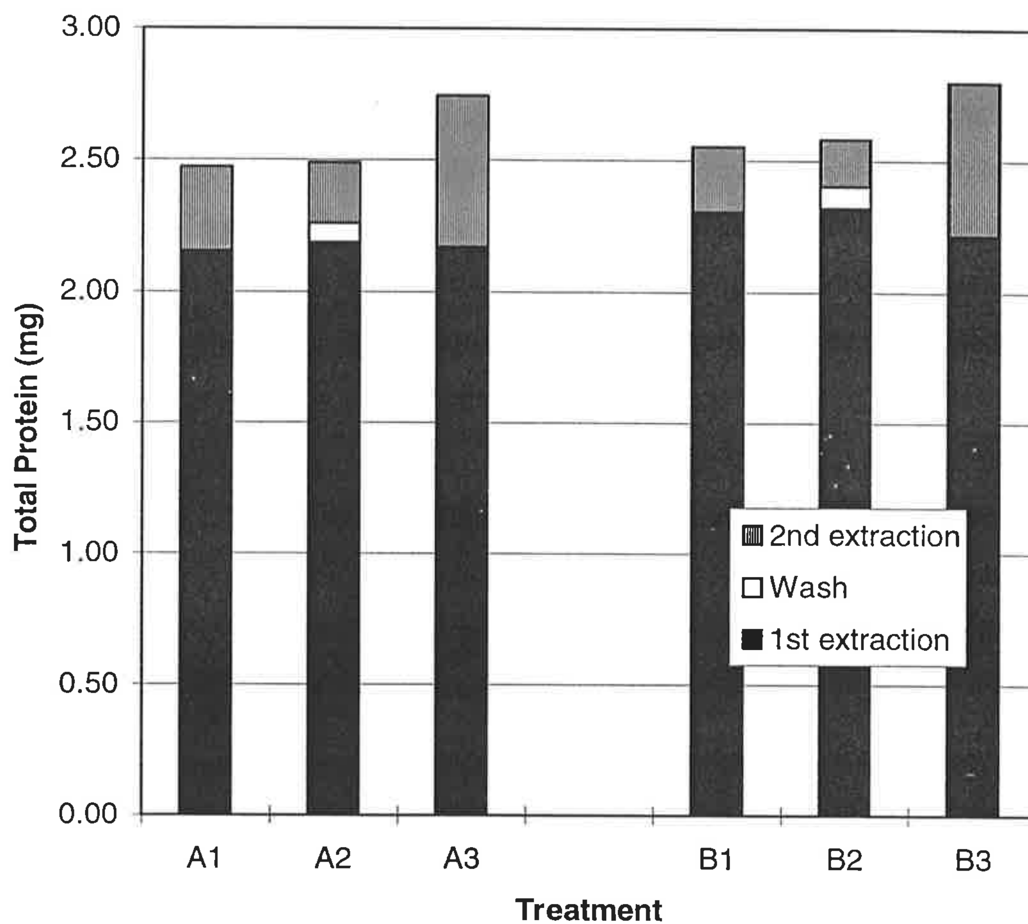


Figure 3.12 Total protein release from *E. coli* during repeated nonselective extraction procedures. The treatment contained 6 M urea, 3 mM EDTA, 0.1 M Tris (pH 9.0), with 0 or 20 mM DTT (groups A and B, respectively), and operated at 37°C. Treatment subgroups A2 and B2 included a wash step and treatment subgroups A3 and B3 included pellet resuspension by sonication, prior to the repeat nonselective extraction step.

3.7 Conclusion

The use of a chemical treatment comprising 6 M urea, 3 mM EDTA, 20 mM DTT at pH 9.0 is effective for the extraction of the recombinant protein Long-R^3-IGF-I from intact *E. coli* cells. This process has a series of advantages and disadvantages that influence its suitability for incorporation into manufacturing applications. As shown in Table 3.1, the process compares favourably with the traditional approach (mechanical disruption, centrifugation followed by *in-vitro* dissolution) in terms of recombinant protein recovery. The purity of the end product is, however, significantly lower due to the presence of endogenous host-cell proteins.

The lower purity is a significant draw back for the processing of many recombinant products. The addition of extra chromatography steps into a process (to overcome the poorer purity of the starting material) would add significantly to the overall process costs. The extraction process for interferon-γ described in USP 4,476,049, overcomes the lower purity of the extraction product by the use of a highly-selective purification step (affinity chromatography) in subsequent processing (Kung, 1984). Incorporation of a crude purification step such as precipitation or aqueous two-phase extraction after the direct extraction step could be utilised to raise the purity of the recombinant protein without rendering the process uneconomic.

This technique could find direct application in the extraction of recombinant protein from intact *E. coli* for analysis by techniques such as HPLC, PAGE, ELISA, and RIA. This could be used for monitoring recombinant protein expression levels during fermentation.

The benefits and shortcomings of this technology for the extraction of the recombinant protein Long-R^3-IGF-I have been illustrated. Application of this technology to other recombinant proteins could result in different process characteristics. The requirements for solubilisation of inclusion bodies are dependant on the specific protein and the culture conditions, and will impact on the direct extraction process. Only after application of the

technology described in this chapter to a wide range of recombinant proteins will the broad applicability be proven.

CHAPTER 4

SELECTIVE EXTRACTION OF RECOMBINANT PROTEIN

Summary

A novel selective extraction procedure was developed by modifying the nonselective procedure described in chapter 3 that was used to extract the recombinant protein Long-R^3-IGF-I from intact *E. coli* cells. The selective extraction procedure consisted of two stages. Both stages use the basic permeabilisation solution (6 M urea, 3 mM EDTA, 0.1 M Tris at pH 9.0) developed in chapter 2. In the first stage the cells are permeabilised without release of the recombinant protein (Long-R^3-IGF-I). This was achieved by the addition of 2-hydroxyethyldisulphide (2-HEDS), a promoter of disulphide bond formation, to the basic solution. This prevented solubilisation of the Long-R^3-IGF-I while allowing the release of the soluble cytoplasmic protein. The soluble fraction was discarded after centrifugation and the Long-R^3-IGF-I was then solubilised by the addition of permeabilisation solution containing dithiothreitol (DTT). This breaks the disulphide bonds and frees the protein. The 2-HEDS concentration must exceed 10 mM during the first stage for maximal retention of the Long-R^3-IGF-I in the insoluble fraction. Effective resuspension of the insoluble pellet is important for maximal release of Long-R^3-IGF-I into the extracellular phase during the second stage.

The selective extraction procedure recovered 83% (w/w) of Long-R^3-IGF-I with 46% (w/w) purity (results comparable to conventional extraction). Conventionally and selectively extracted material differed in their protein profile, as illustrated by PAGE analysis. Reduced contamination by outer-membrane components was apparent for the selective technique.

4.1 Introduction

Selective release of recombinant protein from its bacterial host provides an obvious advantage over a non-selective release process such as that described in the previous chapter. The purity of the starting material entering downstream processing can affect purification costs as it dictates the number purification steps required, the cost of chemical reagents, and the total process time.

The traditional strategy for recombinant protein recovery provides a degree of selectivity, as its separates inclusion bodies from the bulk of the host cell components using centrifugation. The recovery does result in the coextraction of some contaminants from the host bacteria. Contaminants include components of the inclusion body, materials that adhere to the surface of the inclusion body, and cell debris that cosediments with the inclusion body preparation. The contaminants of most concern that commonly coextract with inclusion bodies include the outer-membrane components, including a protease that can degrade many recombinant products (OmpT), and the endotoxin lipopolysaccharide. Protein and lipopolysaccharide contaminants are not desirable in parenteral recombinant protein therapeutic products. The level of the adhering and the cosedimenting contaminants can be reduced by the incorporation of a washing steps prior to inclusion body solubilisation. Processes that rely on multiple washing steps to remove key contaminants can prove awkward to implement cost-effectively at commercial scale.

An alternative to this traditional approach is the recovery of recombinant protein that has accumulated in the bacterial periplasmic space. Secretion of the product into the periplasm is possible where the gene for the protein of interest is ligated to an endogenous secretion signal sequence. A degree of selectivity in the recovery process is possible as periplasmic material can be recovered without contamination by the cytoplasmic contents. This approach has been applied to the extraction of IGF-I from *E. coli* (Hart *et al.*, 1994). The construct was designed so the recombinant IGF-I was secreted to the bacterial periplasm. Chemical *in-situ* solubilisation was applied to the periplasmic inclusion bodies, and aqueous two-phase extraction was used to purify the resulting solution.

In-situ cytoplasmic inclusion body dissolution as described in chapter 3 of this thesis is a non-selective procedure. The purity of recombinant protein in the resulting *in-situ* solubilisation solution is low compared with that achieved using the traditional extraction processes. The research detailed in this section describes the modification of *in-situ* solubilisation to provide selectivity to the procedure. This improves the purity of the recombinant protein extracted from the cells.

4.1.1 Research goals

The research presented in this chapter aims to:

- develop a technique that stabilises cytoplasmic inclusion bodies while permitting cell wall permeabilisation and the release of soluble cell components;

- develop a technique for releasing the recombinant protein from the stabilised inclusion bodies as monomeric protein;

- determine the role of the chemicals on inclusion body stabilisation;

- develop a selective release procedure for the extraction of Long-R^3-IGF-I from the cytoplasm of *E. coli* and determine the process yield and the product purity.

4.2 Development of a two-stage extraction procedure

This study investigated various modifications of the *in-situ* inclusion body solubilisation procedure to achieve selective release of the recombinant product. Modification of the levels of urea, EDTA, DTT, and pH (described in chapter 3) failed to provide any significant increase in purity of the extracted recombinant protein.

A two-stage chemical extraction procedure is proposed for the selective release of the recombinant protein. During the first stage permeabilisation of the bacteria is accomplished without solubilisation of the recombinant inclusion body (releasing the host cell protein into the aqueous phase). Selectivity is achieved by separating the insoluble and soluble fractions using a procedure such as centrifugation. During the second stage, the inclusion bodies are solubilised to release the recombinant protein into the aqueous phase.

To achieve cell wall permeabilisation without inclusion body solubilisation, the following stratagem was employed. Chemicals known to promote disulphide bond formation where added to the permeabilisation buffer. During treatment the formation of intermolecular (rather than intramolecular) disulphide bonds would be favoured due to the high recombinant protein concentration within the cell (whether as inclusion bodies or recently-solubilised material). This would result in either stabilised inclusion bodies or the formation of insoluble aggregates that could be separated from the soluble fraction by centrifugation.

Disulphide bonds are easily broken by the addition of a reducing agent, providing a simple procedure for the solubilisation of recombinant protein during the second stage.

The formation of disulphide bonds is a common feature in the renaturation (or refolding) of proteins and several methods of promoting disulphide bond formation have been used. In renaturation of a protein the aim is to achieve correct intramolecular disulphide

bonding. The following promoters of disulphide bond formation were considered as they are represent different approaches for the renaturation of proteins:

1. Air-oxidation in the presence of Cu^{2+} catalyst (Ahmed *et al.,* 1975);

2. Mild oxidising agent dehydroascorbic acid (Steiner and Clark, 1968);

3. Disulphide reagent 2-hydroxyethyldisulphide (2-HEDS) (King *et al.,* 1992).

Air oxidation is a widely-used method for the renaturation of thiol-containing proteins. The reduced cysteine residues are oxidised by oxygen to form disulphide bonds. The presence of the catalyst Cu^{2+} enhances the air-oxidation of many proteins, including RNase (Ahmed *et al.,* 1975). Cu^{2+} concentrations of 0.1 to 1 μM were shown to be effective. Cu^{2+}-catalysed air oxidation is, however, a slow and low yielding method when used to refold some proteins. It is also incompatible with the use of EDTA in the lysis buffer developed in Chapter 3.

The mild oxidising agent dehydroascorbic acid is used as an alternative to oxygen to oxidise the reduced cysteine residues. This technique is an unconventional method of refolding proteins that has been used in the renaturation of rat proinsulin (Steiner and Clark, 1968). Renaturation of rat proinsulin was achieved using 1 mM dehydroascorbic acid.

Disulphide reagents are commonly used in the renaturation of many recombinant proteins. Usually they are used in conjunction with the reduced form of the reagent where they aid in facilitating thiol-disulphide exchange. The refolding of IGF-I and its analogues has been successfully achieved using 2-HEDS in the presence of its reduced form (β-mercaptoethanol) (King *et al.,* 1992). The concentration of 2-HEDS used to refold the IGF-I's was 0.1 mM. Later refolding strategies for Long-R^3-IGF-I used 1 mM 2-HEDS with only trace quantities of the reducing agent DTT (Greenwood *et al.,* 1994).

Alternative reagents for disulphide bond formation that were not tested include air oxidation with Co^{2+} catalyst, the disulphide reagents oxidised glutathione and cysteine (either alone or in combination with their reduced form), Na-tetrathionate, and possibly other mild oxidising agents. These reagents could be effective at stabilising the recombinant protein within the bacterial cytoplasm by promoting disulphide bond formation, and are worthy of consideration if no success was achieved using the stated chemicals for a given protein.

4.2.1 Method

The method screened for use in the selective extraction of Long-R^3-IGF-I was comprised of two stages. The first was a general protein release step, and the second a recombinant protein release step.

The test solutions tested for the first stage contained the following: 6 M urea, 3 mM EDTA, and 0.1 M Tris buffer, plus for treatment A, 2-HEDS, (0, 5, 10, 20, 50 mM); treatment B, 5 µM Cu^{2+} (0, 0.5, 1.5, 3, 5 µM) and no EDTA, and treatment C, dehydroascorbic acid (0, 5, 10, 20, 50 mM). The pH was adjusted to 9.0 using conc. HCl. The test solution (4.75 mL) was held in a 25 mL MacCartney bottle. *E. coli* used in this experiment were induced for the expression of Long-R^3-IGF-I which accumulated as inclusion bodies in the host cytoplasm (Appendix C2). *E. coli* sample (0.25 mL) was added to each test solution which was then agitated in a shaking incubator set at 37°C. Reactions continued for 30 min. The test solution was centrifuged at 10,000 xg for 15 min and the supernatant removed from the pellet.

In the second stage the pellet was suspended in 5 mL of 6 M urea, 3 mM EDTA, 20 mM DTT, and 0.1 M Tris. The pH value was adjusted to 9.0 using conc. HCl. The centrifuge tubes containing the solution were agitated in a shaking incubator set at 37°C. Reactions continued for 30 min. The test solution was centrifuged at 10,000 xg for 15 min and the supernatant removed from the pellet.

Supernatant samples were analysed for total protein release into the extracellular phase (Appendix A3) and recombinant protein release into the extracellular phase (Appendix A4).

4.2.2 Results and Discussion

4.2.2.1 Air-oxidation in the presence of Cu^{2+} catalyst

E. coli cells were treated with disruption buffer (minus EDTA) plus the Cu^{2+} catalyst (in the first stage) and with disruption buffer plus DTT (in the second stage). The recombinant and total protein release during the each stage is shown in Figures 4.1 and 4.2, respectively.

The presence of Cu^{2+} catalyst to enhance the air oxidation of cysteine residues had little effect on the levels of either Long-R^3-IGF-I or total protein released into the extracellular phase during the first stage of treatment. There is no evidence of any stabilisation of the inclusion bodies by disulphide bond formation. Air oxidation is often slow at producing disulphide bonds in recombinant proteins (De-Bernardez and Georgiou, 1991). It is possible that solubilisation of the recombinant protein is much faster than the formation of intermolecular disulphide bonds by this procedure. The Cu^{2+}-catalytic air oxidation process had too little time to operate in a measurable manner. The lack of EDTA could also have reduced the effectiveness of cell-wall permeabilisation sufficiently, thus minimising the entry of the catalyst into the bacterial cytoplasm.

The lack of inclusion body stabilisation due to intermolecular disulphide bond formation was confirmed during the second stage of the process as there was no significant difference in the level of Long-R^3-IGF-I released between treatments with and without the Cu^{2+} catalyst. Total protein levels were slightly different, but this result is not relevant to this project.

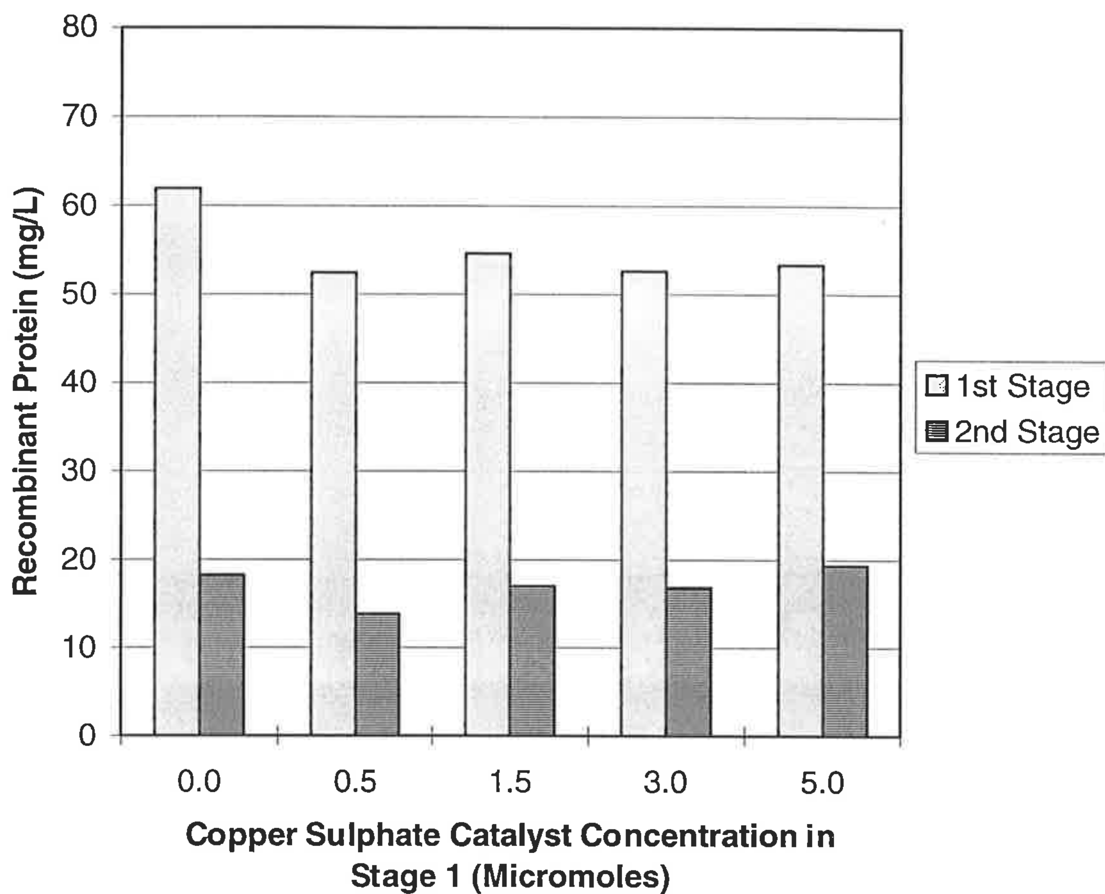


Figure 4.1 Recombinant protein release from *E. coli* during the first stage and second stage of the selective release trial using the catalyst Cu^{2+} as a promoter of disulphide bond formation in the first stage. The treatment groups contained $CuSO_4$ (0, 0.5, 1.5, 3, or 5 μM) during the first stage, and 20 mM DTT and 3 mM EDTA during the second stage. Both stages included 6 M urea, 0.1 M Tris, and operated at 37°C for 30 min.

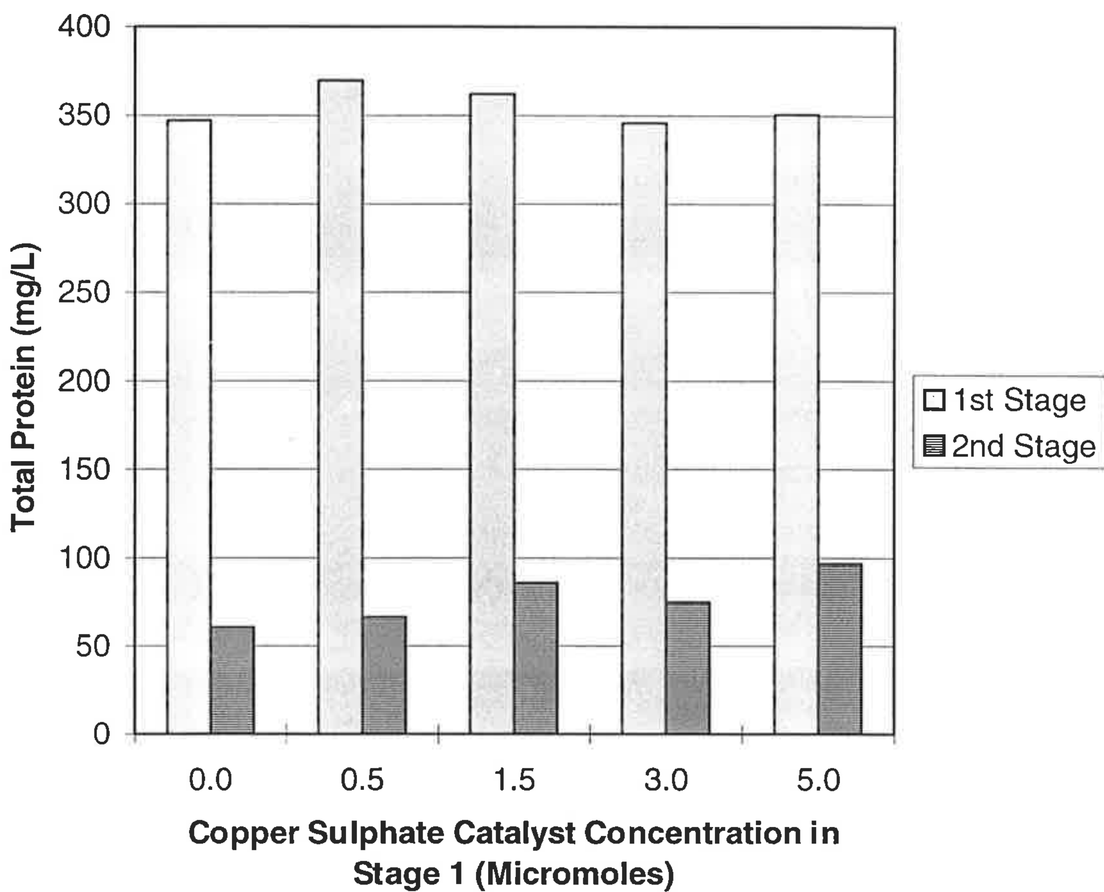


Figure 4.2 Total protein release from *E. coli* during the first stage and second stage of the selective release trial using the catalyst Cu^{2+} as a promoter of disulphide bond formation in the first stage. The treatment groups contained $CuSO_4$ (0, 0.5, 1.5, 3, or 5 μM) during the first stage, and 20 mM DTT and 3 mM EDTA during the second stage. Both stages included 6 M urea, 0.1 M Tris, and operated at 37°C for 30 min.

4.2.2.2 Dehydroascorbic acid

E. coli cells were treated with disruption buffer plus the mild oxidising agent dehydroascorbic acid (DHA) (in the first stage) and with disruption buffer plus DTT (in the second stage). Recombinant and total protein release during each stage is shown in Figures 4.3 and 4.4, respectively.

During the first stage of treatment DHA resulted in a significant reduction in the level of recombinant protein released into the extracellular phase. At DHA concentrations of 5, 10, and 20 mM a significant reduction in recombinant protein release was achieved without any reduction in total protein release. The reduction in recombinant protein release with no reduction in total protein release implies that a proportion of the material in the inclusion body is not being released from the *E. coli* cells. 50 mM DHA, however, reduced both recombinant and total protein release. This could be due to interference with permeabilisation of the cell, or the precipitation of both recombinant and cellular proteins.

During the second stage of the treatment the reducing agent DTT was not successful at releasing the recombinant protein into its soluble monomeric form. The inability of this procedure to solubilise the recombinant protein implies that it was not stabilised by the formation of intermolecular disulphide bond formation. The recombinant protein insolubility is possibly due to protein precipitation. The technique could be used in a selective release procedure if a suitable procedure for resolubilisation of the protein is discovered.

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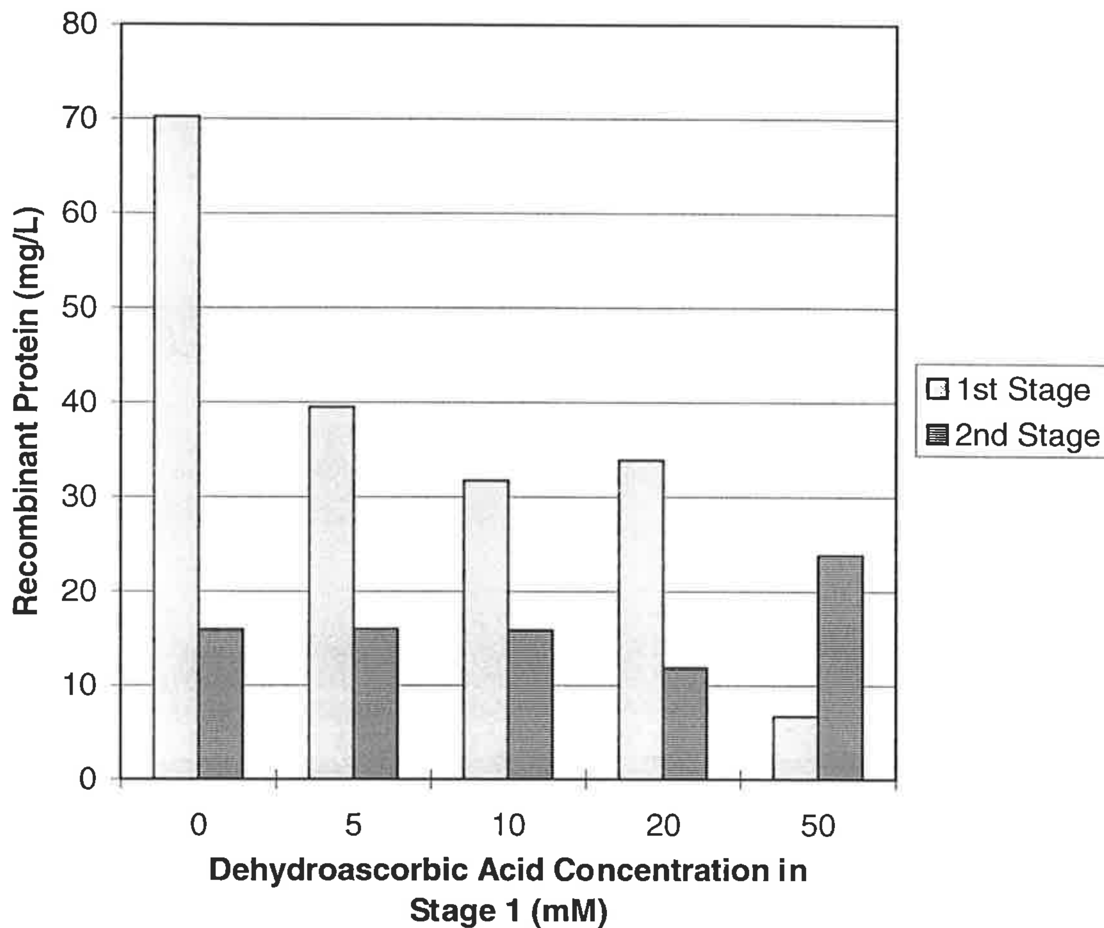

Figure 4.3 Recombinant protein release from *E. coli* during the first stage and second stage of the selective release trial using the mild oxidising agent dehydroascorbic acid (DHA) as a promoter of disulphide bond formation in the first stage. The treatment groups contained DHA (0, 5, 10, 20, or 50 mM) during the first stage and 20 mM DTT during the second stage. Both stages included 6 M urea, 3 mM EDTA, 0.1 M Tris, and operated at 37°C for 30 min.

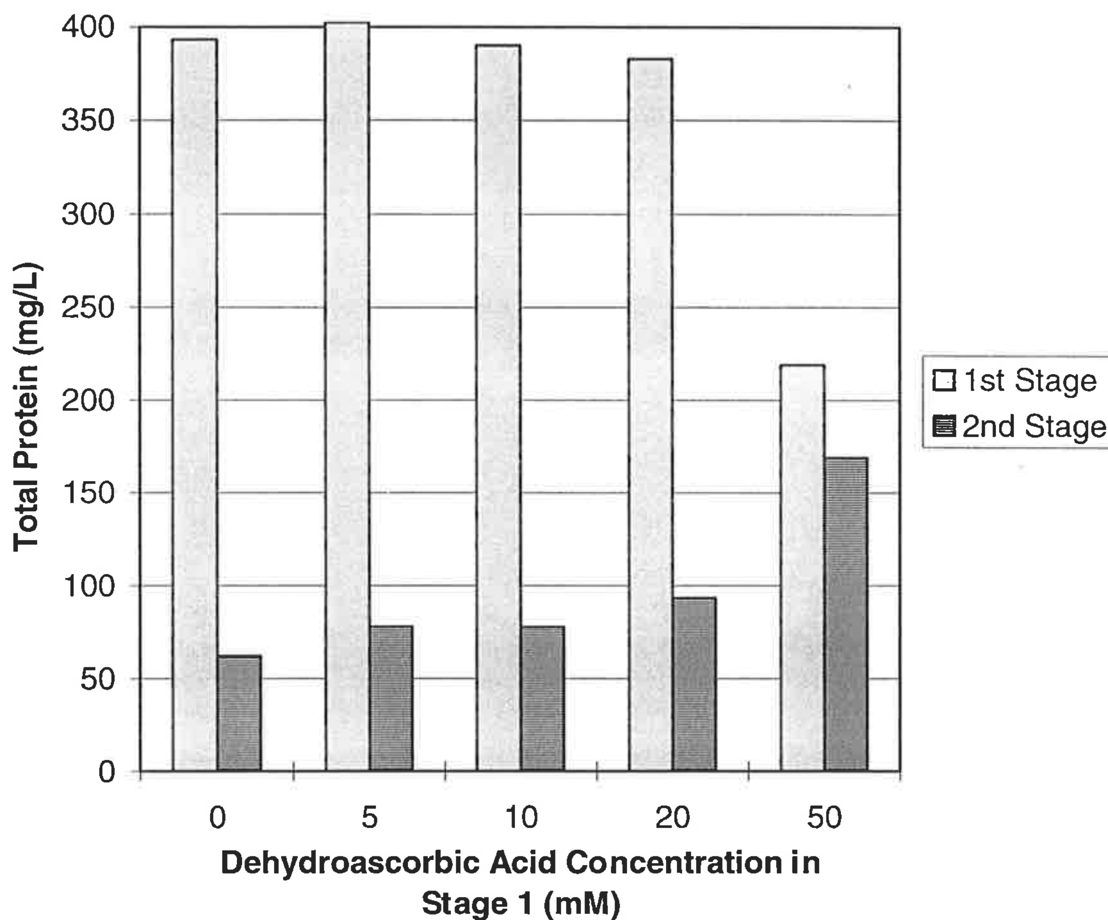

Figure 4.4 Total protein release from *E. coli* during the first stage and second stage of the selective release trial using the mild oxidising agent dehydroascorbic acid (DHA) as a promoter of disulphide bond formation in the first stage. The treatment groups contained DHA (0, 5, 10, 20, or 50 mM) during the first stage and 20 mM DTT during the second stage. Both stages included 6 M urea, 3 mM EDTA, 0.1 M Tris, and operated at 37°C for 30 min.

4.2.2.3 2-hydroxyethyldisulphide

E. coli cells were treated with disruption buffer plus the disulphide reagent 2-hydroxyethyldisulphide (2-HEDS) (in the first stage) and with disruption buffer plus DTT (in the second stage). The recombinant and total protein release during each stage is shown in Figures 4.5 and 4.6, respectively.

During the first stage of treatment 2-HEDS resulted in a significant reduction in the level of recombinant protein released into the extracellular phase. 2-HEDS treatment caused only a minor reduction in the levels of total cellular protein release. It did not interfere with cell wall permeabilisation, but did maintain the recombinant protein in the insoluble fraction.

The treatment of the insoluble fraction with disruption buffer plus the reducing agent DTT resulted in the release of a considerable proportion of monomeric recombinant protein into the extracellular phase (as demonstrated in Figure 4.5). The evidence indicates that the presence of 2-HEDS in the disruption buffer does result in the formation of intermolecular disulphide bonds which are readily broken by the addition of reducing agent. This treatment shows much promise as a method of stabilising recombinant protein inclusion bodies during the first stage of a selective release procedure.

The levels of recombinant protein and total protein in solution after the second stage of the treatment with 10 mM 2-HEDS were 60 mg/L and 130 mg/L, respectively. The purity of this material was 46% (w/w) and the overall recovery was 75% (w/w). The purity of material released by treatment of the *E. coli* cells with disruption buffer containing 20 mM DTT was 18% (w/w). The resulting improvement in purity resulting from this selective release procedure was 2.6 fold.

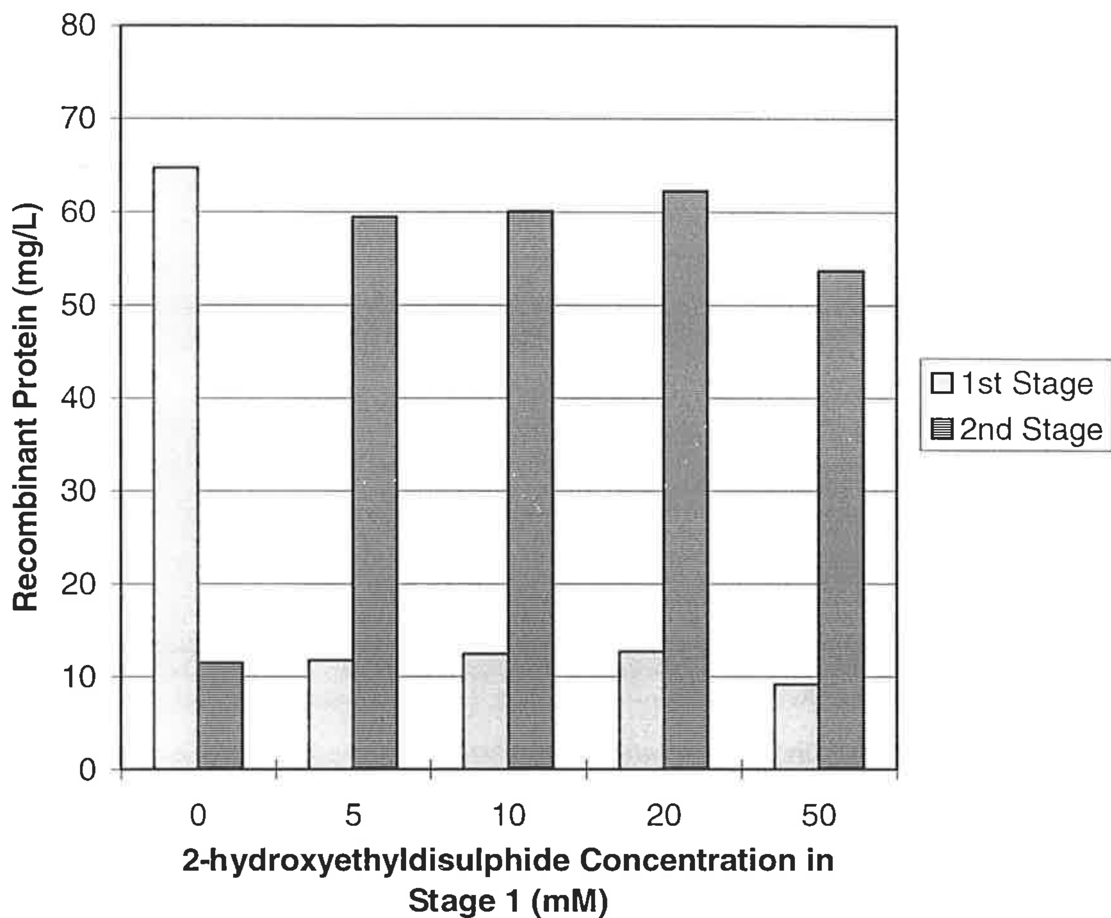

Figure 4.5 Recombinant protein release from *E. coli* during the first stage and second stage of the selective release trial using the disulphide reagent 2-hydroxyethyldisulphide (2-HEDS) as a promoter of disulphide bond formation in the first stage. The treatment groups contained 2-HEDS (0, 5, 10, 20, or 50 mM) during the first stage and 20 mM DTT during the second stage. Both stages included 6 M urea, 3 mM EDTA, 0.1 M Tris, and operated at 37°C for 30 min

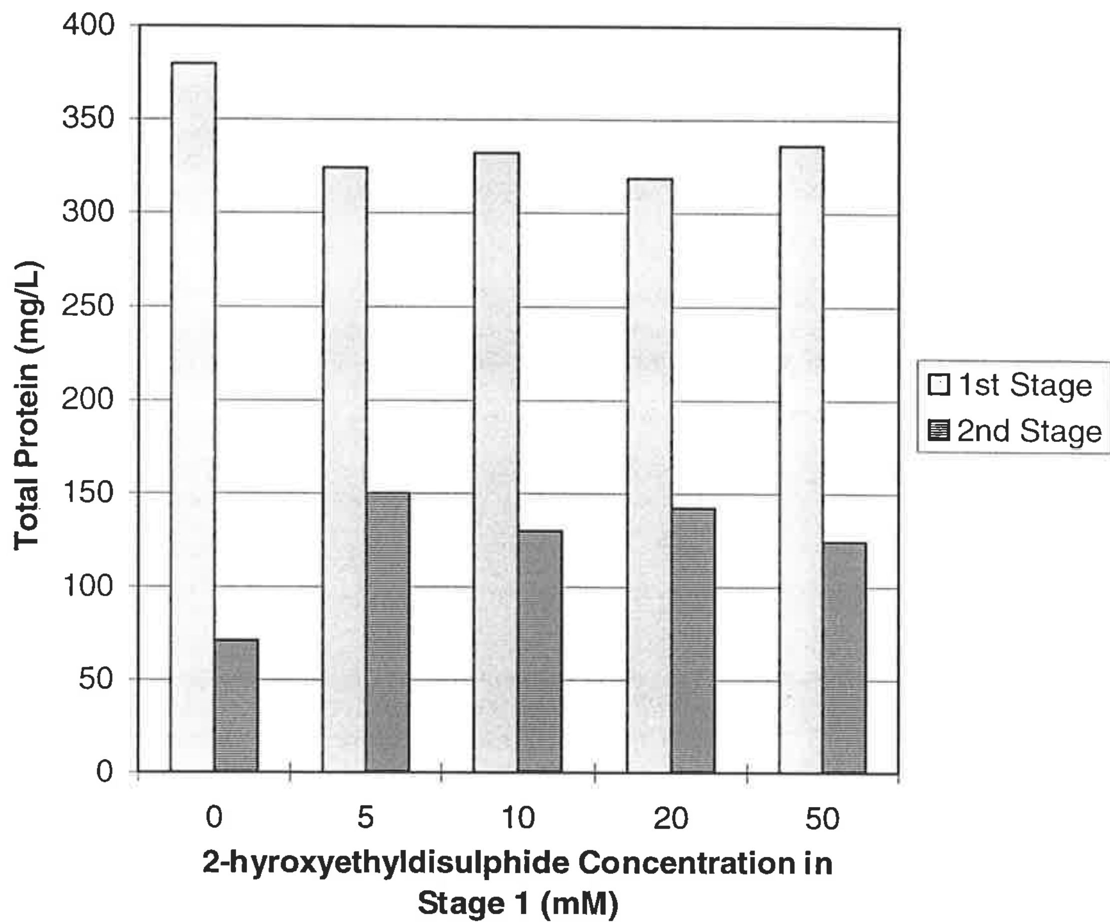

Figure 4.6 Total protein release from *E. coli* during the first stage and second stage of the selective release trial using the disulphide reagent 2-hydroxyethyldisulphide (2-HEDS) as a promoter of disulphide bond formation in the first stage. The treatment groups contained 2- HEDS (0, 5, 10, 20, or 50 mM) during the first stage and 20 mM DTT during the second stage. Both stages included 6 M urea, 3 mM EDTA, 0.1 M Tris, and operated at 37°C for 30 min.

4.2.3 Conclusion

2-HEDS proved to be the most promising promoter of disulphide bond formation that was tested for the stabilisation of recombinant protein inclusion bodies *in-vivo* (Table 4.1). 2-HEDS is known to promote intramolecular disulphide bond formation in Long-R^3-IGF-I during the renaturation of this protein (King *et al.*, 1992). The results of this experiment indicate that 2-HEDS promotes intermolecular disulphide bond formation at the high Long-R^3-IGF-I concentrations found within the *E. coli* cytoplasm. Stabilisation by intermolecular disulphide linkage is a promising method of retaining recombinant protein in the insoluble fraction, as the disulphide bond can be subsequently eliminated by the simple addition of a reducing agent, thus allowing easy recovery of the product.

Table 4.1 Comparison of the two-stage procedures tested using the different treatments to promote disulphide bond formation.

	1st Stage		2nd Stage	
Disulphide bond promoter	Total Protein (mg/L)	Rec. Protein (mg/L)	Total Protein (mg/L)	Rec. Protein (mg/L)
None	393	67	67	11
Cu^{2+} (5μM)	350	53	81	19
DHA (20 mM)	383	34	93	11
2HEDS (5mM)	*324*	*12*	*150*	*60*

4.3 Modification of the first stage

The disulphide reagent 2-HEDS was able to limit the release of the recombinant protein Long-R^3-IGF-I during the permeabilisation of *E. coli*. Addition of DTT was able to subsequently release the recombinant protein into the aqueous-phase (see section 4.2). To develop this into a selective release method the impact of the 2-HEDS concentration on the stabilisation of recombinant protein must be quantified. This variable could have an effect on product purity and recovery.

4.3.1 Method

Two experiments were conducted to cover the wide range of 2-HEDS concentrations used in this experiment.

First experiment

The test solutions used for the first stage contained the following: 6 M urea, 3 mM EDTA, 0.1 M Tris buffer, plus the following concentrations of 2-HEDS (1 µM, 3 µM, 10 µM, 30 µM, 100 µM, 300 µM, 1 mM, 3 mM, or 10 mM). pH was adjusted to 9.0 using conc. HCl. The test solution (4.75 mL) was held in a 25 mL MacCartney bottle. *E. coli* used in this experiment were induced for the expression of Long-R^3-IGF-I which accumulated as inclusion bodies in the host cytoplasm (Appendix C2). *E. coli* sample (0.25 mL) was added to each test solution which was then agitated in a shaking incubator set at 37°C. Reactions continued for 30 min. The test solution was centrifuged at 10,000 xg for 15 min and the supernatant removed from the pellet.

In the second stage the pellet was suspended in 5 mL of 6 M urea, 3 mM EDTA, 20 mM DTT, and 0.1 M Tris. The pH was adjusted to 9.0 using conc. HCl. The centrifuge tubes containing the solution were agitated in a shaking incubator set at 37°C. Reactions

continued for 30 min. The test solution was centrifuged at 10,000 xg for 15 min and the supernatant removed from the pellet.

Supernatant samples were analysed for total protein release into the extracellular phase (Appendix A3) and recombinant protein release into the extracellular phase (Appendix A4).

Second experiment.

Differs from the first experiment in that different 2-HEDS concentrations were used for the first stage (300 μM, 1 mM, 3 mM, 6 mM, 10 mM, 15 mM, 20 mM, 25 mM, or 30 mM).

4.3.2 Results and Discussion

The levels of recombinant and total protein released during the first and second stages of the extraction procedure are clearly affected by the concentration of 2-HEDS present during the first stage of the procedure.

The presence of 2-HEDS had the desired negative effect on the level of Long-R^3-IGF-I released from the permeabilised cells in the first stage (Figure 4.7). 2-HEDS concentrations greater than 15 mM had no additional effect on the reduction of Long-R^3-IGF-I release. Between 0.1 and 15 mM 2-HEDS concentration, additional reagent further reduced Long-R^3-IGF-I release. With a 2-HEDS concentration of less than 0.1 mM, the reagent had no measurable effect.

Long-R^3-IGF-I release during the second stage of the extraction process was enhanced by the addition of 2-HEDS during the first stage. Figure 4.8 shows Long-R^3-IGF-I release during the second stage, along with a fitted line for the level of Long-R^3-IGF-I expected assuming complete recovery of the insoluble material (determined using the values for the first stage of the process). The results for Long-R^3-IGF-I release are variable between

treatment groups and do not match the levels of Long-R^3-IGF-I release expected assuming complete release of the material stabilised during the first stage. The incomplete recovery is possibly due to the difficulty in effectively resuspending the insoluble material after the centrifugation step. The resultant poor mixing during the second step might reduce the effectiveness of solubilisation. The presence of strands of nucleic acid in the insoluble material probably stabilises the pelleted material and makes resuspension difficult.

Total protein release during the first stage is shown in Figure 4.9. The 2-HEDS concentration during the first stage of the procedure only had a minor impact on total protein release. The minor effect is possibly due to recombinant protein retention during the first stage of the process.

The process as it stands does not recover all of the Long-R^3-IGF-I that should be available for solubilisation during the second stage of the process (determined by mechanical disruption). The Long-R^3-IGF-I concentrations recovered during the second stage were also highly variable. The recovery and relative concentration of Long-R^3-IGF-I (as a percentage of total protein) were calculated and compared to the maximum expected values (assuming complete solubilisation of the stabilised Long-R^3-IGF-I during the second stage of the procedure). The results from the second stage of the selective release procedure are shown in Table 4.2. Of the Long-R^3-IGF-I present in the *E. coli* cell 74% (w/w) is available for recovery following optimisation of the second stage of the procedure. The figures also show that an appreciable purification of the recombinant protein is achieved during the procedure (improving from approximately 18% to 51% (w/w) of the total protein). The purification of the product using this procedure matches levels commonly reached using traditional recovery processes (Table 3.1).

Optimisation of the second stage of the procedure to achieve recovery levels closer to the maximum possible is important.

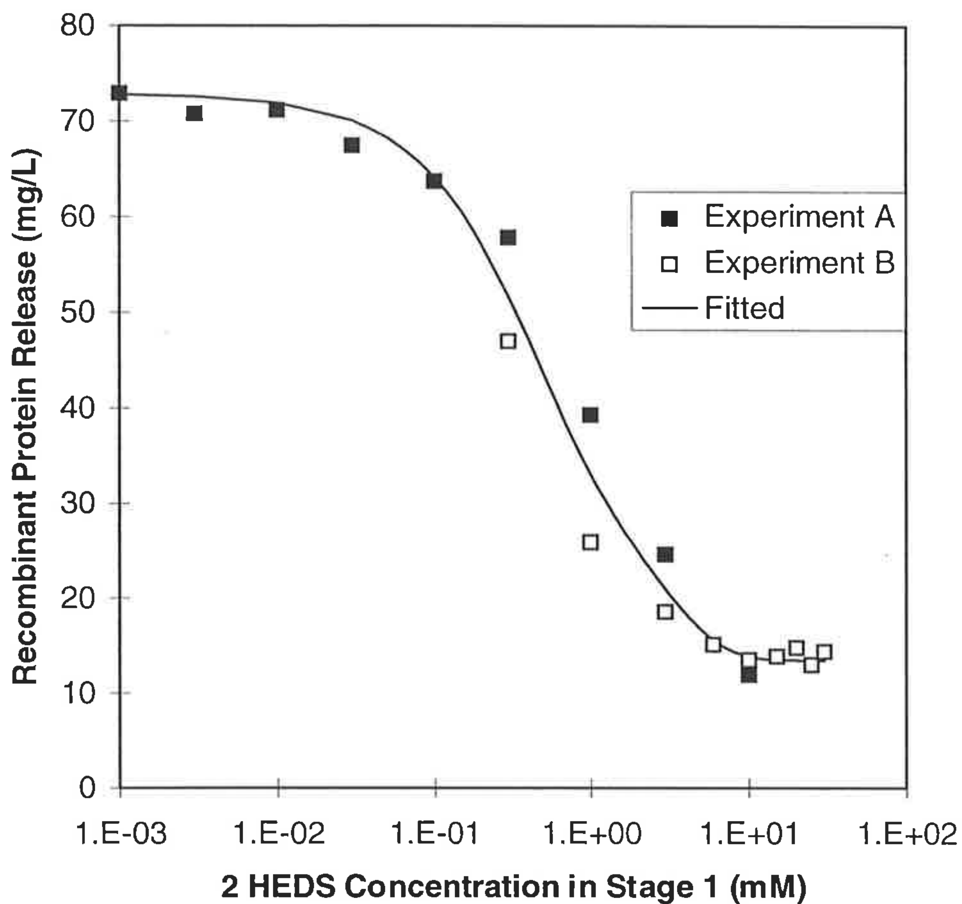

Figure 4.7 Recombinant protein release from *E. coli* during the first stage of treatment with various 2-hydroxyethyldisulphide (2-HEDS) concentrations included in the stage 1 disruption buffer. Treatment groups contained 2-HEDS (10^{-3} to 30 mM) during the first stage and 20 mM DTT during the second stage. Both stages included 6 M urea, 3 mM EDTA, 0.1 M Tris, and operated at 37°C.

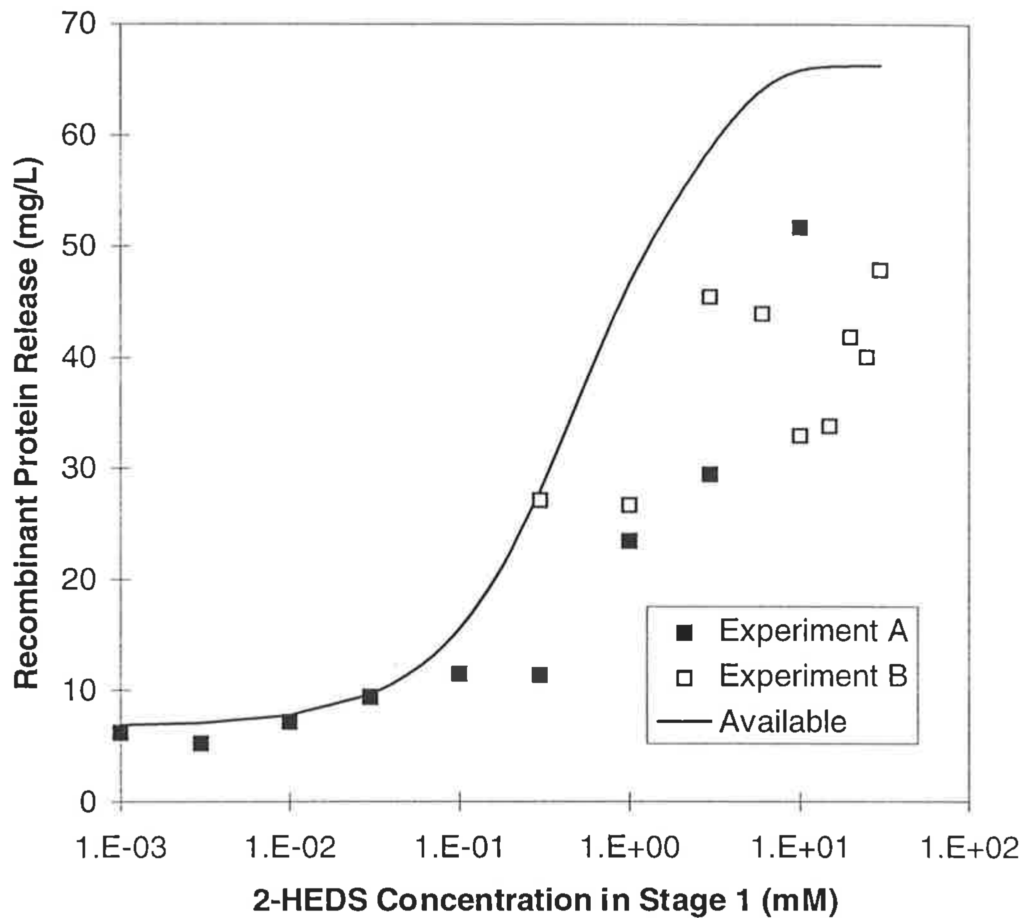

Figure 4.8 Recombinant protein release from *E. coli* during the second stage of the treatment with various 2-hydroxyethyldisulphide (2-HEDS) concentrations included in the stage 1 disruption buffer. Treatment groups contained 2-HEDS (10^{-3} to 30 mM) during the first stage and 20 mM DTT during the second stage. Both stages included 6 M urea, 3 mM EDTA, 0.1 M Tris, and operated at 37°C.

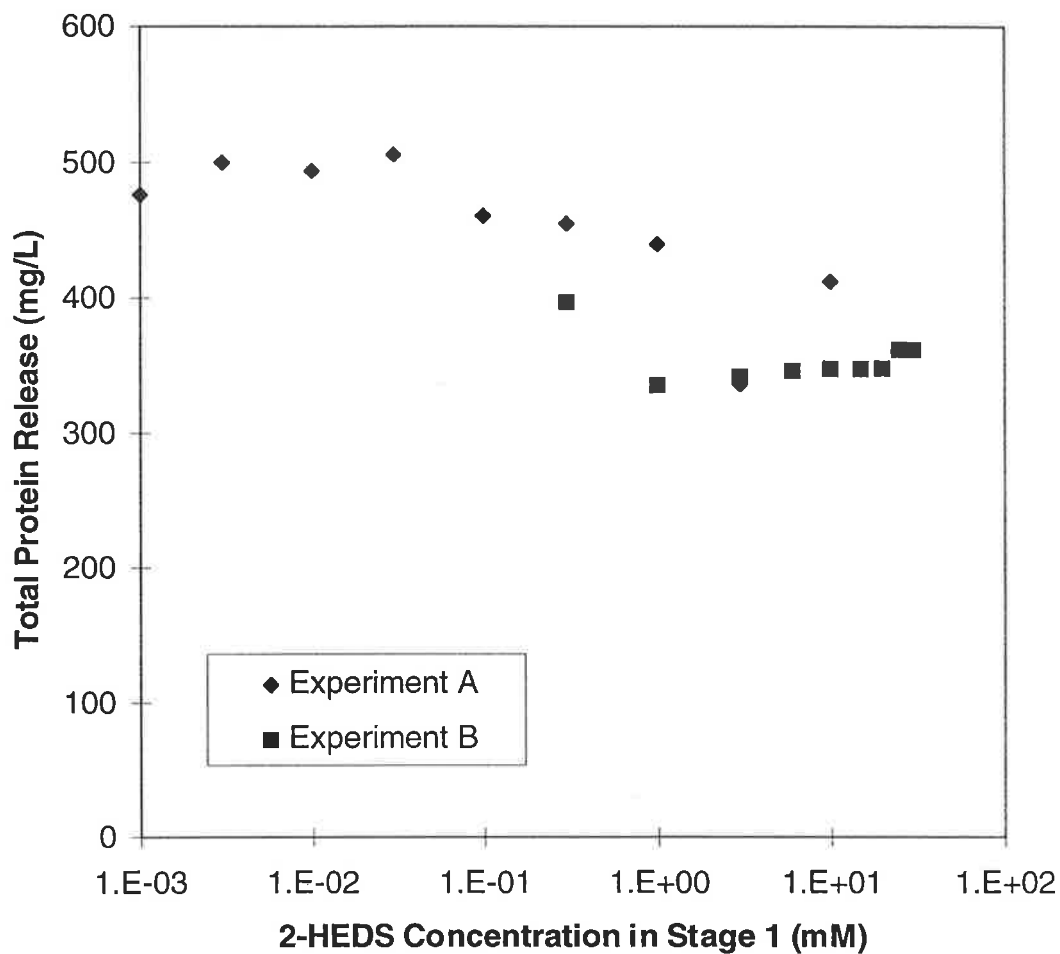

Figure 4.9 Total protein release from *E. coli* during the first stage of the selective extraction procedure. The treatment groups contained 2-hydroxyethyldisulphide (0.3 to 30 mM) during the first stage and 20 mM DTT during the second stage. Both stages included 6 M urea, 3 mM EDTA, 0.1 M Tris, and operated at 37°C.

Table 4.2 Actual and theoretical recombinant protein purity (expressed as relative concentration) and recovery levels from the second stage of the selective release process.

2-HEDS Concentration in Stage 1 (mM)	Relative Concentration of Recombinant Protein in Extracellular Phase after Stage 2. (% w/w)		Recovery of Recombinant Protein in Extracellular Phase after Stage 2. (% w/w)	
	Actual*	Available**	Actual*	Available**
0	14	14	10	10
0.1	28	25	30	39
1.0	32	52	35	57
3.0	51	62	57	69
10	38	67	42	74
30	51	67	57	74

* Actual release after stage 2.

** Maximum possible based on stage 1 results (i.e., total Long-R^3-IGF-I extracted using nonselective extraction less the amount extracted at the stated 2-HEDS concentration.

4.4 Refinement of the second stage

The research detailed in section 4.3 demonstrated that the addition of 10 mM or more 2-HEDS during the first stage resulted in maximal Long-R^3-IGF-I released during the second stage. The levels of Long-R^3-IGF-I released during the second stage were, however, variable and the recovery was incomplete. To improve the selective extraction procedure the recovery of Long-R^3-IGF-I during the second stage must be maximised.

The research detailed in this section attempts to maximise Long-R^3-IGF-I release during the second stage of the extraction process. The following modifications were tested to determine whether they improved the recovery of the retained recombinant protein. Resuspension of the insoluble pellet was conducted using the relatively mild vortex mixing and by the more vigorous action of a sonicator. Improvement in pellet dispersion will presumably aid mixing and increase the surface area to volume ratio of the insoluble fraction, enhancing solubilisation. A wash step was included prior to the second stage to ensure adequate removal of the soluble fraction. The third stage was a repeat of the second stage to check that the dissolution step was effective.

4.4.1 Method

E. coli used in this experiment were induced for the expression of Long-R^3-IGF-I which accumulated as inclusion bodies in the host cytoplasm (Appendix C2).

Standard solutions

Solution A consisted of 8 M urea, 4 mM EDTA, 20 mM 2-HEDS, 0.1 M Tris at pH 9.0. Solution B consisted of 8 M urea, 4 mM EDTA, 27 mM DTT, 0.1 M Tris at pH 9.0.

The *E. coli* sample (0.25 mL) was added to 20 mL centrifuge tubes. The following treatment steps were taken and samples taken for analysis.

Treatment 1: Standard Protocol

1. Commence the first stage with the addition of 4.75 mL of solution A, agitate at 37°C for 30 min.
2. Centrifuge at 10,000 xg for 15 min.
3. Remove supernatant (sample collected).
4. Resuspend in 1.25 mL of 0.1 M Tris.
5. Commence the second stage with the addition of 4.75 mL of solution B, agitate at 37°C for 30 min.
6. Centrifuge at 10,000 xg for 15 min.
7. Remove supernatant (sample collected).
8. Steps 5 to 7 were repeated for the additional third stage.

Treatment 2: Standard Protocol plus a 0.1M Tris Wash Step

1. Commence the first stage with the addition of 4.75 mL of solution A, agitate at 37°C for 30 min.
2. Centrifuge at 10,000 xg for 15 min.
3. Remove supernatant (sample collected).
4. Resuspend in 2 mL of 0.1 M Tris and mix for 5 min.
5. Centrifuge at 10,000 xg for 15 min.
6. Remove supernatant (sample collected).
7. Resuspend in 1.25 mL of 0.1 M Tris.
8. Commence the second stage with the addition of 4.75 mL of solution B, agitate at 37°C for 30 min.
9. Centrifuge at 10,000 xg for 15 min.
10. Remove supernatant (sample collected).
11. Steps 8 to 10 were repeated for the additional third stage.

Treatment 3: Standard Protocol plus Pellet Dispersion by Sonication

1. Commence the first stage with the addition of 4.75 mL of solution A, agitate at 37°C for 30 min.
2. Centrifuge at 10,000 xg for 15 min.
3. Remove supernatant.
4. Resuspend in 1.25 mL of 0.1 M Tris.
5. Homogenise with sonicator for 2 seconds.
6. Commence the second stage with the addition of 4.75 mL of solution B, agitate at 37°C for 30 min.
7. Centrifuge at 10,000 xg for 15 min.
8. Remove supernatant (sample collected).
9. Steps 6 to 8 were repeated for the additional third stage.

Treatment 4: Standard Protocol plus 0.1M Tris Wash and Pellet Dispersion by Sonication

1. Commence the first stage with the addition of 4.75 mL of solution A, agitate at 37°C for 30 min.
2. Centrifuge at 10,000 xg for 15 min.
3. Remove supernatant (sample collected).
4. Resuspend in 2 mL of 0.1 M Tris and mix for 5 min.
5. Centrifuge at 10,000 xg for 15 min.
6. Remove supernatant (sample collected).
7. Resuspend in 1.25 mL of 0.1 M Tris.
8. Homogenise with Sonicator for 2 seconds.
9. Commence the second stage with the addition of 4.75 mL of solution B, agitate at 37°C for 30 min.
10. Centrifuge at 10,000 xg for 15 min.
11. Remove supernatant (sample collected).
12. Steps 9 to 11 were repeated for the additional third stage.

Analysis

Samples were analysed for total protein release (Appendix A3) and recombinant protein release (Appendix A4).

4.4.2 Results and discussion

Recombinant and total protein release from the various treatment groups are shown in Figures 4.10 and 4.11, respectively. The results are expressed as the total micrograms of protein released into the extracellular phase (to enable an overall mass balance to be easily constructed).

First stage

The first stage of treatment resulted in the release of 70-80 μg (equivalent to 15-16 mg/L) Long-R^3-IGF-I. Total protein release was 1660-1670 μg (equivalent to 332-334 mg/L). These levels are similar to those achieved in section 4.3 of this thesis.

Intermediate steps

Washing of the insoluble fraction after centrifugation by resuspension of the insoluble fraction in 0.1 M Tris buffer followed by recentrifugation (treatments 2 and 4) resulted in the release of little Long-R^3-IGF-I and total protein from any of the treatment groups. This procedure does not provide any significant advantage to this procedure. A wash protocol using a another solution could not provide a reduction in total protein without sacrificing much Long-R^3-IGF-I.

Suspension of the insoluble fraction in 0.1 M Tris buffer followed by brief sonication was effective at dissipating the insoluble fraction though the solution. Visual observation

showed the solution transformed from a irregular pellet to a homogenous cloudy suspension following approximately 2 seconds of treatment. Non-sonicated pellets were difficult to resuspend and could not be evenly resuspended by agitation.

Second stage

The second stage of treatment resulted in the release of varying quantities of recombinant protein into the extracellular phase. The major difference within the treatment groups was due to the resuspension of the pellet. The two non-sonicated treatment groups (1 and 2) released 190-280 μg (equivalent to 37-57 mg/L) Long-R^3-IGF-I compared to the 360-380 μg (equivalent to 72-76 mg/L) Long-R^3-IGF-I for the two sonicated treatment groups (3 and 4). The non-sonicated groups (1 and 2) only recovered 81% and 60% (w/w), respectively of the available Long-R^3-IGF-I during stages 1 and 2. This indicates that the remaining Long-R^3-IGF-I is still located in the insoluble fraction after completion of the second stage.

Third stage

Some of the missing Long-R^3-IGF-I was recovered by the repetition of the second stage, demonstrating that the material was still in the insoluble fraction and could be solubilised by solution B. The major difference within the treatment groups was due to the resuspension of the pellet. The two non-sonicated treatment groups (1 and 2) released 70-110 μg (equivalent to 14-22 mg/L) Long-R^3-IGF-I during the third stage compared to only 16-19 μg (equivalent to 3-4 mg/L) Long-R^3-IGF-I for the two sonicated treatment groups (3 and 4).

Discussion

This experiment demonstrates that the selective extraction procedure can result in the recovery of 82 to 87% (w/w) of the Long-R^3-IGF-I during the second stage of the procedure (Table 4.4). Effective resuspension of the insoluble fraction prior to the second

stage proved important for the efficient recovery of the insoluble Long-R^3-IGF-I. Sonication is very effective at resuspending the insoluble fraction. This operation could be satisfactorily undertaken by other procedures such as high shear mixing or by avoiding pellet formation in the first instance.

The purity of the Long-R^3-IGF-I extracted during the second stage is 42 to 50% (w/w) based on total protein (Table 4.5) and constitutes a purification factor of 2.5 to 2.9 compared to the nonselective extraction method.

The selective extraction procedure can be used for the recombinant protein Long-R^3-IGF-I at the laboratory scale with recovery of the bulk of the recombinant protein and with significant purification. The procedure as reported is not amenable to direct scale-up, as operations such as batch centrifugation and sonication are included. The procedure still requires development before it can be considered a practical commercial alternative.

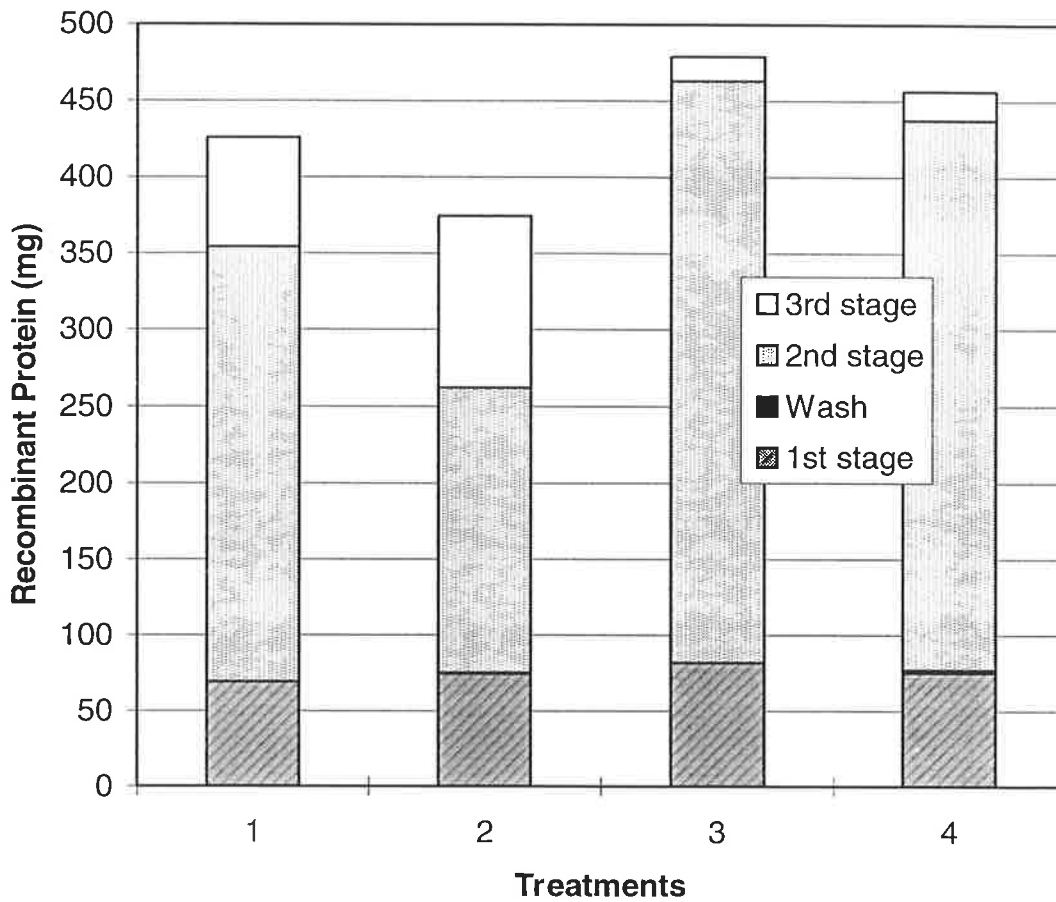

Figure 4.10 Recombinant protein release from *E. coli* during the various treatments (described in section 4.4.1). Both stages of treatment used 6 M urea, 3 mM EDTA, , 0.1 M Tris buffer (pH 9.0), and operated at 37°C. The solution contained 15 mM 2-HEDS for the first stage and 20 mM DTT for the second and third stages.

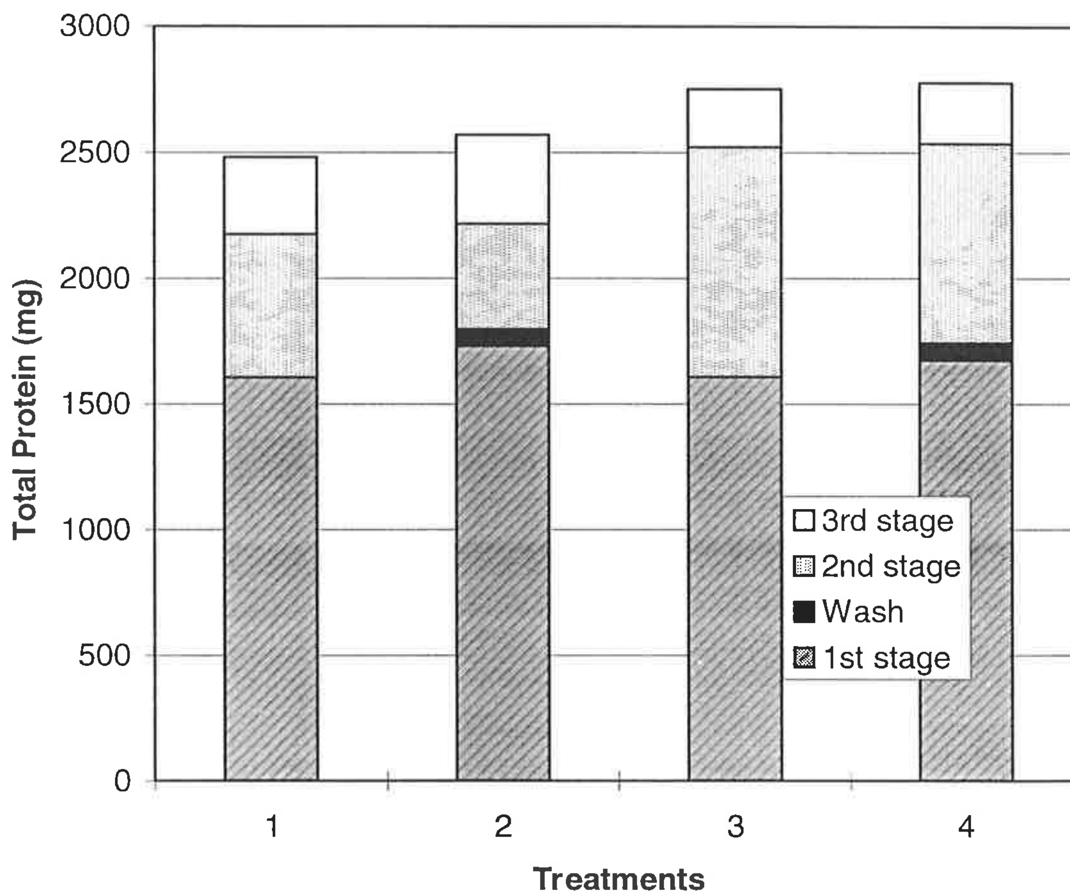

Figure 4.11 Total protein release from *E. coli* during the various treatments (described in section 4.4.1). Both stages of treatment used 6 M urea, 3 mM EDTA, , 0.1 M Tris buffer (pH 9.0), and operated at 37°C. The solution contained 15 mM 2-HEDS for the first stage and 20 mM DTT for the second and third stages.

Table 4.3 Percentage recovery of Long-R^3-IGF-I during the processing of *E. coli* cells treated with 15 mM 2-HEDS during the first stage.

Treatment	Recovery of Long-R^3-IGF-I (%w/w)		
	1st Stage	2nd Stage	3rd Stage
Standard Protocol (1)	16	65	16
Standard Protocol plus 0.1M Tris Wash (2)	17	43	26
Standard Protocol plus Pellet Dispersion (3)	17	80	3
Standard Protocol plus 0.1M Tris Wash and Pellet Dispersion (4)	16	79	4

Table 4.4 Relative concentration of Long-R^3-IGF-I (expressed as a percentage of total protein) during the processing of *E. coli* cells treated with 15 mM 2-HEDS during the first stage.

Treatment	Relative Concentration of Long-R^3-IGF-I (%w/w)		
	1st Stage	2nd Stage	3rd Stage
Standard Protocol (1)	4	50	23
Standard Protocol plus 0.1M Tris Wash (2)	4	47	32
Standard Protocol plus Pellet Dispersion (3)	5	42	7
Standard Protocol plus 0.1M Tris Wash and Pellet Dispersion (4)	5	46	8

4.5 Comparison of selective, nonselective, and conventional extraction

In this section, the two extraction techniques, namely nonselective extraction (chapter 3) and selective extraction (this chapter), were compared to conventional extraction. The conventional extraction technique used here includes the following steps: mechanical disruption using a high-pressure homogeniser, centrifugation, and *in-vitro* inclusion body solubilisation. The cell samples all originated from the same fermentation. The extraction procedures were carried out simultaneously using the same reagents to allow the techniques to be directly compared. The samples were analysed quantitatively for recombinant (HPLC) and total protein (Bradford), and qualitatively by PAGE. The qualitative analysis provides visual confirmation as to the relative purity of the preparations. It also can be used to estimate the presence of particular bacterial proteins associated with the outer-membrane such as OmpA, OmpC, and OmpF. As the outer-membrane is the source of the important contaminants OmpT and lipopolysaccharide, a measure of removal is useful.

4.5.1 Method

E. coli used in this experiment were induced for the expression of Long-R^3-IGF-I which accumulated as inclusion bodies in the host cytoplasm (Appendix C2).

Selective extraction

E. coli cells (the equivalent of 5 mL at A_{600} 4) were centrifuged (10,000 xg, 15 min, 4°C), and the pellet resuspended in 1.25 mL of 0.1 M Tris-HCl and 3.75 mL of 8 M urea, 4 mM EDTA, 0.1 M Tris-HCl, and 20 mM 2-HEDS (adjusted to pH 9.0) to promote inclusion body stability. The solution was agitated in a shaking incubator set at 37°C for 30 min then centrifuged at (10,000 xg, 15 min, 4°C). The second stage involved taking the pellet (from stage 1) resuspending it in 1.25 mL of 0.1 M Tris-HCl (using 1 second of sonication) and 3.75 mL of 8 M urea, 4 mM EDTA, 0.1 M Tris-HCl, and 27 mM DTT (adjusted to pH 9.0) to promote inclusion body solubilisation. The solution was agitated in

a shaking incubator set at 37°C for 30 min and was then centrifuged at (10,000 xg, 15 min, 4°C). The recombinant protein was located in the supernatant.

Nonselective extraction

E. coli cells (the equivalent of 5 mL at A_{600} 4) were centrifuged (10,000 xg, 15 min, 4°C), and the pellet resuspended in 1.25 mL of 0.1 M Tris-HCl and 3.75 mL of 8 M urea, 4 mM EDTA, 0.1 M Tris-HCl, and 27 mM DTT (adjusted to pH 9.0). The solution was agitated in a shaking incubator set at 37°C for 30 min. The solution was then centrifuged at 10,000 xg for 15 min at 4°C. The recombinant protein was located in the supernatant.

Conventional Extraction

E. coli suspension (1L) was homogenised by three passes through an APV Gaulin 15MR high-pressure homogeniser (CD valve) set at 56 MPa. Cell homogenate (the equivalent of 5 mL at A_{600} 4 prior to homogenization) was centrifuged (10,000 xg, 15 min, 4°C) and the pellet resuspended in 1.25 mL of 0.1 M Tris-HCl and 3.75 mL of 8 M urea, 4 mM EDTA, 0.1 M Tris-HCl, and 27 mM DTT (adjusted to pH 9.0). The solution was agitated in a shaking incubator set at 37°C for 30 min. The solution was then centrifuged (10,000 xg, 15 min, 4°C). The recombinant protein was located in the supernatant.

Analysis

Samples of the supernatant fractions collected during operation of the extraction processes were subjected to analysis for recombinant protein by HPLC (Appendix A4), total protein by Bradford analysis (Appendix A3), and PAGE analysis (Appendix A5).

4.5.2 Results and Discussion

The levels of recombinant and total protein recovered during the operation of conventional, nonselective, and selective extraction procedures are shown in Table 4.5. These results can be directly compared as the extraction procedures were carried out using the same fermentation broth, using the same stock solutions, at the same time, and using the same volumetric measurements.

The levels of recombinant protein (Long-R^3-IGF-I) extracted by each procedure are comparable. Conventional extraction results in a soluble fraction and an insoluble fraction. The combined Long-R^3-IGF-I from the two fractions is 105 mg/L. Nonselective extraction has a single extract containing 113 mg/L of Long-R^3-IGF-I. The combined extracts from selective extraction gave 110 mg/L. This confirms that the results for the recombinant protein extraction techniques can be directly compared. The figure of 113 mg/L was used as the estimation of 100% (w/w) recovery for the following assessment.

Table 4.5 Recombinant and total protein release during conventional, nonselective, and selective extraction procedures operated in parallel.

	Total Protein Conc. (mg/L)	Recombinant Protein Conc. (mg/L)
Conventional Extraction Soluble Fraction	474	6
Conventional Extraction Insoluble Fraction	242	99
Nonselective Extraction	663	113
Selective Extraction 1st Stage	421	19
Selective Extraction 2nd Stage	197	91

The conventional extraction procedure achieved an 88% (w/w) recovery of the recombinant protein at a purity of 41% (w/w) in the solubilised insoluble fraction. Nonselective extraction achieved 100% (w/w) recovery of the recombinant protein at a purity of 17% (w/w). Selective extraction achieved 83% (w/w) recovery of the recombinant protein at a purity of 46% (w/w) in the second-stage extract. The selective extraction has a purification factor of 2.7 compared to the conventional extraction purification factor of 2.4. This indicates that, by these criteria, selective extraction provides an extract with comparable purity and recovery to that achieved by the conventional approach.

The product of the extraction techniques can be seen in the PAGE analysis shown in Figure 4.12. The bands corresponding to the soluble fraction from conventional extraction (A1), to nonselective extraction (B), and to the first stage of selective extraction (C1) are loaded at the same level to allow direct comparison. The lanes corresponding to the insoluble fraction from conventional extraction (A2) and to the second stage of selective extraction (C2) are loaded at six times the level of the others to allow visualisation of the contaminating proteins. These lanes can be directly compared with each other.

The PAGE analysis of the nonselective extract shows that it is similar to the soluble fraction from conventional extraction, and to the first stage of selective extraction with the addition of a band corresponding to Long-R^3-IGF-I. This is not surprising as this technique does not provide any significant selectivity.

The lanes in Figure 4.12 corresponding to the insoluble fraction from conventional extraction and to the second stage of selective extraction show that the protein profiles are reasonably similar. The most obvious difference between the two lanes is the significantly lower levels of the bands corresponding to OmpC/F and OmpA in the selective extract. This decrease in the levels of outer-membrane protein (Omp) could be due to the inability of the procedure to solubilise Omp's from the outer-membrane fraction. OmpT is a serine protease located in the outer-membrane that degrades many recombinant proteins during and after their extraction. If further work proves that selective extraction results in reduced levels of OmpT in the extract, then this method would offer a significant advance

over current technology for the recovery of susceptible recombinant proteins such as IGF-2.

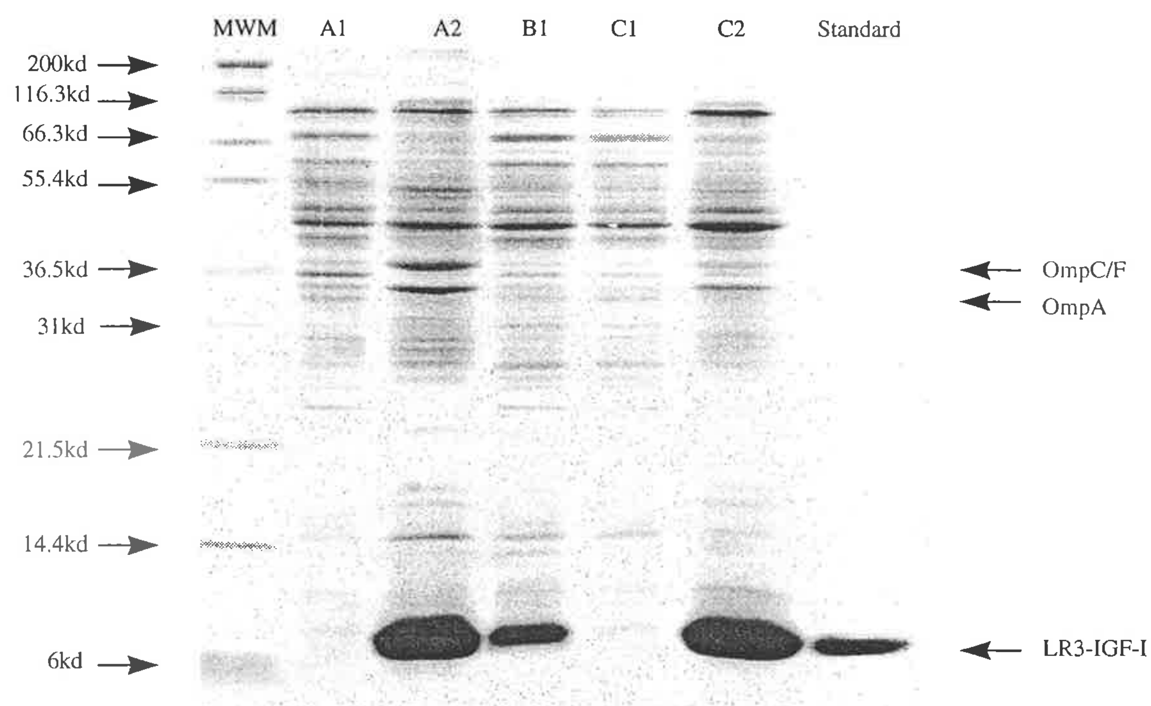

Figure 4.12 PAGE analysis of fractions for the three extraction methods operated in parrallel.

A1: conventional extraction, soluble fraction;

A2: conventional extraction, insoluble fraction;

B1: nonselective extract;

C1: selective extraction, first stage extract;

C2: selective extraction, second stage extract.

Note samples A2 and C2 were loaded at 6x the amount to allow clear visualisation of the contaminating protein bands.

4.6 Conclusion

Addition of 2-HEDS to the basic permeabilisation solution (6 M urea, 3 mM EDTA, 0.1 M Tris at pH 9.0) was effective at limiting the release of the recombinant protein (Long-R^3-IGF-I) while not compromising the cell permeabilisation process. 2-HEDS probably promotes intermolecular disulphide bond formation that maintains the Long-R^3-IGF-I in an insoluble form. It is unclear whether the inclusion bodies are maintained or new aggregates are formed. The product-containing insoluble fraction is easily separated from the soluble fraction by centrifugation. Addition of the reducing agent DTT to the basic solution allowed subsequent Long-R^3-IGF-I solubilisation from the insoluble fraction.

The two-stage process using 15 mM 2-HEDS in the first stage and 20 mM DTT in the second stage was able to recover 83% (w/w) of the available Long-R^3-IGF-I with a purification factor of 2.7. This performance is comparable to conventional extraction.

The two-stage selective extraction process described in this chapter uses laboratory equipment (such as a batch centrifuge and sonicator) and was carried out at a small scale (5 mL). As a laboratory procedure this techniques shows immediate promise and would be of interest to research personnel carrying out the small scale extraction of recombinant proteins from *E. coli*. It has a great advantage that it does not require expensive pieces of equipment used for mechanical disruption. Resuspension of the insoluble fraction after centrifugation is the only factor that can present a problem. Sonication is effective but probably leads to the release of material entrapped inside the remaining cell structures. A milder procedure could prove superior.

Selective extraction is significantly different from conventional extraction. The procedures rely on different principles to achieve selectivity. Conventional extraction is selective as the soluble fraction (under the conditions in the homogenate) is removed by centrifugation. Selective release separates the recombinant protein from the bulk of the contaminants using the recombinant protein's ability to form intermolecular disulphide bonds and thus remain insoluble during permeabilisation of the cell wall. Contaminants in

the selective release procedure are likely to be those that are trapped in the inclusion body (or formed aggregates), inefficiencies in the permeabilisation procedure, and material that is also able to form intermolecular disulphide bonds. The protein profile as illustrated by PAGE analysis is significantly different for the two extraction procedures. The most important difference is the reduced levels of outer membrane proteins in the selectively-released material. If further work proves that the level of the outer membrane protease OmpT is also reduced, this process could prove invaluable for the extraction of OmpT-sensitive proteins such as IGF-2 (Wong, 1996).

The selective extraction process as it stands is not amenable to scale-up for industrial application. Batch centrifugation and sonication are not procedures that are easily scaled-up. These need to be replaced with alternative units before pilot or production scale application can be envisaged.

CHAPTER 5

PROTOTYPE PILOT-SCALE SELECTIVE EXTRACTION PROCESS

Summary

The selective extraction procedure developed in Chapter 4 was modified to make the procedure amenable to scale-up. A prototype pilot-scale system was developed to test the procedure. Diafiltration was used to replace batch centrifugation. The permeabilisation and solubilisation steps were carried out in a stirred reactor with a 1.45 L working volume (in place of the tubes with a 5 mL working volume).

The recovery and purity of the recombinant protein extracted using the prototype system did not match the levels achieved using the lab-scale procedure. A recovery of 51% (w/w) of product at a purity of 32% (w/w) was obtained without any optimisation studies. On optimisation, it was estimated that this procedure has the potential to recover 79% (w/w) Long-R^3-IGF-I at a purity of 69% (w/w). The improved purity relative to the small-scale procedure is due to the selectivity provided by the final filtration step. The optimised pilot-scale selective extraction process would be capable of processing large quantities of Long-R^3-IGF-I at high efficiency

5.1 Introduction

The two-stage procedure developed for the extraction of the recombinant protein Long-R^3-IGF-I from intact *E. coli* cells (described in chapter 4 of this thesis) is an effective laboratory procedure. It is effective at the extraction of recombinant protein from intact *E. coli* recovering 83% (w/w) of the recombinant protein, while providing a degree of selectivity with a purification factor of 2.7.

The laboratory-scale selective extraction procedure needs to be modified to give a procedure that is amenable to scale-up. Equipment such as batch centrifuges and sonicators are satisfactory for laboratory work but are not generally suitable for industrial applications. At the industrial scale, separation of insoluble and soluble fractions is often performed using a continuous-flow centrifuge (e.g., a disc-stack centrifuge) or by diafiltration. Disc-stack centrifuges separate the fractions according to their differences in size and buoyant density, while diafiltration separates material according to size. The effectiveness of centrifugation is reduced by increases in solution viscosity, a scenario that is likely with permeabilisation as the cell concentration is increased to achieve an economic process. Diafiltration is less sensitive to increases in viscosity and was deemed the most appropriate separation technology for incorporation into a pilot-plant prototype.

The use of sonication to resuspend insoluble material is impractical at an industrial scale. However, pellet resuspension should not be necessary. Both continuous centrifugation and diafiltration are unlikely to lead to tightly-packed pellets of insoluble material generated during laboratory batch centrifugation.

The prototype system described in this chapter uses a stirred tank in place of the 25 mL MacCartney bottle used in the earlier experiments. Separation is achieved using a 100 kilodalton molecular weight cut-off tangential flow filter in place of batch centrifugation. The compositions of the permeabilisation buffer and the solubilisation buffer are identical to those used in the laboratory process. An in-line high-shear mixer was employed to break the nucleic acid polymers and reduce solution viscosity. However, this proved unnecessary at

the low cell concentrations used to test the system. The laboratory-scale and pilot-scale selective extraction procedures are shown in detail in Figure 5.1, allowing comparison of the corresponding process steps.

5.1.1 Research goals

The research presented in this chapter aims to:

- design a procedure (based on work in the preceding chapters) that will selectively extract the recombinant protein Long-R^3-IGF-I from intact *E. coli* cells using technology that can be applied at a commercial scale;

- build a prototype rig for the selective extraction of Long-R^3-IGF-I from intact *E. coli* cells;

- determine the operational characteristics of the prototype rig;

- modify the system to reach the performance achieved in the laboratory-scale procedure.

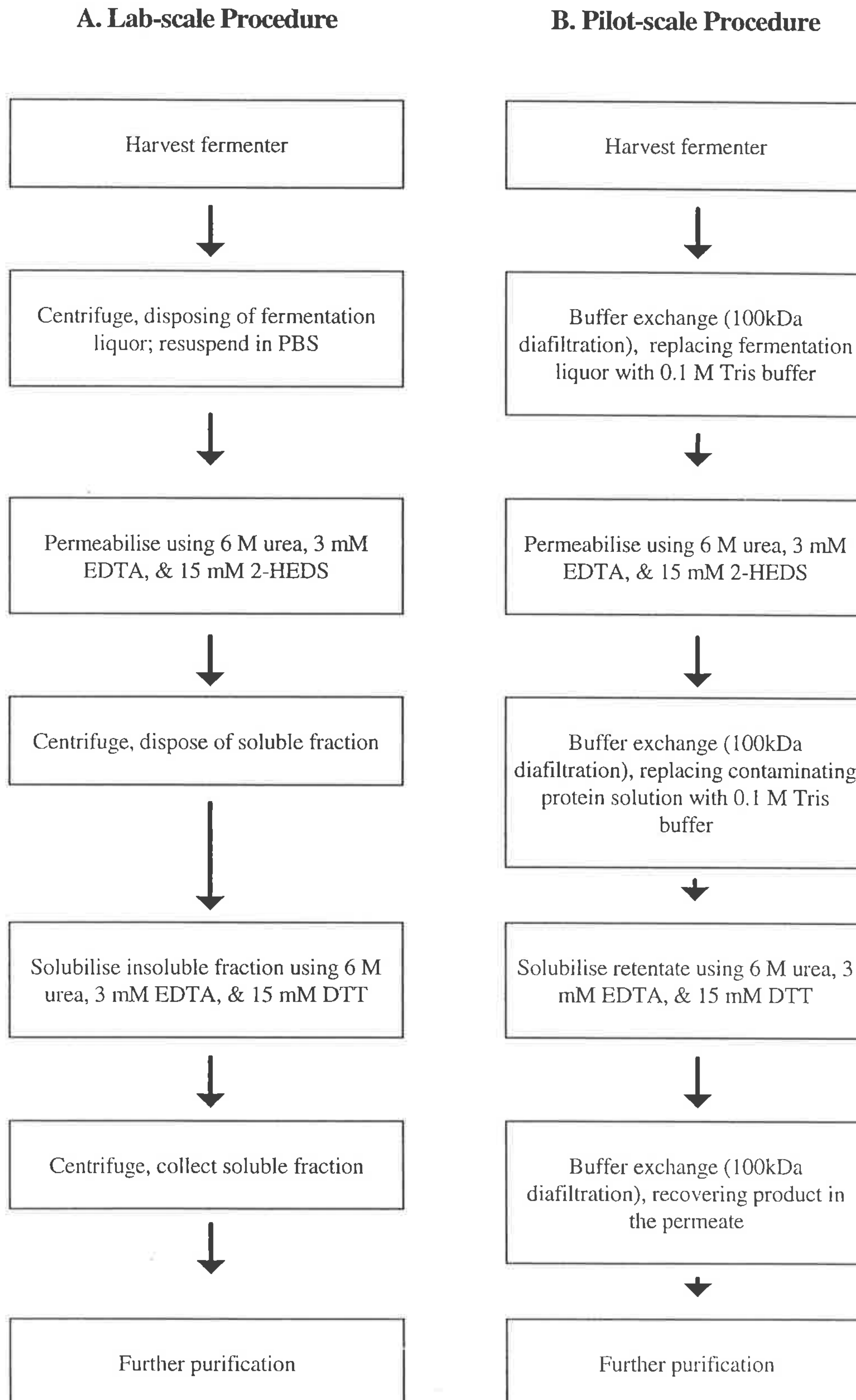

Figure 5.1 Comparison of the laboratory and the pilot-scale selective extraction procedures.

5.2 Design of the selective extraction prototype

5.2.1 Equipment specifications

The following equipment was used to construct the selective extraction pilot-scale system. The process flowsheet is detailed in Figure 5.2.

Stirred tank

New Brunswick Scientific Microferm® fermenter (5 L vessel).

Diafiltration equipment

Pall Filtron Minisette stainless steel cassette hardware

Pall Filtron Minisette cassette

- open channel
- 100 kilodalton molecular weight cut-off
- polyethersulfone
- 0.07 m^2 surface area

In-line mixer

Rushton turbine 10 mm diameter (4 blades)

Electric motor (192 Watts)

Speed 1400 rpm

Peristaltic pump

Masterflex Easy load model 7529-20

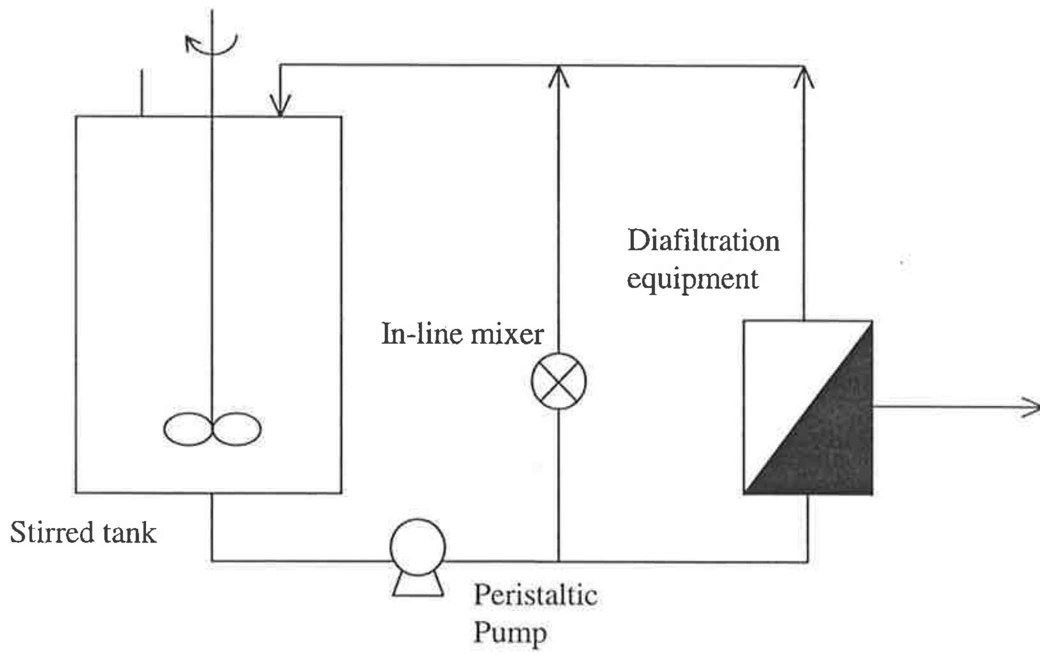

Figure 5.2 Layout of the prototype selective extraction rig.

5.3 Operational qualification

The operation of the prototype pilot-scale selective extraction procedure is studied in detail in this section. A mass balance was constructed for the procedure and the individual process steps were studied in detail.

5.3.1 Method

E. coli used in this experiment were induced for the expression of Long-R^3-IGF-I that accumulated as inclusion bodies in the host cytoplasm (Appendix C2). The fermentation broth received no treatment prior to its use in the operation of the prototype.

5.3.1.1 Operation of the prototype selective extraction equipment

Step 1 Diafiltration 1

0.1 M Tris (1 L) was added to the reactor, pumped through the lines and over the filter membrane. Fermentation broth was added to the reactor (a volume equivalent to 1.45 L at A_{600} 4.0). The volume in the reactor was reduced to 100 mL by ultrafiltration (permeate to waste). Buffer exchange was carried out (with 2 L of 0.1 M Tris) by reducing the reactor volume to 100 mL and then increasing to 600 mL with 0.1 M Tris, repeatedly. The volume in the reactor was reduced to 100 mL (i.e., 450 mL total volume due to the additional volume remaining in the pipe work). Samples of the pooled spent broth were collected for recombinant protein and total protein estimation.

Step 2 Permeabilisation

Permeabilisation solution A (8 M urea, 4 mM EDTA, 0.1 M Tris, at pH 9.0) plus 20 mM 2-HEDS was added to the reactor via the pipe work to initiate permeabilisation. The reactor was mixed by the Rushton turbine rotating at 100 rpm and by pumping the solution through the in-line mixer at 3 L/min. Samples were collected from the reactor at time 5, 10, 15, and

30 min (for recombinant protein and total protein estimation). After 30 min, the next step was commenced.

Step 3 Diafiltration 2

The volume in the reactor was reduced to 100 mL by ultrafiltration. Buffer exchange was carried out using 1 L of permeabilisation solution B (6 M urea, 3 mM EDTA, 0.1 M Tris) by reducing the reactor volume to 100 mL and then increasing to 600 mL with permeabilisation solution B, repeatedly. Buffer exchange was then repeated using 1 L of 0.1 M Tris. The volume in the reactor was reduced to 100 mL. The permeate was collected during diafiltration providing four 0.5 L pools which were sampled for recombinant protein and total protein estimation. The filtrate samples were then pooled and a sample collected for recombinant protein and total protein estimation.

Step 4 Solubilisation

Permeabilisation solution A (8 M urea, 4 mM EDTA, 0.1 M Tris, at pH 9.0) plus 27 mM DTT was added to the reactor via the pipe work to initiate solubilisation, and the solution was mixed using the Rushton turbine operating at 100 rpm for 30 min. Samples were collected from the reactor at time 5, 10, 15, and 30 min (for recombinant protein and total protein estimation).

Step 5 Diafiltration 3

The volume in the reactor was reduced to 100 mL by ultrafiltration. Buffer exchange was carried out using 2 L of permeabilisation solution B (6 M urea, 3 mM EDTA, 0.1 M Tris) plus 5 mM DTT by reducing the reactor volume to 100 mL then increasing to 600 mL with permeabilisation solution B, repeatedly. The permeate contained the extracted Long-R^3-IGF-I. The retentate was sent to waste. Permeate was collected during diafiltration providing four 0.5 L pools, and samples were collected for analysis. The filtrate pools were combined and a sample was collected. A sample of the retentate was also collected. All samples were assayed for recombinant protein and total protein.

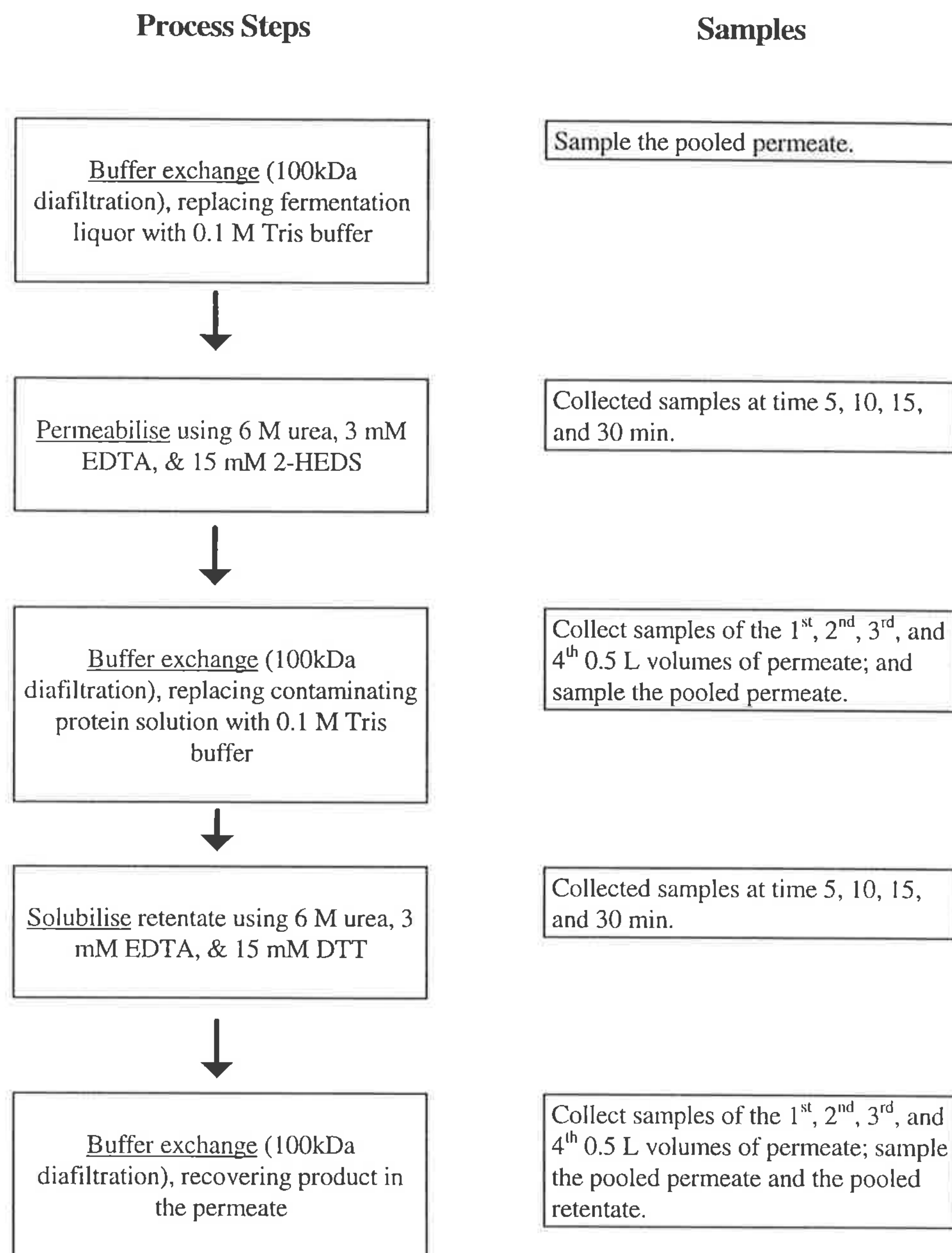

Figure 5.3 Samples collected during the operation of the prototype pilot-scale procedure.

Analysis

The samples collected during the operation of the prototype are detailed in Figure 5.3.

Samples collected during the operation of the extraction process were subjected to analysis for recombinant protein by HPLC (Appendix A4), total protein by Bradford analysis (Appendix A3), and PAGE analysis (Appendix A5).

5.3.1.2 Control 1 - Laboratory-scale selective extraction

This control allows direct comparison of the prototype with the laboratory-scale selective extraction procedure developed in Chapter 4.

E. coli cells (the equivalent of 5 mL at A_{600} 4) were centrifuged (10,000 xg, 15 min, 4°C), and the pellet resuspended in 1.25 mL of 0.1 M Tris-HCl and 3.75 mL of 8 M urea, 4 mM EDTA, 0.1 M Tris-HCl, and 20 mM 2-HEDS (adjusted to pH 9.0) to promote inclusion body stability. The solution was agitated in a shaking incubator set at 37°C for 30 min then centrifuged (10,000 xg, 15 min, 4°C). The second stage involved taking the pellet (from stage 1) resuspending it in 1.25 mL of 0.1 M Tris-HCl (using 1 second of sonication) and 3.75 mL of 8 M urea, 4 mM EDTA, 0.1 M Tris-HCl, and 27 mM DTT (adjusted to pH 9.0) to promote inclusion body solubilisation. The solution was agitated in a shaking incubator set at 37°C for 30 min and was then centrifuged at (10,000 xg, 15 min, 4°C). The recombinant protein was located in the supernatant.

Samples of the supernatant fractions collected during the operation of the extraction process were subjected to analysis for recombinant protein by HPLC (Appendix A4) and total protein by Bradford analysis (Appendix A3).

5.3.1.3 Control 2 - conventional extraction

This control allows direct comparison of the prototype with the conventional extraction procedure used for the extraction of recombinant proteins from inclusion bodies in the cytoplasm of *E. coli* (mechanical disruption, centrifugation, and dissolution).

E. coli suspension (1L) was homogenised by three passes through an APV Gaulin 15MR high-pressure homogeniser (CD valve) set at 56 MPa. Cell homogenate (the equivalent of 5 mL at A_{600} 4 prior to homogenization) was centrifuged (10,000 xg, 15 min, 4°C) and the pellet resuspended in 1.25 mL of 0.1 M Tris-HCl and 3.75 mL of 8 M urea, 4 mM EDTA, 0.1 M Tris-HCl, and 27 mM DTT (adjusted to pH 9.0). The solution was agitated in a shaking incubator set at 37°C for 30 min. The solution was then centrifuged (10,000 xg, 15 min, 4°C). The recombinant protein was located in the supernatant.

Samples of the supernatant fractions collected during the operation of the extraction process were subjected to analysis for recombinant protein by HPLC (Appendix A4) and total protein by Bradford analysis (Appendix A3).

5.3.2 Results and discussion

5.3.2.1 Mass balance

The details of the mass balance are shown in Figure 5.3. The total values of recombinant and total protein used here are the combined values for the soluble and insoluble fractions resulting from conventional extraction (Control 2). The protein component of the *E. coli* cells that is measured in this experiment is separated during the three diafiltration steps.

Diafiltration 1 (spent media removal)

During the first diafiltration step the *E. coli* cells are intact. The aim of this step is to remove the salt component of the spent fermentation broth that could interfere with subsequent permeabilisation. The protein concentration in the permeate in this step was below the sensitivity of the Bradford assay.

Diafiltration 2 (bacterial protein removal)

The second diafiltration step followed the permeabilisation step and aimed to remove soluble protein from the treated cell suspension prior to solubilisation of the inclusion bodies. The permeate from this step contained 19% (w/w) of the total protein (162 mg) and 4% (w/w) of the recombinant protein (5 mg).

Diafiltration 3 (separation of soluble product from cell debris)

The third diafiltration step followed solubilisation of the inclusion bodies. The aim was to collect the recombinant protein in the permeate, hence separating it from the remaining cell debris. The permeate from this step contained 60% (w/w) of the recombinant protein (75 mg) and 28% (w/w) of the total protein (236 mg). The retentate contained 8 % (w/w) of the recombinant protein (10 mg) and 38% (w/w) of the total protein (323 mg).

A significant amount of the recombinant and total protein was not accounted for in this mass balance (33% (w/w) of the recombinant protein (33 mg) and 15% (w/w) of the total protein (123 mg)). This material was probably adsorbed to equipment surfaces. Ultrafiltration membranes are commonly associated with protein adsorption. It may also remain as insoluble material in the cell debris (the retentate) following the third diafiltration step.

PAGE analysis results of permeates from the second and third diafiltration steps, and the retentate from the third diafiltration step, are shown in Figure 5.5. The permeate from the second diafiltration step showed only weak protein bands (some of the material detected by the Bradford assay may not have been detected by the PAGE assay). The Long-R^3-IGF-I in the permeate from the third diafiltration step was free of gross protein contamination and had virtually no outer-membrane protein (OmpA/F and OmpC) contamination. The bulk of the host-cell protein remained in the retentate of the third diafiltration step.

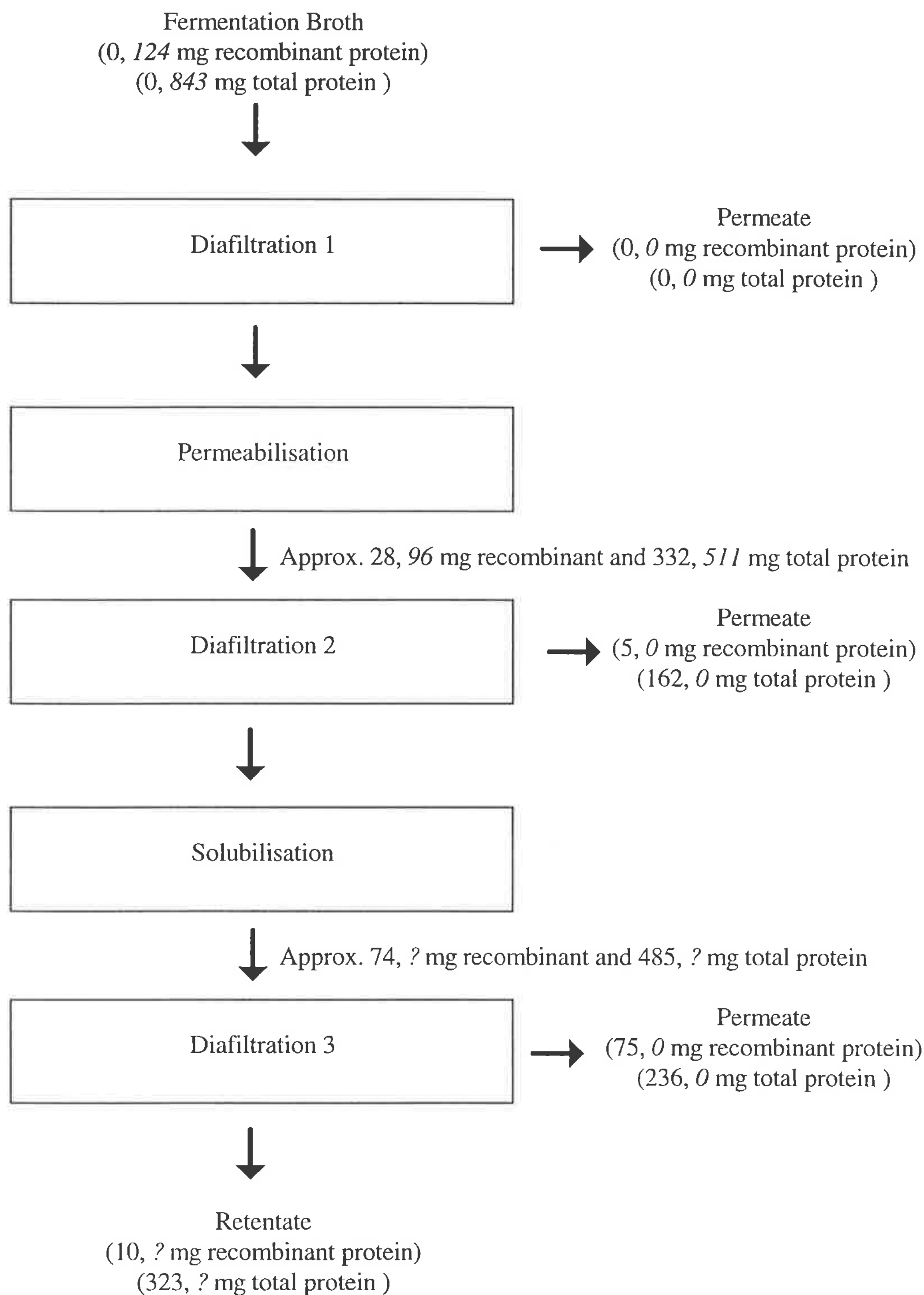

Figure 5.4 Mass balance for the selective extraction prototype. The quantity of intracellular protein is show in italics and extracellular protein in normal letters. (Note that 33 mg of the recombinant protein (26% w/w) and 123 mg of the total protein (15% w/w) is unaccounted for).

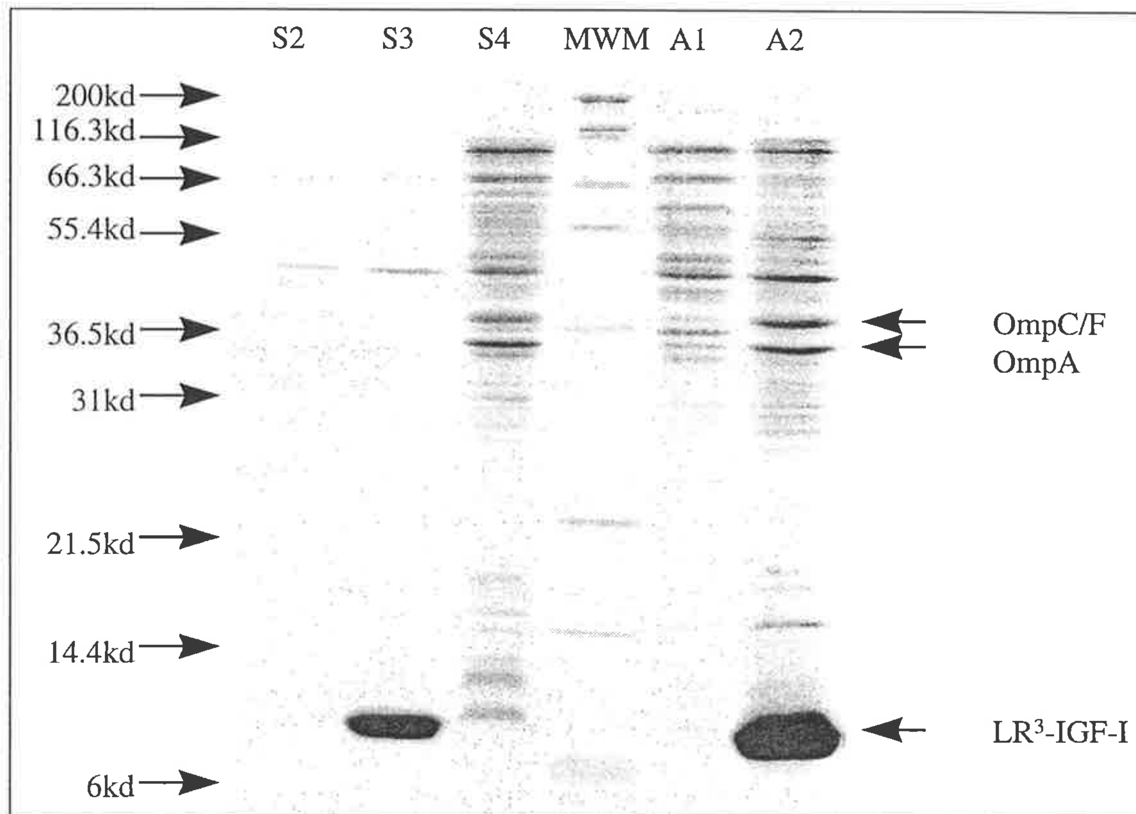

Figure 5.5 PAGE analysis of samples from the operation of the prototype pilot-scale selective extraction rig. The samples are as follows:

S2 permeate from the 2nd diafiltration step

S3 permeate from the 3rd diafiltration step

S4 retentate from the 3rd diafiltration step

MWM molecular weight markers

A1 soluble fraction from conventional extraction

A2 insoluble fraction from conventional extraction

5.3.2.2 Procedure steps

Diafiltration 1

Fermentation broth (1.178 L) was added to the reactor and the volume reduced to 450 mL by diafiltration, 500 mL of 0.1 M Tris was then added to the cell concentrate and the volume reduced to 450 mL (i.e., 100 mL in the reactor and 350 mL in the pipe work). This procedure was repeated 4 times providing a 20-fold dilution of the spent fermentation broth. The aim was to remove the salts, sugars, and metabolites from the extracellular phase as these components might retard the action of urea and EDTA during permeabilising of the *E. coli* cells.

Permeabilisation

The permeabilisation step was designed to release the intracellular bacterial protein while minimising the solubilisation of recombinant protein. Permeabilisation was commenced by the addition of permeabilisation solution containing 8 M urea, 4 mM EDTA, 20 mM 2-HEDS, and 0.1 M Tris at pH 9.0 to the 450 mL of washed cells suspended in 0.1 M Tris buffer at pH 9.0. The reactor was stirred using the Rushton turbine (set at approx. 100 rpm) and the solution was pumped through the inline mixer at 3 L/min. The solution formed a stable foam due to air becoming entrapped in the liquid phase.

Protein release from the bacteria inside the reactor can be described using equation 5.1, a variant of equation 2.3, used to describe permeabilisation kinetics (Section 2.5).

$$P = (P_0 - P_{max}) \exp (-t / \tau) + P_{max} \qquad - (5.1)$$

where P is the amount of protein in the extracellular phase, P_0 is the initial amount of protein in the extracellular phase, P_{max}, is the maximum amount of protein in the extracellular phase, t is time, and τ is the time constant.

Recombinant and total protein concentration were analysed during the permeabilisation step (Figures 5.4 and 5.5, respectively). The curves shown in Figures 5.4 and 5.5 were determined by regression to equation 5.5. The parameters for equation 5.1 describing the release of total protein during permeabilisation are provided in Table 5.1.

Table 5.1 Parameters for equation 5.1 describing protein release from *E. coli* cells during the permeabilisation step of the operation of the prototype selective extraction procedure (standard error is in brackets).

	Parameter*	Value
Recombinant protein	$P_{r,0}$	0 mg
	$P_{r,max}$	28.5 (1.6) mg
	τ_r	5.9 (1.0) min
Total Protein	$P_{t,0}$	0 mg
	$P_{t,max}$	332 (6) mg
	τ_t	5.0 (0.3) min

* Where $P_{r,0}$, $P_{r,max}$, and τ_r are the protein level at time zero, the maximum protein level, and the time constant for recombinant protein release, respectively; and $P_{t,0}$, $P_{t,max}$, and τ_t are the protein level at time zero, the maximum protein level, and the time constant for total protein release, respectively.

The concentration of protein in the reactor can be directly compared to the levels released during the first stage of laboratory-scale selective extraction (the first control). The prototype released 332 mg total protein and 28 mg recombinant protein compared to the control release of 492 mg total protein and 22 mg recombinant protein. Total protein release by the prototype was significantly lower than the control (only 67% w/w of the amount released by the control). This indicates that the cells were not fully permeabilised during this step. The build up of foam during the prototype operation is a key change between the two processes that could be partly responsible for this difference.

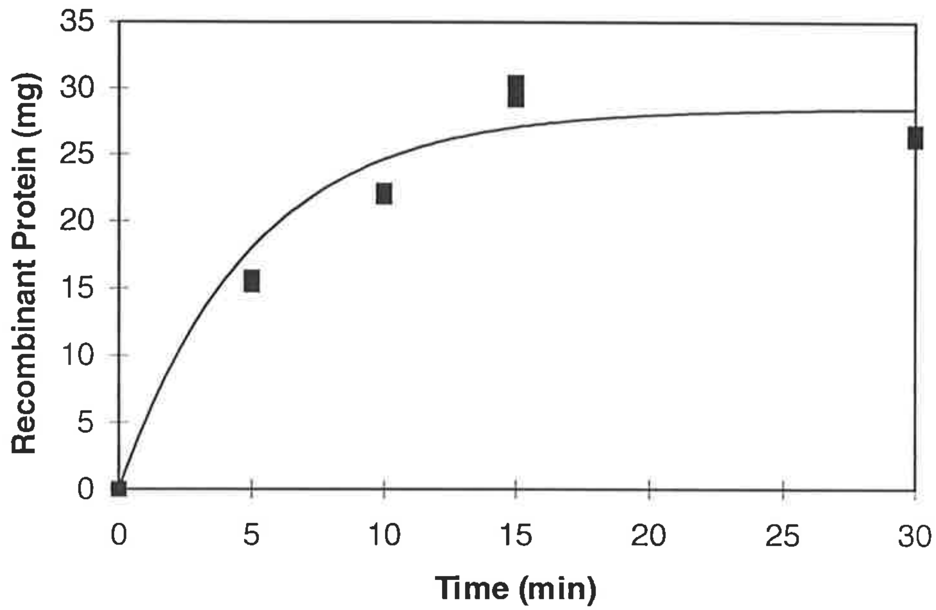

Figure 5.6 Recombinant protein release into the extracellular phase during the permeabilisation step.

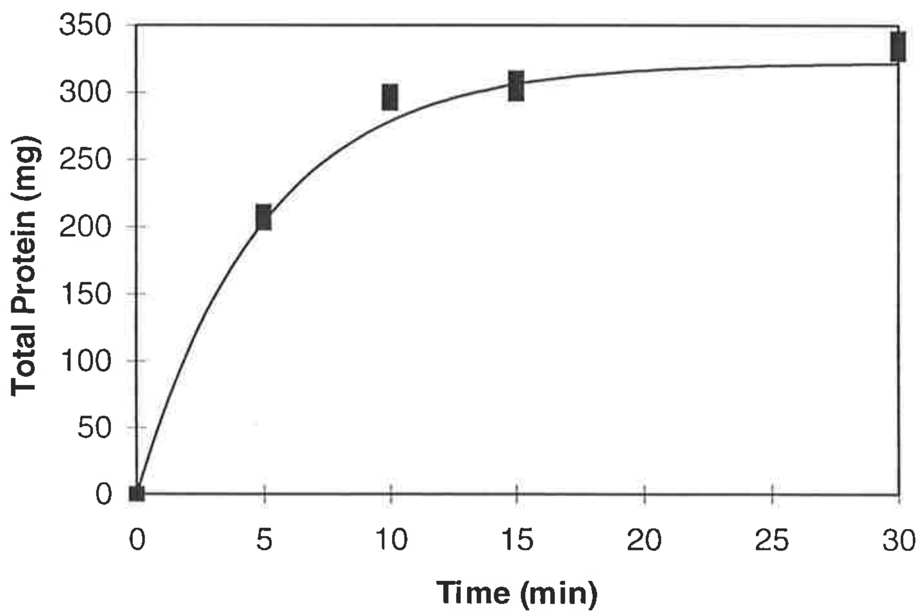

Figure 5.7 Total protein release into the extracellular phase during the permeabilisation step.

Diafiltration 2

The second diafiltration step aimed to remove the protein-rich extracellular phase and replace it with 0.1 M Tris buffer. The diafiltration used in this experiment used stepwise reduction of the volume by tangential flow filtration followed by replacement of the lost volume with fresh solution.

Equation 5.2 can be used to describe the operation of diafiltration where a constant volume is maintained in the reactor:

$$\ln(C_0/C) = (1-R)\ (V/V_0) \qquad - (5.2)$$

where C_0 is the initial concentration in the reactor, C is the concentration in the reactor after the diafiltration, V_0 is the initial volume in the reactor, V is the cumulative volume of permeate, and R is the retention factor (Costa and Cabral, 1991).

The diafiltration procedure used in this experiment used cycles of concentration by diafiltration followed by dilution of the contents of the reactor. Several changes to equation 5.1 were made to make it applicable for the cyclic diafiltration procedure. The V/V_0 is replaced with a dilution factor calculated using equations 5.3 and 5.4. The dilution factor for each step of the cyclic diafiltration procedure is defined as:

$$X_1 = V_0/V_1 \qquad - (5.3)$$

where X_1 is the dilution factor for the first diafiltration cycle. The accumulative dilution factor is:

$$X = X_1 \text{ x } X_2 \text{ x } X_3 \ldots\ldots \qquad - (5.4)$$

where X_1, X_2, X_3 etc. are the dilution factors calculated for each diafiltration cycle and X is the accumulative dilution factor.

The concentration in the reactor constantly changes during cyclic diafiltration, so equation 5.5 expresses the operation of the procedure in terms of the quantity of protein in the reactor.

Equation 5.5 is the modified version of equation 5.2 which is applicable for cyclic diafiltration:

$$\ln P = \ln P_0 - (1\text{-}R) \ln X \qquad - (5.5)$$

where P_0 is the initial quantity of protein in the reactor and P is the quantity of protein in the reactor after diafiltration. The retention factor used in this equation is the average retention factor during the diafiltration operation.

At the start of the second diafiltration step the protein level in the reactor was 332 mg total protein and 28.5 mg recombinant protein. The amount of recombinant and total protein in the filtrate was measured for each dilution step. This amount was subtracted from the amount previously in the reactor to determine the amount remaining. The levels in the reactor are shown in Figures 5.8 and 5.9. The curves shown in the Figures 5.8 and 5.9 were determined by regression to equation 5.5. The parameters for equation 5.5 describing the second diafiltration are shown in Table 5.2.

Table 5.2 Parameters for equation 5.5 describing the second diafiltration step during operation of the prototype selective extraction procedure (standard error is in brackets).

	Parameter *	Value
Total Protein	$P_{t,o}$	332 mg
	R_t	0.78 (0.03)

* Where $P_{t,o}$ and R_t are the initial quantity of total protein in the reactor and retention of total protein, respectively.

The total protein retention factor during the second diafiltration stage was 0.78. The molecular weight of most *E. coli* proteins in their monomeric form falls between 30 and 100 kilodalton (as determined by PAGE analysis with a denaturing gel). While the protein does pass through the membrane the proportion crossing the membrane is lower than the solute. The slow removal of the total protein from the retentate slows the operation of this step and requires a large amount of buffer exchange. To achieve a 5-fold reduction of total protein in the soluble phase in the reactor buffer exchange would be required using 7.3 reactor volumes of solution. To render this operation more economic a different membrane could be used (with a larger pore size), or solution recycling could be employed. Recycling could be achieved using diafiltration with a low molecular weight cut-off membrane (around 5 kilodalton cut-off). The permeate would be returned to the reactor and the retentate (containing the macromolecules) sent to waste.

The level of recombinant protein measured in the permeate was only 5.3 mg, a significantly lower amount than expected based on the quantity of recombinant protein found in the reactor after the permeabilisation step (approximately 28 mg). The molecular weight of Long-R^3-IGF-I is 9 kilodaltons and it should therefore pass freely through a 100 kilodalton cut-off filter. Retention is unlikely to explain the low Long-R^3-IGF-I concentration in the permeate. It is quite probable that the recombinant protein loss is due to adsorption to the filter membrane during diafiltration.

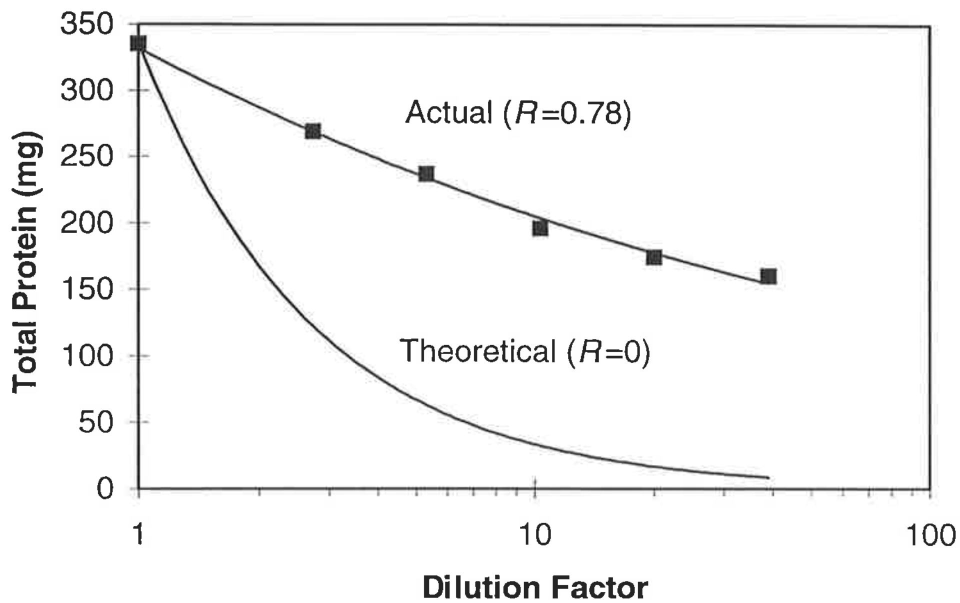

Figure 5.8 Reduction of total protein in the reactor during the second diafiltration step.

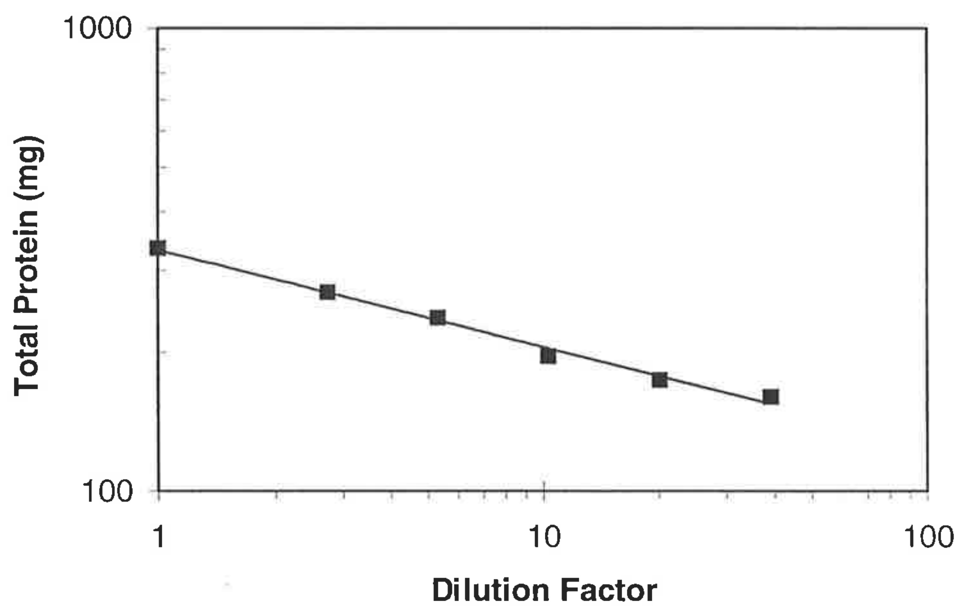

Figure 5.9 Reduction of total protein in the reactor during the second diafiltration step. Using a log/log scale.

Solubilisation

The solubilisation step aimed to release the recombinant protein from the insoluble fraction. Solubilisation was started by the addition of the solubilisation solution containing 8 M urea, 4 mM EDTA, 27 mM DTT, and 0.1 M Tris at pH 9.0 to the 450 mL of washed permeabilised cells suspended in 0.1 M Tris buffer at pH 9.0. The reactor was stirred using the Rushton turbine (set at approx. 100 rpm).

Recombinant and total protein concentration were analysed during the permeabilisation step (Figures 5.8 and 5.9, respectively). The curves shown in Figures 5.8 and 5.9 were determined by regression to equation 5.1. The parameters for equation 5.1 describing the release of total protein during the solubilisation step are provided in Table 5.3.

Table 5.3 Parameters for equation 5.1 describing protein release from *E. coli* cells during the solubilisation step of the prototype pilot-scale selective extraction procedure (standard error is in brackets).

	Parameter*	Value
Recombinant protein	$P_{r,0}$	0 mg
	$P_{r,max}$	74.3 (2.3) mg
	τ_r	1.8 (1.0) min
Total Protein	$P_{t,0}$	170 mg
	$P_{t,max}$	485 (16) mg
	τ_t	1.3 (1.8) min

- Where $P_{r,0}$, $P_{r,max}$, and τ_r are the protein level at time zero, the maximum protein level, and the time constant for recombinant protein release, respectively; and $P_{t,0}$, $P_{t,max}$, and τ_t are the protein level at time zero, the maximum protein level, and the time constant for total protein release, respectively.

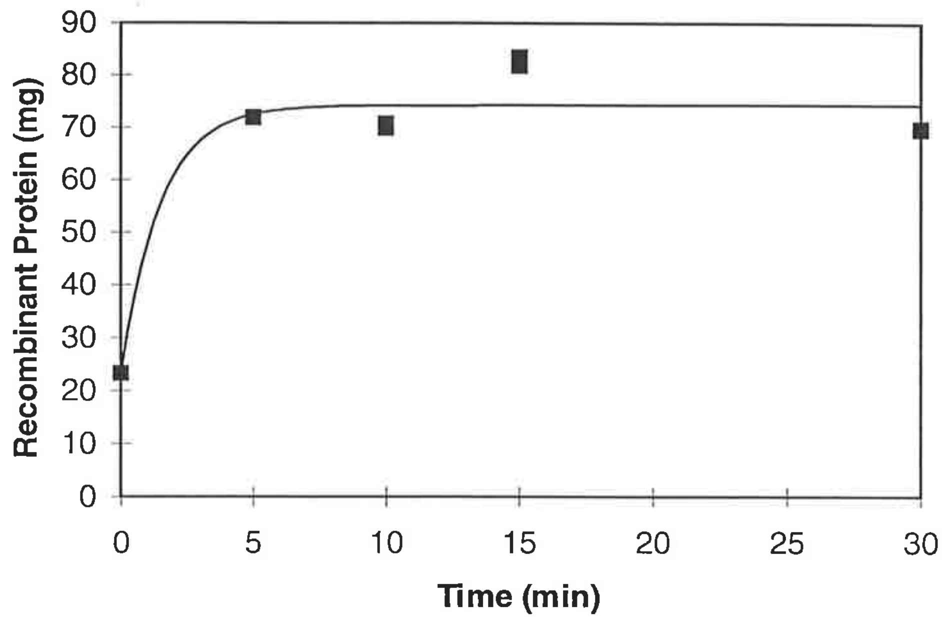

Figure 5.10 Recombinant protein release into the extracellular phase during the solubilisation step.

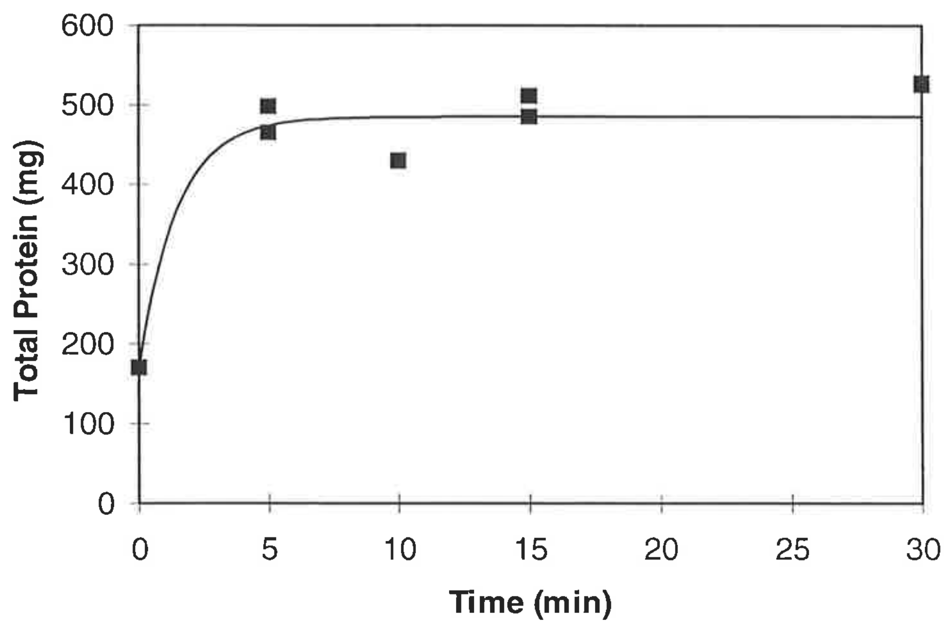

Figure 5.11 Total protein release into the extracellular phase during the solubilisation step.

The concentration of the protein in the reactor can be directly compared to the levels released during the second stage of laboratory-scale selective extraction (the first control). The prototype released 315 mg total protein and 74 mg recombinant protein compared to the control release of 236 mg total protein and 107 mg recombinant protein. A major factor affecting the difference in the levels of total protein release was incomplete permeabilisation. A greater amount of total protein remained in the *E. coli* cells and was extracted during the solubilisation step. The level in the reactor was also supplemented by soluble material remaining after the second diafiltration (170 mg of total protein).

Diafiltration 3

The third diafiltration step aimed to extract the solubilised recombinant protein from the residual solid material derived from the *E. coli* cells.

At the start of the third diafiltration step the protein levels in the reactor were 485 mg total protein and 74 mg recombinant protein. The levels in the reactor are shown in Figures 5.10, 5.11, and 5.12. The curves shown in Figures 5.10, 5.11, and 5.12 were determined by regression to equation 5.5. The parameters for equation 5.5 describing diafiltration are provided in Table 5.4.

A total of 74 mg recombinant protein was present in the reactor at the end of solubilisation. Recombinant protein measured in the permeate resulting from the third diafiltration was 75 mg. The retention factor for Long-R^3-IGF-I was 0.05 (close to the theoretical minimum), showing that the membrane did not significantly retard the protein's progress. Long-R^3-IGF-I molecular weight is only 9 kilodaltons and would be expected to pass freely through a 100 kilodalton cut-off filter. The total protein retention factor during the third diafiltration stage was 0.76 which was similar to the second diafiltration stage. The difference in retention for recombinant and total protein allows the recombinant protein to be removed while the majority of the total protein remains in the retentate.

Table 5.4 Parameters for equation 5.5 describing the third diafiltration step during operation of the prototype selective extraction procedure (standard error is in brackets).

	Parameter	Value
Recombinant Protein	$P_{r,o}$	74 mg
	R_r	0.05 (<0.01)
Total Protein	$P_{t,o}$	485 mg
	R_t	0.76 (0.04)

* Where $P_{r,o}$ and R_r are the initial quantity of recombinant protein in the reactor and retention of recombinant protein, respectively; and $P_{t,o}$ and R_t are the initial quantity of total protein in the reactor and retention of total protein, respectively.

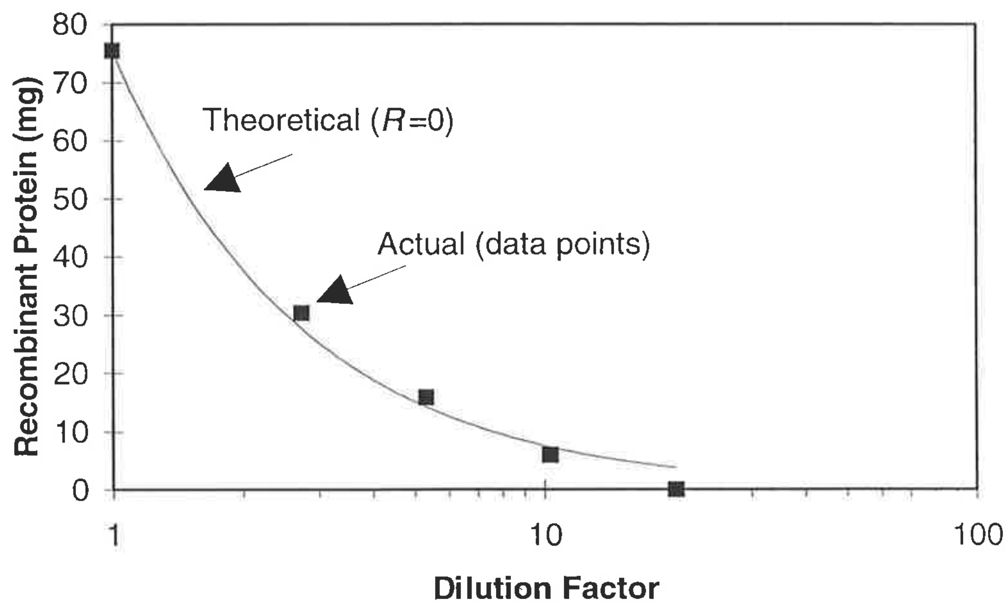

Figure 5.12 Reduction of recombinant protein in the reactor during the third diafiltration step.

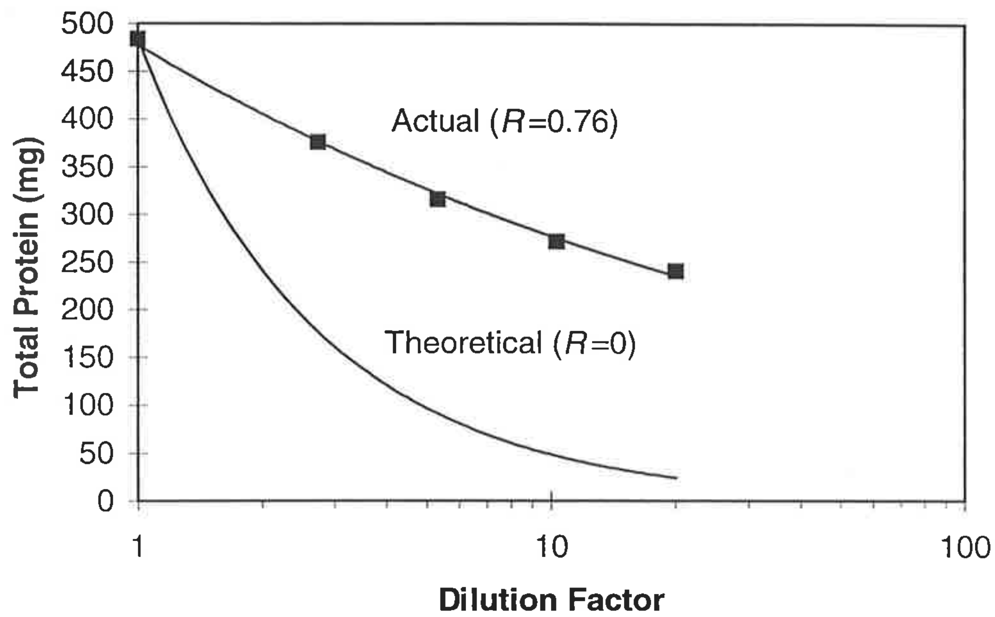

Figure 5.13 Reduction of total protein in the reactor during the third diafiltration step.

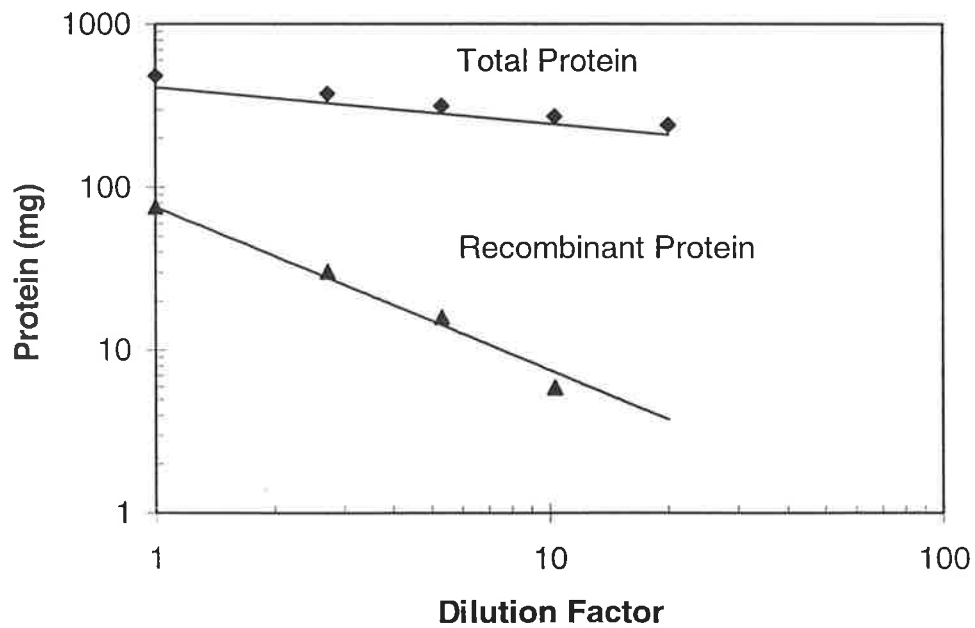

Figure 5.14 Reduction of recombinant and total protein in the reactor during the third diafiltration step. Using a log/log scale.

Control Extraction Procedures

The amounts of recombinant and total protein in controls (lab-scale selective extraction) and control 2 (conventional extraction) can be directly compared to those in the prototype reactor as shown in Table 5.5 (all the figures are converted to a 1 L working volume with a cell concentration equivalent to A_{600} of 4.0).

The recovery and the relative concentration of recombinant protein resulting from the control procedures were superior to those achieved by the prototype pilot-scale selective extraction procedure.

The reasons for the inferior performance of the prototype pilot-scale selective extraction procedure are as follows:

- incomplete protein release during the permeabilisation step (possibly due to foam formation preventing uniform mixing of the cell suspension);

- incomplete protein removal during the 2nd diafiltration step. Retention of the bacterial protein rendered the diafiltration step inefficient at the volumes used.

- loss of recombinant protein during operation of the prototype, possibly due to adsorption to membrane surfaces.

The prototype pilot-scale selective extraction procedure did recover 60% (w/w) of the recombinant protein with a purification factor of 2.2, but should be able to match the performance of the lab-scale procedure. The recombinant protein purity is superior to that achieved by nonselective extraction. The operation of the prototype is open to optimisation that should improve its performance significantly.

Table 5.5 Performance of the control extraction procedures carried out in parallel with the prototype selective extraction procedure. To allow direct comparison of the results for the prototype selective extraction procedure with the lab-scale selective extraction and conventional extraction the results are calculated for a 1 L working volume with an initial cell concentration of A_{600} of 4.0.

		Total Protein (mg/L)	Recombinant Protein (mg/L)
Control 1 - Lab-scale Selective Extraction	1st Stage	339	16
	2nd Stage	162	74
	Recovery	83% (w/w)	
	Purity	46% (w/w)	
Control 2 - Conventional Extraction	Soluble Fr.	385	5
	Insoluble Fr.	197	80
	Recovery	94% (w/w)	
	Purity	40% (w/w)	
Prototype Selective Extraction	2nd Diafiltr. Permeate	111	4
	3rd Diafiltr. Permeate	163	52
	3rd Diafiltr. Retentate	230	7
	Recovery	60% (w/w)	
	Purity	32% (w/w)	

5.4 Potential of the prototype pilot-scale system

5.4.1 Optimisation of the existing procedure

The potential performance of the prototype pilot-scale system can be estimated using the results of the laboratory technique and from operational data of the prototype equipment. The release of recombinant and total protein during the laboratory permeabilisation and solubilisation steps should be achievable using the prototype equipment. Modification to eliminate foam formation and the implementation of temperature control would aid optimisation. The performance of the diafiltration steps determined by the data recorded in sections 5.3 can be extrapolated to the operation of an optimised prototype system. Alternative membranes with reduced protein binding can also be examined.

Assumptions used to determine the possible performance parameters after optimisation of the prototype system include the following:

- the release of recombinant and total protein from the permeabilisation and solubilisation steps match that achieved by the laboratory procedure;
- the filtration rates for recombinant and total protein diafiltration follow the relationships determined in Sections 5.3.3.3 and 5.3.3.5;
- the second diafiltration step achieves an 80% (w/w) reduction of total protein (requiring 7.3 reactor volumes of washing solution);
- the third diafiltration step achieves 90% (w/w) of the available recombinant protein in the permeate, with approximately 50% (w/w) of the available total protein also passing into the permeate (requiring 2.3 reactor volumes of washing solution);
- the protein loss observed during operation of the prototype selective extraction rig is eliminated.

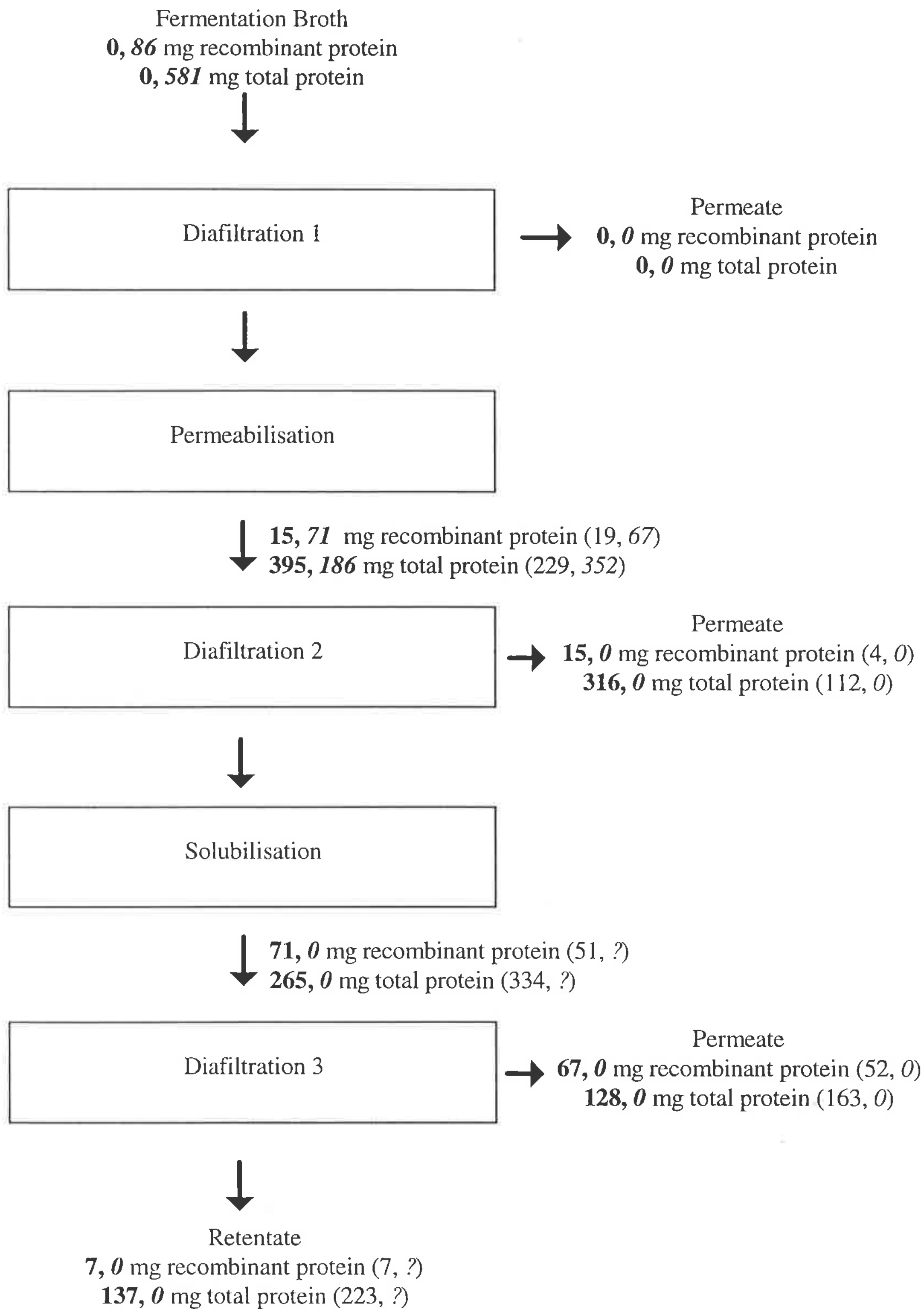

Figure 5.15 Mass balance for the selective extraction prototype showing the potential performance of the system (in bold type). The mass balance assumes a operational reactor volume of 1 litre and a cell concentration equivalent to A_{600} of 4.0. Intracellular protein is

show in italics and extracellular protein in normal type. The actual performance of the prototype is shown in brackets.

Actual performance of the prototype pilot-scale selective extraction procedure had a low level of recombinant protein recovery and purity when compared to the lab-scale procedure (Table 5.5). The potential of the prototype was estimated using the assumptions on page 158. This illustrates the potential the prototype procedure has following optimisation.

As shown in Table 5.6, the prototype system has the potential to extract 75% (w/w) of the recombinant protein at a purity of 61% (w/w) compared to total protein. The recovery levels almost match those achieved by lab-scale selective extraction (Table 5.5). The purity of the product derived from the prototype system has the potential to exceed that achieved by lab-scale selective extraction and conventional extraction (Table 5.5). This is due to the selectivity of the third diafiltration step. The recombinant protein experiences almost no retention by the membrane, whereas the total protein is significantly retained. This allows 95% (w/w) recovery of the available recombinant protein with only approximately 40% (w/w) of the total protein entering the permeate.

Table 5.6 The actual and the potential performance of the prototype selective extraction procedure showing the recovery of the recombinant protein and its purity measured as the relative concentration against total protein.

	Potential Performance	Actual Performance
Recovery	64/86 = 75%	52/86= 60%
Purity	64/105 = 61%	52/163 = 32%

5.4.2 Development of a commercial procedure

Several modifications could be made to the prototype pilot-scale system to improve the effectiveness and economy of operation. The concentration of the starting material processed in the prototype system is an important variable as it affects the ratio of product to reagent used and the reactor volume required. The ratio of product to reagent affects the operational cost of the procedure and the reactor volume affects equipment costs. Operational costs associated with reagent purchase and disposal could be minimised by the recycle of solutions during the second and third diafiltration steps. Membrane selection for the diafiltration steps can have a significant impact by improving product purity and minimising protein loss through membrane binding.

5.4.2.1 Membrane selection

As viewed on a PAGE gel the bulk of the bacterial protein range is of molecular weight between 30 and 100 kilodaltons. The choice of membrane will play an obvious role in the retention of host-cell protein. As the nominal molecular weight cut-off (NMWC) is reduced the retention of host cell proteins will be increased. The test recombinant protein used in this thesis was Long-R^3-IGF-I, which has a molecular weight of approximately 9 kilodaltons. It passes through a 100 kilodalton NMWC filter freely (its retention factor being close to zero) and would be likely to pass through a filter with a smaller cut-off (e.g., 20-30 kilodaltons). The reduction in cut-off would prevent a significant proportion of the host proteins from passing through the membrane and would provide a significant purification factor, but at the expense of reduced flux rates. The requirement for a permeabilisation step could be negated by this development making the nonselective extraction process suitable for smaller proteins and peptides.

Application of this prototype selective extraction procedure to larger molecular weight recombinant proteins would be unlikely to present any purification factor during the third diafiltration step as the size difference between the protein of interest and the bacterial proteins would not allow any selectivity.

Selection of a membrane that has low protein binding characteristics is important to minimise this source of loss during operation of the system. Many membranes are commercially available that are designed to minimise protein binding. These would need to be screened to determine their applicability for filtration of specific recombinant proteins.

5.4.2.2 Recycle

The diafiltration steps require multiple reactor volumes of solutions to be effective (the example used in Table 5.6 required about 10 reactor volumes during its operation). The cost of purchase and disposal of these reagents (and for the associated storage tanks and mixing equipment) could represent a significant fraction of the overall costs.

Recycle of the solutions used during the second and third diafiltration steps could be achieved using another diafiltration system. This second diafiltration system would be designed to separate the bacterial contaminants from solution. A low nominal molecular weight cut-off membrane (such as 5 kilodaltons) could separate the solution from the macromolecules. All the reagents are of low molecular weight and would pass through such a membrane freely. The permeate could then be recycled into the reactor. Low molecular weight components of the bacteria such as metabolites and salts could limit the extent of recycle. These components could interfere with the solubilisation step if in high enough concentration (the action of chaotropic agents like urea can be compromised by the presence of salts).

5.4.2.3 Cell concentration

The amount of starting material added to the system directly affects the required reactor volume. Reactor volume will have an impact on equipment cost and also affects the amount of solution required for each process step. The proportion of material lost due to adsorption to surfaces or membranes drops as the amount of starting material relative to membrane surface area increases.

The cell concentration that can be added to the reactor at the start of the procedure will firstly affect the permeabilisation step. The effect of cell concentration on permeabilisation was tested for the nonselective extraction of recombinant protein (section 3.4). Increasing the cell concentration from A_{600} 4 to 20 had a negligible affect on permeabilisation efficiency. The cell concentration should be tested for A_{600} levels of up to 100 as these concentrations are commonly encountered in commercial fermentations. The ability of 2-HEDS to stabilise the recombinant protein will also need to be demonstrated at these higher cell concentrations.

The cell concentration that is possible will be limited by one of a series of factors. Urea is known to bind macromolecules in solution. An increase in cell concentration will increase the concentration of a wide range of macromolecules that could bind sufficient urea to compromise the permeabilisation process. The available EDTA concentration will also be reduced if the concentration of divalent cations in the media is too high. 2-HEDS availability might also be too low at high cell concentrations.

5.5 Conclusion

The prototype system used in this chapter was able to extract recombinant Long-R^3-IGF-I from *E. coli* cells. It recovered 60% (w/w) of the Long-R^3-IGF-I at a purity of 32% (w/w) (compared to total protein).

The performance of the prototype was hampered by incomplete protein release during the permeabilisation step, the incomplete removal of bacterial protein during the 2nd diafiltration step, and the loss of recombinant protein possibly due to adsorption to the membrane.

A study of the diafiltration steps illustrated their performance and demonstrated the modifications that are required to optimise these steps. The recombinant protein Long-R^3-IGF-I (9 kilodaltons) was not significantly retained by the 100 kilodalton NMWC membrane. Bacterial protein (mainly between 30 and 100 kilodaltons) was, however, significantly retained by the membrane. A sizeable volume of buffer will therefore be needed during the second diafiltration step to make it effective at reducing the total soluble protein concentration in the reactor. The third diafiltration is able to use this phenomenon to provide a degree of selectivity, as the bulk of the recombinant protein can be recovered with some retention of the contaminating total protein.

The permeabilisation step was not as effective as that achieved during the lab-scale selective extraction procedure. This step should be able to match the performance of the lab-scale procedure. Elimination of foam formation and implementation of temperature control could resolve this problem.

With optimisation the prototype selective extraction system is capable of 75% (w/w) recovery of the recombinant product at a purity of 61% (w/w) purity. While optimisation will provide significant improvements to performance, the existing prototype design did demonstrate the concept and potential. It recovered most of the recombinant protein and provided a purification factor that provides significant advantages over systems based on nonselective extraction. The recombinant protein is recovered in the permeate from the 3rd

permeabilisation step. It is soluble and separated from the remaining cell debris, and is in a form suitable for subsequent purification by adsorption to an ion-exchange resin using a previously-published purification procedure (Falconer *et al.*, 1994).

To create a commercially viable selective extraction procedure from the pilot-scale system described in this chapter the following developments may be necessary. Buffer recycle could be implemented during the operation of the second and third diafiltration steps to reduce the cost of chemicals and minimise waste-treatment costs. The concentration of cells during the permeabilisation and solubilisation steps could also be increased to reduce the reactor size and the amount of chemicals required to process a quantity of recombinant protein. The choice of membrane is also important to enhance the selectively of the diafiltration steps, to reduce the protein loss due to adsorption to the membrane surface, and to improve the physical performance of the diafiltration steps.

CHAPTER 6

CONCLUSION

The procedures developed in this thesis are for the extraction of recombinant proteins from inclusion bodies located in the cytoplasm of *E. coli* cells. They represent an effective set of alternative procedures to the methods commonly used at laboratory and commercial-scale. The advantages and disadvantages of the new procedures are discussed and comparison is made with the traditional approaches. Applications for the new technology are described along with any further developments that could be required before full commercial exploitation.

Traditional extraction

The production of recombinant proteins is commonly carried out in the host bacterium *Escherichia coli*. Following expression, the recombinant protein needs to be extracted from the cell prior to renaturation and purification. Extraction of recombinant proteins from inclusion bodies located in the cytoplasm of *E. coli* cells is usually undertaken using a conservative set of process steps. Traditional extraction uses mechanical disruption, centrifugation and dissolution to release monomeric denatured protein from the bacteria. This protocol has been applied to the extraction of a wide range of recombinant proteins. It provides a degree of selectivity as most of the soluble host cell components are removed during the centrifugation step. It is usually reliable and uses widely available technology.

During traditional extraction some host-cell macromolecules are extracted along with the recombinant product. The most important of these are the outer-membrane components lipopolysaccharide and the serine-protease OmpT. OmpT is responsible for the degradation of a significant number of recombinant proteins containing dibasic residues. Traditional extraction procedures have been modified to reduce the coextraction of outer-membrane components. Multiple centrifugation steps or wash steps using a range of

components have also been used. Multiple homogenisation steps are often included to ensure complete cell disruption and adequate cell debris fragmentation to aid centrifugation. The resultant procedure becomes reliant on over-sized process units when the batch schedule is constrained by the fermentation cycle time.

High-pressure homogenisation is usually used to disrupt the bacteria. It is comprised of one or more piston pumps and a disruption valve. Centrifugation is usually undertaken with a disc-stack centrifuge. Production of veterinary or medical recombinant proteins has to comply with the code of Good Manufacturing Practice (GMP) and various guidelines for handling genetically manipulated organisms. This equipment needs to be contained, steam sterilise-able in-place, clean-able in-place, its component parts composed of suitable materials, and its operation validated. This grade of equipment is expensive to purchase and validate.

Alternative procedures that are less costly to establish and operate will become increasingly important as the manufacture of agricultural, industrial, and generic medical recombinant proteins becomes more competitive. The procedure provided in this thesis aims to provide such an alternative. Simpler technology could also provide the techniques for use by scientists or engineers at the laboratory-scale without the need for specialised equipment. This has obvious advantages for under-equipped laboratories world-wide.

Permeabilisation

The barrier presented by the bacterial cell wall has to be overcome for extraction of intracellular proteins. This was achieved by chemical treatment of the *E. coli* cells with a combination of the chaotropic agent urea and the chelating agent EDTA. By themselves, each chemical has a modest capacity to release protein from *E. coli*. In combination, synergistic effects enhances intracellular protein release. The combination of 6 M urea, 3 mM EDTA, 0.1 M Tris buffer, at pH 9.0 became the standard permeabilisation buffer used throughout this thesis due to its ability to release all of the intracellular protein from both exponential and stationary-phase *E. coli*.

EDTA's ability to permeabilise the outer-membrane of *E. coli* is well documented (Leive, 1974, Marvin *et al.*, 1989). It weakens the outer-membrane by chelating the divalent cations that stabilise the lipopolysaccharide structure. The resultant outer-membrane is permeable to a range of chemicals including urea. Urea gains access to the inner-membrane and is able to disrupt its structure, allowing the intracellular components to leak into the extracellular phase.

Experimental results showed that the interaction between urea and EDTA is complex. The choice of buffer had little impact on permeabilisation. The kinetics of protein release were first-order. The reaction was relatively quick with a first-order time constant of 2.5 min.

Treatment of *E. coli* cells with a combination of urea and EDTA performs differently to previously published permeabilisation techniques. Historically, most permeabilisation techniques were developed for the release of native proteins from bacteria. The aim was to selectively release the protein of interest without denaturing the protein. Treatment with urea and EDTA in this study was designed with recombinant protein extraction in mind, where total cell wall permeabilisation (measured by total protein release) was the goal and denaturation of the protein accepted.

Nonselective extraction

Nonselective extraction of recombinant protein from intact *E. coli* cells was developed for a strain of *E. coli* K12 that expressed an analogue of insulin-like growth factor 1 (Long-R^3-IGF-I).

The urea concentration used to permeabilise the *E. coli* cells is known to dissolve Long-R^3-IGF-I inclusion bodies *in-vitro* (Greenwood *et al.*, 1994). The permeabilisation procedure was applied for the nonselective release of recombinant protein from inclusion bodies located in the cytoplasm of *E. coli*. The permeabilisation buffer was effective at extracting recombinant protein from the bacteria. This was enhanced by the addition of the reducing buffer dithiothreitol. The combination of permeabilisation buffer and 20 mM DTT was able to extract comparable levels of recombinant protein to the traditional

procedure based on mechanical disruption and centrifugation. The purity was much lower due to the coextraction of all the bacterial intracellular protein.

EDTA played no role in solubilisation of inclusion body *in-vitro,* but was required for permeabilisation of the bacterial cell wall and hence urea's access to the inclusion body. Urea, however, played a dual role in permeabilisation and dissolution of the inclusion body. Solution pH proved important for permeabilisation with a value of less than 9 resulting in reduced protein release. The kinetics of recombinant and total protein release were first-order with time constants of approximately 3 min.

Nonselective extraction of recombinant proteins other than the model protein Long-R^3-IGF-I does require testing before its effectiveness can be guaranteed. Some recombinant protein inclusion bodies may require the use of a stronger chaotropic agent such as guanidine-HCl or urea concentrations greater than 6 M for complete solubilisation. The nonselective extraction procedure would require modification for successful use with these recombinant proteins.

This nonselective procedure has direct application for scientists or engineers wanting to analyse recombinant protein levels in intact cells during fermenter operation. This procedure can extract recombinant protein making it available for analysis by methods such as PAGE, HPLC, ELISA, and RIA.

Commercial application of this procedure is limited by the low purity of the recombinant protein after extraction. The procedure would need to be combined with a selective separation method. The process described in USP 4,476,049 (Kung, 1984) used affinity chromatography to improve product purity after the nonselective extraction of immune interferon using guanidine-HCl. Clever usage of tangential flow filtration could provide significant improvement of purity for small proteins (less than 10 kilodaltons) and peptides. The bulk of the host cell proteins are between 30 and 100 kilodaltons (as estimated by PAGE analysis) and could be radically reduced in concentration by the use of an appropriate nominal molecular weight cut-off membrane.

Selective extraction - laboratory procedure

Addition of the disulphide reagent 2-hydroxyethyldisulphide (2-HEDS) to the standard permeabilisation buffer reduced the level of recombinant protein release into the aqueous-phase while allowing permeabilisation to occur. This phenomenon allowed the development of a selective two-stage extraction procedure. The procedure used permeabilisation buffer plus 15 mM 2-HEDS during the first stage, centrifugation to separate the soluble and insoluble fractions, and permeabilisation buffer plus 20 mM DTT during the second stage. The recovery and purity of the material extracted in the second stage was comparable to that extracted by the traditional procedure. Levels of outer-membrane components (OmpA and OmpC/F) in the selective extract were significantly lower for extraction by the traditional procedure, as observed by PAGE analysis. If this is true for the troublesome outer-membrane protease OmpT, then this procedure may prove invaluable for the extraction of OmpT susceptible proteins like IGF-2 (Wong, 1996).

Selective extraction of recombinant proteins other than the model protein Long-R^3-IGF-I by the procedures developed here must be tested prior to use. The action of 2-HEDS at preventing solubilisation of the recombinant protein relies on the presence of cysteine residues in the protein. This procedure has a reduced probability of success for recombinant proteins where the proportion of cysteine residues is low and no chance of success for proteins with no cysteine residues. Some recombinant protein inclusion bodies may also require the use of a stronger chaotropic agent such as guanidine-HCl or higher concentrations of urea for complete solubilisation during the second stage. The selective extraction procedure may require some modification for the successful extraction of different recombinant proteins.

Selective release has immediate application for the recovery of recombinant proteins at the laboratory-scale. This procedure can be easily adapted for the extraction of small amounts of recombinant protein produced from experiments at the shake-flask scale. The traditional procedure would rely on a laboratory having mechanical disruption equipment such as a lab-scale high-pressure homogeniser or a large French press, as well as a

centrifuge. The selective extraction procedure only requires a centrifuge to separate the insoluble from the soluble fractions after the first stage.

Selective extraction - scaleable procedure

The scaleable selective extraction procedure differed from the lab-scale procedure in the use of tangential flow filtration rather than batch centrifugation as the method for separating the insoluble and soluble fractions after the permeabilisation and solubilisation steps. The permeabilisation and solubilisation reactions were carried out using a 1.45 L stirred reactor rather than the 5 mL tubes used in the previous experiments. The reactor and tangential flow filtration are amenable to scale-up and could be utilised at an industrial-scale.

The prototype system effectively extracted Long-R^3-IGF-I from *E. coli* cells. It did not match the recovery nor the purity achieved at the lab-scale, but the performance parameters allowed the potential of the system to be estimated. The prototype's potential was estimated using the operating parameters from the experimental tests and the values from the lab-scale selective extraction procedure. It has the potential to recover nearly the same level of recombinant protein as the lab-scale procedure but with a higher level of purity. The improved purity is due to the selectivity that can be attained with the third diafiltration step.

For a new separation or extraction step to be competitive with the existing procedures it has to have the following characteristics:

- high recovery of the recombinant product
- relatively high product purity
- ability to reduce the concentration of specific problem contaminants (e.g. OmpT)
- reduced operation costs
- be amenable to integration with other process steps
- utilise equipment that is amenable to cGMP operation

For the recovery of Long-R^3-IGF-I from *E. coli* cells, selective release can meet some of these requirements. It is can recover a significant proportion of the product at a relatively high level of purity (when compared to the conventional extraction procedure). The operation costs are yet to be proven to be competitive with conventional extraction. Selective release does not rely of expensive pieces of equipment such as disc-stack centrifuges, but the costs associated with reagents could be high especially if operation at high cell concentration and reagent recycle is not possible. Selective release would integrate easily with the existing purification process used for Long-R^3-IGF-I (Falconer *et al.*, 1994) and has the potential to significantly increase the throughput of the existing process. The filtrate from the third diafiltration stage could be concentrated on a ion-exchange resin affording purification and concentration prior to refolding and further purification. The equipment for selective release includes a stirred reactor and tangential flow filtration. Both these pieces of equipment are simple to validate, easy to scale-up, and can be operated in compliance with cGMP.

Like the lab-scale procedure this operation would have to be modified for specific recombinant proteins. It is also dependant on the presence of cysteine residues for the effectiveness of 2-HEDS. The molecular weight of the protein will influence the choice of the nominal molecular weight cut-off of the tangential flow membrane. With peptides or low molecular weight proteins of less than 30 kilodalton, a significant selectivity can be gained during the third diafiltration step.

The quantity of cells that can be processed by the selective extraction procedure without compromising recovery or product purity will affect the cost effectiveness of this procedure. Solution recycle could also reduce operational costs of the system reducing the amount of reagents required and the size of tanks required for mixing and holding the solutions. An industrial process could use on-line absorbance measurements (A_{600}) to monitor the permeabilisation reaction, as a correlation between protein release and A_{600} has been observed.

Summary

Three procedures are described in this thesis. They have potential applications at both the laboratory and the industrial scale. These processes are shown in Table 6.1.

Table 6.1 Procedures arising from the research in this thesis.

Procedure	**Potential applications**
Nonselective extraction (Chapter 3)	Preparation of samples for analysis. Extraction of rec. protein (in combination with a selective step).
Selective extraction - lab-scale (Chapter 4)	Small-scale extraction of rec. protein (containing cysteine residues).
Selective extraction - scaleable (Chapter 5)	Large-scale extraction of rec. protein (containing cysteine residues).

These procedures have been demonstrated with the recombinant protein Long-R^3-IGF-I. The procedures need to be tested for the extraction of wide range of recombinant proteins before the full potential of this approach will be realised.

NOMENCLATURE

a	Constant (eq. 2.2)
b	Constant (eq. 2.2)
C	Protein concentration in the extracellular phase (mgL^{-1})
C_{max}	Maximum protein concentration in the extracellular phase (mgL^{-1})
C_0	Initial protein concentration in the extracellular phase (mgL^{-1})
c	Constant (eq. 2.2)
d	Particle diameter (μm)
g	Gravitational acceleration (ms^{-2})
K	Relative centrifugal force (N)
P	Protein in the extracellular phase (mg)
P_{max}	Maximum protein in the extracellular phase (mg)
P_0	Initial protein in the extracellular phase (mg)
R	Retention factor
r	Radial position of the particle (m)
r_o	Particle start radius (m)
r_i	Radius at the detector (m)
t	Time (s)
x	Absorbance at 600nm, (eq. 2.2), (-)
X_1	Dilution factor for the first diafiltration cycle
X	Accumulative dilution factor
Y	Fractional protein release
Y_{max}	Maximum fractional protein release
Y_0	Initial fractional protein release

Greek Symbols

$\Delta\rho$	Difference between the density of the particle and the medium ($kg\ m^{-3}$)
τ	Time constant (s)

μ	Viscosity of the medium (Pa s)
υ_g	Velocity (ms^{-1})
ω	Angular velocity (s^{-1})

References

Ahmed, A.K., Schaffer, S.W., Wetlaufer, D.B. 1975. Nonenzymic reactivation of reduced bovine pancreatic ribonuclease by air oxidation and by glutathione oxidoreduction buffers. *J. Biol. Chem.,* **250**, 8477-8482.

Ames, G.F-L., Prody, C., Kustu, S. 1984. Simple, rapid and quantitive release of periplasmic proteins by chloroform. *J Bacteriol.*, **160**, 1181-1183.

Asenjo, J.A., Patrick, I. 1990. Large-scale protein purification, in *Protein Purification Approaches*, IRL Press, Oxford, UK, 1-28.

Babbitt, P.C., West, B.L., Buechter, D.D., Kuntz, I.D., Kenyon, G.L. 1990. Removal of a proteolytic activity associated with aggregates formed from expression of creatine kinase in *Escherichia coli* leads to improved recovery of active enzyme. *Bio/technol.*, **8**, 945-949.

Barnacal, P.A. 1995. Editor's introduction: Biotechnology comes of age. *Pharmaceutical Manufacturing International*, **1995**, 9-12.

Birdsell, D.C., Cota-Robles, E.H. 1968. Lysis of spheroplasts of *Escherichia coli* by a non-ionic detergent. *Biochem. Biophys. Res. Commun.*, **31**, 438-446.

Bowden, G.A., Paredes, A.M., Georgiou, G. 1991. Structure and morphology of protein inclusion bodies in *Escherichia coli*. *Bio/technol.*, **9**, 725-730.

Bradford, M.M. 1976. A rapid and sensitive method for quanititation of microgram quantities of protein utilising the principle of protein-dye binding. *Anal. Biochem.*, **72**, 248-254.

Cerny, G., Teuber, M. 1971. Differential release of periplasmic *versus* cytoplasmic enzymes from *Escherichia coli* B by polymyxin B. *Arch. Mikrobiol.*, **78**, 166-179.

Chang, J.Y., Swartz, J.R. 1993. Single-step solublisation and folding of IGF-1 aggregates from *Escherichia coli. ACS Symposium Series,* **526**, 178-188.

Santos, J.A.L., Mateus, M., Cabral, J.S. 1991. Pressure-driven membrane processes, in *Chromatographic and Membrane Processes in Biotechnology.* Kluyver Academic Publisher, Dordrecht, The Netherlands, 197-199.

Cronan, J.E., Gennis, R.B., Maloy, S.R. 1987. Cytoplasmic membrane, in *Escherichia coli and Salmonella typhimurium. Cellular and Molecular Biology.* Am. Soc. Microbiol., Washington, D.C., USA, 31-55.

Datar, R.V., Cartwright, T., Rosen, C-G. 1993. Process economics of animal cell and bacterial fermentations: A case study analysis of tissue plasminogen activator. *Bio/technol.*, **11**, 349-357.

Datar, R.V., Rosen, C-G. 1993. Cell and cell debris removal: centrifugation and crossflow filtration, in, *Biotechnology,* Vol. 3 Bioprocessing, VCH, Weinheim, Germany, 472-503.

De Bernardez-Clark, E., Georgiou, G. 1991. Inclusion bodies and recovery of proteins from the aggregated state. *ACS Symposium Series*, **470**, 1-20.

De Smet, M.J., Kingma, J., Witholt, B. 1978. The effect of toluene on the structure and permeability of the outer membrane and cytoplasmic membranes of *Escherichia coli. Biochim. et Biophys. Acta*, **506**, 64-80.

Emtage, J.S. 1985. DNA makes protein makes money. *Nature*, **317**, 185-186.

Enger, C.R. 1985. Disruption of microbial cells, in *Comprehensive Biotechnology: The principles, applications and regulations of biotechnology in industry, agriculture and medicine*. Vol. 2, Pergamon Press, Oxford, UK, 305-324.

Falconer, R.J., Jarvinen, J.E., Francis, G., O'Neill, B.K., Middelberg, A.P.J. 1994. Improvements to a process for producing insulin-like growth factors. 3rd Asia-Pacific Biochemical Engineering Conference, Singapore, 384-386.

Filip, C., Fletcher, G., Wulff, J.L., Earhart, C.F. 1973. Solublisation of the cytoplasmic membrane of *Escherichia coli* by the ionic detergent Sodium-Lauryl Sarcosinate. *J. Bacteriol.*, **115**, 717-722.

Fischer, B., Sumner, I., Goodenough, P. 1993. Isolation & renaturation of bio-active proteins expressed in *Escherichia coli* as inclusion bodies. *Arzneim.-Forsch./Drug Res.*, **42**, 1512-1515.

Forman, S.M., DeBernardez, E.R., Feldberg, R.S., Swartz, R.W. 1990. Crossflow filtration for the separation of inclusion bodies from soluble proteins in recombinant *Escherichia coli* cell lysate. *J. Mem. Sci.*, **48**, 263-279.

Georgiou, G., Valax, P. 1996. Expression of correctly folded proteins in *Escherichia coli. Curr. Opinion. Biotechnol.,* **7**, 190-197.

Greenwood, M., Kotlarski, N., O'Neill, B.K., Falconer, R.J., Francis, G.L., Middelberg, A.P.J. 1994. Dissolution and refolding of Long-R^3-IGF-I, in, *Proceedings of the 1994 IChemE Research Event, University College London, UK.*, Institution of Chemical Engineers, Rugby, UK., 250-252.

Grodberg, J., Dunn, J.J. 1988. OmpT encodes the *Escherichia* coli outermembrane protease that cleaves t RNA polymerase during purification. *J. Bacteriol.*, **170**, 1245-1253.

Hart, R.A., Lester, P.M., Reifsnyder, D.H., Ogez, J.R. & Builder, S.E. 1994. Large scale, *In-situ* isolation of periplasmic IGF-1 from *E.coli. Bio/technol.*, **12**, 1113-1117.

Hart, R.A., Rinas, U., Bailey, J.E. 1990. Protein composition of *Vitreoscilla* hemoglobin inclusion bodies produced in *Escherichia coli. J. Biol. Chem.*, **265**, 12728-12733.

Hartley, D.L., Kane, J.F. 1987. Properties of inclusion bodies from recombinant *Escherichia coli. Biochem. Soc. Trans.*, **16**, 101-102.

Hatefi, Y., Hanstein, W.C. 1974. Destabilisation of membranes with chaotropic agents. *Methods in Enzymology*, **31**, 770-790.

Hejnaes, K.R., Bayne, S., Norskov, L., Sorensen, H.H., Thomsen, J., Schaffer, L., Wollmer, A., Skriver, L. 1992. Development of an optimal refolding process for recombinant Ala-Glu-IGF-I. *Protein Eng.* **5**, 797-806.

Hettwer, D., Wang, H. 1989. Protein release from *Escherichia coli* cell permeablised with guanidine-HCl and triton X-100. *Biotechnol. Bioeng*, **44**, 886-895.

Hibbard, L.S., Tulinsky, A. 1978. Expression of functionality of α-chymotrypsin. Effects of guanidine hydrochloride and urea in the onset of denaturation. *Biochem.,* **17**, 5460-5468.

Hoare, M., Dunnill, P. 1989. Biochemical engineering challenges of purifying useful proteins. *Phil. Trans. R. Soc. Lond.* B, **324**, 497-507.

Ingram, I.O. 1981. Mechanism of lysis of *Escherichia coli* by ethanol and other chaotropic agents. *J. Bacteriol.*, **146**, 331-336.

Irvin, R.T., MacAlister, T.J., Costerton, J.W. 1981. Tris(hyroxymethyl)-aminomethane buffer modification of *Escherichia coli* outer-membrane permeability. *J. Bacteriol.*, **145**, 1397-1403.

Jackson, R.W., Demoss, J.A. 1965. Effects of toluene on *Escherichia coli. J. Bacteriol.*, **90**, 1420-1425.

Janson, J-A, Ryden, L. 1993. Protein separation and purification, in *Biotechnology 2nd Edition*, Vol. 3, Bioprocessing, VCH, Weinheim, Germany, 618-640.

Kamoun, P.P. 1988. Denaturation of globular proteins by urea: breakdown of hydrogen or hydrophilic bonds? *TIBS*, **13**, 424-425.

Keshavarz-Moore, E., Oldrich, M., Hoare, M., Dunnill, P. 1991. Application of biochemical engineering principles to develop a recovery process for protein inclusion bodies. *Annal. NY Acad. Sci.*, **646**, 307-314.

King, R., Wells, J.R.E., Krieg, P., Snoswell, M., Brazier, J., Bagley, C.J., Wallace, J.C., Ballard, F.J., Ross, M., Francis, G.L. 1992. Production and characterisation of recombinant insulin-like growth factor-I and potent analogues of IGF-I, with Gly or Arg substituted for Glu^3, following their expression in *Escherichia coli* as fusion protein. *J. Molecular Endocrinol.*, **8**, 29-41.

Kleinig, A.R., Middelberg, A.P.J. 1996. The correlation of cell disruption with homogeniser valve pressure gradient determined by computational fluid dynamics. *Chem. Eng. Sci.*, **51**, 5103-5510.

Kung, H-F. 1984. Method for the extraction of immune interferon, USP 4,476,049.

Langley, K.E., Berg, T.R, Strickland, T.W., Fenton, D.M., Boone, T.C., Wypych, J. 1987. Recombinant-DNA-derived bovine growth hormone from *Escherichia coli* 1. Demonstration that the hormone is expressed in reduced form, and isolation of the hormone inoxidised, native form. *Eur. J. Biochem.*, **163**, 313-321.

Leduc, M., Frehel, C., Siegel, E., Van Heijennoort, J. 1989. Multilayered distribution of peptidoglycan in the periplasmic space of *Escherichia coli. J. Gen. Microbiol.*, **135**, 1243-1254.

Leive, L. 1974. The barrier function of the gram-negative envelope. *Annal. NY Acad.* Sci., **235**, 109-129.

Marston, F.A.O., Hartley, D.L. 1990. Solubilisation of protein aggregates. *Methods in Enzymol.*, **182**, 264-276.

Marvin, H.J., Ter Beest, M.B.A., Witholt, B. 1989. Release of outer membrane fragments from wildtype *Escherichia coli* and from several E.coli lipopolysaccharide mutants by EDTA and heat shock treatments. *J. Bacteriol.*, **171**, 5262-5267.

Meagher, M.M., Bartlett, R.T., Rai, V.R. & Khan, F.R. 1994. Extraction of rIL-2 inclusion bodies from *Escherichia coli* using cross-flow filtration. *Biotechnol. Bioeng.*, **43**, 969-977.

Middelberg, A.P.J., O'Neill, B.K., Bogle, I.D.L. 1992. A new model for the disruption of *Escherichia coli* by high-pressure homogenisation. Part I. Model development and verification. *Trans IChemE,* **70**, 205-212.

Middelberg, A.P.J. 1992. A model for the disruption of *Escherichia coli* by high-pressure homogenisation, Ph.D. thesis, University of Adelaide, Australia.

Middelberg, A.P.J. 1995. Process-scale disruption of microorganisms. *Biotechnol. Advances*, **13**, 491-551.

Miller, C.G. 1996. Protein degradation and proteolytic modification, in, *Escherichia coli and Salmonella typhimurium. Cellular and molecular biology.* Am. Soc. Microbiol., Washington, D.C. , USA, 938-954.

Mitraki, A., King, J. 1989. Protein refolding intermediates and inclusion body formation. *Bio/technol.,* **7**, 690-697.

Naglak, T.J., Hettwer, D.J., Wang, H.Y. 1990. Chemical permeabilisation of cells for intracellular product release. *In, Separation Processes in Biotechnology*, Marcel Dekker, Inc., NY, USA, 177-205.

Naglak, T.J., Wang, H.Y. 1991. Rapid protein release from *Escherichia coli* by chemical permeabilisation under fermentation conditions. *Biotechnol. Bioeng*, **39**, 732-740.

Neu, H.C., Heppel, L.A. 1964. Some observations on the latent ribonucleases of *Escherichia coli. Proc. Natl. Acad. Sci.* USA, **51**, 1267-1274.

Nikaido, H. 1996. Outer membrane, in, *Escherichia coli and Salmonella typhimurium. Cellular and molecular biology.* Am. Soc. Microbiol., Washington, D.C. , USA, 29-47.

Novella, I.S., Fargues, C., Grevillot, G. 1994. Improvement of the extraction of penicillin amylase from *Escherichia coli* cells by a combined use of chemical methods. *Biotechnol. Bioeng*, **33**, 379-382.

Pace, C.N. 1986. Determination and analysis of urea and guanidine hydrochloride denaturation curves. *Methods in Enzymol.*, **131**, 266-280.

Park, J.T. 1987. Murein sacculus, in, *Escherichia coli and Salmonella typhimurium. Cellular and molecular biology.* Am. Soc. Microbiol., Washington, D.C. , USA, 23-30.

Price, H.S. 1993. Appendix. in, *The Biotechnology Report 1993/94*, Camden Publishing Ltd., London, UK, 277-287.

Puri, N.K., Crivelli, E., Cardamone, M., Fiddes, R., Bertolini, J., Ninham, B., Brandon, M.R. 1992. Solubilisation of growth hormone and other recombinant proteins from *Escherichia coli* inclusion bodies by using a cationic surfactant. *Biochem. J*, **285**, 871-879.

Rudolph, R., Lilie, H. 1996. In vitro folding of inclusion body proteins. *FASEB J.*, **10**, 49-56.

Russell, A.D., Morris, A., Allwood, M.C. 1973. Methods for assessing damage to bacteria induced by chemical and physical agents. *Methods in Microbiol.*, **8**, 96-174.

Quirk, A.V., Woodrow, J.R. 1984. Investigation of the paramenters affecting the separation of bacterial enzymes from cell debris by tangential flow filtration. *Enzyme Microb. Technol.*, **6**, 201-206.

Schnaitman, C.A. 1971a. Solublization of the cytoplasmic membrane of *Escherichia. coli* by Triton X-100. *J. Bacteriol.*, **108**, 545-552.

Schnaitman, C.A. 1971b. Effect of ethylenediaminetetraacetic acid, Triton X-100, and lysozyme on the morphology and chemical composition of isolated cell walls of *Escherichia coli*. *J. Bacteriol.*, **108**, 553-563.

Simmons, L.C., Yansura, D.G. 1996. Translational level is a critical factor for the secretion of heterologous proteins in *Escherichia coli*. *Nature Biotechnol.*, **14**, 629-634.

Steiner, D.F., Clark, J.L. 1968. The spontaneous reoxidation of reduced beef and rat proinsulin. *Biochem.*, **60**, 622-629.

Tandon, S., Horowitz, P.M. 1987. Detergent-assisted refolding of guanidinium chloride-denatured rhodanese. *J. Bio. Chem.*, **262**, 4486-4491.

Taylor, G., Hoare, M., Gray, D.R., Marston, F.A.O. 1986. Size and density of protein inclusion bodies. *Bio/Technol.*, **4**, 553-557.

Teuber, M. 1970. Release of the periplasmic penicillinases from *Escherichia coli* by toluene. *Arch. Mikrobiol.*, **73**, 61-64.

Thomson, J.A., Schurtenberger, P., Thurston, G.M., Benedek, G.B. 1987. Binary liquid phase separation and critical phenomena in a protein/water solution. *Proc. Natl. Acad. Sci.* USA, **84**, 7079-7083.

Tuggle, C.K., Fuchs, J.A. 1985. Glutathione reductase is not required for maintenance of reduced glutathione in *Escherichia coli* K-12. *J. Bacteriol.*, **162**, 448-450

Vaara, M. 1992. Agents that increase the permeability of the outer membrane. *Microbiol. Revs.*, **56**, 395-411.

Valax, P., Georgiou, G. 1993. Molecular characterisation of β-lactamase inclusion bodies produced in *Escherichia coli*. 1. Composition. *Biotechnol. Prog.*, **9**, 539-547.

Whittaker, J.J., Jackson, J.H. 1980. A permeabilised-cell assay for transaminase B activity in *Escherichia coli* K-12. *Anal. Biochem*, **105**, 133-140.

Woldringh, C.L. 1970. Lysis of the cell membrane of *Escherichia coli* K12 by ionic detergents. *Biochim. et Biophys. Acta*, **224**, 288-290.

Wong, H.H. 1996. Modelling studies of the interaction between homogenisation, centrifugation, and inclusion body dissolution. Ph.D. thesis, University of Adelaide, Australia.

APPENDICES

Appendix A Analytical procedures

A1 Particle size analysis

The Joyce-Loebl analytical disc centrifuge has been used for particle-size analysis. Details on the operation of this procedure are recorded in Middelberg, (1992). When operated with a 10% (w/w) glycerol spin solution it can be used to measure the size of bacteria.

Solutions

Spin fluid was 10% (w/w) glycerol in RO water.
Buffer fluid was RO water.
Sample fluid was 20 mM sodium phosphate and 30 mM sodium chloride (pH 7.0).

Operation

Set the disc speed at 8000 rpm and the gain at 6.0. When the disc is rotating inject 15 mL spin solution into the rotor. At time 60 seconds inject 1.0 mL buffer fluid. At time 300 seconds inject 0.5 mL of sample.

Calculation

The Stokes diameter is calculated using equation 8.1.

$$\tau = 18\eta \ln (r_d/r_o) / (d_s \times 10^{-6})^2 \Delta\rho\varpi^2 \quad - (8.1)$$

Where τ is the time for the particle to reach the detector, d_s is the Stokes diameter (μm), $\Delta\rho$ is the density difference between the particle and the fluid (84 kg m^{-3}), ϖ is the disc

angular velocity, η is the fluid viscosity (1.36 cP), r_o is the particle start radius (4.30 cm), and r_d is the detector radius (4.82 cm). Absorbance is plotted against Stokes diameter.

A2 Optical density

The concentration of bacterial suspensions were estimated by measurement of absorbance at 600 nm using a Unicam 8625 UV/Vis Spectrophotometer. Note that bacterial suspensions with an optical density of greater than 1 required dilution with PBS prior to measurement.

A3 Total protein estimation (Bradford assay)

Total soluble protein was estimated using a commercial kit based on the Bradford assay (Bio-Rad, Sydney, Australia) with bovine serum albumin as the standard. Samples for analysis were diluted 1:10 with PBS + 0.02% formaldehyde (the dilution halts the reaction), then centrifuged for 15 min at 10,000 xg and the supernatant collected. 0.8 mL of the supernatant was mixed with 0.2 mL of dye reagent and the adsorption read at 595 nm after 5 min. Each sample was analysed in duplicate.

Note. Trails demonstrated that interference to the Bradford assay by reagents such as urea and EDTA was negligible after the 1:10 dilution with PBS + 0.02% formaldehyde.

A4 Recombinant protein estimation (HPLC analysis)

Solvents

0.1% TFA. Add approximately 200 mL of MQ water to a 500 mL volumetric flask. Add 500 μL of TFA to the volumetric flask. Make up the volume to 500 mL with MQ water. Immediately prior to use filter the reagents using the Millipore filtration rig and 0.2 μm GV filter paper.

80% Acetonitrile / 0.08% TFA. MQ water (100 mL) was added to a 500 mL volumetric flask. TFA (400 μL) was then added to the volumetric flask. The volume was made up to 500 mL with Acetonitrile. Immediately prior to use the reagents were filtered using a Millipore filtration rig and 0.2 μm GV filter paper.

Sample preparation.

The samples were centrifuged after collection (10,000 xg for 2 min), and stored frozen till HPLC analysis took place. DTT (0.5 mg) was added to 150 μL of thawed sample, mixed and left for 5 min. Immediately prior to injection 5 μL TFA was added and the sample mixed.

HPLC operation.

The column was a C4, aquapore, reverse phase column, (Brownlee Laboratories, Santa Clara, CA). A 20 to 50% (v/v) acetonitrile gradient over 30 min was used to separate the proteins. Protein was detected using a UV/Vis in-line spectrophotometer set at 215 nm. A Long-R^3-IGF-I standard (GroPep, Adelaide, Australia) was used to calculate the conversion factor for the HPLC analysis.

A5 Polyacrylamide gel electrophoresis (PAGE)

Polyacrylamide gel electrophoresis (PAGE) is able to separate proteins according to their molecular weight. It is a qualitative assay that can be rendered semi-quantitative when combined with image analysis or densitometry.

Solutions

Buffer A. Tris base (45.4 g) was dissolved in 250 mL MQ water and the pH adjusted to 8.8 with conc. HCl.

Buffer B. Tris base (6 g) was dissolved in 100 mL MQ water and the pH adjusted to 6.8 with conc. HCl.

10% SDS. Sodium dodecyl sulphate (SDS) (5 g) was dissolved in 50 mL MQ water.

10% Ammonium persulphate. Ammonium persulphate (100 mg) dissolved in 1 mL MQ water (immediately prior to use).

5 x Running buffer. Tris base (15 g), glycine (72 g), and SDS (5 g) were dissolved in 1 L MQ water.

Stain. Coomassie Blue R-250 (0.5 g), methanol (200 mL), and acetic acid (50 mL) made up to 500 mL with MQ water.

De-stain. Methanol (400 mL) and acetic acid (50 mL) made up to 1 L with MQ water.

Sample buffer. Buffer B (12.5 mL), glycerol (20 mL), β-mercaptoethanol (5 mL), and bromophenol blue (0.5 g) made up to 100 mL with MQ water.

Gel preparation

The clean glass plates and spacers were assembled, and clamped in place onto a rubber gasket. Acrylamide/bis (50 mL), buffer A (25 mL), 10% SDS (1 mL), and MQ water (23.5 mL) were combined to form the separating gel and degassed by application of a vacuum for 15 min. 10% Ammonium persulphate (0.5 mL) and TEMED (50 µL) were added to the solution immediately prior to pouring the slab. Butanol (approximately 10 mL) was run onto the top of the separating gel to provide a linear edge. The gel was given 30 min to set. Buffer B (2.5 mL), acrylamide/bis (1.3 mL), 10% SDS (0.1 mL), and MQ water (6.1 mL) were combined to form the stacking gel and degassed by application of a vacuum for 15 min. 10% Ammonium persulphate (50 µL) and TEMED (10 µL) were added to the solution immediately prior to pouring the stacking gel. The butanol was tipped off the gel and the top of the separating gel rinsed with MQ water. A comb was placed above the separating gel and the stacking gel poured to fill the space above the separating gel. The gel was given 30 min to set. The comb was removed and the wells rinsed with MQ water. The PAGE gel assembly was removed from the gel setting platform and fixed to the electrophoresis tank cooling core. Running buffer (1.5 L) was added to the electrophoresis tank.

Sample preparation

To 2 parts of the sample 1 part of 30% trichloroacetic acid was added and the solution left for 5 min. The solution was then centrifuged at 10,000 xg for 5 min. The supernatant discarded and the pellet resuspended in sample buffer. The sample was then heated to 100°C for 5 min. The sample was then cooled prior to loading in the wells in the stacking gel.

PAGE operation

The complete with PAGE gels were attached to the electrophoresis tank cooling core. Approximately 100 mL running buffer was added to the top of the cooling core. Samples were then loaded to the wells in the stacking gel using an autopipette. After loading is finished the lid is placed on the electrophoresis tank and the cables attached to the 1000/500V power supply. The power supply was set to maximum voltage and power, and the current set to 40 Amps (for 2 gels). Run was pressed to start electrophoresis. It ran for approximately 4.5 h. On completion the gels were removed from the electrophoresis tank, then from the cooling core and from the glass plates. The gels were place in Coomassie Blue stain for over 4 h. To destain the gels they were placed in 500 mL destain solution for 30 min (this process was repeated till the background of the gel became clear).

Appendix B Media composition

B1 Shake flask media

D-Glucose (3.125 g/L), NH_4Cl (2.42 g/L), KH_2PO_4 (2.38 g/L), Na_2HPO_4 (3.9 g/L), K_2SO_4 (1.82 g/L), $MgSO_4.7H_2O$ (0.625 g/L), $FeSO_4.7H_2O$ (20 mg/L), $MnSO_4.H_2O$ (5.1 mg/L), $ZnSO_4.7H_2O$ (8.6 mg/L), $CuSO_4.5H_2O$ (0.76 mg/L), trisodium citrate (88mg/L), and Thiamine (40 mg/L).

The D-glucose and the $MgSO_4.7H_2O$ were dissolved in RO water (half the final volume), and stored in a Schotte bottle. The remaining components except thiamine were dissolved in RO water (half the final volume), and 25 mL added to each 100 mL conical shake flask. Cotton wool bungs were placed in the mouths of the flasks, and the top wrapped with aluminium foil. The glucose/ $MgSO_4$ solution and the flasks were autoclaved at 121°C for 20 min. The glucose/ $MgSO_4$ solution (25 mL) was added aseptically to each flask. The thiamine solution was filter sterilised and aseptically added to each flask to give a final concentration of 88 mg/L.

B2 Fermentation media

The fermentation media used the same recipe as the shake flask media except D-Glucose was 6.25 g/L.

B3 PBS

NaCl (6.5 g/L), KH_2PO_4 (1.37 m/L). pH was adjusted to 6.9 with 2 M NaOH.

Appendix C Fermentation operation parameters

C1 Standard fermentation procedure - uninduced

A glycerol stock of *E. coli* strain JM101 ($lacI^q$) containing the strictly-regulated plasmid p[Met^1]-pGH(11)-Val-Asn-[Arg^3]-IGF-I was streaked onto a C1 agar (plus 100 mg/l Amp) plate and incubated at 37°C for 24 h. A shake flask containing 50 mL of sterile modified C1 media (Appendix B1) was inoculated with a colony from the plate and then incubated at 37°C for 8 h. A volume from the shake flask was used to inoculate the fermenter (Chemap CF2000) containing 12 L of modified C1 media (Appendix B2) to give an initial absorbance at 600 nm (A_{600}) of 0.00025. The pH of the fermenter was controlled at 6.9 using 2 M NaOH, the temperature at 37°C, and the dissolved oxygen above 50% saturation. Samples were collected from the fermenter and the A_{600} measured. At an A_{600} of 4.0 (logarithmic phase cells) and 4 h after the culture exhausted the glucose in the media (stationary phase cells), the *E. coli* samples were harvested. The samples were centrifuged at 10000 xg, 4°C, for 30 min, and the cell pellet resuspended in phosphate-buffered saline (Appendix B3) to give an A_{600} of 80.

C2 Standard fermentation procedure - induced

A glycerol stock of *E. coli* strain JM101 ($lacI^q$) containing the strictly-regulated plasmid p[Met^1]-pGH(11)-Val-Asn-[Arg^3]-IGF-I was streaked onto a C1 agar (plus 100 mg/L Amp) plate and incubated at 37°C for 24 h. A shake flask containing 50 mL of sterile modified C1 media (Appendix B1) was inoculated with a colony from the plate and then incubated at 37°C for 8 h. A volume from the shake flask was used to inoculate the fermenter (Chemap CF2000) containing 12 L of modified C1 media (Appendix B2) to give an initial absorbance at 600 nm (A_{600}) of 0.00025. The pH of the fermenter was controlled at 6.9 using 2 M NaOH, the temperature at 37°C, and the dissolved oxygen above 50% saturation. Samples were collected from the fermenter and the A_{600} measured. At an A_{600} value of 2.0, recombinant protein expression was induced by the addition of 16 mg/L IPTG. The fermentation proceeded for another 4 h then the cells were harvested.

The samples were centrifuged at 10000 xg, at 4°C, for 30 min, and the cell pellet resuspended in phosphate-buffered saline (Appendix B3) to give an A_{600} value of 80.

C3 Preparation of an inclusion body extract.

The cells produced following the procedure detailed in Appendix C2 were collected after 4 h induction prior to the centrifugation step (note, these were used immediately after harvesting, they were not treated with a killing agent nor frozen prior to use). The harvested cells were homogenised using 3 passes through an APV-Gaulin model 15MR-8TBA with an operating pressure of 56 MPa. The homogenate was centriguged at 10000 xg, 4°C, for 30 min, and the cell pellet resuspended in phosphate-buffered saline (Appendix B3) to give a cell concentration equivalent to A_{600} value of 80. This resulted in a suspension of inclusion bodies (plus cell debris) at the same proportions as in the whole cell preparation in Appendix C2.