Chromatography refolding of recombinant alpha-fetoprotein for high refolding productivity and intensified bioprocessing

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**CHROMATOGRAPHY REFOLDING OF RECOMBINANT
ALPHA-FETOPROTEIN FOR HIGH REFOLDING
PRODUCTIVITY AND INTENSIFIED BIOPROCESSING**

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ABSTRACT

Alpha-fetoprotein (AFP) is a commercially valuable biopharmaceutical candidate for autoimmune indications. Transgenically-derived recombinant AFP has recently successfully completed a Phase Two clinical trial study for rheumatoid arthritis indications at Merrimack Pharmaceuticals (Cambridge, MA, USA). The launch of this protein on market shelves in the future will subsequently demand cheaper second-generation product when product patent expires, thus necessitating new processes that can reduce product cost. The production of AFP as inclusion bodies (IBs) in *Escherichia coli* (*E. coli*) is advantageous for process-scale commercial manufacture due to speed, simplicity and cost reasons but conversion of the inactive protein aggregate into biologically active protein requires an efficient refolding step. The use of dilution refolding in previously reported recombinant human AFP (rhAFP) laboratory processes has resulted in low refolding yields, which negatively impacts the overall process yield and productivity. A superior refolding and bioprocessing route is clearly needed to facilitate efficient and rapid product delivery to market, if a commercial process for rhAFP is to be possible.

In this thesis, chromatography refolding was researched to address the poor refolding performance of rhAFP in previous ‘dilution refolding’-based rhAFP processes. The successful development of two chromatography refolding platforms, i.e. (i) on-column anion exchange chromatography (AEC) and (ii) size exclusion chromatography (SEC), is reported.

To facilitate rhAFP chromatography refolding, an optimised laboratory process based on mechanical disruption was developed to efficiently recover and purify rhAFP for direct loading into the chromatography column without the need for complex pre-treatment steps. On-column AEC refolding achieved a rhAFP refolding yield of 28% at 1 mg/ml protein refolding concentration, which increased rhAFP refolding productivity and purity by 9- and 10-folds, respectively, compared to dilution refolding. The distinct advantage of AEC refolding is its simultaneous purification capability, where refolded rhAFP could be recovered at 95% purity in a single

chromatography refolding step. NaCl-controlled surface refolding of AEC was found to be important in increasing molecular flexibility during refolding on AEC matrices.

The development of a novel pulse-fed SEC (PF-SEC) refolding platform further increased rhAFP refolding yield to 53% at 1 mg/ml refolding concentration. The unique integration of multiple feed loads and the establishment of a urea gradient along the axial direction of the column increased rhAFP refolding productivity by 8- and 64-fold using triple pulse feeds compared with on-column AEC refolding and pulse dilution refolding, respectively, at 1 mg/ml rhAFP refolding concentration.

The basis for a ‘chromatography refolding’-based rhAFP process that resolves low refolding yield and process productivity challenges is provided through this thesis. The process intensification contributions of chromatography refolding coupled with the well-documented scalability of chromatography columns from laboratory to industrial scales will ease process validation to deliver rhAFP at affordable costs.

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LIST OF ABBREVIATIONS

15Q	SOURCE 15Q
AEC	Anion exchange chromatography
AFP	Alpha-fetoprotein
BSA	Bovine serum albumin
CD	Circular dichroism
CE	Chemical extraction
CE ₀	Chemical extraction processing without PEI treatment
CE _p	Chemical extraction processing with PEI treatment
CV	Column volume
DCE	Direct chemical extraction
DEAE (S)	Fractogel EMD DEAE (S)
DEAE-FF	HiTrap DEAE Sepharose Fast-Flow ion exchange columns
DTT	Dithiothreitol
<i>E. coli</i>	<i>Escherichia coli</i>
EDTA	Ethylenediaminetetraacetic acid
FPLC	Fast protein liquid chromatography
GSH	Oxidised glutathione
GSSG	Reduced glutathione
GST	Glutathione S transferase
HCl	Hydrochloride
HIC	Hydrophobic interaction chromatography
HPLC	High performance liquid chromatography
IB	Inclusion body
IBs	Inclusion bodies
IEC	Ion exchange chromatography
IMAC	Immobilised metal affinity chromatography
IPTG	Isopropylthio-β-D-galactoside
KCl	Potassium chloride
KH ₂ PO ₄	Potassium phosphate monobasic
LPS	Lipopolysaccharide

MBP	Maltose binding protein
MD	Mechanical disruption
MD ₀	Mechanical disruption processing without a washing step
MD _W	Mechanical disruption processing with a Triton X-100 washing step
Na ₂ HPO ₄	Sodium phosphate dibasic
NaCl	Sodium chloride
PALS	Phase analysis light scattering
PBS	Phosphate-buffered saline
P-CAC	Preparative continuous annular chromatography
PEG	Polyethylene glycol
PEI	Polyethyleneimine
PF-SEC	Pulse-fed size exclusion chromatography
pI	Isoelectric point
Q-FF	HiTrap Q Sepharose Fast-Flow ion exchange columns
rhAFP	Recombinant human alpha-fetoprotein
RP-HPLC	Reversed phase high performance liquid chromatography
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SEC	Size exclusion chromatography
SMB	Simulated moving bed
std-AFP	Standard AFP
Tris	Tris(hydroxymethyl)aminomethane

CHAPTER 1 : INTRODUCTION

Proteins are essential components of life and participate in virtually every cellular activity. They serve as enzymes that catalyse biochemical reactions, function as transport molecules, form receptors and channels, and have important mechanical functions to maintain cell shape (Nelson and Cox, 2004). Importantly, proteins display important therapeutic functions. Protein molecules are more advantageous than small molecule drugs in terms of their (i) high specificity and more complex functions that cannot be replicated by smaller molecule drugs, (ii) lower tendency to cause adverse side effects or elicit human responses due to their highly specific functions and endogenous origin in the human body, and (iii) faster clinical development and regulatory approval time. From a cost perspective, the unique primary structure of each protein renders more extensive patent protection for protein drugs compared to their small molecule counterparts (Leader *et al.*, 2008). Their effective treatment of many diseases and their role as the only approved therapy for some diseases such as diabetes mellitus (Clark *et al.*, 1982; Banting *et al.*, 1991; Bach, 1994), haemophilia (Bray *et al.*, 1994) and growth disorders such as acromegaly (Trainer *et al.*, 2000; van der Lely *et al.*, 2001) make protein-based drugs indispensable healthcare essentials today.

The advent of recombinant DNA technology coupled with the completion of human genome sequencing efforts have contributed to the remarkable growth in the global market for protein therapeutics, currently estimated to be worth approximately USD 50 billion¹, where demand for first and second generation protein pharmaceuticals continues to increase. Recombinant DNA technology has enabled the over-expression of important proteins in host cells such as bacteria, yeast and insect cells, leading to the successful production of several blockbuster drugs such as insulin, tumor necrosis factor-alpha human monoclonal antibody, interleukin-1, and β -glucocerebrosidase^{1,2} (Johnson, 1983; Grabowski *et al.*, 1995; Leader *et al.*, 2008). However, the

¹ <http://www.researchandmarkets.com/reports/c50905>

² <http://www.biopharma.com/approvals.html>

widespread use of recombinant biopharmaceuticals across all patient segments continues to be hindered by the high cost of producing these molecules, compared to small molecule drugs or traditional medicine. A huge portion of the production cost is attributed to product recovery costs.

1.1 Challenges in recombinant protein production

Escherichia coli (*E. coli*) is expected to remain as the favoured expression host for recombinant protein production due to ease of host manipulation and cultivation, as well as cost advantages (Li *et al.*, 2004b; Jana and Deb, 2005; Sahdev *et al.*, 2008). Despite these advantages, protein production in *E. coli* still faces a number of challenges that are product-specific. For example, heterologous protein expression in *E. coli* does not always allow the protein to be produced in the correctly folded three dimensional conformations. Very often, *E. coli*-derived proteins are expressed as inclusion bodies (IBs) which are dense, insoluble and inactive protein aggregates (Taylor *et al.*, 1986; Mitraki *et al.*, 1991). IB formation can be advantageous in terms of the high expression yield and product protection from proteolysis degradation (Taylor *et al.*, 1986; Valax and Georgiou, 1993) but necessitates an *in vitro* refolding step to convert the protein aggregates to their biologically active forms. A high refolding yield to convert the insoluble proteins to biologically active proteins is critical to maintain a high overall product recovery (Choe *et al.*, 2006). Conventional refolding methods such as dilution refolding can be readily used for laboratory scale refolding. However, dilution refolding which requires refolding to be conducted at very low protein concentrations to minimise off-pathway aggregation, is difficult to scale-up due to high solvent and vessel requirements. Furthermore, as protein refolding yields vary in a contaminant-dependent fashion, the need for pre- and post-purification steps to be coupled with dilution refolding necessitates an increased number of unit operations in a process, which will increase process complexity leading to more difficult process validation for industrial applications. In recent years, refolding in chromatography columns has attracted industrial interest due to the ability to perform refolding at much higher protein concentrations than dilution (Kim *et al.*, 1997; Negro *et al.*, 1997; Qiu *et al.*, 1997). Chromatography column scalability from lab to industrial stages has also been well-documented (De Bernardez Clark, 2001;

Jungbauer *et al.*, 2004; Jungbauer and Kaar, 2007), rendering it attractive as an enabling technology to intensify protein pharmaceutical manufacturing. Improvements in conventional refolding methodology that can improve refolding productivity and contribute towards bioprocess intensification are important to increase the production efficiency of clinically-important proteins in *E. coli*, while keeping a low cost metric. Success in these efforts will ensure that protein biopharmaceuticals can be marketed at cheaper prices, thus making drugs more affordable in the future.

1.2 Alpha-fetoprotein: a biopharmaceutical candidate

The focus of this Ph.D. project is to research and develop new refolding strategies leading to increased overall process yield and productivity for the production of a biopharmaceutical candidate, i.e. alpha-fetoprotein (AFP), in *E. coli*. AFP is currently one of the lead investigational drugs at Merrimack Pharmaceuticals (Cambridge, MA, USA) for treating multiple sclerosis and myasthenia gravis (Dudich, 2007). Pre-clinical safety and efficacy studies of AFP in animal models have shown convincing evidence of the immunomodulatory potential of AFP in multiple indications, and clinical trials are currently underway to test the effectiveness of AFP in treating rheumatoid arthritis and psoriasis in human patients¹ (Dudich, 2007). A potentially huge market demand for AFP is envisaged upon regulatory approval of this protein, where AFP is expected to address medical needs for those diseases which cannot be effectively treated today. AFP for clinical evaluation at Merrimack is obtained from the milk of transgenic goats¹. The transgenic production route is disadvantaged by (i) the unproved processing route, which may also be possibly more costly, and (ii) the increased risk of patient exposure to animal diseases. The launch of AFP on market shelves in the near future will subsequently demand cheaper second-generation product when product patent expires, thus necessitating new processes that can reduce product cost.

¹ <http://www.merrimackpharma.com/>

The production of recombinant human AFP (rhAFP) as IBs in *E. coli* has been recently reported in two separate studies (Boismenu *et al.*, 1997; Leong and Middelberg, 2007). In the first study (Boismenu *et al.*, 1997), the use of an expensive lysozyme-induced cell lysis step embedded in a laboratory process that comprises many unit operations is reported. The complex process flowsheet and the lack of absolute refolding yield quantification increases the scale-up difficulty of that process. In the second study (Leong and Middelberg, 2007), the use of a ‘chemical extraction’-based process was reported for production of rhAFP. Despite the simplicity and ease of operation of the chemical extraction process, the non-selective nature of chemical extraction resulted in the simultaneous release of other host proteins, which necessitates a series of feed pre-treatment steps (i.e. DNA removal and two chromatography steps) prior to refolding. Importantly, the use of dilution refolding in both processes resulted in low rhAFP refolding yields and low refolded protein concentrations. Confronted with the limitations of the two earlier AFP production processes, research into the development of superior refolding strategies and improved bioprocessing for more efficient and higher productivity manufacturing of AFP is clearly needed, and forms the overall goal of this Ph.D. research.

1.3 Research objectives and scope

The overall objective of this Ph.D. research project is to develop new refolding strategies to improve rhAFP refolding yields and overall process productivity. These refolding platforms should be readily integrated into the bioprocess without the need for multiple or expensive pre- and post-refolding conditioning or purification requirements. Considering the process intensification advantages that chromatography refolding offers, the development of chromatography refolding methods for rhAFP will be researched to enhance refolding productivity, leading to improved process economics. The overall objective of this Ph.D. study will be guided by three specific aims as follows.

- (i) To develop a simple, chromatography-free and effective rhAFP laboratory process that can deliver rhAFP at sufficiently high purity for direct use in chromatography refolding studies.

- (ii) To develop an ion exchange chromatography (AEC) refolding strategy for high productivity refolding of rhAFP. The effects of protein-resin interaction, resin chemistry and mobile phase physicochemical environment on rhAFP will be studied. The capacity for bioprocess intensification in the form of simultaneous protein refolding, purification and concentration *via* a single chromatography refolding step will be researched.
- (iii) To develop an alternative ‘non-adsorptive’ chromatography refolding strategy for rhAFP refolding using size exclusion chromatography (SEC). Adsorptive chromatography refolding methods require the adsorption of proteins to column matrices, where such adsorption often induces a strong protein-matrix interaction thus restricting the flexibility of protein molecule and ease of refolding. A non-adsorptive refolding strategy is advantageous in removing steric constraints to refolding, since SEC refolding allows unfolded protein molecules to refold in the column matrix pores. Strategies to overcome low load and productivity constraints inherent in existing SEC refolding methodologies will also be researched.

1.4 Thesis outline

This thesis consists of 7 chapters.

Chapter 2 reviews AFP’s molecular characteristics and therapeutic functions, and the protein’s potential role as a biopharmaceutical candidate for autoimmune diseases. Conventional and current methodologies for bioprocessing *E. coli*-derived proteins are presented, and their advantages and disadvantages with respect to yield, product quality and cost are discussed. The mechanisms of protein refolding are also discussed at process and molecular levels, and AFP refolding literature is reviewed. Recent developments in chromatography tools are presented. This chapter aims to guide the development of an AFP-focused chromatography refolding platform and bioprocess that will improve rhAFP process yield and throughput.

In Chapter 3, the development of a pre-refolding purification process of AFP for IB release and removal of soluble contaminants, to provide rhAFP at high purity for chromatography refolding studies was investigated. ‘Mechanical disruption’- and ‘chemical extraction’-based laboratory processes for rhAFP were developed and a parallel comparison of these two processes in terms of product purity, recovery, process flowsheet complexity, and non-proteinaceous host cell contaminant carry-over were compared.

In Chapter 4, the development of an AEC refolding platform for rhAFP refolding is presented. Systematic studies of chromatographic refolding design and operating parameters to improve rhAFP yields were studied. The use of biophysical and biochemical analytical tools to evaluate the success of rhAFP refolding is also reported.

Chapter 5 reports the investigation of the effect of NaCl on surface refolding of rhAFP on anion exchange matrices. The effects of salt-induced variation in protein and resin surface charges on the on-column refolding performance of rhAFP were investigated. rhAFP adsorption isotherms in the presence of salt were also studied to elucidate the role of ionic strength in governing protein adsorption on the anion exchange matrices.

Chapter 6 reports the development of a novel SEC refolding strategy for improved rhAFP refolding. The use of a new pulse sample feeding strategy to overcome poor refolding productivity associated with the low sample to bed volume ratio inherent in SEC refolding operations was researched. Parallel comparison of the refolding performance achieved by SEC, AEC and dilution refolding was performed.

In Chapter 7, conclusions of the overall research achieved from this study are summarised, and the potential avenues for future work are presented.

CHAPTER 2 : LITERATURE REVIEW

2.1 Alpha-fetoprotein (AFP)

This chapter starts with the review of literature knowledge on AFP molecular characteristics and clinical functions which is critical for the development of an AFP-oriented refolding platform.

2.1.1 AFP structure

AFP is a large glycoprotein with a molecular mass of 68 kDa. It consists of a single polypeptide chain with 590 amino acid residues (Morinaga *et al.*, 1983a), and is glycosylated at the N-terminus with ~3 kDa oligosaccharides (Morinaga *et al.*, 1983b). AFP has 15 disulfide bonds which contribute to the conformational stability of the molecule and drive the formation of the molecule's 'V'-shaped structure (Figure 2.1) (Luft and Lorscheider, 1983). The AFP molecule has 3 domains (i.e. domain I, II and III) (Morinaga *et al.*, 1983a); each domain has approximately 195 amino acid residues with 4, 5, 6 disulfide bonds positioned in domain I, II and III respectively (Morinaga *et al.*, 1983a). The binding of natural ligands such as long chain unsaturated fatty acids, including arachidonic acid, docosahexaenoic acid, oleic acid and linoleic acid (Parmelee *et al.*, 1978; Carlsson *et al.*, 1980) to AFP affects the molecule's net charge properties; AFP isolated from human fetal tissues has been reported to either have isoelectric points (pIs) of 4.7 or 5.3 depending on the fatty acid content, where pI of 5.3 corresponds to fat-free AFP while a pI of 4.7 corresponds to AFP with bound fatty acids (Parmelee *et al.*, 1978).

Figure 2.1: Spatial structure of human AFP as elucidated by electron microscopy (Luft and Lorscheider, 1983).

2.1.2 Clinical importance of AFP

AFP is an important molecule for diagnostic and therapeutic applications, where fluctuation in AFP levels in the plasma of both embryo and adults has critical medical significance (Markku and Ruoslahti, 1973; Brock *et al.*, 1975; Markku, 1975; Ruoslahti and Seppala, 1979; Deutsch, 1991). AFP belongs to the family of albumin-like proteins (Deutsch, 1991), and is normally only produced in the fetus by the liver and yolk sac during fetal development. AFP is therefore present at high concentration in the fetus serum, but is almost substituted by serum albumin at the end of the second month of postpartum (Bergstrand and Czar, 1956; Gitlin and Pericelli, 1970; Ruoslahti and Seppala, 1972; Deutsch, 1991; Gillespie and Uversky, 2000). Only small AFP traces are detected in the serum of healthy adults. Elevated AFP concentrations in pregnant women are indicative of developmental abnormalities in the baby, such as Down's syndrome and open neural tube defects. AFP is also a tumor marker for diseases such as liver cancer and germ cell tumors of the ovary and testis (Abelev, 1971; Okuda *et al.*, 1975; Yoshiki *et al.*, 1976; Deutsch, 1991). It has also been reported that increased AFP levels in the serum of pregnant women is correlated with the remission of rheumatoid arthritis, multiple sclerosis, and other autoimmune disorders (Silva and Spector, 1992; Buyon *et al.*, 1996; Confavreux *et al.*, 1998; Nelson, 1998; Agarwal *et al.*, 1999; Parker *et al.*, 2004; Dudich, 2007). The

hypothesised role of AFP in modulating the mothers' immune system to protect the developing fetus from autoimmune disorders such during pregnancy, has been verified by animal model studies (Brenner and Abramsky, 1981; Abramsky *et al.*, 1982; Abramsky and Brenner, 1983; Brenner *et al.*, 1984; Brenner *et al.*, 1985; Yamashita *et al.*, 1994; Ogata *et al.*, 1995; Matsuura *et al.*, 1999). AFP is currently under clinical development by Merrimack Pharmaceuticals Inc. (Boston, MA, USA) as a biopharmaceutical candidate for the treatment of autoimmune diseases. The use of recombinant AFP derived from the milk of transgenic goats for rheumatoid arthritis indications has recently been successfully completed in a Phase Two clinical trial study by Merrimack¹.

In addition to diagnostic applications, AFP possesses extracellular and intracellular transport functions. It was shown that AFP could interact with estrogens, has high affinity to fatty acids, bilirubin, and can bind Cu²⁺, Ni²⁺ ions (Aoyagi *et al.*, 1978; Parmelee *et al.*, 1978; Gillespie and Uversky, 2000). The participation of AFP in regulating cell multiplication and metabolism, as well as its interaction with microphages (Lu *et al.*, 1984) and T-lymphocytes has been as associated with its intracellular transport function.

To cost-effectively commercialise AFP for autoimmune therapy and extend the use of AFP for diagnostic applications, an efficient bioprocess that can economically produce kilograms of bioactive protein is needed. Neither glycosylation nor ligand binding was reported to be necessary for AFP biological activity (Semeniuk *et al.*, 1995), thus opening the way for the use of the *E. coli* inclusion body (IB) route to produce AFP (Boismenu *et al.*, 1997; Leong and Middelberg, 2007). The two rhAFP bioprocesses reported, however, are still encumbered by low process productivity and yield due to sub-optimal refolding and downstream processing strategies. A thorough understanding of bioprocessing requirements which encompass detailed knowledge in recombinant expression systems, refolding and purification strategies, and process integration is essential to enhance production output, as will be reviewed in Sections 2.2 and 2.3 of this chapter.

¹ <http://www.merrimackpharma.com/>

2.2 Recombinant protein expression

2.2.1 Overview of protein expression systems

The two most well-established protein expression systems for heterologous protein expression are (i) cell-based and (ii) cell-free expression systems.

Cell-based protein expression systems are very widely used and involve transforming host cells with vectors that carry cloned encoding gene of different target proteins. The vector will then direct expression of the recombinant proteins in the host cells at high amounts, usually under the regulation of a strong promoter. Both prokaryotic and eukaryotic expression systems are used for recombinant production of proteins. Commonly used host cells include bacteria, yeast, insect and mammalian systems. Each expression system has its advantages and limitations. The choice of the expression system depends on the desired properties of the target protein such as solubility, speed, yield, functionality and post-translational modification requirements (Andersen and Krummen, 2002; Schmidt, 2004). Bacteria expression systems are popular because bacterial cells grow rapidly on cheap media, are easy to culture and scale up. However, formation of the target protein as insoluble protein aggregates which necessitates an *in vitro* refolding step, and the inability of bacteria to perform complex post-translational modifications which may be required for protein functions are major shortcomings for this expression system. Yeast cells can efficiently express recombinant proteins in the soluble form and avoid insolubility problems, but post-translational modifications of the target protein are not always correct (Boer *et al.*, 2007). Although more complex post-translational modification can be carried out by insect cells expression system, the cost is very high compared with bacteria and yeast expression systems (Wickham *et al.*, 1992). Mammalian cells can produce recombinant proteins with the correct post-translational patterns (e.g., glycosylation). Although secreted levels of recombinant proteins from mammalian cells are regularly reported above 1 g/L, most mammalian expression yields are still lower compared to *E. coli*. However, it has been recently reported that the use of glutamine synthetase (GS) in Chinese hamster ovary (CHO) could reach a 5 g/l expression yield (Park *et al.*,

2010). The main drawbacks of mammalian cell expression like slow growth of mammalian cells, and high costs of cell culture media still puts them at a slight disadvantage compared to bacteria systems for large scale production of proteins (Choe *et al.*, 2006).

The cell-free protein expression system synthesises proteins *in vitro* using cellular biomolecular translation machinery components which comprises RNA polymerase, ribosomes, transcription factors, regulatory protein factors and tRNAs (Jermutus *et al.*, 1998; Nakano and Yamane, 1998; Katzen *et al.*, 2005). This expression system can rapidly synthesise small amounts of the protein of interest in a cell-free environment for identification. The amount of proteins produced, however, is insufficient for detailed structure-function characterisation. Cell-free expression systems are therefore unsuitable for large scale production of proteins due to the exorbitant scale-up cost of the cell extracts.

2.2.2 *E. coli* as a host system for protein expression

The *E. coli* expression system was successfully used to express recombinant AFP at relatively high yields in earlier studies (Boismenu *et al.*, 1997; Leong and Middelberg, 2007). High protein expression yields will readily facilitate high cell density fermentation, which renders this expression system suitable for large-scale AFP production. The absence of glycosylation has also been found to have no effect on AFP *in vitro* refolding (Leong and Middelberg, 2006b). For these reasons, *E. coli* was chosen as the host system for AFP expression.

However, the formation of AFP as IBs due to high protein over-expression necessitates efficient conversion of the IBs to the biologically active state by *in vitro* refolding (Jana and Deb, 2005; Sahdev *et al.*, 2008). The exact reason for IB formation in *E. coli* remains unclear and has been suggested to be related to changes in kinetic competition between folding and aggregation caused by the changing rate of protein synthesis, and insufficient supply of chaperones to support correct protein refolding. IBs, however, can be advantageous in terms of (i) high protein expression levels, (ii) protection from host proteolytic degradation, (iii) easy separation from

soluble contaminants, and (iv) low risk of protein denaturation in the reducing cytoplasmic environment or inactivation during product release (Fischer *et al.*, 1992b; Datar *et al.*, 1993; Walsh and Headon, 1994; Rudolph and Lilie, 1996; Swartz, 2001; Li *et al.*, 2004b). The development of an optimal and intensified bioprocessing and refolding strategy to maximise recovery of bioactive proteins while minimising production cost is crucial. Current techniques for IB processing are reviewed in Section 2.3, and the process advantages and disadvantages of the different methods are presented.

2.3 Bioprocess development for recombinant protein production in *E. coli*

The goal of downstream processing is to recover as much of the target protein in the purest possible state using a streamlined and cost-effective process. Bioprocess design strategies are primarily governed by the solubility of the expressed proteins (Jungbauer and Kaar, 2007). Solubility of the expressed protein can often be enhanced with fusion tags, which are solubility-enhancing protein partners that are genetically fused to the target protein. Examples of fusion tags which are commonly used to enhance protein solubility are maltose binding protein (MBP) and thioredoxin (Trx). Glutathione S transferase and polyhistidine are commonly used in tandem with MBP and Trx to ease protein purification (Jana and Deb, 2005; Sahdev *et al.*, 2008). However, fusion tag removal either by chemical or enzymatic means can significantly complicate bioprocessing due to the need for additional purification steps to purify the target protein from the cleaved fragments. The low specificity of chemical cleavage methods as well as poor efficiency and high cost of enzymatic cleavage systems can also decrease the overall yield and cost-effectiveness of the process.

The bioprocessing for both soluble and insoluble proteins generally includes target protein release, contaminant separation and purification of the target protein, followed by further polishing steps, if necessary (Figure 2.2). A few additional steps are required for processing of IB proteins following product release such as IB washing, resolubilisation and refolding. Section 2.3.1 reviews current techniques for IB isolation prior to protein refolding.

2.3.1 IB isolation

IBs are typically recovered from the cell cytoplasm by cell lysis through chemical or mechanical means. At lab scale, degradation of cell walls using mild non-ionic detergents or *via* enzymatic means using lysozyme has been reported (Georgiou and Valax, 1999; Vallejo and Rinas, 2004b; Jungbauer and Kaar, 2007). Optimising detergent concentrations for cell lysis is important to maintain protein integrity and minimise problems in downstream processing, for example, during membrane filtration/concentration (De Bernardez Clark, 2001). Although effective at small scale, the use of lysozyme for membrane permeabilisation is prohibitively expensive at large scale, and also necessitates rigorous purification efforts to completely remove the lysozyme. To date, the two IB isolation methods that are generally accepted as scalable and easily validated are (i) mechanical disruption (Suhail Alam and Agrawal), and (ii) chemical extraction (CE) (Chaudhuri, 1994).

Figure 2.2: Generic bioprocess flowsheets for processing of soluble and insoluble recombinant proteins derived from *E. coli*.

2.3.2 Mechanical disruption and solubilisation

Common MD techniques which have been developed for releasing IB proteins are (i) sonication, (ii) French press and (iii) high pressure homogenisation (Vallejo and Rinas, 2004b; Jungbauer and Kaar, 2007). Sonication employs ultrasound energy (20-50 kHz) which is generated by high frequency oscillation of the sonicator probe, to disrupt the cells and release cellular contents (Le and Trotta, 1991). French press and high pressure homogeniser induce cell lysis by subjecting the cells to high shear and viscous stresses, which lead to cell disruption (Vanderheiden *et al.*, 1970; Le and Trotta, 1991).

After MD, the soluble protein fraction is separated from the insoluble fraction by centrifugation. To improve the purity of the IB proteins, the cell pellets obtained post-centrifugation are often washed with selective buffers to remove non-specifically bound cellular contaminants such as bacterial cell debris and outer membrane proteins (Keith *et al.*, 1987; Valax and Georgiou, 1993; Batas *et al.*, 1999; Rathore *et al.*, 2003; Venkiteshwaran *et al.*, 2007). Washing can be performed with detergents, denaturants (used at non-denaturing concentrations) and reducing agents (Lim *et al.*, 1989; Guisez *et al.*, 1993; Oneda and Inouye, 1999; Sunitha *et al.*, 2000). Effective removal of contaminants from the IB pellets is important as refolding efficiency has been shown to decrease with poor IB purity (Leong and Middelberg, 2007).

The recovered IBs are then solubilised in high concentrations of chaotropic agents such as 8 M urea or 6 M guanidine hydrochloride, to disrupt non-covalent bonds in the protein aggregates (Fischer *et al.*, 1992b). Reducing agents such as 2-mercaptoethanol and DTT are also often added in the resolubilisation step to reduce disulfide bonds and to recover the IBs in a monomeric and soluble form (Ryden, 1989; De Bernardez Clark *et al.*, 1999; Tran-Moseman *et al.*, 1999; Danek and Robinson, 2004; Lee *et al.*, 2006b). Any remaining insoluble material can be removed by another centrifugation step, if required.

2.3.3 Chemical extraction and DNA removal

More recently, IB release by CE which involves the use of chemicals to permeabilise the bacteria cell membrane has also been reported (Figure 2.3). The use of an economical chemical fomulation which comprises high concentrations of urea (6 M) coupled with ethylenediaminetetraacetic acid (EDTA) and DTT has been shown to yield good protein extraction efficiency (Falconer *et al.*, 1997; 1998; 1999), and this method is now commonly known as direct chemical extraction (DCE). A high urea concentration is required to solubilise the IBs and render them monomeric for refolding. EDTA functions to enhance the permeabilisation of the bacterial cell wall by chelating divalent metal ions that crosslinks with the lipopolysaccharide (LPS) molecules in the outer membrane of *E. coli*, thus releasing the LPS layer and increasing permeability across the outer membrane (Leive, 1974). EDTA therefore facilitates free access of the denaturant to the cytoplasmic membrane, to weaken the hydrophobic interaction of lipid bilayer and enables the denaturant-solubilised protein to exit the cytoplasm. DTT enhances the solubilisation of IBs after extraction from their bacterial hosts (Chang and Swartz, 1993), by reducing mispaired inter- and intra-molecular disulfide bonds, and prevents further cysteine oxidation in the oxidising environment during cell lysis. The choice of pH used during chemical permeabilisation has been shown to also affect protein release from treated cells (Falconer *et al.*, 1997; 1998). At acidic pH values, the chemicals had nearly no effect on bacterial cell wall permeability. Under an alkaline pH environment (i.e. at pH 7.0 and above), however, a much higher total protein release was observed (Falconer *et al.*, 1998). The kinetics of direct extraction was reported to have a time constant of minutes, which significantly reduces IB release time (Falconer *et al.*, 1997).

Figure 2.3: Schematic of direct chemical extraction which releases all intracellular components non-selectively in the solubilised form.

An important limitation in the use of CE is the non-selective release of both proteins and cellular DNA from the cell cytoplasm. The concurrent release of DNA can substantially increase the viscosity of the chemical extract mixture, which increases difficulty in chromatographic purification. Anion exchange chromatography (AEC) is a common method for purification of recombinant proteins since most *E. coli* proteins have a pI of 4-7 (Han and Lee, 2006), and will be mostly negatively charged at physiological pH. However, since DNA is also strongly negatively charged, the loading of a 'cellular DNA'-rich sample mixture onto AEC supports will result in competition for binding between DNA and the target protein, thus reducing the adsorption capacity of the column for the latter. To address DNA carry-over problems, the use of polycationic polymers such as 30 to 90 kDa polyethyleneimine (PEI) has been reported to effectively remove DNA contaminants in CE mixtures (Burgess and Robert, 1991). Since the pKa value of the imino group within the PEI polymer is 10-11, PEI remains positively charged in solutions of neutral pH and will neutralise anionic molecules including DNA. Based on a recently reported study, PEI can also behave as a chelating agent to permeabilise bacterial cell walls, and thus eliminates the need for EDTA during CE (Nian *et al.*, 2008). However, the disadvantage of using PEI is that PEI can also interact non-specifically with extracted proteins which are negatively charged in the CE mixture. Therefore, the PEI concentration used for DNA removal must be optimised to allow substantial removal of host cell DNA while minimising the loss of the target protein. A cationic polyamine (i.e. spermine) has also been effectively used to remove DNA (Choe and Middelberg, 2001) but is not widely used due to cost reasons.

2.3.4 Comparison of mechanical disruption and chemical extraction processing of IB proteins

Figure 2.4 presents the general bioprocess flowsheet schematics for MD- and CE-based processing for IB release and purification. Compared to MD, IB recovery by CE requires less processing steps, where repetitive centrifugation and washing steps which can be laborious and cost-intensive, if unoptimised, are eliminated (Choe *et al.*, 2006; Jungbauer and Kaar, 2007). The reduction in the number of processing steps

reduces yield loss, and enhances the simplicity and economic benefit of the CE technique. A major disadvantage of CE, however, is the non-selective extraction of a myriad of cell-derived proteins and DNA, which significantly reduces target protein purity compared to MD-processed proteins (Falconer *et al.*, 1997; 1998; Lee *et al.*, 2006a; Leong and Middelberg, 2007; Chen and Leong, 2009). Since chromatographic supports are intolerant to viscous crude samples, the chemical extract must be pre-treated for DNA removal before a pre-refolding purification step to purify the target protein from host cell contaminants before refolding (Leong and Middelberg, 2007; Chen and Leong, 2009). MD processing, on the other hand, readily yields high purity IBs, where the centrifugation and wash steps are effective in removing most of the cell debris, contaminant proteins and cellular DNA, thus eliminating the need for any pre-refolding chromatographic purification step (Jungbauer and Kaar, 2007; Chen and Leong, 2009). MD also has the advantage of recovering proteins at higher concentration compared to CE, where the re-suspension volumes of the pellet can be readily adjusted, which eases the subsequent refolding step (Chen and Leong, 2009).

Clearly, the choice of employing MD or CE for rhAFP bioprocessing will depend on the product quality post-IB release and purification, as well as process complexity. Both MD- and CE-based processing will be studied and optimised for rhAFP pre-refolding processing in this thesis.

Figure 2.4: Process flowsheet schematics of ‘mechanical disruption’- and ‘chemical extraction’-based bioprocesses.

2.4 Protein refolding

Following the attainment of denatured monomeric IBs in the soluble form, the denatured proteins will be subjected to an *in vitro* refolding step to recover the biological activity of the protein. The feasibility of using an IB route for protein production often hinges on the refolding yield obtained. Therefore, understanding refolding from molecular, kinetic and process perspectives is crucial to enhance refolding yield. This section reviews published theories that elucidate the dynamics of protein folding, leading to the discussion of molecular and process factors that must be considered during the selection of refolding parameters.

2.4.1 Protein folding theories and models

Protein folding is a physical self-organization process of which the single polypeptide chain folds into its characteristic functional three-dimensional structure. Each protein is translated from a corresponding sequence of mRNA to form a linear chain of amino acids, known as the protein primary structure (Alberts *et al.*, 2002). This linear chain then folds to form the secondary structure, which generally comprises alpha helices and beta sheets that are stabilised by hydrogen bonds (Alberts *et al.*, 2002). The formation of a tertiary structure with functional regions or domains usually follows secondary structure formation (Alberts *et al.*, 2002). The tertiary structure of a protein is stabilised by both covalent and non-covalent bonds between the diverse amino acid side chains present within the polypeptide (Pace *et al.*, 1996; Deechongkit *et al.*, 2004). Unlike the secondary structure which is limited to a few fixed conformations, the variation in tertiary structure is unlimited and dependent on the amino acid sequence of the polypeptide (Alberts *et al.*, 2002). In general, the driving forces involved in protein folding (e.g., van der Waals, electrostatic, hydrophobic and covalent bond formation) are directed at maintaining a stable and rigid protein conformation (Pace *et al.*, 1996). Quaternary structure formation does not occur in all proteins, and involves the "co-assembly" of folded polypeptide subunits.

In the 1950s, using the enzyme ribonuclease as a model protein, Christian Anfinsen showed that the three-dimensional structure of a protein is determined by its amino acid sequence (Anfinsen, 1973; Nelson and Cox, 2004). In that work, it was observed that the ribonuclease could refold to its original functional state when high concentrations of urea and reducing agents were removed. It was thus concluded that protein denaturation is reversible and the protein spontaneously folds into its native or energetically stable state when present in an optimal physicochemical environment. In 1969, Levinthal proposed that there are too many possible conformations for a protein to sample in search of its native state, and hence folding must occur as a result of a 'directed' process instead of a random conformational search (Nelson and Cox, 2004).

Different theoretical models of protein folding have followed the Levinthal paradox, including 'nucleation-propagation' model, 'diffusion-collision' model and

‘hydrophobic collapse’ model (Yon, 2001). The classical ‘nucleation-propagation’ model suggests that nucleation, followed by a fast propagation of folding around it, is the limiting step during refolding. The ‘diffusion-collision’ model which was established in 1976 by Karplus (Karplus and Weaver, 1994) proposes that nucleation occurs concurrently in different sections of the polypeptide chain resulting in the formation of different microstructures which will diffuse, associate and coalesce to assemble sub-structures with a native conformation. This view differs slightly from the ‘hydrophobic collapse’ model, which hypothesised that the first event that occurs during protein refolding is a collapse caused by the complex hydrophobic interactions *via* the long polypeptide chain before the formation of secondary and tertiary structures (Dill, 1985).

A new view which is more commonly known as the ‘energy landscape’ model, perceives protein folding as a diffusion-like process. This model proposes that fluctuations in the conformation of an unfolded or incompletely folded polypeptide chain would enable native-like interactions between the residues of the polypeptide to be formed, leading to correct refolding by trial and error (Wolynes *et al.*, 1995; Dobson, 2003). This new view describes protein folding using specific terms such as ‘folding funnels’ and ‘energy landscapes’, where a protein is believed to fold to the native state through a large number of intermediates having decreasing energy states (Figure 2.5).

Figure 2.5: The thermodynamics of protein folding depicted as a free-energy funnel (Alberts *et al.*, 2002).

The ‘energy landscape’ model proposes that unfolded proteins in non-denaturing environments are unstable structures having high energetic states. As the polypeptide chain travels toward the bottom of the funnel during folding, the intrachain free energy decreases with increasing compactness, hydrophobic core development, intrachain hydrogen bonding and salt bridge formation. The polypeptide chain’s conformational choices also become more narrowed, leading to the formation of one native conformation. Semi-stable intermediates, that can briefly slow the folding process, are represented by small depressions along the sides of the free-energy funnel (Nelson and Cox, 2004). The kinetics of protein refolding can therefore be expressed in a simplified manner as:

where U represents the unfolded protein; I_n represents refolding intermediates having decreasing energy states in the descending order, and N represents the native protein (Ozkan *et al.*, 2002).

Refolding assumes first-order reaction kinetics while aggregation assumes a higher-order reaction kinetics, where the interaction between two or more molecules are involved (De Bernardez Clark *et al.*, 1998). The changes in kinetic competition between correct folding and aggregate formation can therefore be represented by:

where A represents aggregates, and k_1 and k_2 represent first and second or higher order aggregation rate constants, respectively (Ajit, 1995; De Bernardez Clark *et al.*, 1998).

In reality, the refolding kinetics are often more complex, where in some cases, both refolding intermediates and the native protein can form aggregates (Buswell and Middelberg, 2002), as shown by the following reaction scheme:

where I_n and N_n are refolding and native aggregates, respectively (Mannall *et al.*, 2006).

From the ‘energy landscape’ model, it is clear that the refolding efficiency of a protein is directly dependent on the topography of the free-energy landscape. The external environment that governs the energy topography includes denaturant type and concentration, redox and the presence of refolding additives. To achieve efficient refolding *in vitro*, these parameters must be carefully optimised. The best refolding environment will often differ from one protein to another as different proteins have varying amphipathicity and will self-associate to different extents (De Bernardez Clark, 1998). Section 2.4.2 discusses important refolding parameters that govern protein refolding behaviour.

2.4.2 Refolding parameters

2.4.2.1 Redox potential

Good control of the redox environment is critical to enhance refolding yields of cysteine-containing proteins such as AFP (Lyles and Gilbert, 1991; Leong and Middelberg, 2006b). The redox potential of the refolding buffer directly affects the oxido-shuffling efficiency of cysteine residues to form disulfide bonds, which affects the rate of correct refolding. Depending on whether a net reducing or oxidising environment is optimal for refolding, the redox potential in the refolding environment can be controlled by adjusting the ratio and concentration of redox couples such as reduced and oxidised glutathione (i.e. GSH and GSSG) or reduced and oxidised cysteine residues.

2.4.2.2 Time

Refolding time also plays an important role in protein *in vitro* refolding processes especially for proteins that are prone to form aggregates even in the native state (Langenhof *et al.*, 2005; Leong and Middelberg, 2007). Since refolding is a unimolecular reaction, a low refolding concentration minimises higher-order aggregate formation reactions and hence increases refolding yield. However, refolding at low protein concentrations reduces overall process productivity and necessitates large refolding tanks and refolding buffer volumes upon scale up, which is uneconomic.

2.4.2.3. Refolding additives

Refolding additives such as L-arginine, sugars, denaturants at low concentrations (e.g., urea and guanidinium chloride), detergents (e.g., Triton X-100, lauryl maltoside), and soluble polymers (e.g., polyethylene glycol) have been reported to enhance protein refolding yields (Middelberg, 2002; Jungbauer and Kaar, 2007). Refolding additives inhibit aggregation through different mechanisms which are molecule-specific.

More recently, the use of protein chaperones as refolding additives has also been reported. Molecular chaperones are specialised proteins that guide the correct folding of proteins *in vivo* by selectively binding to stretches of hydrophobic amino acids that tend to be exposed in non-native proteins but are otherwise buried in native proteins (Lee and Tsai, 2005; Melnikov and Rotanova, 2010). Many chaperones are also known as heat shock proteins in eukaryotic organisms. Although some globular proteins are able to form their native state unassisted, chaperone-assisted folding is often essential in the crowded intracellular environment to prevent protein aggregation and misfolding which may occur as a consequence of exposure to heat or other changes in the cellular environment (Ben-Zvi and Goloubinoff, 2001; Glover and Tkach, 2001; Weibezahn *et al.*, 2005; Liberek *et al.*, 2008; Tutar and Tutar, 2010). Examples of helper proteins and artificial chaperones that have been effectively used to improve *in vitro* refolding yields of proteins include minichaperones (Altamirano *et al.*, 1997), GroEL, GroES (Preston *et al.*, 1999) and oxidoreductases (Rozema and Gellman, 1996; Machida *et al.*, 2000). However, due to their high costs, the use of these molecules is often restricted to small scale refolding studies (Jungbauer and Kaar, 2007; Tsumoto *et al.*, 2003b).

2.5 Conventional *in vitro* refolding methods

The most common techniques for protein refolding *in vitro* which are widely used in industry and academic laboratories are dilution and dialysis (Clark, 2001).

2.5.1 Dilution refolding

Dilution refolding is achieved by rapidly diluting the denatured protein into the refolding buffer. Refolding at dilute protein concentrations (i.e. $\leq 10 \mu\text{g/ml}$) is aimed at reducing the propensity for protein self-association, thus minimising aggregation. However, performing refolding at low protein concentrations requires large refolding buffer volumes and subsequent post-refolding purification steps, which significantly increase process cost upon scale-up. To overcome problems associated with low refolded protein concentrations, pulse dilution, which involves the intermittent

addition of denatured proteins to the refolding buffer to increase protein refolding concentration, has been developed (Buchner *et al.*, 1992; Fischer *et al.*, 1992a; Terashima *et al.*, 1996b; Katoh and Katoh, 2000; Vallejo and Rinas, 2004b; a). Although pulse dilution can effectively increase refolding protein concentration, the intermittent introduction of denatured protein into the refolding buffer can gradually increase denaturant concentration in the refolding system, and destabilise the refolded proteins present (Tsumoto *et al.*, 2003; Vallejo and Rinas, 2004b). Moreover, pulse dilution does not resolve the problem of large refolding buffer volume requirements, which still limits scale up feasibility of dilution refolding methods.

2.5.2 Dialysis refolding

In dialysis refolding, denaturant is removed by dialysing the denatured protein against the refolding buffer in a dialysis cartridge (Maeda *et al.*, 1995; Varnerin *et al.*, 1998; West *et al.*, 1998; Yoshii *et al.*, 2000). Although simple to conduct, dialysis refolding which performs buffer exchange *via* ion diffusion through the dialysis membrane can be extremely time-consuming (De Bernardez Clark, 1998). Protein loss to the hydrophobic dialysis cartridge membranes is also commonly observed. Additionally, the large volumetric requirements of the refolding buffer during dialysis makes the refolding process uneconomical and difficult to scale-up.

2.5.3 Diafiltration refolding

Similar to dialysis refolding, diafiltration refolding involves the transfer of denatured proteins into a refolding buffer through a membrane. Unlike dialysis, which relies on passive diffusion, diafiltration involves forcing solution through the membrane by pressure, which will largely reduce the operation time. In contrast to dilution refolding, the removal of denaturant during diafiltration refolding is conducted gradually. However, this method causes more aggregation during refolding compared to dilution refolding due to non-specific adsorption of proteins to the membrane (Vallejo and Rinas, 2004b).

2.6 Chromatographic refolding

In view of the scale-up difficulties and productivity problems associated with dilution and dialysis refolding methods, alternative refolding methods that are fit for industrial bioprocessing requirements are clearly needed. Liquid chromatography is a well-established method for purification of proteins. Recently, protein refolding on chromatography columns has been identified as a promising refolding strategy which facilitates refolding at significantly higher protein concentrations than allowable by dilution or dialysis whilst minimising aggregation (Jungbauer *et al.*, 2004; Choe *et al.*, 2006). Chromatography refolding is also expected to be readily scaled using scale-up tools and technology that are well-established for chromatography protein purification.

In general, chromatography refolding can be categorised into two operations depending on the nature of protein-resin interaction, i.e. (i) non-adsorptive refolding and (ii) adsorptive refolding. Non-adsorptive refolding encompasses refolding using a size exclusion chromatography (SEC) column, where proteins do not adsorb to the column chromatographic support and refold while passing through the column, and are separated by size differences during elution. Adsorptive refolding involves refolding on functionalised chromatographic supports such as ion exchange, hydrophobic interaction and affinity chromatography columns. The advantages and limitations of different chromatography refolding methods are reviewed in Sections 2.6.1 to 2.6.2.

2.6.1 Size exclusion chromatographic refolding

Protein refolding in SEC columns relies on a buffer exchange mechanism. The SEC column is first equilibrated with the refolding buffer. When the denatured-reduced protein is introduced into the column, the smaller molecular weight denaturant and reducing agent will enter the resin pores and be separated from the larger molecular weight unfolded protein, which then induces the denatured protein to refold (Werner *et al.*, 1994; Batas and Chaudhuri, 1996; Hamaker *et al.*, 1996; Batas *et al.*, 1997; Gauthier and Patston, 1997; Batas and Chaudhuri, 1999; Muller and Rinas, 1999; Fahey *et al.*, 2000a; Fahey *et al.*, 2000b; García-Sáez and Plasterk, 2000; Batas and

Chaudhuri, 2001; Fahey and Chaudhuri, 2001; Gu *et al.*, 2001; Gu *et al.*, 2002; Goa *et al.*, 2003; Gu *et al.*, 2003; Harrowing and Chaudhuri, 2003; Schlegl *et al.*, 2003; Lanckriet and Middelberg, 2004). As the denatured protein molecules enter the pores of the SEC matrix, they are also temporarily isolated from other protein molecules, thereby reducing the opportunity for protein-protein interaction (Batas and Chaudhuri, 1996; Jungbauer *et al.*, 2004; Li *et al.*, 2004b; Vallejo and Rinas, 2004b; Sahdev *et al.*, 2008).

The first attempt to refold proteins using SEC was reported by Werner and co-workers in 1994 (Werner *et al.*, 1994). In that study, denatured recombinant human ETS-1 protein and bovine ribonuclease were introduced into a Superdex 75 HR 10/30 column that was equilibrated with refolding buffer. The successful refolding of those proteins led to the use of SEC to refold numerous types of proteins (Batas and Chaudhuri, 1996; Hamaker *et al.*, 1996; Batas *et al.*, 1997; Fahey *et al.*, 2000b) including several purified IB proteins (Batas and Chaudhuri, 1999; Muller and Rinas, 1999). Since it was generally believed that protein refolding in SEC columns are completed in the pores (Chaudhuri, 1994), the column geometry, resin properties, sample concentration and viscosity, as well as mobile phase flow rates that will dominate mass transfer effects, are important parameters that must be carefully studied to enhance refolding efficiency.

Introduction of a gradual buffer exchange step from denaturing to native-favouring in the SEC column has been shown to improve the refolding yield of pure proteins (Batas and Chaudhuri, 1996). For example, the development of a urea gradient mobile phase buffer system to improve SEC refolding was reported in 2001 (Gu *et al.*, 2001). This strategy allows a gradual linear reduction in urea concentration in the mobile phase which was found to improve the refolding yield of lysozyme compared to refolding in the absence of a urea gradient. The development of a dual-gradient system, which combined a decreasing denaturant concentration with an increasing pH-gradient for recombinant single-chain antibody Fv fragments (scFvs) refolding on a HiLoad 16/60 Superdex 30 prep grade column was also recently reported (Gu *et al.*, 2002). Other non-adsorptive chromatography refolding systems that have been exploited for protein refolding include preparative continuous annular chromatography (P-CAC) and simulated moving bed (SMB) chromatography. The

development of P-CAC for continuous refolding of pure proteins has been reported (Schlegl *et al.*, 2003; Schlegl *et al.*, 2005). However, the complexity of the P-CAC system set-up which is comparable to that of simulated moving bed (SMB) chromatography (Park *et al.*, 2005), increases scale-up difficulty of these continuous SEC refolding platforms.

The active development in SEC refolding methodology improvement shows the broad recognition of SEC's potential as a refolding tool. However, the disadvantages of SEC which include (i) low feed to column volume ratio, (ii) low product concentration and (iii) low refolding productivity are still unaddressed to date. These limitations, if unresolved, will continue to hinder the use of SEC in process-scale refolding strategies.

2.6.2 Adsorptive chromatographic refolding

Adsorptive chromatographic refolding allows reversible adsorption of unfolded proteins to the chromatographic solid support, where the switching of mobile phase from denaturing to native-favouring will initiate protein refolding on-column. Spatial isolation of the adsorbed proteins on the chromatographic resin reduces the tendency for protein-protein interaction and hence protein aggregation (Kim *et al.*, 1997; Negro *et al.*, 1997; Qiu *et al.*, 1997). Adsorptive refolding on chromatographic supports also provides the opportunity to integrate a reducing agent removal step and simultaneous purification of proteins. Additionally, the refolded protein can subsequently be eluted in a concentrated and purified form, thus eliminating the need for further concentration steps (Geng and Chang, 1992; Muller and Rinas, 1999; Zouhar *et al.*, 1999; Cho *et al.*, 2001; Rehm *et al.*, 2001; Geng and Bai, 2002; Bai *et al.*, 2003; Gong *et al.*, 2004; Wang *et al.*, 2004). Such elegant integration of processing steps eliminates the need for multiple unit operations and can significantly simplify the bioprocess flowsheet to improve overall process yield and economics. Nonetheless, to fully exploit the use of chromatography stationary phase as an interface for refolding, good understanding of the influence of the physicochemical environment on protein-resin interaction is critical.

Commonly used chromatographic supports for reversible adsorptive refolding include ion exchange chromatography (Yin *et al.*), hydrophobic interaction chromatography (Wolynes *et al.*) and affinity chromatography supports.

2.6.2.1 Refolding by ion exchange chromatography

IEC is very commonly used for protein purification. IEC resins separate proteins based on differences in their net charges, which is a function of a protein's isoelectric point (pI) and mobile phase composition. As such, for refolding applications, denatured proteins are applied on the column at a low salt concentration and eluted at a high salt concentration after refolding is complete. An increasing number of studies on IEC refolding method development reflects significant interest in IEC as a refolding platform (Suttnar *et al.*, 1994; Stempfer *et al.*, 1996; Cho *et al.*, 2001; Li and Su, 2002; Li *et al.*, 2002; Li *et al.*, 2003; Kweon *et al.*, 2004; Langenhof *et al.*, 2005). The first study of IEC on-column refolding was reported in 1986 by Creighton (Creighton, 1986a; b; 1990), using a three buffer system (i.e. binding buffer, refolding buffer and elution buffer). In 1994, Suttnar and co-workers (Suttnar *et al.*, 1994) successfully refolded an IB-derived papilloma virus HPV16 E7MS2 fusion protein on an IEC column. In 2002, a dual-gradient system in IEC refolding was developed by Gu and co-workers (Li *et al.*, 2002) to facilitate the formation of correct disulfide bonds in lysozyme, which combined an increasing pH gradient with decreasing urea concentration gradient. Using bovine serum albumin (BSA) as a model protein, Langenhof and co-workers (Langenhof *et al.*, 2005) showed that IEC on-column refolding was effective in integrating protein refolding and purification in a single chromatography step.

2.6.2.2 Refolding by hydrophobic interaction and affinity chromatography

HIC has also been successfully used to refold denatured proteins with concurrent removal of contaminating proteins (Geng and Chang, 1992; Guo, 2001; Geng and Bai, 2002; Bai *et al.*, 2003; Gong *et al.*, 2004; Wang *et al.*, 2004). HIC columns adsorb denatured proteins under high ionic strength, and elute the refolded proteins at low salt conditions. Protein adsorption on the HIC stationary phase is driven by the entropy increase due to the removal of water molecules that form hydration shells

around the protein and hydrophobic functional groups of the HIC matrix, at high ionic strength. Micro domains of proteins will form from the hydrophobic regions of the adsorbed proteins on the hydrophobic matrix, and finally co-organise to form the native conformation. However, the use of HIC as a refolding platform is confronted by several problems. Firstly, it has been reported that protein adsorption on a HIC matrix at high salt concentrations can destabilise the protein's secondary structures (Ueberbacher *et al.*, 2008), but this phenomenon is likely to be protein-specific. Solubilising denatured proteins under high salt concentrations may also increase protein loss due to salting-out effects. Additionally, viscosity problems may arise from the combined use of high salt and denaturant concentration (e.g. 8 M urea) during HIC refolding. Unless addressed, these problems could limit the use of HIC matrices as refolding platforms.

Adsorbing unfolded proteins on functionalised solid supports as employed in IEC and HIC refolding procedures can often constrain the protein molecule's flexibility due to multi-point protein-matrix interaction, where one domain may interact more strongly with the matrix compared to another domain. Consequently, the ability of the molecule to refold to its correct conformation is hindered. The use of affinity chromatography is aimed at reducing multi-point protein-matrix interaction, where only a specific region of the protein is targeted to interact with the resin, thus increasing molecule flexibility for refolding. Fusion protein tags such as 6×Histidine, glutathione S transferase (Bergstrand and Czar) and maltose binding protein (MBP) are often used to direct specific protein interaction with affinity ligands on chromatography supports for purification purposes. However, for refolding applications, only the polyhistidine tag can be effectively used as a fusion partner because its ligand-binding property is unaffected by the denaturing environment. (Hunt, 2005). Fusion partners like GST and MBP which have more structured conformations will lose their biological activity and ligand-binding ability when denatured. The successful refolding of many proteins using immobilised metal affinity chromatography (<http://www.merrimackpharma.com/>) with concomitant purification has been reported in numerous studies (Sinha *et al.*, 1994; Itoh *et al.*, 1996; Zahn *et al.*, 1997; Colangeli *et al.*, 1998; Rogl *et al.*, 1998; Zouhar *et al.*, 1999; Rehm *et al.*, 2001; Panagabko *et al.*, 2002; Saini *et al.*, 2002; Glynou *et al.*, 2003; Lemercier *et al.*, 2003;

Schauer *et al.*, 2003; Vincent *et al.*, 2004). The polyhistidine tag, however, may need to be subsequently cleaved from the target protein as the presence of the tag may impede the biological activity of some refolded proteins.

2.6.3 Current status of chromatography refolding

Survey of recent literature indicates that adsorptive and non-adsorptive chromatographic refolding is still largely limited to pure protein samples (Tables 2.1 and 2.2). The use of commercially purchased pure proteins eliminates ‘cellular contaminant’-related bottlenecks that could affect protein refolding behaviour on-column. To truly verify the potential of packed chromatography columns for protein refolding and demonstrate its relevance in bioprocessing of biopharmaceuticals, the use of ‘host cell’-derived protein extracts as model proteins is necessary. There is a clear need for detailed studies to develop and advance chromatographic refolding techniques that can increase both refolding and bioprocessing productivities of IB proteins, as will be investigated in this Ph.D. thesis. The use of IEC and SEC will be investigated for their suitability to refold rhAFP, a model IB protein. IEC is chosen over HIC and IMAC as an adsorptive chromatography platform for rhAFP refolding studies in this thesis, to eliminate (i) high salt loading requirements which may precipitate proteins, and (ii) protein tagging requirements which may impair protein biological activity.

Table 2.1: Summary of recent adsorption chromatography refolding studies.

Model protein	Molecular weight (kDa)	Disulfide bonds	Sample load (mg)	Column type and column volume	Protein sample origin	Refolding yield (%)	Reference
Hen egg-white lysozyme	14.7	4	8	IEC (5 ml)	Commercially purchased	62-100	(Li <i>et al.</i> , 2002)
Hen egg-white lysozyme	14.7	4	1	HIC (1 ml)	Commercially purchased	52-56	(Li <i>et al.</i> , 2004a)
Bovine α – lactalbumin	14	4	3	IEC (2ml)	Commercially purchased	15	(Machold <i>et al.</i> , 2005)
Bovine serum albumin (BSA)	66	17	0.5-20	IEC (1 ml)	Commercially purchased	20-55	(Langenhof <i>et al.</i> , 2005)
Consensus interferon (C-IFN)	19	2	3	HIC (13.6 ml)	IB 90% purity	80	(Wang <i>et al.</i> , 2006)
His6-tagged glutathione S-transferase (GST-(His6))	27.6	N.R*	N.R	Affinity (1 ml)	IB 95% purity	N.R Recovery or activity	(Hutchinson and Chase, 2006)

*N.R: not reported.

Table 2.2: Summary of recent SEC refolding studies.

Model protein	Molecular weight (kDa)	Disulfide bonds	Sample load (mg)	Protein sample origin	Refolding yield (%)	Reference
Hen egg-white lysozyme	14.7	4	10	IBs	35 (Relative specific activity)	(Batas <i>et al.</i> , 1999)
Hen egg-white lysozyme	14.7	4	0.5-6	Commercially purchased	84-90 (Activity yield)	(Gu <i>et al.</i> , 2001)
Hen egg-white lysozyme	14.7	4	17-38	Commercially purchased	71-53	(Lanckriet and Middelberg, 2004)
Recombinant human interferon- γ	18	N.R*	1.6	IBs	65-78	(Guan <i>et al.</i> , 2005)
Recombinant human granulocyte colony-stimulating factor (rhG-CSF)	N.R	2	0.2-2	IBs	32-53 (Mass recovery)	(Wang <i>et al.</i> , 2008)
New thrombolytic agent (NTA)	N.R	N.R	10-40	IBs	15-45 (Activity recovery)	(Fan <i>et al.</i> , 2008)

*N.R: not reported.

CHAPTER 3 : DEVELOPMENT OF A LABORATORY BIOPROCESS TO RECOVER AND PURIFY rhAFP FOR CHROMATOGRAPHY REFOLDING STUDIES

3.1 Introduction

This Ph.D. study aims to research the feasibility of a ‘chromatography refolding’-based bioprocessing route in *E. coli* to enhance the refolding productivity and bioprocess yield of recombinant human AFP (rhAFP). To facilitate rhAFP chromatography refolding studies which are reported in Chapters 4 to 6 of this thesis, it is important to first develop an optimised laboratory bioprocess that can yield sufficiently pure rhAFP, which forms the aim of the study reported in this chapter. Chromatography column loads must be sufficiently pure, particulate-free, and have minimum viscosity to minimise mass transfer effects which will affect protein adsorption and column performance. Since rhAFP refolding yield has been found to be strongly correlated with the contaminant spectrum (Leong and Middelberg, 2007), maintaining a high pre-refolding product purity will be important to enhance refolding yields.

Since rhAFP is expressed as inclusion bodies (IBs) in *E. coli*, the recovery of rhAFP will necessitate (i) cell breakage to recover the IBs, (ii) isolation of the IBs from soluble contaminants, (iii) resolubilisation of the IBs, and (iv) refolding of the denatured rhAFP protein. Mechanical disruption (Suhail Alam and Agrawal) and chemical extraction (CE) are two most commonly used techniques for IB recovery in laboratory scale processing, as reviewed in Chapter 2. The use of enzymatic cell lysis and CE to isolate and recover rhAFP were reported in two earlier studies (Boismenu *et al.*, 1997; Leong and Middelberg, 2007). Figure 3.1 shows the bioprocess flowsheets of these two processes. In the first process developed by Boismenu and co-workers, rhAFP was recovered by lysozyme- and detergent-induced cell lysis, followed by repetitive centrifugation and wash steps (Boismenu *et al.*, 1997). The use of cost-prohibitive lysozyme for cell lysis and protein extraction, coupled with the

addition of DNase, RNase and protease inhibitors can significantly elevate production costs. Additionally, no absolute yield was reported in that process, which increases the difficulty of process scale-up. In the second study, rhAFP was directly recovered by urea-induced CE (Leong and Middelberg, 2007). The CE-based process was successful in addressing cost issues associated with IB recovery by the use of a simple, fast and easily automated extraction method, which was found to readily release soluble denatured rhAFP. However, the simultaneous release of other host cell contaminants such as cellular proteins, lipids and DNA fragments significantly lowered the purity of the recovered rhAFP. As such, CE-derived rhAFP cannot be directly used for chromatography refolding without several pre-refolding chromatography purification steps which will further increase product losses. A new laboratory process that can address (i) process cost/complexity constraints reflected in the Boismenu process, and (ii) rhAFP purity constraints to yield sufficiently pure rhAFP for direct chromatography refolding studies is therefore developed in this chapter.

Figure 3.1: Process flowsheet schematics of the Boismenu *et al.* (A) and Leong *et al.* (B) bioprocesses for the production of rhAFP.

3.2 Materials and methods

Recombinant *E. coli* BL21(DE3)RIL strain containing plasmid EP334-001 pET24D 3.1A was used to express rhAFP.

Standard AFP (std-AFP) (i.e. glycosylated AFP derived from human amniotic fluid) was purchased in a lyophilized form (purity >96%) from Fitzgerald Industries International (Concord, MA, USA).

Tryptone, yeast extract, agar, kanamycin, isopropylthio- β -D-galactoside (IPTG), glycerol, Laemmli sample buffer (2 $\times$ concentrate), acetonitrile (HPLC grade), polyethyleneimine (PEI), urea, concentrated hydrochloric acid (HCl), sodium chloride (NaCl), potassium chloride (KCl), sodium phosphate dibasic (Na₂HPO₄), potassium phosphate monobasic (KH₂PO₄), tris(hydroxymethyl)aminomethane (Tris), ethylenediaminetetraacetic acid (EDTA), dithiothreitol (DTT) and Triton X-100 were purchased from Sigma-Aldrich (Singapore). Amberlite was purchased from GE Healthcare (Singapore).

3.2.1 Expression of rhAFP

To prepare glycerol stocks of the *E. coli* strain for rhAFP expression, recombinant *E. coli* BL21(DE3)RIL strains harbouring the pET24D-rhAFP plasmid were grown on an LB agar plate (Tryptone 10 g/l, Yeast extract 5 g/l, NaCl 10 g/l, pH 7.0, solidified with 15 g/l agar), supplemented with 50 μ g/ml kanamycin, and incubated overnight at 37 °C. A single colony was picked and used to inoculate a 30 ml 2x YT medium (Tryptone 16 g/l, Yeast extract 10 g/l, NaCl 5 g/l, pH 7.0) containing 50 μ g/ml kanamycin. This 30 ml culture was mixed by agitation for 10-12 h using a horizontal shaker set at 200 rpm and 37 °C, and harvested when culture optical density at 600 nm (OD₆₀₀) reached 1.5 $\pm$ 0.1, as measured using a UV spectrophotometer (Eppendorf BioPhotometer, Singapore). These cells were used to create 15% (v/v) glycerol stocks, which were stored in 1.5 ml eppendorf tubes at -80 °C.

For rhAFP expression, an aliquot of the above glycerol stock was streaked on an LB agar plate containing 50 μ g/ml kanamycin and incubated overnight at 37 °C. A single colony was used to inoculate a 30 ml 2x YT medium containing 50 μ g/ml kanamycin. The 30 ml cell culture was mixed by agitation for 10-12 h using a horizontal shaker set at 200 rpm and 37 °C. 5 ml of the cell culture was inoculated into 500 ml 2x YT medium containing 50 μ g/ml kanamycin, and the cells were grown at 37 °C, 200 rpm shaking conditions for 1-2 h. The cells were induced with 0.4 mM IPTG when OD₆₀₀ reached 1.0 $\pm$ 0.1. After induction, the cell culture was further incubated at 37 °C, 200 rpm shaking conditions for 2 h until OD₆₀₀ of 2.0 $\pm$ 0.1 was reached. Cells were then

harvested by centrifugation (10,000g, 10 min) and resuspended in 20 mM Tris-HCl buffer (pH 7.4) for sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) analysis and Bradford quantitative protein determination assay. The final cell suspension was divided into 250 ml aliquots and each aliquot was centrifuged (4,000g, 4 °C, 20 min). Cell pellets were washed in phosphate-buffered saline (PBS; pH 7.2) and centrifuged (4,000g, 4 °C, 20 min). Washed cell pellets were stored at -80 °C (for a maximum period of 2 weeks), and thawed before use for product release or directly used for product release.

3.2.2 Amberlite-treatment of urea to reduce carbamylation

To reduce the occurrence of protein carbamylation, all urea solutions used in this study were treated with Amberlite. Amberlite (1% (w/v)) was added into 9 M stock urea solutions in distilled H₂O and incubated for 1 h at room temperature on the shaker, after which the resin was removed by filtration (0.45 µm, Millipore, Singapore). Tris, EDTA and DTT stock solutions were added to the 9 M stock urea solution to prepare all buffers used for refolding experiments.

3.2.3 rhAFP IB release by mechanical disruption using sonication

Intracellular proteins were first released by sonication using a digital sonifier (Branson[®], Danbury, CT, USA). The harvested cells from a 250 ml culture were aliquoted into four fractions, each containing 62.5 ml culture. After centrifugation, each cell pellet from a 62.5 ml culture was re-suspended in 12.5 ml 50 mM Tris buffer (pH 8.0) and sonication of the re-suspended cell pellets was conducted on an ice bath for 180 s (at 35% amplitude, 18 cycles). The sonicated cells were centrifuged (10,000 g, 4 °C, 20 min), and the pellets were recovered and washed with Washing Buffer (2 M urea, 50 mM Tris, 3 mM EDTA, 10 mM DTT, 0.5% (w/v) Triton X-100, pH 8.0) at room temperature (21 °C) under gentle shaking condition for 2 h. The cells were centrifuged (10,000 g, 4 °C, 20 min) and the supernatant was discarded.

3.2.4 Resolubilisation of rhAFP IBs post-sonication

The washed cell pellets were solubilised in 2 to 50 ml Denaturation Buffer (8 M urea, 20 mM Tris, 3 mM EDTA, 20 mM DTT, pH 8.5) to achieve different final rhAFP protein concentrations (i.e. ranging from 0.23 to 5.8 mg/ml), and incubated under gentle shaking conditions for 5 h, at room temperature, to allow complete solubilisation of the pellet. Protein concentration was determined by reversed phase high performance liquid chromatography (RP-HPLC), SDS-PAGE analysis, and a chip-based electrophoresis method performed on an Agilent 2100 Bioanalyzer (Agilent) to determine protein concentration and purity.

3.2.5 rhAFP release by chemical extraction

A thawed cell pellet from a 250 ml cell culture was re-suspended in 25 ml Chemical Extraction Buffer (8 M urea, 20 mM Tris, 3 mM EDTA, 20 mM DTT, pH 9.0). Extraction was performed for 2 h at room temperature under gentle shaking conditions using a roller mixer.

3.2.6 DNA removal using PEI from chemical extract

Various PEI concentrations (0.001%-0.2% (w/v)) were investigated to selectively precipitate cellular DNA contaminants from the chemical extract. 1% (w/v) and 5% (w/v) PEI stock solutions were mixed with the chemical extract and Chemical Extraction Buffer at different volumes proportions to achieve different final PEI concentrations ranging from 0.001% to 0.2% (w/v) in 2 ml Eppendorf tubes. The mixtures were incubated for 30 min under gentle shaking conditions at room temperature. The samples were centrifuged for 10 min, at 10,000g. Blank controls were prepared for each sample at different PEI concentrations. DNA and protein contents in the supernatant were estimated by absorbance measurement using a UV spectrophotometer (Beckman Coulter DU 800 Spectrophotometer, Singapore) at 260 nm and 280 nm wavelengths, respectively. The Chemical Extraction Buffer was used

to blank the spectrophotometer prior to absorbance measurement of the different samples. Bradford assays were also performed on the samples pre- and post-PEI treatment to estimate the loss of protein due to co-precipitation with PEI, if any.

3.2.7 Analytical methods

3.2.7.1 Reversed phase high performance liquid chromatography (RP-HPLC)

RP-HPLC analysis was performed on a Shimadzu LC-20AVP high performance liquid chromatography (HPLC) system using a C5 Jupiter reversed phase column which has 5 μm particle size, 300 Å pore size, and a column dimension of 150 $\times$ 4.6 mm (Phenomenex, Singapore). The column was first equilibrated with 10 ml 40% (v/v) acetonitrile, followed by a 47 to 57% (v/v) acetonitrile in water gradient over 30 min. 0.05% (v/v) TFA was added to all RP-HPLC buffers. Absorbance was measured at 214 nm at room temperature. Protein mass was determined by peak integration, based on a standard curve attained by calibration using native and denatured-reduced std-AFP (Appendix 1). Peak tailing appearing in the eluted protein traces was excluded from peak integration, which was not present in RP-HPLC traces of std-AFP.

3.2.7.2 Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) analysis

Reducing SDS-PAGE was performed using precast 4-12% gradient NuPAGE Bis-Tris polyacrylamide gels (Invitrogen, Singapore). Protein samples were mixed with Laemmli sample buffer 2 $\times$ concentrate at a ratio of 1:1 (v/v) and heated for 10 min at 100 °C. 7.5 μl of the protein sample was loaded into each well. Electrophoresis was conducted for 50 min at 200 V. Protein bands were detected using SimpleBlueTM SafeStain (Invitrogen, Singapore) staining and the gel was destained using distilled water. Protein purity was evaluated using densitometry (GS-800 gel densitometer, Bio-rad, USA), where the intensity of the rhAFP band relative to those of other proteins in the SDS-PAGE gel was measured, and used to determine rhAFP purity (%). Protein purity of each sample was analysed in triplicate.

3.2.7.3 Bradford quantitative protein determination assay

Total protein concentration was determined using Bradford quantitative protein determination assay (Fermentas, Singapore), and bovine serum albumin standards were employed to plot the calibration curve. Samples and reagents were prepared according to the protocol provided by the supplier.

3.2.7.4 Agilent 2100 Bioanalyzer

Total protein concentration and rhAFP purity were also determined using Bioanalyzer in combination with the Protein 230 Chip kit (Agilent, Singapore). The Agilent 2100 Bioanalyzer detection is based on laser-induced fluorescence of an intercalating dye, which interacts with protein-SDS complexes. Since the quantitation accuracy of the Protein 230 assay depends on the staining efficiency of the protein, absolute rhAFP quantitation was enabled by the generation of a calibration curve of std-AFP (Appendix 2). Chips were prepared using the protocol provided with the Protein 230 Plus LabChip[®] kit, where 10 protein samples could be analysed concurrently per chip, over a period of 30 min. 4 µl sample was injected per well for analysis.

3.2.7.5 Determination of DNA concentration

Contamination of protein by DNA in the chemical extract supernatant was estimated by measuring the 260 nm to 280 nm ratios of samples using a UV spectrophotometer (Section 3.2.6). 260 nm absorbance readings (OD_{260}) were used to estimate DNA concentrations in the sample; OD_{260} of 1 was estimated to correspond to 50 µg/ml double stranded DNA and 33 µg/ml single stranded DNA, using a 1 cm path length cuvette (Sambrook and Russell, 2001). All measurements were conducted at room temperature.

3.3 Results and discussion

3.3.1 Expression of rhAFP

The rhAFP laboratory bioprocess development study was preceded by a rhAFP expression study. Following cell harvest at OD_{600} of 2.0 ± 1 , rhAFP expression was qualitatively and quantitatively determined by SDS-PAGE analysis. The native unglycosylated rhAFP molecule has a predicted theoretical molecular mass of 66.6 kDa (Leong and Middelberg, 2007). SDS-PAGE analysis shows a characteristic rhAFP band with the correct predicted molecular mass in the induced cells (lane 3, Figure 3.2A). A rhAFP expression yield of 55 ± 3 mg rhAFP/L culture with $35 \pm 3\%$ purity was achieved after 2 h of induced expression, as determined by SDS-PAGE analysis and Bradford assay. $13 \pm 4\%$ AFP was expressed in the soluble form while $87 \pm 4\%$ was expressed in the insoluble form, as determined by SDS-PAGE and Bradford assay (Figure 3.2B).

Considering the high over-expression yield of rhAFP in the insoluble IB form, MD processing was the apparent choice for bioprocessing to recover rhAFP at a sufficiently pure form for chromatography refolding studies. A MD-based rhAFP bioprocess was therefore first developed, as reported in Section 3.3.2. A CE-based bioprocess which is advantaged by a simpler flowsheet was also developed in parallel, as reported in Section 3.3.3, to allow comparison between the two process designs in terms of (i) rhAFP recovery, (ii) rhAFP purity, and (iii) cellular DNA removal (sample viscosity).

Figure 3.2: SDS-PAGE analysis of rhAFP expression in *E. coli* BL21(DE3)RIL. A: Lane 1, molecular weight marker, 4 μ l; lane 2, whole cell lysate before induction, 15 μ l (cell concentration of $OD_{600}=4$); lane 3, whole cell lysate after induction, 15 μ l (cell concentration of $OD_{600}=4$). B: Lane 1, molecular weight marker, 4 μ l; lane 2, supernatant after sonication and centrifugation, 15 μ l (cell concentration of $OD_{600}=10$); lane 3, resolubilised pellet after sonication and centrifugation, 15 μ l (cell concentration of $OD_{600}=20$).

3.3.2 Mechanical disruption

The use of sonication to release IB-derived rhAFP from a 250 ml culture was studied. After sonication, the cell lysate was centrifuged and the cell pellet was washed with Triton X-100, prior to resolubilisation in Denaturation Buffer as described in Sections 3.2.3 and 3.2.4. Although the separation of soluble contaminants can be readily achieved by centrifugation, it is often reported that the IBs can still be significantly contaminated with outer membrane proteins and cell envelope materials, which co-precipitate with the insoluble fractions after sonication and centrifugation (Rinas and Bailey, 1992; Valax and Georgiou, 1993). Considering the hydrophobic nature of

these contaminants, IB washing with Triton X-100, which is a weak detergent (Vallejo and Rinas, 2004a), was studied to further improve rhAFP purity.

The effect of washing the cell pellets with Triton X-100 following post-sonication centrifugation on rhAFP purity and recovery was studied. A Triton X-100 concentration ranging from 0 to 2% (w/v) was employed, where it was observed that increasing the detergent concentration to 2% (w/v) did not further improve rhAFP purity, as determined by SDS-PAGE analysis. Therefore, 0.5% (w/v) Triton X-100 was used in all the subsequent MD processing studies reported in this thesis to minimise Triton X-100 carry over in subsequent processing steps. Table 3.1 shows that employing a Triton washing step improved rhAFP purity, albeit at a small yield loss.

Table 3.1: Comparison of rhAFP IBs recovered by sonication with and without 0.5% (w/v) Triton X-100 washing.

Materials	Sonication without washing step	Sonication with washing step
Total rhAFP expressed (mg)	13.8±0.8	13.8±0.8
rhAFP recovered in the insoluble fraction after sonication and centrifugation (mg) or percentage recovery (%)	12±0.5 (87±4%)	12±0.5 (87±4%)
rhAFP recovered after washing step and centrifugation (mg) or percentage recovery (%)	N.A.	11.5±0.4 (83±3%)
rhAFP recovery	87±4%	83±3%
rhAFP purity	68±3%	74±4%

This study is based on a cell concentration of OD₆₀₀=20 originating from a 250 ml cell culture.

The effect of Triton X-100 washing on rhAFP purification from cellular DNA was also investigated. The extent of protein contamination by cellular DNA was estimated by measuring the 260 nm to 280 nm absorbance ratio of the protein fractions, where pure proteins have an estimated 260 nm/280 nm ratio of 0.6 (Glase, 1995; Sambrook

and Russell, 2001). Table 3.2 shows that cellular DNA carry over was significantly reduced by Triton washing and a subsequent centrifugation step. After sonication, most of the cellular DNA remained in the soluble fraction, while Triton washing of the pellets further reduced DNA load to yield a 260 nm/280 nm absorbance ratio that was comparable to that of DNA-free protein solution. Substantial removal of cellular DNA is important to reduce (i) sample viscosity and (ii) competitive DNA binding on the chromatography resin in subsequent adsorptive chromatography refolding studies.

Table 3.2: Comparison of cellular DNA amounts in the rhAFP fraction at different processing steps in the MD processing route.

Processing steps	Volume (ml)	260/280 ratio
(a) After sonication and centrifugation (supernatant)	50	1.76±0.07
(b) After sonication and centrifugation (resolubilised pellet in Denaturation Buffer)	25	1.15±0.05
(c) After (b), followed by 0.5% (w/v) Triton X-100 washing of cell pellet and centrifugation (resolubilised pellet in Denaturation Buffer)	25	0.62±0.04

This study is based on a cell concentration of $OD_{600}=20$ originating from a 250 ml cell culture.

Solubilised rhAFP following (c) in Table 3.2 yielded a rhAFP purity and concentration of $74\pm3\%$ and 0.45 ± 0.03 mg/ml, respectively, as determined by Bioanalyzer, SDS-PAGE analysis and Bradford assay (Figure 3.3). This outcome opens the way for direct use of the solubilised rhAFP for chromatography refolding without the need for further pre-refolding chromatography purification or concentration steps.

Figure 3.3: rhAFP release and purification by sonication and Triton washing. Lane 1, molecular weight marker; lane 2, supernatant after sonication and centrifugation; lane 3, resolubilised pellet after sonication, washing and centrifugation.

3.3.3 Chemical extraction

A CE processing route for rhAFP recovery and purification was also studied in parallel to assess if comparable rhAFP recovery and purification could be achieved by fewer unit operations. 8 M urea, 3 mM EDTA, and 20 mM DTT were successfully used to chemically permeabilise the cellular membranes of *E. coli* cells to release rhAFP (Figure 3.4).

Figure 3.4: SDS-PAGE analysis of chemical extract. Lane 1, molecular weight marker, 4 μ l; lane 2, chemical extract, 15 μ l (cell concentration of OD₆₀₀=20); lane 3, whole cell lysate after induction, 15 μ l per lane (cell concentration of OD₆₀₀=4).

However, the viscosity of the chemical extract was found to be high, which is not unexpected, considering the huge co-release of many intracellular DNA. The use of PEI to selectively remove DNA from the chemical extract was subsequently studied. The change in DNA load with varying PEI concentrations, as determined by the 260 nm/280 nm absorbance ratio of the chemical extract is shown in Figure 3.5. Sample absorbance at 260 nm showed a general reducing trend with increasing PEI concentration from 0 to 0.03 % (w/v), and stabilised at PEI concentrations beyond 0.03% (w/v). A reduction in 260 nm sample absorbance indicates that PEI was effective in DNA removal. SDS-PAGE analysis, however, showed that co-precipitation of proteins to PEI was rather significant, especially at PEI concentrations above 0.05 % (w/v) (Figure 3.6). An optimised PEI concentration of 0.03 % (w/v) was therefore used in all subsequent CE buffers.

Figure 3.5: Chemical extract absorbance at 260 nm and 280 nm with the addition of 0 to 0.20 % (w/v) PEI. All samples contained 8 M urea, 20 mM Tris, 3 mM EDTA and 20 mM DTT.

Figure 3.6: SDS-PAGE analysis of chemical extract following the addition of PEI at various concentrations. Lane 1, molecular weight marker, 4 μ l; lanes 2-13, supernatant of chemical extract after 0.5 h of incubation with PEI at different concentrations (i.e. 0, 0.001, 0.003, 0.005, 0.007, 0.009, 0.01, 0.02, 0.03, 0.04, 0.05 and 0.20% (w/v)).

The recovery and purity of rhAFP from CE processing with 0.03% (w/v) PEI is shown in Table 3.3. The low rhAFP recovery was attributed to protein loss as insoluble particulates due to incomplete resolubilisation of the cell pellet in the CE Buffer. An increase in extraction time from 2 h to 24 h coupled with optimisation of centrifugation speed and time did not further improve rhAFP recovery. A 2 h chemical extraction incubation time was therefore used throughout this study to limit the protein exposure time to high urea concentrations, which may increase the propensity for protein carbamylation. Although simpler and faster to conduct than MD bioprocessing, it was clear that rhAFP bioprocessing by CE significantly compromised protein recovery and purity.

Table 3.3: Comparison of rhAFP recovery using CE with PEI treatment.

	Chemical extraction with 0.03% (w/v) PEI treatment
Total rhAFP expressed (mg)	13.8±0.8
rhAFP recovered after CE (mg) or percentage recovery (%)	4.4±0.4 (32±3%)
rhAFP recovered after CE in the presence of 0.03 % (w/v) PEI (mg) or percentage recovery (%)	3.7±0.4 (27±3%)
rhAFP recovery	27±3%
rhAFP purity	21±3%

This study is based on a cell concentration of $OD_{600}=20$ originating from a 250 ml cell culture. Protein recovery and purity were determined by Bioanalyzer and Bradford analyses.

3.3.4 Comparison of MD- and CE-based bioprocesses for rhAFP production

In this study, the development of two different process flowsheets based on MD (sonication) and CE were compared for preparation of purified and concentrated denatured rhAFP for chromatography refolding studies. A performance evaluation of both processing routes with respect to rhAFP purification, concentration and recovery was studied using a 250 ml cell culture base case. Figure 3.7 compares the effect of different process design choices on rhAFP protein recovery and purity obtained from the MD and CE bioprocesses. The MD bioprocesses clearly outperformed the CE processes in terms of rhAFP recovery, where a 3-fold higher rhAFP recovery was obtained using MD₀ (MD processing without a Triton X-100 washing step) and MD_w (MD processing with a Triton X-100 washing step) processes compared to CE₀ (CE processing without PEI treatment) and CE_p (CE processing with PEI treatment). In fact, there was almost no rhAFP loss from the sonication and Triton X-100 washing steps, and only rhAFP expressed in the soluble form could not recovered. Unlike MD processes where the soluble target proteins had to be discarded due to the high contaminant load, CE has the advantage of recovering both soluble and insoluble fraction of the target protein. However, the sub-optimal recovery of rhAFP from CE is

attributed to the tight packing of the cell pellet which proved difficult to subsequently re-suspend completely, despite lengthening extraction time and optimising centrifugation conditions. Consequently, the efficient recovery of rhAFP in the denatured-soluble form was hindered. From Figure 3.7, it is also clear that MD_W yielded significantly higher rhAFP purity (i.e. 74±4%) compared to CE₀ and CE_P (i.e. 21±3%). The MD_W process was clearly more effective in reducing DNA contamination of the rhAFP fraction compared to CE_P.

Figure 3.7: Comparison of rhAFP recovery, purity and DNA contamination for the MD and CE bioprocesses. MD₀ and MD_W represent MD processing without and with a Triton X-100 washing step; CE₀ and CE_P represent CE processing without and with PEI treatment.

Figure 3.8 shows the MD and CE bioprocesses flowsheets which were developed in parallel for rhAFP IB recovery and pre-refolding purification in this study. Although CE processing was simpler to conduct and comprised fewer unit operations compared to MD processing, the recovery and quality of the rhAFP product obtained from the CE bioprocess were inferior to those obtained using the MD process. Consequently, pre-refolding purification step(s) will still need to follow CE processing to obtain

sufficiently pure denatured rhAFP for chromatography refolding, leading to potentially more yield losses. The high rhAFP purity obtained from the optimised MD_w process opens the way for direct loading of the rhAFP protein on chromatography columns for refolding studies. Additionally, the flexibility to readily adjust the re-suspension volumes of the cell pellet after sonication, washing and centrifugation allows a higher concentration of pure rhAFP to be obtained for refolding *via* the MD processes (i.e. up to 6 mg/ml rhAFP) compared to CE processes (i.e. 0.15 mg/ml rhAFP) with a 250 ml culture base case.

Figure 3.8: (A) MD and (B) CE laboratory bioprocess flowsheets to recover and purify rhAFP for chromatography refolding studies.

3.4 Conclusions

A successful laboratory MD bioprocess which involved sonication and Triton X-100 washing has been developed to efficiently recover purified rhAFP for chromatography refolding studies. A parallel comparison study of MD and CE processing showed that MD processing was superior to CE processing in purifying rhAFP at higher step yields, protein purity and concentration. These results clearly show that upstream

process design is important and will significantly influence downstream processing requirements. Using the MD_w process, almost all of the expressed rhAFP IBs were recovered by sonication followed by a Triton washing step. Adequate optimisation of this MD process to integrate protein release and purification eliminates the need for any chromatography pre-conditioning prior to refolding. The high rhAFP purity (i.e. 74%) and the excellent cellular DNA removal ability obtained from the MD_w process facilitates the direct use of the rhAFP for refolding in chromatography modes, which will be investigated in Chapters 4, 5 and 6 of this thesis.

CHAPTER 4 : rhAFP REFOLDING BY ION EXCHANGE CHROMATOGRAPHY

4.1 Introduction

The relatively high insoluble AFP expression yields in *E. coli* (i.e. ~ 50 mg per L of culture) from the laboratory scale expression study reported in Chapter 3, renders the IB route potentially advantageous for process-scale commercial manufacture of rhAFP, if high throughput refolding can be achieved. The main bottleneck for rhAFP production using the *E. coli* route lies in the low refolding yield associated with the use of dilution refolding, which necessitates refolding to be conducted at low protein concentrations. An earlier AFP refolding study using batch dilution showed that refolding at a low protein concentration of 0.04 mg/ml was required to achieve a refolding yield of 40% (Leong and Middelberg, 2007). Such low refolding concentration imposes large solvent and reactor volumes upon scale up, which increases process cost. Compared to batch dilution refolding, chromatography refolding offers a high degree of process intensification which includes the possibility to perform refolding, purification and concentration in a single unit operation, as reviewed in Section 2.6 of Chapter 2. These advantages will remove the refolding roadblocks imposed by dilution refolding, leading to process intensification and improved overall process productivity. Therefore, the aim of the study described in Chapter 4 is to develop a new adsorptive chromatography refolding strategy for rhAFP using anion exchange chromatography (AEC).

The successful development of an optimised laboratory bioprocess involving sonication and Triton X-100 washing reported in Chapter 3 yielded soluble denatured rhAFP with 74% purity, and allows direct protein loading into chromatography columns for refolding studies. The significant reduction of protein and cellular DNA contaminants achieved is critical for minimising competitive contaminant binding and viscous fingering in packed chromatography columns. AEC was employed in this refolding study because AFP, which has a theoretical pI of 5.3 (Parmelee *et al.*, 1978),

will have a net negative charge and adsorb to the positively charged chromatography matrix under the alkaline refolding environment of pH 8.5. Q Sepharose Fast Flow (Q-FF) and DEAE Sepharose Fast Flow (DEAE-FF), which are strong and weak anion exchangers will be investigated for surface refolding of rhAFP. Both refolding and chromatography operation parameters (i.e. resin chemistry, column geometry, redox potential and feed conditioning) are important parameters that will impact rhAFP refolding, and will therefore be studied and optimised for efficient refolding of rhAFP on-column. Studying on-column chromatography refolding of rhAFP will also improve the understanding of AFP refolding from a molecular perspective, where the effect of protein adsorption on the oxidative shuffling of the 32 cysteines can be studied. The outcome of this study will provide an indication of the feasibility of an ‘adsorptive chromatography refolding’-based process for rhAFP production at higher process yields. A successful chromatography refolding outcome for rhAFP will also extend the application of chromatography refolding to other complex proteins which suffer from poor refolding yields using conventional refolding methods such as dilution and dialysis.

4.2 Materials and methods

Reduced (GSH) and oxidised glutathione (GSSG) were purchased from Sigma-Aldrich (Singapore). HiTrap Q Sepharose Fast Flow (Q-FF) (1 ml and 5 ml), HiTrap DEAE Sepharose Fast Flow (DEAE-FF) (1 ml and 5 ml), and Sephadex G-25 PD-10 columns were purchased from GE Healthcare (Singapore).

Other chemicals were sourced as detailed in Chapter 3.

4.2.1 rhAFP recovery and purification

rhAFP was expressed and harvested using the same method as reported in Section 3.2.1. rhAFP IBs were released by sonication and washed with Triton X-100 using the optimised MD_w process reported in Section 3.2.2. The washed pellet from a 250 ml culture was solubilised in 5 to 50 ml Denaturation Buffer (8 M urea, 20 mM Tris, 3

mM EDTA, 20 mM DTT, pH 8.5) to achieve different final rhAFP protein concentrations ranging from 0.2 to 2 mg/ml. The solubilised IBs were incubated under gentle shaking conditions for 5 h at room temperature (21 °C). RP-HPLC analysis and chip-based electrophoresis performed on an Agilent 2100 Bioanalyzer were employed to determine protein concentration and purity, as described in Sections 3.2.7.1 and 3.2.7.4, respectively.

4.2.2 Determination of rhAFP adsorption isotherm

Adsorption isotherms of denatured-reduced rhAFP on Q-FF and DEAE-FF resins were determined by static batch adsorption studies performed at room temperature. The AEC resins were resuspended in Denaturation Buffer, and mixed with denatured-reduced rhAFP to obtain different final rhAFP concentrations ranging from 0.5 to 4.8 mg/ml in 2 ml Eppendorf tubes. The ‘denatured-reduced protein’-resin mixtures were incubated under shaking condition at room temperature for 24 h to achieve adsorption equilibrium. The protein-resin mixture was then centrifuged (2000 g, 10 min) and the supernatant was filtered through a 0.45 µm micro filter unit (Millipore), and analysed by RP-HPLC to determine the amount of adsorbed protein at equilibrium per ml resin, q (mg/ml), and free protein concentration at equilibrium, c (mg/ml). The Langmuir equation (Equation 4.1) was used to fit the adsorption data:

$$q = \frac{q_m c}{K_d + c} \quad (\text{Equation 4.1})$$

where q_m is the maximum binding capacity per ml resin (mg/ml) and K_d is the dissociation constant (mg/ml).

4.2.3 Binding recovery of denatured-reduced rhAFP on AEC columns

All AEC experiments were performed on an ÄKTA explorer fast protein liquid chromatography (FPLC) workstation (GE Healthcare, Singapore) at room temperature.

Four AEC columns were studied: (i) HiTrap Q Sepharose Fast Flow 1 ml column (1 ml Q-FF column), (ii) HiTrap DEAE Sepharose Fast Flow 1 ml column (1 ml DEAE-FF column), (iii) HiTrap Q Sepharose Fast Flow 5 ml column (5 ml Q-FF column), and (iv) HiTrap DEAE Sepharose Fast Flow 5 ml column (5 ml DEAE-FF column). DEAE-FF is a weak anion exchanger which is functionalised with diethylaminoethyl groups while Q Sepharose FF is a strong anion exchanger which is functionalised with quaternary ammonium groups.

To determine the optimum total protein load for chromatography refolding experiments, the binding recovery of denatured-reduced rhAFP was first determined. 1 ml and 5 ml Q-FF and DEAE-FF columns were equilibrated with 12 column volumes (CV) of Denaturation Buffer and then loaded with up to 18 ml protein sample containing 0.23 mg/ml denatured-reduced rhAFP at constant flow rate of 0.5 CV/min (which is equivalent to 0.5 and 2.5 ml/min for 1 ml and 5 ml columns, respectively). After loading, the columns were washed with 8 CV and 2 CV of Denaturation Buffer for the 1 ml and 5 ml columns, respectively, to remove any unbound proteins. Protein adsorbed on the column was eluted by a linear NaCl gradient over 10 CV and 4 CV from Denaturation Buffer to AEC Stripping Buffer (8 M urea, 20 mM Tris, 3 mM EDTA, 20 mM DTT, 1 M NaCl, pH 8.5) for the 1 ml and 5 ml columns, respectively. Eluted fractions were collected and analysed by RP-HPLC to determine rhAFP binding recovery, which was determined as the mass ratio of adsorbed rhAFP to total rhAFP loaded (Equation 4.2).

$$R = \frac{M_A}{M_L} \quad (\text{Equation 4.2})$$

where R is binding recovery (%), M_A is the mass of adsorbed rhAFP (mg), and M_L is the mass of total denatured-reduced rhAFP loaded (mg).

4.2.4 rhAFP refolding on AEC columns

On-column refolding conditions were first systematically studied and optimised with 1 ml Q-FF and DEAE-FF columns. The effects of protein load, redox environment,

and column incubation time on rhAFP refolding yield were studied. A constant flow rate of 0.5 CV/min was used throughout. The column was first equilibrated with 12 CV of Denaturation Buffer and then loaded with up to 12 ml protein extract containing 0.23 mg/ml denatured-reduced rhAFP. Refolding was initiated by switching buffer from Denaturation Buffer to AEC Refolding Buffer (3 M urea, 20 mM Tris, 1 mM EDTA, 0 to 5 mM GSH, and 0 to 3.6 mM GSSG, pH 8.5) over 10 CV. The bound proteins were incubated on-column for 0 to 24 h. Protein elution was initiated by replacing the AEC Refolding Buffer with AEC Elution Buffer (20 mM Tris, 1 mM EDTA, 1 M NaCl, pH 8.5) over 10 CV. Flow through and wash fractions after buffer switching to AEC Refolding Buffer, and elution fractions after buffer switching to AEC Elution Buffer, were collected and analysed by SDS-PAGE, RP-HPLC and Bioanalyzer to determine rhAFP recovery, refolding yield and purity. The column was washed and regenerated with 10 CV of AEC Stripping Buffer after each refolding cycle.

rhAFP refolding yield was calculated as the mass ratio of final refolded rhAFP to total denatured-reduced rhAFP loaded, as described by Equation 4.3. Protein mass was determined by RP-HPLC as described in Section 3.2.7.1.

$$Y = \frac{M_R}{M_T} \quad (\text{Equation 4.3})$$

where Y is rhAFP refolding yield (%), M_R is the mass of the refolded rhAFP (mg), and M_T is the mass of total denatured-reduced rhAFP in the refolding system (mg).

Refolding productivity was determined according to Equation 4.4:

$$P = \frac{YM_T}{V_R t} \quad (\text{Equation 4.4})$$

where P is rhAFP refolding productivity (mg/ml/h), V_R is the volume of the refolding reactor (ml) (i.e. V_R is the AEC column volume for AEC refolding and refolding buffer volume for dilution refolding), and t is the refolding incubation time (h).

4.2.5 The effect of AEC column dimensions on rhAFP AEC refolding

To investigate the effect of column geometry on refolding yield, rhAFP refolding was conducted on 1 ml and 5 ml AEC columns, and the refolding outcomes were compared. On-column refolding was performed on 5 ml Q-FF and DEAE-FF columns using the same refolding conditions which were optimised for the 1 ml columns, as described in Section 4.2.4. The column was first equilibrated with 2.5 CV of Denaturation Buffer and then loaded with up to 12 ml protein extract containing 0.23 mg/ml denatured-reduced rhAFP, at a constant flow rate of 0.5 CV/min (1.3 cm/min). Refolding was initiated by switching buffer from Denaturation Buffer to the AEC Refolding Buffer (3 M urea, 20 mM Tris, 1 mM EDTA, 4.5 or 2.7 mM GSH, and 0.9 or 2.7 mM GSSG for Q-FF or DEAE-FF, respectively, pH 8.5) over 4 CV. The protein was then incubated on-column for 6 h. Protein elution was initiated by a linear NaCl gradient over 4 CV from AEC Refolding Buffer to AEC Elution Buffer. The column was washed and regenerated with 5 CV AEC Stripping Buffer after each refolding cycle. Samples from the flow through, buffer switching and elution fractions were collected and analysed by SDS-PAGE, RP-HPLC and Bioanalyzer to determine rhAFP recovery, refolding yield and purity, as described in Section 4.2.4.

4.2.6 Off-column AEC refolding of rhAFP

To compare the effect of protein adsorption on rhAFP refolding yield, off-column refolding was also conducted in parallel, where the rhAFP protein was induced to refold only after elution from the column. Off-column refolding of rhAFP was studied on a 1 ml Q-FF column. Column equilibration and sample loading procedures were the same as those reported for on-column refolding in 1 ml Q-FF columns, described in Section 4.2.4. DTT and unbound proteins were removed with 10 CV Off-column Equilibration Buffer (8 M urea, 20 mM Tris, 3 mM EDTA, pH 8.5). For off column refolding, the mobile phase flow rate was reduced from 0.5 CV/min to 0.25 CV/min, and elution was initiated by a buffer switch from Off-column Equilibration Buffer to Off-column Refolding Buffer (20 mM Tris, 1 mM EDTA, 4.5 mM GSH, 0.9 mM GSSG, 0.15 M NaCl, pH 8.5) over 10 CV. The effect of introducing 0.5 M L-arginine into the Off-column Refolding Buffer on rhAFP refolding yield was also investigated.

The column was washed and regenerated with 10 CV AEC Stripping Buffer. Eluted protein fractions were incubated at 4 °C for 3 h and subsequently analysed by RP-HPLC and Bioanalyzer to determine rhAFP refolding yield and purity.

4.2.7 Batch dilution refolding of rhAFP

Solubilised rhAFP IBs for batch dilution refolding studies were prepared using the same method described in Section 4.2.1. DTT was removed from the denatured-reduced protein using a PD-10 column, which was first equilibrated in 25 ml PD-10 Equilibration Buffer. 2.5 ml of denatured-reduced rhAFP was loaded onto the column and 3.5 ml PD-10 Equilibration Buffer was added to elute the rhAFP in DTT-free denaturant. 1.92 ml of DTT-free denatured protein in 8 M urea, 20 mM Tris, 3 mM EDTA (pH 8.5) was immediately 2.6-fold diluted into 3.08 ml refolding buffer (20 mM Tris, 3 mM EDTA, 4.4 mM GSH and 4.4 mM GSSG, pH 8.5) and incubated under gentle shaking conditions at 4 °C for 6 h. The final composition of the Dilution Refolding Buffer was 3 M urea, 20 mM Tris, 3 mM EDTA, 2.7 mM GSH and 2.7 mM GSSG (pH 8.5).

4.2.8 Analytical methods

RP-HPLC analysis was performed using the same procedure as described in Section 3.2.7.1 in Chapter 3. SDS-PAGE analysis, Bradford quantitative protein determination assay, and Agilent 2100 Bioanalyzer analysis were conducted to determine protein concentration and protein purity using the same methods reported in Section 3.2.7.

4.2.8.1 Enzyme linked immunosorbent assay (ELISA)

The correct appearance of AFP epitopes in the refolded rhAFP fractions following AEC and dilution refolding was verified by the AFP ELISA diagnostic kit (Leinco Technologies, USA). Two unique antibodies (goat polyclonal and mouse monoclonal) were used to recognize distinct antigenic determinants on the rhAFP molecule. The

plastic wells were supplied pre-coated with murine monoclonal anti-AFP. Refolded rhAFP (20 μ l) diluted with bovine serum to concentrations ranging from 10 to 350 ng/ml was added to the wells prior to incubation (5 min). Goat polyclonal anti-AFP horseradish peroxidase conjugate (200 μ l) was added to each well. After 60 min incubation, wells were washed to remove unbound labeled-antibody. Enzyme substrate-chromogen (100 μ l) (hydrogen peroxide, H₂O₂, and tetramethylbenzidine, TMB) was added to each well and incubated for 30 min at room temperature. H₂SO₄ (1.0 N) was then added to each well to stop the reaction, and product concentration was read at 450 nm using a microplate reader (Bio-Rad xMarkTM Absorbance Microplate Spectrophotometer). The absorbance of Refolding Buffer, enzyme-labeled goat polyclonal anti-AFP and denatured rhAFP at 450 nm were tested as controls. Bioactive AFP mass was determined based on a standard curve attained by calibration using native std-AFP (Appendix 3).

4.2.8.2 Circular dichroism (CD) spectroscopy

Far and near UV CD spectra of denatured, native and refolded AFP were measured on a ChirascanTM CD spectrometer (Applied Photophysics, UK) at room temperature. 1 and 10 mm pathlength quartz cuvettes were used for far and near UV CD measurements, respectively. Denatured rhAFP was prepared by denaturing-reducing the protein in 8 M urea, 20 mM DTT for 12 h. DTT was subsequently removed by a PD-10 column, since DTT has a strong absorbance in the region of spectrum used. The CD spectra of native and refolded rhAFP were measured in 10 mM sodium phosphate buffer (pH 7.5). Protein concentrations were 0.1 and 0.5 mg/ml for far and near UV CD spectra measurements, respectively, as determined by RP-HPLC. All spectra were averaged from a total of 10 scans and the buffer baseline was subtracted for spectra correction.

4.3 Results and discussion

Two types of anion exchangers having cross-linked agarose matrices (i.e. Q-FF and DEAE-FF) were investigated for on-column refolding of rhAFP. Early studies have indicated that agarose gels showed higher binding capacities for pure proteins

compared to synthetic polymer matrices which are sold under commercial names such as Source, Toyopearl and Fractrogel[®] EMD (Machold *et al.*, 2005). It is hypothesised that the low charged state of agarose beads minimises the occurrence of ionic repulsion between the resin and protein, thus improving protein adsorption. The use of both strong (Q-FF) and weak (DEAE-FF) anion exchangers was aimed to study if differing extents of protein-matrix interaction due to different ionisation states of the functional groups, would affect rhAFP refolding.

4.3.1 Adsorption isotherm

Adsorption isotherms of denatured-reduced rhAFP on the two anion exchangers were first investigated through a static batch adsorption study. The adsorption data were regressed using Equation 4.1 to provide the best fit Langmuir adsorption isotherm (Figure 4.1). The fitting of adsorption data to Langmuir adsorption isotherm was performed by the OriginPro 7.0 software. The maximum resin adsorption capacity, q_m , and dissociation constant, K_d of denatured-reduced rhAFP on Q-FF and DEAE-FF were estimated from curve fitting. A comparable q_m was obtained for Q-FF and DEAE-FF under identical binding conditions, which were 33 mg/ml and 30 mg/ml, respectively. The estimated K_d values derived from curve fitting for Q-FF and DEAE-FF were 0.23 mg/ml and 0.25 mg/ml, respectively, which suggests only slightly stronger rhAFP binding on the Q-FF resin compared to the DEAE-FF resin under the specified denaturation environment.

Figure 4.1: Adsorption isotherms for denatured-reduced rhAFP on Q-FF and DEAE-FF anion exchange resins.

4.3.2 Binding recovery of denatured-reduced rhAFP

The binding recovery of denatured-reduced rhAFP on Q-FF and DEAE-FF columns were thereafter investigated to determine the dynamic binding recovery under various protein loads. Both Q-FF and DEAE-FF columns showed comparable dynamic binding capacities for denatured-reduced rhAFP for protein load between 0.15 to 3 mg at a loading flow rate of 0.5 CV/min, where an average rhAFP recovery of $66 \pm 2.1\%$ and $73 \pm 2.5\%$ were obtained for Q-FF and DEAE-FF 1 ml columns, respectively (Figure 4.2). Extending the rhAFP binding studies to 5 ml Q-FF and DEAE-FF columns showed that binding recovery did not further improve with increasing column volumes, where rhAFP recovery of $64 \pm 2.6\%$ and $73 \pm 2\%$ was achieved at rhAFP load from 0.4 to 3 mg for Q-FF and DEAE-FF 5 ml columns, respectively (Figure 4.2).

Figure 4.2: Binding recovery of denatured-reduced rhAFP at different loads using 1 ml and 5 ml Q-FF and DEAE-FF columns.

4.3.3 AEC on-column refolding

With an almost constant denatured-reduced rhAFP binding recovery achieved using both 1 ml and 5 ml AEC columns, the subsequent study on the effect of varying refolding parameters such as (i) redox environment, (ii) incubation time and (iii) protein load on rhAFP refolding yield, was therefore only performed with the 1 ml columns. A rhAFP refolding concentration of 1 mg/ml was chosen to form the basis of all chromatography refolding studies reported in this chapter, except for the protein load variation study.

4.3.3.1 Redox optimisation

Refolding a highly disulfide-bonded protein such as AFP while adsorbed on chromatographic supports will inevitably require an optimum redox environment to ensure efficient shuffling of the cysteines. The influence of redox landscape on rhAFP on-column refolding was first investigated by different concentrations and ratios of

the GSH and GSSG redox couple in the AEC Refolding Buffer. On-column refolding was performed as described in Section 4.2.4. Omission of GSH and GSSG in the AEC Refolding Buffer gave rhAFP on-column refolding yields of less than 5% but refolding yields increased with increasing GSH and GSSG concentrations at a fixed GSH:GSSG ratio of 1:1 (Figure 4.3).

Figure 4.3: The effect of different GSH to GSSG concentrations (at a fixed GSH:GSSG ratio of 1:1) on rhAFP on-column refolding yield, at 1 mg/ml rhAFP refolding concentration on 1 ml Q-FF and DEAE-FF columns.

Given that a GSH and GSSG concentration of 2.7 mM, respectively, gave the highest rhAFP refolding yield, the use of a total GSH and GSSG concentration of 5.4 mM was fixed in the following study to investigate the effect of varying GSH:GSSG ratios on rhAFP on-column refolding yields. A net reducing redox environment contributed by a GSH:GSSG ratio of 5:1 (i.e. 4.5 mM/0.9 mM) in the AEC Refolding Buffer gave the highest rhAFP refolding yield on the Q-FF column, while a 1:1 GSH:GSSH ratio (i.e. 2.7 mM/2.7 mM) was most optimal in maximising rhAFP refolding yield on the DEAE-FF column (Figure 4.4). The different redox requirements of the two columns suggest different protein-matrix interaction behaviours which will affect disulfide

shuffling efficiency of rhAFP while adsorbed on the column matrix. This result reflects the importance of selecting a matrix that interacts favourably with the protein to support efficient refolding in the adsorbed mode. A 4.5 mM/0.9 mM and 2.7 mM/2.7 mM GSH:GSSG ratio was therefore used for subsequent on-column rhAFP refolding studies on Q-FF and DEAE-FF, respectively.

Figure 4.4: The effect of different GSH to GSSG ratios on on-column rhAFP refolding yield, at rhAFP refolding concentration of 1 mg/ml.

The success of Q-FF and DEAE-FF AEC on-column refolding was validated by RP-HPLC analysis which compared the retention time of refolded rhAFP with that of std-AFP. Figure 4.5 shows the RP-HPLC chromatography profile of on-column refolded rhAFP using optimised GSH:GSSG ratios. Both Q-FF and DEAE-FF ‘on-column’-refolded fractions (peak D and E, Figure 4.5) eluted from the reversed phase column at the same retention time were compared with native std-AFP (peak A, Figure 4.5), indicating that the refolded rhAFP has comparable hydrophobic characteristics and disulfide conformational patterns with native std-AFP.

Figure 4.5: RP-HPLC analysis of 'AEC on-column'-refolded rhAFP. A, native std-AFP (0.1 mg/ml, 10 μ l); B, denatured-reduced std-AFP (0.1 mg/ml, 20 μ l); C, denatured-reduced rhAFP (AEC on-column refolding load, 0.23 mg/ml, 10 μ l); D, 'Q-FF on-column'-refolded rhAFP (20 μ l); E, 'DEAE-FF on-column'-refolded rhAFP (20 μ l).

Bioactivity of the refolded AFP was also verified by ELISA. Positive responses were observed with 'on-column'-refolded rhAFP using both Q-FF and DEAE-FF columns, while controls comprising denatured-reduced AFP yielded negative responses (Appendix 3). This result indicates that the on-column refolded rhAFP exhibits comparable bioactivity to human-derived AFP. Estimation of rhAFP refolding yields using ELISA gave comparable refolding yields as those obtained by RP-HPLC analysis (Table 4.1).

Table 4.1: Mass balance for rhAFP on-column refolding on 1 ml Q-FF and DEAE-FF columns after 6 h refolding incubation on-column.

Column	rhAFP load (mg)	rhAFP in flow through (mg)	rhAFP in wash fraction during buffer switch from Denaturation to AEC Refolding Buffer (mg)	Refolded rhAFP in 'AEC Elution Buffer'-eluted fraction (mg) ^a	rhAFP in 'AEC Stripping Buffer'-eluted fraction (mg) ^b	rhAFP refolding yield determined by RP-HPLC (%)	rhAFP refolding yield determined by ELISA (%)
Q-FF 1 ml	1	0	0	0.15	0.7	15±1.3%	12±3%
DEAE 1 ml	1	0	0	0.19	0.62	19±1.5%	17±2.5%

^a determined by RP-HPLC analysis.

^b determined by SDS-PAGE analysis and Bradford quantitative protein determination assay.

The secondary and tertiary structures of the refolded rhAFP were also analysed by CD spectroscopy. The secondary and tertiary structures of ‘AEC on-column’-refolded rhAFP were determined by measuring the protein’s CD spectrum in the far UV region (190-250 nm) and near UV region (250-310 nm), respectively. Figure 4.6 shows that the ‘on-column’-refolded rhAFP had comparable far- and near-UV spectra fingerprints as the native std-AFP, indicating that the refolded protein adopts a conformational state that is comparable with the native protein. Importantly, this result shows that adsorptive refolding of rhAFP on chromatography columns did not compromise protein conformation.

Figure 4.6: (A) Far and (B) near UV CD spectra of (1) denatured rhAFP, (2) native std-AFP and (3) AEC-refolded rhAFP.

4.3.3.2 Optimisation of on-column rhAFP incubation time

All the on-column refolding studies reported thus far were based on a refolding on-column incubation time of 6 h. On-column refolding incubation time is an important parameter to optimise since process productivity is directly dependent on refolding time. When elution of the column-bound rhAFP was initiated immediately after buffer exchange from Denaturation Buffer to AEC Refolding Buffer, no refolded rhAFP was detected in the eluted fractions, as determined by RP-HPLC analysis. However, as on-

column refolding incubation time increased, a corresponding increase in rhAFP refolding yield from 0% to $15 \pm 1.3\%$ for Q-FF, and $19 \pm 1.5\%$ for DEAE-FF, was observed (Figure 4.7). DEAE-FF appeared to have a more positive influence on rhAFP refolding kinetics, where a higher rhAFP refolding yield was achieved in a shorter refolding incubation time compared to Q-FF. As a strong anion exchanger, Q-FF is charged across a wide range of pH levels and may be more strongly ionised at a fixed pH condition, compared to DEAE-FF. Therefore, a potentially stronger rhAFP-matrix interaction could restrict the free movement of protein molecule in the adsorbed state, thereby reducing refolding efficiency. A weaker protein-matrix interaction on DEAE-FF, on the other hand, could yield the adsorbed rhAFP protein molecule more flexibility, thus improving rhAFP refolding yields. From these results, the optimum on-column refolding incubation time of 6 h was used for subsequent AEC on-column refolding studies.

Figure 4.7: The effect of on-column refolding incubation time on rhAFP refolding yield, when refolded at 1 mg/ml rhAFP concentration on 1 ml Q-FF and DEAE-FF.

4.3.3.3 Optimisation of denatured-reduced rhAFP load

Following optimisation of the redox environment and incubation time, the effect of protein load on rhAFP refolding yield and productivity were next investigated. Varying protein load is expected to alter the spatial distribution of the adsorbed protein on the column, which is hypothesised to influence the extent of intermolecular protein interaction during refolding and hence refolding yield. To determine an optimum rhAFP load that will maximise on-column refolding yield, different amounts of denatured-reduced rhAFP (i.e. 0.15 to 2.4 mg) were loaded onto 1 ml Q-FF and DEAE-FF columns, and refolded under optimised redox environments (i.e. GSH:GSSG ratio of 4.5 mM/0.9 mM and 2.7 mM/2.7 mM for Q-FF and DEAE-FF column, respectively). Although increasing denatured-reduced rhAFP protein load up to 2.5 mg did not affect binding recovery, as reported in Section 4.3.2, it clearly had a strong influence on rhAFP refolding yields. rhAFP on-column refolding yields decreased by 64 % and 62 % for Q-FF and DEAE-FF, respectively, with increasing rhAFP load (Figure 4.8). It is hypothesised that increasing protein load will induce a high local protein concentration at different sections of the column, which can increase protein aggregation tendencies. RP-HPLC and Bioanalyzer analyses of the 'AEC Elution Buffer'-eluted protein fractions revealed that > 95% of the protein recovered were correctly refolded.

Figure 4.8: The effect of denatured-reduced rhAFP load on refolding yield for 1 ml Q-FF and DEAE-FF column.

A mass balance was performed on the AEC on-column refolding operation at 1 mg rhAFP load and the results are summarised in Table 4.1. It is clear that a significant amount of rhAFP was lost to irreversible binding on the column during refolding, as indicated by the substantial amount of rhAFP recovered from the stripping step (i.e. ‘AEC Stripping Buffer’-eluted fraction). The strongly bound rhAFP aggregates could only be desorbed using AEC Stripping Buffer which contained high urea and salt concentrations. Based on these results, a 1 mg denatured-reduced rhAFP load was employed to achieve a reasonable trade-off between productivity and on-column aggregation.

Figure 4.9 shows the FPLC chromatogram of rhAFP on-column refolding performed on a 1 ml DEAE-FF column. SDS-PAGE analysis of the protein fraction from Peak 1 (Figure 4.10) revealed no protein content, and the absorbance at 280 nm was attributed to absorbance by GSH and GSSG in the AEC Refolding Buffer. RP-HPLC and Bioanalyzer analyses of protein fractions from Peak 2 showed a high rhAFP content present at a protein concentration of 0.12 mg/ml and 95% purity. Compared to

std-AFP (lane 2), a slightly lower molecular weight of rhAFP observed in Figure 4.10 is due to the unglycosylated structure of rhAFP. A higher rhAFP concentration would be readily obtained by substituting gradient elution with a step elution during process scale-up.

Figure 4.9: FPLC chromatogram of rhAFP on-column refolding performed on a 1 ml DEAE-FF column. A, rhAFP loading and wash; B, buffer switching from Denaturation Buffer to AEC Refolding Buffer; C, on-column incubation in AEC Refolding Buffer; D, elution.

Figure 4.10: SDS-PAGE analysis of the AEC on-column refolding fractions at 1 mg rhAFP load for 1 ml DEAE-FF column. Lane 1, molecular weight marker, 4 μ l; lane 2, std-AFP (0.1 mg/ml) 15 μ l; lane 3, denatured-reduced rhAFP (load) (0.23 mg/ml) 15 μ l; lane 4, flow through fractions, 15 μ l; lane 5, wash fractions (following buffer switch from Denaturation Buffer to AEC Refolding Buffer), 15 μ l; lane 6, 'AEC Elution Buffer'-eluted fraction from Peak 1 (Figure 4.9), 15 μ l; lanes 7-8, 'AEC Elution Buffer'-eluted fraction from Peak 2 (Figure 4.9), 15 μ l; lane 9, 'AEC Stripping Buffer'-eluted fraction, 15 μ l.

4.3.4 The effect of column dimensions on rhAFP refolding

Results presented in Sections 4.3.2 and 4.3.3 have shown that increasing protein load had negligible effects on rhAFP binding recovery, but significantly reduced refolding yield. It is likely that on-column rhAFP aggregation could subsequently induce the formation of precipitate layers, especially at the top section of the column, which may impede the free movement of protein molecules across the matrix and halt further increase in refolding yield. This limitation could be overcome by increasing column diameter to allow protein diffusion in the radial and axial directions. Therefore, the

effect of increased column geometry on rhAFP refolding yield was next studied, with the aim to improve refolding yield.

On-column refolding studies were extended to 5 ml Q-FF and DEAE-FF columns using the same refolding method and conditions as those for 1 ml columns. On-column refolding was performed at a fixed volumetric mobile phase flow rate, denatured-reduced rhAFP load and refolding incubation time as that used for 1 ml columns (i.e. 1.3 cm/min, 1 mg denatured-reduced rhAFP, and 6 h respectively). The use of 5 ml columns improved rhAFP refolding yields by 66% and 68% for both Q-FF and DEAE-FF columns, respectively (Table 4.2). This result suggests that for the same protein load, the 5 ml columns which offered a larger matrix surface area facilitated better dispersion of the protein across the column, and hence reduced the occurrence of a high local protein concentration at a given region of a matrix. Consequently, inter-molecular protein interaction could be minimised, and higher refolding yields were achieved.

Table 4.2: The column dimensions of 1 and 5 ml Q-FF and DEAE-FF as provided by the supplier and the corresponding rhAFP refolding yields, achieved at 1 mg denatured-reduced rhAFP load.

Column type	Internal diameter (mm)	Length (mm)	Internal area (mm ²)	Bed volume (ml)	rhAFP refolding yield (%)
Q-FF 1 ml	7	25	38.5	0.96	15±1.3 %
Q-FF 5 ml	16	25	201	5.02	25±3 %
DEAE-FF 1 ml	7	25	38.5	0.96	19±1.5 %
DEAE-FF 5 ml	16	25	201	5.02	32±2 %

A further increase in the denatured-reduced rhAFP load from 0.5 mg to 2.8 mg, however, decreased rhAFP refolding yields by 61% and 63 % for the 5 ml Q-FF and DEAE-FF columns (Figure 4.11).

Figure 4.11: The effect of protein load on on-column rhAFP refolding yields for 5 ml Q-FF and DEAE-FF columns.

4.3.5 AEC off-column refolding

Following systematic studies and optimisation of on-column AEC refolding parameters, the best rhAFP refolding yields obtained from refolding on 1 ml DEAE-FF and Q-FF, were 19 ± 1.5 % and 15 ± 1.3 %, respectively, as reported in Table 4.2. The significant loss of rhAFP refolding yield due to on-column aggregation raises the question of whether resin interference was the cause of the observed reduced refolding efficiency. To answer this question, off-column refolding was subsequently investigated in parallel with on-column refolding to compare rhAFP refolding yields without subjecting the protein molecules to on-column incubation with the refolding buffer. Instead, the adsorbed denatured-reduced rhAFP proteins were eluted immediately with AEC Off-column Refolding Buffer containing 0.15 M NaCl following adsorption and wash steps, and incubated for 3 h. Q-FF 1 ml was used for all the off-column rhAFP refolding studies reported in this chapter.

Off-column rhAFP refolding yields were comparable to that obtained by on-column refolding (where on-column protein incubation was also performed for 3 h to normalise refolding time) (Figure 4.12). Off-column rhAFP refolding yields could be significantly increased to reach $42\pm4\%$ by the addition of 0.5 M L-arginine to the AEC Off-column Refolding Buffer. L-arginine is a refolding additive that stabilises and enhances the solubility of refolding intermediates by shielding the hydrophobic regions of the partially folded polypeptide chains, thereby suppressing aggregation (De Bernardez Clark *et al.*, 1999; Vallejo and Rinas, 2004b). This result suggests that a high extent of off-column aggregation is hydrophobic-driven, which could be minimised by the addition of L-Arginine. During on-column refolding, the occurrence of hydrophobic-driven protein-resin and protein-protein interaction in addition to electrostatic interaction, cannot be ruled out. Despite the positive effect of L-Arginine shown by the off-column refolding results, the use of L-arginine to enhance rhAFP on-column refolding is unpractical because the high buffer conductivity contributed by L-arginine will interfere with protein adsorption on the positively charged matrix at physiological pH. Furthermore, the use of L-arginine in the refolding buffer will also increase scale-up costs. An improved understanding of protein-resin interaction leading to an improved on-column refolding platform design that will minimise surface-induced misfolding is therefore critical to leverage the simultaneous purification and concentration advantage of adsorptive chromatography refolding for large scale applications, as will be investigated in Chapter 5.

Figure 4.12: Comparison of rhAFP refolding yields achieved with AEC on-column (3 h on-column incubation) and off-column refolding on a 1 ml Q-FF column.

4.3.6 Comparison of AEC and dilution refolding methods

The performance of on-column AEC refolding was also directly compared to the most conventional and commonly used batch dilution refolding method. 0.45 mg/ml was the highest rhAFP refolding concentration attainable with batch dilution in this study due to protein solubility and viscosity constraints in the upstream sample preparation step. Therefore, in the parallel comparison study between batch dilution and 1ml DEAE-FF on-column refolding, the rhAFP refolding concentration was fixed at 0.45 mg/ml. A rhAFP refolding yield of 22% (Figure 4.8) was obtained when rhAFP was refolded on DEAE-FF and incubated for 3 h, while a 14% refolding yield was achieved by batch dilution following 6 h incubation (Figure 4.13). When the same refolding incubation time of 3 h was used for dilution refolding, no refolding yield could be detected, showing how on-column refolding readily outperformed dilution refolding in terms of refolding efficiency.

Figure 4.13: The effect of rhAFP refolding concentration on refolding yield for batch dilution refolding.

Figure 4.14 shows the RP-HPLC chromatogram profiles of refolded rhAFP fractions following dilution and AEC on-column refolding. The significant ‘peak shouldering’ observed in peak D compared to peak E suggests a lower refolding efficiency and refolded product purity by batch dilution compared to on-column refolding, after 6 h of refolding incubation time. Both batch- and column-refolded rhAFP gave positive responses in the ELISA test, with controls comprising denatured-reduced rhAFP, yielding negative responses (Tables 4.3 and 4.4).

Figure 4.14: RP-HPLC chromatograms comparing dilution- and 'DEAE-FF on-column refolded'-rhAFP. A, native std-AFP (0.1 mg/ml, 10 μ l); B, denatured-reduced std-AFP (0.1 mg/ml, 20 μ l); C, denatured-reduced rhAFP (on-column refolding load, 0.23 mg/ml, 10 μ l); D, 'dilution'-refolded rhAFP (20 μ l); E, 'DEAE-FF on-column'-refolded rhAFP (20 μ l).

A parallel comparison of the refolding performance of on-column AEC and batch dilution refolding methods was performed. Tables 4.3 and 4.4 provide the optimised experimental parameters and refolding performance indicators for both dilution and AEC column refolding. Despite a refolding concentration that was two-fold higher than dilution refolding, the refolding productivity for on-column DEAE-FF refolding was 6-fold higher compared to dilution refolding. This superior on-column refolding productivity was attributed to the higher protein refolding concentrations possible, and the reduced refolding incubation time required compared to batch dilution refolding.

At 1 mg/ml rhAFP refolding concentration, the concentration of rhAFP recovered from on-column DEAE-FF refolding was 2-fold higher than that achieved by dilution refolding at half the protein refolding concentration. Further increase in 'on-column'-refolded rhAFP concentration is expected to be readily achieved by replacing gradient

elution with a step elution upon scale-up, thereby eliminating the need for a separate post-refolding protein concentration step. Another clear advantage of on-column refolding over dilution refolding is the simultaneous protein purification achieved, where rhAFP purity after on-column refolding was enhanced from $74\pm4\%$ (achieved in batch dilution) to $95\pm1\%$, as determined by Bioanalyzer analysis (Table 4.4). This result demonstrates that column refolding was able to purify the correctly refolded rhAFP protein from misfolded, incompletely folded proteins and other contaminant proteins. By comparing dilution and on-column refolding, the contributions of on-column refolding toward bioprocess intensification is clear, where its capability to simultaneously purify and refold proteins in a single, simple and readily automated step shows suitability for large-scale IB refolding applications. Tables 4.3 and 4.4 also show that the solvent requirement for AEC refolding processes was comparable to dilution refolding despite the higher refolded rhAFP concentration achieved by the former. The refolding buffer requirement to scale column refolding will be lower than that for dilution refolding, where a proportional increase in buffer consumption is required for the latter but not the former.

Off-column refolding (without L-arginine) of rhAFP also achieved a refolding productivity that was 4-fold higher compared to dilution refolding. However, off-column refolding is less suitable for large-scale applications since this method induces a higher extent of protein dilution during the slower elution-refolding step. Compared to the best on-column refolding results which was achieved with 1 ml DEAE-FF, off-column refolding showed no apparent advantages over on-column refolding in terms of rhAFP yield, purity, concentration or productivity.

Table 4.3: Experimental parameters in dilution refolding.

Denatured-reduced rhAFP concentration (mg/ml)	1.64
Denatured-reduced rhAFP volume (ml)	2.5
Denatured-reduced rhAFP mass (mg)	4.1
rhAFP concentration after PD-10 desalting (mg/ml)	1.17
rhAFP refolding concentration (mg/ml)	0.45
Dilution-fold	2.6
rhAFP sample for refolding (ml)	1.92
Refolding buffer (ml)	3.1
Refolding volume (ml)	5
Total buffer used (ml)	32
Refolding incubation time (h)	6
Refolded rhAFP concentration (mg/ml)	0.063
Refolding yield determined by RP-HPLC (%)	14
Refolding yield determined by ELISA (%)	10
Refolded rhAFP purity (%)	9.5
Refolding productivity (mg/ml/h)	0.0105

Table 4.4: Experimental parameters and refolding performance indicators in AEC on-column refolding.

	DEAE-FF on- column refolding	Q-FF	
		On-column refolding	Off-column refolding
Initial rhAFP concentration (mg/ml)	0.23	0.23	0.23
Load volume (ml)	6	6	6
Mass of rhAFP load (mg)	1	1	1
Denaturation buffer for column equilibration (ml)	12	12	12
Equilibration buffer for DTT removal (ml)	0	0	10
AEC Refolding buffer (ml)	10	10	10
AEC Elution buffer (ml)	10	10	0
Total buffer used (ml)	32	32	32
Time (h)	3	6	3
Eluted refolded rhAFP concentration (mg/ml)	0.12	0.07	0.04
Column volume (ml)	1	1	1
Refolding yield determined by RP-HPLC (%)	19	15	12
Refolding yield determined by ELISA (%)	17	12	10
Refolded rhAFP purity (%)	95	93	92
Productivity (mg/ml/h)	0.063	0.025	0.04

4.4 Conclusions

In this chapter, the development of an AEC-based matrix refolding strategy for rhAFP was reported. The dependence of rhAFP refolding efficiency on chromatography operating parameters such as resin chemistry, column geometry, protein load, incubation time and refolding redox potential was studied. Upon systematic optimisation of the aforementioned parameters, rhAFP was successfully refolded on both Q-FF and DEAE-FF chromatography column matrices at higher protein concentrations than those allowable by dilution refolding (i.e. > 0.5 mg/ml rhAFP). The simultaneous separation of correctly 'on-column'-refolded rhAFP from refolding aggregates and contaminant proteins further simplifies bioprocessing and enhances overall product recovery.

rhAFP adsorption on the chromatography matrix enhanced refolding productivity by reducing the time required to attain refolding equilibrium compared to dilution refolding. Increase in rhAFP refolding productivity by on-column refolding was most evident with the use of a DEAE matrix, where a $19 \pm 1.5\%$ refolding yield was readily attained at 1 mg/ml rhAFP refolding concentration, after 3 h incubation. On-column DEAE refolding of rhAFP improved refolding productivity and refolded protein purity by 6- and 10-fold, respectively. Off-column refolding, on the other hand, improved rhAFP refolding productivity by 4-fold compared to dilution refolding, where refolding occurred in the eluate instead of on the column matrix. These results clearly demonstrate that column refolding is not limited to pure and/or simple proteins, but is also readily applicable for high-throughput refolding of IB-derived proteins with complex molecular structures.

Despite these process improvements compared to dilution refolding, the sub-optimal on-column refolding yields due to significant aggregation on-column may continue to limit the usage of on-column refolding in large scale applications. It is hypothesised that on-column aggregation is the result of a strong protein-matrix interaction, which reduces flexibility of the protein molecule to refold. Confronted with this limitation, a proper understanding of factors which influence protein-resin interaction is important to improve AEC on-column refolding yields, and forms the aim of the next chapter of

this thesis. In Chapter 5, the role of salt in controlling the behaviour of protein-resin interaction and surface refolding of rhAFP on AEC matrices will be researched.

CHAPTER 5 : THE ROLE OF NaCl IN ANION EXCHANGE CHROMATOGRAPHY REFOLDING

5.1 Introduction

In Chapter 4, the successful development of an anion exchange chromatography (AEC) on-column refolding platform for rhAFP refolding was reported. Optimised refolding of rhAFP on a DEAE-FF column achieved a 6- and 10-fold improvement in refolding productivity and protein purity compared to dilution refolding. The simultaneous purification ability of on-column DEAE refolding will clearly contribute to streamlined processing of rhAFP. However, the loss of 70% of the rhAFP to aggregation on the AEC matrices reduces refolding and process yield. Adsorption of denatured-reduced rhAFP on the AEC matrix is predominantly driven by electrostatic interaction between charged functional groups of the resin and oppositely charged groups of the protein, although the hydrophobic interaction contributions also cannot be excluded (Vutukuru *et al.*, 2006). However, it is hypothesised that a strong protein-resin interaction due to multi-point interaction can significantly limit the flexibility of the adsorbed protein, and consequently reduce refolding yield.

The aim of the study described in Chapter 5 is to investigate the role of NaCl salt in controlling surface refolding of rhAFP. It is hypothesised that NaCl will incur a charge shielding effect on both the protein molecules and AEC matrix functional groups, thereby weakening electrostatic-driven interaction and increasing protein flexibility to refold. The influence of NaCl in altering the surface charges of the (i) refolded rhAFP protein, (ii) anion exchange resin, and (iii) 'refolded rhAFP'-resin complex will be systematically determined by zeta potential measurements, which will open the way for the investigation of the effects of surface charge variation on rhAFP adsorption and on-column refolding behaviours.

5.2 Materials and methods

Four anion exchange resins were studied: (i) Fractogel EMD DEAE (S) (Merck, Singapore), (ii) SOURCE 15Q (GE Healthcare, Singapore), (iii) Q Sepharose Fast Flow (GE Healthcare, Singapore), and (iv) DEAE Sepharose Fast Flow (GE Healthcare, Singapore). Resins (i) to (iv) will be referred to as (i) DEAE (S), (ii) 15 Q, (iii) Q-FF, and (iv) DEAE-FF, respectively, throughout this chapter. DEAE (S) and DEAE-FF are weak anion exchange resins which are functionalised with diethylaminoethyl groups, and have particle diameters of 30 μm and 90 μm , respectively. 15Q and Q-FF are strong anion exchange resins which are functionalised with quaternary ammonium groups, and have particle diameters of 15 μm and 90 μm , respectively. Tricorn 5/50 empty columns which have a packed bed volume ranging from 0.69-1.16 ml were purchased from GE Healthcare, Singapore.

Other chemicals were sourced as detailed in Chapter 3 and 4.

5.2.1 rhAFP pre-refolding recovery and purification

rhAFP was expressed, recovered and purified using the same method as reported in Sections 3.2.1 and 3.2.2 in Chapter 3. The Triton-washed pellets were solubilised in 2 to 50 ml Denaturation Buffer (8 M urea, 20 mM Tris, 3 mM EDTA, 20 mM DTT, pH 8.5) to achieve different final denatured-reduced rhAFP protein concentrations ranging from 0.23 to 5.8 mg/ml, and incubated under gentle shaking conditions for 5 h, at room temperature (21 $^{\circ}\text{C}$).

5.2.2 Zeta potential measurements

Zeta potential measurements of the refolded rhAFP protein and AEC resins were performed under a refolding-favouring environment at room temperature, on a ZetaPALS zeta potential analyzer (Brookhaven Instruments Corporation, USA) using the phase analysis light scattering (PALS) method (Weiner *et al.*, 1998). The mean electrophoretic mobility, μ ($\text{m}^2/(\text{Vs})$), of each sample was determined from 20

measurements of each sample, and the zeta potential value, ζ (mV), was determined by the ZetaPALS software based on the Smoluchowski equation: $\zeta = \frac{\mu\eta}{\epsilon}$ (Equation 5.1), where η (Pas) and ϵ (F/m) are the viscosity and dielectric constant of the solution, respectively.

5.2.2.1 Zeta potential measurement of AEC resins with varying pH

Since the recommended particle size range for accurate ζ measurements using the PALS method was 1 nm to 30 μm , ζ measurements were performed only with the 15Q and DEAE (S) resins. To study the effect of pH on the ζ values of the AEC resins, 15Q and DEAE (S) resins were added into 20 mM Tris-HCl buffer (pH 9.6) to a final concentration of 0.01 ml resin/ml buffer. The pH values of both resin suspensions were adjusted by adding diluted HCl, and the ζ of each resin suspension at a fixed pH was measured.

5.2.2.2 Zeta potential measurement of AEC resins with varying NaCl concentration

The effect of NaCl on the surface charge of (i) 15Q and DEAE (S) resins, (ii) refolded rhAFP and (iii) 'refolded rhAFP'-resin complex were studied by measuring the change in the measured ζ values of (i) to (iii) with varying NaCl concentration under refolding conditions. For ζ measurements of (i), the resins were added into AEC Refolding Buffer (3 M urea, 20 mM Tris, 1 mM EDTA, 2.7 mM GSH, and 2.7 mM GSSG, pH 8.5) in the presence of 0 to 100 mM NaCl, to a final concentration of 0.01 ml resin/ml buffer. For ζ measurements of (ii), denatured rhAFP (following DTT removal by PD-10 desalting column) was diluted into AEC Refolding Buffer to a final protein concentration of 0.01 mg/ml. ζ measurements were performed on the refolded rhAFP solution after 24 h of protein incubation in AEC Refolding Buffer. For ζ measurements of (iii), denatured-reduced rhAFP and the AEC resins were mixed to achieve a final rhAFP load of 1 mg protein/ml resin under shaking conditions for 24 h in the presence of NaCl concentration from 0 to 50 mM. After 24 h incubation, the protein-resin mixtures were centrifuged at 8,000 g for 10 min and the pellets were washed with AEC Refolding Buffer. The protein-resin mixture was incubated in AEC Refolding Buffer for 6 h prior to ζ measurements.

5.2.3 The effect of NaCl on rhAFP adsorption isotherm

The effect of NaCl on denatured-reduced rhAFP adsorption capacity on the AEC resins was studied by determining the adsorption isotherms of denatured-reduced rhAFP on 15Q, Q-FF, DEAE (S) and DEAE-FF resins. Adsorption isotherms were determined by static batch adsorption experiments at room temperature. The AEC resins were added into 2 ml Eppendorf tubes containing 0.5 to 4.8 mg/ml denatured-reduced rhAFP in the presence of 0 and 50 mM NaCl, to obtain a final denatured-reduced rhAFP load of 4.5 to 37.6 mg protein/ml resin. The protein-resin mixtures were incubated under shaking condition at room temperature for 24 h to achieve adsorption equilibrium. The protein-resin mixture was then centrifuged (2000 g, 10 min) and the supernatant was filtered through a 0.45 μ m micro filter unit (Millipore) and analysed by RP-HPLC to determine the amount of adsorbed protein per ml of resin at equilibrium. The data obtained were fitted into the Langmuir equation (Equation 4.1) to determine the dissociation constant, k_d (mg/ml), and the maximum binding capacity, q_m (mg/ml).

5.2.4 The effect of NaCl on rhAFP adsorption on AEC columns

All AEC chromatography were conducted on an ÄKTA Explorer FPLC workstation (GE Healthcare, Singapore) at room temperature. 15Q and DEAE (S) resins were packed in a Tricorn 5/50 column (1 ml column volume, 5 mm i.d., 50 mm bed height) according to the manufacturer's instruction. Q-FF and DEAE-FF columns were used as purchased.

A constant mobile phase flow rate of 0.5 CV/min was used for column equilibration, sample loading and elution. To determine denatured-reduced rhAFP recovery during the adsorption step, each column was first equilibrated with 12 CV of Denaturation Buffer containing 0 to 75 mM NaCl. 1.38 mg denatured-reduced rhAFP was loaded into the column, and the column was washed with 8 CV Denaturation Buffer containing 0 to 75 mM NaCl to remove any unbound proteins. Adsorbed proteins on

the column were eluted by a linear NaCl gradient over 10 CV from Denaturation Buffer to Stripping Buffer (8 M urea, 20 mM Tris, 3 mM EDTA, 20 mM DTT, 1 M NaCl, pH 8.5). Eluted fractions were collected and analysed by RP-HPLC to determine the amount of rhAFP bound in the presence of 0 to 75 mM NaCl. Denatured-reduced rhAFP binding recovery was calculated using Equation 4.2.

The adsorption strength of denatured-reduced rhAFP on 15Q and DEAE (S) columns was also measured by comparing the elution volumes required to desorb the protein from the column stationary phase. After 1.38 mg denatured-reduced rhAFP was loaded into the column in the presence of 0 to 50 mM NaCl, the column was washed with 12 CV Denaturation Buffer (without NaCl). Elution was initiated by introducing a linear NaCl gradient from Denaturation Buffer (without NaCl) to 15% Stripping Buffer (i.e. 0 to 150 mM NaCl) over 15 CV. The elution volumes required to desorb denatured-reduced rhAFP bound at different NaCl conditions were measured.

5.2.5 The effect of NaCl on AEC on-column rhAFP refolding

1 and 5 ml DEAE-FF columns were used in the on-column rhAFP refolding study. A constant mobile phase flow rate of 0.5 CV/min was used for column equilibration, sample loading and elution. Each column was equilibrated with Denaturation Buffer containing 0 to 50 mM NaCl. 1.38 mg denatured-reduced rhAFP containing 0 to 50 mM NaCl was loaded into the column. Refolding was initiated by a buffer switch from Denaturation Buffer to AEC Refolding Buffer containing 0 to 50 mM NaCl, over 10 CV, and the protein was incubated on-column for 3 h. Elution was initiated by a buffer switch from AEC Refolding Buffer to Elution Buffer (20 mM Tris, 1 mM EDTA, 1 M NaCl, pH 8.5) using a linear gradient over 10 CV. The column was washed and regenerated with 10 CV of Stripping Buffer after each refolding cycle. Flow through, wash and elution fractions were collected and analysed by SDS-PAGE and RP-HPLC to determine rhAFP refolding yield. rhAFP refolding yield and refolding productivity were determined according to Equations 4.3 and 4.4 (please refer to Section 4.2.4, Chapter 4).

5.2.6 The effect of NaCl on rhAFP refolding by batch dilution

rhAFP denaturation-reduction was conducted as described in Section 5.2.1. 0.5 ml DTT-free protein mixture (containing 1.8 mg denatured rhAFP) was rapidly diluted into 4.5 ml refolding buffer containing various NaCl concentrations, to obtain a final rhAFP concentration of 0.36 mg/ml and a final refolding buffer composition of 0.8 M urea, 20 mM Tris, 3 mM EDTA, 2.7 mM GSH and 2.7 mM GSSG, 0 to 75 mM NaCl (pH 8.5). The protein was incubated under gentle shaking conditions at 4°C for 6 h. rhAFP refolding yield was determined by RP-HPLC.

5.2.7 Analytical methods

RP-HPLC analysis was determined using the same method as described in Section 3.2.7.1 (Chapter 3).

5.3 Results and discussion

In this chapter, the role of NaCl in AEC on-column refolding of rhAFP was studied using (i) 15Q, (ii) DEAE (S), (iii) Q-FF and (iv) DEAE-FF columns. The effect of NaCl on varying the surface potential and adsorption isotherms of rhAFP was studied to elucidate the behaviour of protein-resin interaction when NaCl was present in the denaturation and refolding buffers.

5.3.1 Zeta potential measurements of anion exchange resins and rhAFP under refolding conditions

The effect of NaCl on the protein and anion exchanger surface charges was first studied by measuring the zeta potential, ζ , of the resins and rhAFP protein under refolding conditions. Zeta potential is an electric potential at the interface of an electric double layer that exists around each particle, and is a function of the particle surface charge (Hunter, 1981; Weiner *et al.*, 1998). The ζ values of the resins and

proteins therefore provide a quantitative indication of the strength of electrostatic-driven protein-matrix interaction.

Chapter 4 reported the development of rhAFP on-column refolding methods using Q-FF and DEAE-FF packed bed columns. However, in this study, ζ measurements were performed on the 15Q and DEAE (S) resins instead because the particle size range recommended for accurate ζ measurements using the PALS method was 1 nm to 30 μm ¹. Larger particle sizes will reduce the accuracy of ζ measurement due to the increased influence of the electrokinetic effects by other forces such as sedimentation with increasing particle size. Since 15Q and DEAE (S) resins are derivatized with the same functional groups as Q-FF and DEAE-FF, respectively, but have particle sizes within the 1 nm to 30 μm range (Table 5.1), the ζ values of the 15Q and DEAE (S) resins were therefore used to approximate the surface potential of the Q-FF and DEAE-FF resins at varying NaCl concentrations. A comparable dynamic binding capacity of BSA or HSA on DEAE-FF, 15Q and DEAE (S) columns (Table 5.1) suggests a comparable surface charge density for the resins.

¹ <http://www.bic.com/index.html>

Table 5.1: Properties of anion exchange resins from resin product data sheet.

Resin	15Q	DEAE (S)	Q-FF	DEAE-FF
Functional group	Quaternary amine	Diethylaminoethyl	Quaternary amine	Diethylaminoethyl
Type of medium	Strong anion exchange	Weak anion exchange	Strong anion exchange	Weak anion exchange
Mean particle size	15 μm	30 μm	90 μm	90 μm
Dynamic binding capacity	N.A.	100 mg BSA/ml medium	120 mg HSA/ml medium ^a	110 mg HSA/ml medium ^a
Ionic capacity	N.A.	N.A.	0.18-0.25 mmol Cl ⁻ /ml medium	0.11-0.16 mmol Cl ⁻ /ml medium
Matrix base	Polystyrene	Polymethacrylate	Agarose	Agarose

N.A.: not available.

^a: Samples were applied at 75 cm/h until 50% breakthrough.

The ζ variation of 15Q and DEAE (S) resins as a function of pH is shown in Figure 5.1. Both resins remained positively charged over pH 3.5 and 9.5 although the net positive charge decreased with increasing pH. DEAE (S), being a weak anion-exchanger, showed a weaker net positive charge compared to 15Q over the pH range studied. The larger ζ values demonstrated by 15Q resins suggest a potentially stronger interaction with oppositely charged protein regions, as postulated by Coulombs' Law (Staahlberg *et al.*, 1991; Stahlberg *et al.*, 1992; Gerstner *et al.*, 1994).

Figure 5.1: Zeta potential of 15Q and DEAE (S) resins as a function of pH.

The effect of varying NaCl concentration on refolded rhAFP and resin surface charges, and their correlation with 'refolded rhAFP protein'-resin association behaviour was subsequently studied. All ζ measurements were conducted with samples (i.e. resin and/or protein) contained in AEC Refolding Buffer, since the high viscosity imposed by 8 M urea solutions prevents zeta potential measurements under denatured-reduced conditions. Figure 5.2 shows that 15Q and DEAE (S) resins as well as refolded rhAFP experienced reduction in surface charges with increasing NaCl in AEC Refolding Buffer. With a theoretical pI of 5.3 in the native conformation (Parmelee *et al.*, 1978), refolded rhAFP would maintain a net negative charge in the refolding buffer at pH 8.5. As both resins and rhAFP protein maintained opposite charges over the salt

concentration range tested, the polar charged amino acid residues in rhAFP will interact with the positively charged functional groups of the AEC resins by electrostatic and van der Waals interaction.

To mimic on-column AEC rhAFP refolding, as studied in Chapter 4, denatured-reduced rhAFP was mixed with 15Q and DEAE (S) resins in a batch adsorption study to yield a protein load of 1 mg rhAFP/ml resin. After 24 h incubation in Denaturation Buffer, the protein-resin mixture was centrifuged and the pellet containing resins and bound proteins were resuspended in AEC Refolding Buffer. ζ measurements of the 'refolded rhAFP'-resin complex showed a reduction in net surface charge with increasing NaCl concentration (Figure 5.2). Binding of denatured-reduced rhAFP to the resins was hindered by NaCl concentrations above 50 mM NaCl, where all the rhAFP remained in the supernatant after 24 h incubation, as determined by SDS-PAGE analysis. As NaCl concentration increased, the charge shielding effect of the NaCl counter ions on the positively charged resins and negatively charged denatured-reduced rhAFP also increased, as reflected by decreasing ζ values. As such, protein-resin association strength will be reduced. In addition to NaCl counter ion shielding effect, the adsorption of negatively charged denatured-reduced rhAFP to the resin will also reduce the net positive charge of the resin, and thus contribute to lowering the ζ values.

Figure 5.2: Zeta potential of the refolded rhAFP, AEC resins and ‘refolded rhAFP’-resin complex at varying NaCl concentrations.

5.3.2 The effect of NaCl on rhAFP adsorption isotherm

Following the surface potential characterisation of the protein and AEC resins under refolding conditions with varying NaCl concentrations, the effect of NaCl on denatured-reduced rhAFP adsorption isotherm was subsequently studied. For NaCl to be effectively used in on-column refolding of proteins on AEC columns, it is important to ascertain the sensitivity of rhAFP adsorption to increasing salt concentration. The binding capacity of denatured-reduced rhAFP on 15Q, DEAE (S), Q-FF and DEAE-FF resins was determined in a batch adsorption study, where denatured-reduced rhAFP was mixed with the AEC resins under denaturing conditions for 24 h to achieve adsorption equilibrium. The rhAFP adsorption data obtained for both resins were regressed into the Langmuir equation (Figure 5.3 and Figure 5.4) to determine the maximum resin adsorption capacity, q_m , and dissociation constant, K_d , when rhAFP was adsorbed at various NaCl concentrations (Table 5.2).

A higher K_d was observed with increasing NaCl concentration in Denaturation Buffer for all the AEC resins studied, indicating that NaCl reduced the ‘denatured-reduced rhAFP’-resin interaction strength during protein adsorption under denaturing conditions. In the presence of 50 mM NaCl, the maximum binding capacities of denatured-reduced rhAFP on 15Q, DEAE (S), Q-FF and DEAE-FF resins decreased by 26 %, 17%, 20% and 22%, respectively, compared to when NaCl was absent (Table 5.2). This reduced binding is likely to increase the spatial distribution of the adsorbed proteins, thus reducing intermolecular-induced protein aggregation on-column. To verify this hypothesis, the effect of NaCl on AEC on-column refolding of rhAFP was next studied, as reported in Section 5.3.4.

Figure 5.3: Adsorption isotherms for denatured-reduced rhAFP on 15Q and DEAE (S) resins. Solid and dotted line curves are the Langmuir fit to experimental data for denatured-reduced rhAFP adsorption in the absence and presence of NaCl, respectively.

Figure 5.4: Adsorption isotherms for denatured-reduced rhAFP on Q-FF and DEAE-FF resins. Solid and dotted line curves are the Langmuir fit to experimental data for rhAFP adsorption in the absence and presence of NaCl, respectively.

Table 5.2: Parameters of Langmuir adsorption isotherms of rhAFP as a function of NaCl and AEC resin type.

Resin	0 mM NaCl		50 mM NaCl	
	q_m (mg/ml)	K_d (mg/ml)	q_m (mg/ml)	K_d (mg/ml)
15Q	26.1	0.433	19.2	0.664
DEAE (S)	31.0	0.123	25.7	0.341
Q-FF	32.9	0.227	26.1	0.503
DEAE-FF	30.0	0.250	23.4	0.737

5.3.3 The effect of NaCl on rhAFP on-column refolding on DEAE-FF columns

Having demonstrated that NaCl, at 50 mM concentration, weakened denatured-reduced rhAFP adsorption strength on all the resins studied in the batch adsorption mode, the effect of NaCl addition on rhAFP on-column adsorption and refolding using DEAE-FF columns was subsequently studied. DEAE-FF was used for this study because a higher rhAFP on-column refolding yield and productivity was obtained compared to Q-FF, as reported in Chapter 4.

1 and 5 ml DEAE-FF columns were equilibrated with Denaturation Buffer containing 0 to 75 mM NaCl. Denatured-reduced rhAFP was loaded into each column using the same buffer to achieve a final rhAFP load of 1 mg in the presence of 0 to 75 mM NaCl. The adsorption results show that rhAFP binding was generally unaffected by NaCl concentration up to 50 mM (Figure 5.5). An average of 0.93 ± 0.05 mg and 0.94 ± 0.03 mg rhAFP was adsorbed per 1 ml and 5 ml DEAE-FF column, respectively, in the presence of 0 to 50 mM NaCl. Increasing denatured-reduced rhAFP load in the 5 ml DEAE-FF column in the presence of 50 mM NaCl, did not compromise rhAFP adsorption, where an average rhAFP recovery of $71 \pm 1.5\%$ was achieved (Figure 5.6). This result therefore justifies the use of NaCl concentration range of 0 mM to 50 mM and a protein load of up to 3.5 mg for subsequent rhAFP on-column refolding using DEAE-FF columns.

Figure 5.5: The effect of NaCl concentration on denatured-reduced rhAFP binding recovery on 1 ml and 5 ml DEAE-FF columns at 1 mg rhAFP load.

Figure 5.6: Binding recovery of denatured-reduced rhAFP at different loads on a 5 ml DEAE-FF column with and without 50 mM NaCl.

To study the effect of NaCl on rhAFP on-column refolding, denatured-reduced rhAFP containing 0 mM to 50 mM NaCl was loaded into a 1 ml DEAE-FF column which was also equilibrated in the same Denaturation Buffer containing NaCl at various concentrations. The protein was incubated on-column for 3 h in AEC Refolding Buffer also containing the same NaCl concentration as the equilibration and load buffers. Figure 5.7 shows that increasing NaCl concentration up to 50 mM in the load, equilibration and refolding buffers generally increased rhAFP refolding yields for both 1 ml and 5 ml DEAE-FF columns. NaCl at an optimum concentration of 50 mM and 25 mM, improved rhAFP refolding yields by 47% and 56% for the 1 ml and 5 ml DEAE-FF column, respectively, at 1 mg rhAFP load. The increased refolding yield with increasing column volume suggests that as column volume increases, the spatial isolation and distribution of the protein also increases correspondingly, thus reducing aggregation.

As protein load was increased to 3.5 mg, NaCl continued to have a positive effect on rhAFP refolding yields (Figure 5.8). SDS-PAGE analysis of the flow through, wash,

elution and stripping fractions of 5 ml DEAE-FF ‘on-column refolded’-rhAFP indicate that rhAFP loss to irreversible adsorption on the column during refolding was reduced by 25% in the presence of 50 mM NaCl compared to on-column refolding without NaCl. In the AEC resin batch adsorption study to investigate the effect of NaCl on denatured-reduced rhAFP adsorption reported in Section 5.4.2, the increase in NaCl concentration decreased protein adsorption amount per adsorbent volume, as shown by the decreasing q_m values. This in turn will decrease adsorbed protein density on the column, thus decreasing aggregation and improving refolding yield, as reflected by the improved rhAFP on-column refolding yield with NaCl.

Figure 5.7: The effect of NaCl concentration on rhAFP refolding yield using 1 ml and 5 ml DEAE-FF at 1 mg rhAFP load.

Figure 5.8: The effect of denatured-reduced rhAFP load on refolding yield for 5 ml DEAE-FF with 0 and 50 mM NaCl.

rhAFP on-column refolding in the presence of 0 to 50 mM NaCl was also studied in parallel on 1 ml 15Q and DEAE (S) columns at the same rhAFP load of 1 mg. rhAFP refolding yields increased by 36% and 58% for 15Q and DEAE (S) column, respectively, at an optimal NaCl concentration in the load, denaturation and refolding buffer (Figure 5.9). However, the refolding yields were lower than those achieved using DEAE-FF columns, suggesting that variation in resin particle size can influence refolding yield due to different binding steric effects. Besides, smaller bead size also yields higher ligand density which increases the adsorbed protein density and can increase the chance for aggregation formation within a small spatial area.

Figure 5.9: The effect of NaCl concentration on on-column rhAFP refolding yield for 15Q and DEAE (S) columns at 1 mg rhAFP load.

The increase in rhAFP refolding yield with the addition of 50 mM NaCl suggests that salt can relax the protein-resin association to an extent which allows the adsorbed proteins more flexibility for refolding and oxidative shuffling, resulting in more efficient refolding. A study to empirically measure protein adsorption strength on 15Q and DEAE (S) columns was conducted to verify this hypothesis. Denatured-reduced rhAFP containing 0 to 50 mM NaCl was loaded onto 1 ml 15Q and DEAE (S) columns, also equilibrated in the Denaturation Buffer containing 0 to 50 mM NaCl. After protein loading, the columns were washed with Denaturation Buffer without NaCl to remove any unbound proteins and a linear NaCl gradient from 0 mM to 150 mM NaCl in Denaturation Buffer over 15 CV was introduced across the column. Figure 5.10 shows the effect of NaCl concentration on the retention volume of denatured-reduced rhAFP adsorbed on the 15Q and DEAE (S) columns, respectively. As expected, retention volume decreased with increasing NaCl concentration in the load and equilibration buffers, which confirms a weaker protein-resin interaction with increasing NaCl concentration. This result demonstrates that rhAFP adsorption

strength can be directly controlled by adjusting salt concentration in the load and column equilibration buffer.

Figure 5.10: The effect of NaCl concentration on rhAFP retention volume and conductivity on 1 ml 15Q and DEAE (S) columns.

5.3.4 The effect of NaCl on rhAFP dilution refolding

rhAFP refolding by batch dilution in the presence of different NaCl concentrations was conducted as a control study to investigate if NaCl assumed the role of a refolding additive to improve refolding yield. NaCl has been shown to improve solubility of some proteins by enhancing their ‘salting-in’ during refolding (Ladisch, 2001). rhAFP was refolded by dilution at 0.36 mg/ml refolding concentration in the presence of 0 to 75 mM NaCl. The refolding results showed that a consistent refolding yield of 17% was achieved with and without NaCl, as determined by RP-HPLC analysis (Figure 5.11, peaks F and G). This result demonstrates that NaCl does not function as a small molecule additive to improve rhAFP refolding. Instead, NaCl improves rhAFP on-column refolding by (i) weakening protein-resin interaction to increase protein molecule flexibility for refolding, and (ii) decreasing local density of

the protein on the matrix, thus decreasing the tendency for intermolecular protein interaction. These factors contribute towards reducing rhAFP aggregation on-column and increases refolding yield.

Figure 5.11: RP-HPLC chromatograms comparing dilution- and ‘AEC on-column’-refolded rhAFP with 25 mM NaCl. A, native std-AFP (0.1 mg/ml, 10 μ l); B, denatured-reduced std-AFP (0.1 mg/ml, 20 μ l); C, denatured-reduced rhAFP (column refolding load, 0.23 mg/ml, 5 μ l); D, refolded rhAFP on 1 ml 15Q column in the presence of 25 mM NaCl (20 μ l); E, refolded rhAFP on 1 ml DEAE (S) column in the presence of 25 mM NaCl (20 μ l); F, refolded rhAFP from batch dilution refolding without NaCl (20 μ l); G, refolded rhAFP from batch dilution refolding in the presence of 25 mM NaCl (20 μ l).

Table 5.3 compares the performance of rhAFP on-column refolding on a 1 ml DEAE-FF column with and without NaCl (Table 5.3). With the addition of 50 mM NaCl, rhAFP refolding productivity increased by 47% without compromising refolded protein purity and solvent consumption. These results show that the use of NaCl in on-column refolding is practical, economical, easily scalable, and can be readily

integrated into the conventional ion exchange chromatography refolding platforms for improved refolding yields.

Table 5.3: Experimental parameters employed for on-column refolding using 1 ml DEAE-FF column with and without NaCl.

	DEAE-FF	
	On-column refolding with 50 mM NaCl	On-column refolding without NaCl
Initial rhAFP concentration (mg/ml)	0.23	0.23
Load volume (ml)	6	6
Mass of rhAFP load (mg)	1	1
Denaturation buffer for column equilibration (ml)	12	12
Equilibration buffer for DTT removal (ml)	0	0
Refolding buffer (ml)	10	10
Elution buffer (ml)	10	10
Total buffer used (ml)	32	32
Time (h)	3	3
Eluted rhAFP protein concentration (mg/ml)	0.13	0.12
Refolding yield determined by RP-HPLC (%)	28	19
Refolding yield determined by ELISA (%)	30	17
Refolded rhAFP purity (%)	95	95
Productivity (mg/ml/h)	0.093	0.063

5.4 Conclusions

The results presented in this chapter provide qualitative and quantitative evidence for the role of NaCl in controlling the surface refolding of rhAFP on AEC matrices and columns. The best on-column refolding performance was achieved using the DEAE-FF column, where a 1.5-fold improvement in both rhAFP on-column refolding yield and productivity was achieved by the addition of 50 mM NaCl in the column load, equilibration and refolding buffers, compared to the conventional on-column refolding method without NaCl. Scaling up on-column refolding of rhAFP with NaCl using the 5 ml DEAE-FF column showed an increased refolding yield to 50%, at a fixed rhAFP load of 1 mg.

A higher K_d and lower q_m value with increasing NaCl concentration obtained from Langmuir regression, indicated that NaCl weakened the adsorption strength of denatured-reduced rhAFP on the AEC columns. The role of NaCl in weakening

‘denatured-reduced rhAFP’-resin association was experimentally demonstrated by the lower NaCl concentration required to desorb bound denatured-reduced rhAFP protein, with increasing NaCl concentrations in the protein load. Zeta potential measurements of the AEC resins, refolded rhAFP and ‘refolded rhAFP’-resin complex under refolding-favouring environment showed that the presence of NaCl confers a charge shielding effect on both the resin and refolded protein, which will also weaken resin-protein interaction. As the binding strength between the rhAFP protein molecules and ion-exchanger weakens with salt, the adsorbed protein gains more flexibility for refolding as well as oxidative shuffling, thereby improving refolding yield. The outcome of this study proposes a new method for surface-controlled refolding using salt, to readily control protein binding strength and increase protein flexibility for refolding. This improved on-column refolding platform will enhance the refolding performance of existing chromatography refolding methods, and also guide future studies in tailoring resins for specific protein binding requirements.

However, despite the clear role of NaCl in improving on-column refolding performance of rhAFP as discussed in this chapter, the substantial loss of protein to on-column aggregation (even in the presence of NaCl) suggests that a surface refolding strategy can still be yield-limiting. In the next chapter, an alternative chromatography refolding technique using size exclusion chromatography (SEC), which eliminates protein surface adsorption, will be developed and studied for rhAFP refolding. The advantages of a non-adsorptive column refolding method with respect to rhAFP refolding efficiency and bioprocess intensification will be directly compared with the AEC on-column refolding method, in view of the goal of this Ph.D. research to study the feasibility of a ‘chromatography refolding’-centered rhAFP process.

CHAPTER 6 : rhAFP REFOLDING BY PULSE FED SIZE EXCLUSION CHROMATOGRAPHY

6.1 Introduction

The results from Chapter 5 showed the important role of NaCl in improving on-column rhAFP refolding, by weakening protein-resin electrostatic interaction to increase molecule flexibility for refolding. Although NaCl addition clearly improved refolding yield and productivity, the significant loss of protein to aggregation (i.e. ~ 70%) at 1 mg/ml refolding concentration suggests that adsorption of proteins on the column stationary phase can still limit their ability to refold efficiently. It was therefore considered important to investigate other refolding alternatives that could still offer the advantages of chromatography refolding such as high degree of process intensification without the need for protein immobilisation.

In line with this aim, the development and feasibility study of a size exclusion chromatography (SEC) refolding method for rhAFP is studied, as reported in this chapter. Unlike adsorptive chromatography column refolding strategies such as ion exchange chromatography refolding, where proteins are refolded while adsorbed on the chromatography matrix, SEC allows proteins to refold in the unbound state in the matrix pores. SEC refolding has the following advantages:

- (i) elimination of any protein-matrix contact which may hinder refolding, with adequate control of the mobile phase physicochemical environment to minimise non-specific adsorption of the protein to the matrix,
- (ii) ability to perform simultaneous purification of refolded proteins, where larger molecular weight aggregates can be readily separated from the correctly refolded protein monomers,

- (iii) elimination of a salt elution step necessitated in ion exchange refolding, which may compromise protein integrity due to salting-out effects. As such, SEC does not require any post-refolding buffer exchange steps to remove salt, thereby simplifying bioprocessing.

Despite these known advantages of SEC, the widespread use of SEC for preparative refolding applications is limited because the small load to bed volume ratio required reduces refolding productivity and prevents the use of SEC for industrial refolding applications. Confronted by protein adsorption constraints in anion exchange chromatography (AEC) refolding, the overall aim of the study reported in Chapter 6 is to develop a non-adsorptive chromatography refolding method using SEC to improve rhAFP refolding yields and productivity. To facilitate refolding at high productivity in a SEC platform, the low load bottleneck inherent in SEC operations must first be overcome. To address this problem, the development of a pulse feeding strategy for SEC will be studied. Physicochemical optimisation of the SEC mobile phase for efficient rhAFP refolding and non-specific protein-matrix interaction will also be systematically studied. Parallel comparison studies of the rhAFP refolding performance using the newly developed SEC refolding strategy with AEC, dilution and pulse dilution refolding processes at a fixed protein refolding concentration, will be performed to assess the feasibility of a ‘SEC refolding’-based rhAFP bioprocess.

6.2 Materials and methods

The size exclusion column Superdex 200 10/300 GL was purchased from GE Healthcare (Singapore).

Other chemicals were sourced as detailed in Chapters 3 and 4.

6.2.1 rhAFP recovery and purification

rhAFP was cultured, released and purified using the same method as reported in Sections 3.2.1 and 3.2.2 in Chapter 3. The washed pellet was solubilised in 2 to 50 ml

Denaturation Buffer (8 M urea, 20 mM Tris, 3 mM EDTA, 20 mM DTT, pH 8.5) to achieve different final denatured rhAFP protein concentrations ranging from 0.2 to 6 mg/ml, and incubated under gentle shaking conditions for 5 h, at room temperature (21 °C).

6.2.2 Single load SEC refolding of rhAFP

All SEC experiments were performed on an ÄKTA explorer workstation (GE Healthcare, Singapore) at room temperature. rhAFP refolding by SEC was conducted on a Superdex 200 10/300 GL column which has a column volume (CV) of 24 ml. The mobile phase flow rate was set to 0.02 CV/min (i.e. 0.5 ml/min) throughout, and reduced to 0 CV/min only during sample loading. The column was first equilibrated with 2 CV SEC Refolding Equilibration Buffer (0 to 4 M urea, 20 mM Tris, 3 mM EDTA, 2.7 mM GSH and 2.7 mM GSSG, 150 mM NaCl, pH 8.5), followed by a change in equilibration buffer from refolding to denaturing condition. The SEC Denaturation Equilibration Buffer (8 M urea, 20 mM Tris, 3 mM EDTA, 150 mM NaCl, pH 8.5) was introduced into the column at a linear gradient to gradually displace the Refolding Equilibration Buffer and achieve a decreasing urea gradient in the axial direction of the column. The linear gradient employed to reduce urea concentration ranged from 0 CV to 0.63 CV (i.e. 0 ml to 15 ml). 200 µl to 1 ml denatured-reduced rhAFP (containing 2 to 6 mg/ml rhAFP) was loaded into the column *via* a sample loop that was equilibrated with SEC Denaturation Equilibration Buffer. Eluted protein fractions in SEC Refolding Equilibration Buffer were collected and analysed by SDS-PAGE, Bradford assay, densitometry and RP-HPLC to determine product purity, recovery and refolding yield. rhAFP refolding yield was determined according to Equation 4.3 (Section 4.2.4, Chapter 4). Refolding productivity was determined using equation 4.4.

6.2.3 Pulse fed SEC (PF-SEC) refolding of rhAFP

To perform rhAFP refolding by PF-SEC, the column was equilibrated in SEC Refolding Equilibration Buffer and Denaturation Equilibration Buffer using the same

method as reported for single load SEC refolding in Section 6.2.2. The first rhAFP sample load was also introduced into the column using the same method described in Section 6.2.2. Upon complete loading of the first sample, the mobile phase flow rate was resumed at 0.02 CV/min, and then stopped for injection of the second protein load before resuming at 0.02 CV/min when sample load was completed. The same procedure was repeated for loading of the third sample. 500 µl or 1 ml denatured-reduced protein sample (containing 2 to 6 mg/ml rhAFP) was introduced into the column per protein load (pulse), where a total of 3 mg and 17.7 mg rhAFP were loaded for single and triple pulse feeds, respectively. Eluted protein fractions were collected and analysed by SDS-PAGE, Bradford assay, densitometry and RP-HPLC to determine product purity, recovery and refolding yield. rhAFP refolding yield and refolding productivity were determined using the same methods as described in Section 6.2.2.

6.2.4 Batch dilution refolding and pulse dilution refolding of rhAFP

Dilution and pulse dilution refolding were performed as control studies to compare the refolding efficiency with those achieved by single load SEC and PF-SEC refolding. rhAFP IBs were solubilised and denatured-reduced in Denaturation Buffer as described in Section 6.2.1. DTT was removed from the denatured-reduced protein sample using a PD-10 desalting column using the same procedures described in Chapters 4 and 5. For batch dilution refolding, 1.9 ml DTT-free denatured protein (at 1.05 mg/ml) was diluted into 3.1 ml refolding buffer to obtain a protein refolding concentration of 0.4 mg/ml. For pulse dilution refolding, three batches of 0.63 ml DTT-free protein mixture (at 2.5 mg/ml) were consecutively added into 3.1 ml refolding buffer at 8 min intervals under gentle stirring conditions, to obtain a final rhAFP refolding concentration of 0.95 mg/ml. The final refolding buffer composition used in dilution and pulse dilution was 3 M urea, 20 mM Tris, 3 mM EDTA, 2.7 mM GSH and 2.7 mM GSSG (pH 8.5). This refolding buffer will be referred to as Dilution Refolding Buffer throughout this chapter. Diluted protein mixtures were incubated under gentle shaking conditions at room temperature for 6 h, and their respective refolding yields analysed using methods described in Section 6.2.5.

6.2.5 Analytical methods

All analytical methods used including SDS-PAGE, Bradford assay, densitometry, RP-HPLC, ELISA and CD were the same as reported in Sections 3.2.7 (Chapter 3) and 4.2.8 (Chapter 4).

6.3 Results and discussion

6.3.1 Single load rhAFP refolding by SEC

1 mg denatured-reduced rhAFP protein load was used for single load SEC refolding to maintain a consistent rhAFP load as the on-column AEC refolding studies reported in Chapters 4 and 5. To develop a SEC refolding methodology, it was considered important to first optimise the physicochemical environment in the SEC column to enhance refolding efficiency. Previous refolding studies have shown that a gradual change in mobile phase environment from denaturing to native-favouring will reduce the number of “refolding kinetic traps” that the protein may have to encounter in its refolding pathway, thus minimising the occurrence of protein misfolding (Batas and Chaudhuri, 1996; De Bernardez Clark *et al.*, 1999; Leong and Middelberg, 2006a; Huang and Leong, 2009). The SEC column for rhAFP refolding was therefore designed to contain a urea gradient along the axial direction to facilitate more efficient refolding. The SEC column was equilibrated with SEC Refolding Equilibration Buffer followed by SEC Denaturation Equilibration Buffer, which resulted in the partitioning of the column into two sections. The upper section of the column (section 1, Figure 6.1) comprised a linearly decreasing urea gradient in the axial direction, while the lower section (section 2, Figure 6.1) comprised SEC Refolding Equilibration Buffer only.

Figure 6.1: Schematic of the mobile phase component in the SEC column.

Urea, at non-denaturing concentrations, can effectively inhibit hydrophobic-driven aggregation (Cowley and Mackin, 1997; Gu *et al.*, 2001; Fan *et al.*, 2008). Variation of urea concentration in the SEC Refolding Equilibration Buffer from 0 to 4 M corresponded with rhAFP refolding yields that differed by $15\pm4\%$ at 1 mg denatured-reduced rhAFP load. 3 M urea gave the highest refolding yield of 64% (Figure 6.2), and was therefore used in all subsequent SEC Refolding Equilibration Buffers in this study.

Figure 6.2: The effect of urea concentration in SEC Refolding Equilibration Buffer on rhAFP refolding yield, at 1 mg denatured-reduced rhAFP load.

At a molecular level, it has been shown that the refolding process does not involve a series of fixed steps between specific intermediates, but rather a search of many conformations accessible to the polypeptide chain (Wolynes *et al.*, 1995; Dill and Chan, 1997). The number of possible refolding intermediates that needs to be sampled by a protein molecule during its transition from the unfolded to a correctly folded state is suggested to depend on the topography of the free-energy landscape (Chan and Dill, 1998; Dinner *et al.*, 2000; Dobson, 2003). The topography of this landscape is fixed and invariant in dilution refolding, where the landscape is characterised by a steep gradient promoting relatively rapid refolding limited by oxido-shuffling. The incorporation of a urea gradient in SEC refolding is aimed at decreasing the steepness in the energy landscape topography to facilitate more extensive conformational search by protein intermediates during refolding. Since the rate of change of the chemical environment will potentially control the rate of refolding, the rate of change in urea concentration in the axial direction of the SEC column is thus expected to significantly influence rhAFP refolding yield. The effect of gradient length on rhAFP refolding in SEC was therefore first investigated. The SEC column was equilibrated

with SEC Refolding and Denaturation Equilibration buffers at varying urea gradient lengths, where the linear reduction of urea concentration from 8 M to 3 M in the axial direction of the column was performed over a range of 0 to 0.63 CV (i.e. 0 to 15 ml), at a fixed mobile phase flow rate of 0.5 ml/min. rhAFP refolding yields varied with different urea gradient lengths. At 1 mg rhAFP load, rhAFP refolding yield increased from 53% to 70%, as urea gradient length increased from 0 ml to 10 ml (Figure 6.3). A longer urea gradient represents a flatter refolding energy landscape, where a slower transition to a solvent environment that increasingly favours the native state appears to enhance rhAFP refolding yield compared to a steeper transition from denaturing to native environment. Further increase in urea gradient length beyond 10 ml, however, reduced rhAFP refolding yield due to the reduced contact time between the protein and refolding buffer. In the perspective of a higher overall refolding process productivity, a 5 ml gradient was used for subsequent SEC refolding studies instead of a 10 ml gradient, although refolding yield was slightly lower.

Figure 6.3: The effect of urea gradient length on rhAFP refolding yield during SEC refolding, at 1 mg denatured-reduced rhAFP load.

Figure 6.4 shows the FPLC chromatogram of the elution peaks for single load SEC refolding of rhAFP. Peak 1 contained a small amount of large molecular weight rhAFP aggregates, as indicated by a faint rhAFP band in the SDS-PAGE gel in Figure 6.5 (lane 3). RP-HPLC analysis confirmed the absence of refolded rhAFP in peak 1 (panel D, Figure 6.6), which indicates that only a small amount of rhAFP was lost as larger molecular weight aggregates during refolding. Correctly refolded rhAFP was predominantly found in peaks 2a and 2b, as confirmed by RP-HPLC analysis (Figure 6.6, panels E and F). Peak 3 contained only DTT from the denaturant, as determined by a control study, where denaturation buffer was injected into the SEC column and the change in 280 nm absorbance was recorded (Figure 6.5, lanes 6-7). RP-HPLC analyses of the pooled fractions from peaks 2a and 2b gave a rhAFP refolding yield of $64\pm4\%$ (lanes 4 and 5 in Figure 6.5). Although most rhAFP after SEC refolding were recovered in peaks 2a and 2b, approximately 35% of the rhAFP were monomeric misfolded species, as determined from the quantitation of refolding yield by RP-HPLC. Misfolded proteins are often unstable and have limited solubility, and hence would be removed by the HPLC column filter during RP-HPLC analysis. The spectrum of refolding intermediates is believed to be dependent of the choice of refolding methods, which will present different refolding landscape topographies.

Figure 6.4: FPLC chromatogram of the elution peaks following single load SEC refolding of rhAFP at 1 mg denatured-reduced rhAFP load and 5 ml urea gradient. The inset provides a 10 times magnified view of peaks 1, 2a and 2b.

Figure 6.5: SDS-PAGE analysis of SEC-refolded protein fractions. Lane 1, molecular weight marker, 4 μ l; lane 2, SEC load, 0.25 rhAFP mg/ml) 12 μ l; lane 3, protein fraction from FPLC peak 1 (Figure 6.4), 15 μ l; lanes 4-5, protein fractions from FPLC peaks 2a and 2b, respectively (Figure 6.4), 15 μ l each; lanes 6-7, protein fraction from FPLC peak 3 (Figure 6.4), 15 μ l each; lane 8, std-AFP (0.1 mg/ml) 15 μ l.

Figure 6.6: RP-HPLC analysis of single load SEC-refolded rhAFP. A, native std-AFP (0.1 mg/ml, 10 μ l); B, denatured-reduced std-AFP (0.1 mg/ml, 20 μ l); C, denatured-reduced rhAFP (SEC load, 0.25 mg/ml), 5 μ l; D, fraction from FPLC Peak 1 (Figure 6.4), 20 μ l; E and F, fractions from FPLC Peaks 2a and 2b (Figure 6.4), respectively, 20 μ l each.

The bioactivity of SEC-refolded rhAFP was further confirmed by ELISA. SEC-refolded AFP exhibits comparable bioactivity to native human-derived AFP (positive controls), while denatured-reduced rhAFP yielded negative responses (Appendix 3). CD spectroscopy was also employed to compare the secondary and tertiary protein conformations of SEC-refolded rhAFP against native std-AFP. Figure 6.7 shows comparable far- and near-UV spectra fingerprints as the native std-AFP, indicating that the refolded protein adopts a conformational state that is comparable with the native protein.

Figure 6.7: (A) Far and (B) near UV CD spectra of (1) denatured rhAFP, (2) native std-AFP and (3) SEC-refolded rhAFP.

6.3.2 Single load SEC refolding versus batch dilution refolding of rhAFP

A parallel performance comparison of single load SEC refolding and batch dilution refolding requires that a fixed rhAFP refolding concentration is used for the refolding studies. As the highest refolding protein concentration that could be practically attained using dilution refolding was 0.4 mg/ml, SEC refolding was also performed at the same rhAFP concentration. Preliminary studies to optimise rhAFP dilution refolding yield showed that increasing refolding time from 6 h to 24 h (where steady state refolding yield was attained) increased refolding yield from 15±3% to 21±2%, but decreased refolding productivity from 0.01 to 0.003 mg/ml/h, at 0.4 mg/ml refolding concentration. 6 h refolding incubation time was therefore used in all subsequent dilution refolding studies reported in this chapter to increase refolding throughput.

rhAFP refolding by SEC using a 5 ml urea gradient length readily achieved a rhAFP refolding yield of 60% in 0.4 h (Table 6.1). No dilution-refolded rhAFP could be detected by RP-HPLC analysis after 0.4 h. A 10-fold increase in refolded rhAFP

concentration was also obtained with single load SEC refolding compared to batch dilution. The superior refolding yield achieved by SEC refolding compared to dilution refolding is attributed to: (i) the availability of SEC matrix pores which can separate the denatured proteins during refolding, thus minimising any undesirable interaction between refolding intermediates; (ii) the more gradual change in urea concentration during SEC refolding which is facilitated by a urea gradient (Li *et al.*, 2004b).

Despite these improvements in rhAFP refolding performance, the low sample to bed volume ratio bottleneck in SEC operations must be resolved to further enhance refolding productivity. To address this problem, the development of a pulse-fed SEC (PF-SEC) refolding strategy is subsequently researched, as reported in Section 6.3.3.

Table 6.1: Comparison of rhAFP refolding parameters and performance indicators of single load SEC refolding and batch dilution refolding at 0.4 mg/ml refolding rhAFP concentration.

	Single load SEC refolding	Batch dilution refolding
Mass of rhAFP load (mg)	5	2
rhAFP load purity (%)	74	74
SEC elution volume (ml)/Dilution refolding buffer (ml)	12.5	5
Refolding volume (ml)	12.5	5
Refolding concentration (mg/ml)	0.4	0.4
Refolding incubation time (h)	0.4	6
Refolding yield determined RP-HPLC analysis (%)	60	15
Refolding yield determined by ELISA (%)	59	13
Refolded rhAFP purity (%) ^a	46	11
Refolded rhAFP concentration (mg/ml)	0.61	0.06
Productivity (mg/ml/h)	0.60	0.01

^a determined by Bradford assay, densitometry and RP-HPLC analyses.

6.3.3 rhAFP refolding by PF-SEC

Pulse dilution refolding was developed to improve batch dilution refolding throughput by controlling the rate of denatured protein addition into the refolding buffer, to keep the concentration of refolding intermediates low (Fischer *et al.*, 1992a; Terashima *et al.*, 1996a; Vallejo and Rinas, 2004a). Based on the same principle, a novel pulse-fed SEC refolding strategy that employs multiple feed loading was developed in this study to enhance the refolding performance in SEC columns for improved scale-up potential. Multiple feed loading necessitates re-optimisation of the urea gradient length to ensure that the protein has sufficient contact time with SEC Refolding Equilibration Buffer to maximise refolding yield (Figure 6.8).

Figure 6.8: Schematic diagram of a PF-SEC refolding process.

The effect of multiple feed loads on rhAFP PF-SEC refolding yields was first investigated. Figure 6.9 shows that with the use of 2 ml to 5 ml urea gradient lengths, rhAFP refolding yields decreased slightly (i.e. by 5-10 %) with increasing feed loads. A more significant decrease in refolding yields (i.e. by 40-47%) was apparent when

longer urea gradient lengths (i.e. 7.5 ml and 10 ml) were used, due to insufficient protein incubation time in the refolding-favouring environment.

Figure 6.9: The effects of urea gradient length and number of pulse feeds on rhAFP refolding yield (1 mg denatured-reduced rhAFP per load).

The final urea concentration at column exit for different urea gradient lengths is shown in Figure 6.10. Final urea concentrations were maintained at 3 M when three feed pulses were employed using 2 ml and 3.5 ml urea gradients, but an increase in urea gradient from 5 ml to 7.5 ml resulted in the third feed pulse exiting the column at non-native favouring urea concentrations (i.e. 4 M and 5.3 M, respectively), hence reducing refolding yields. The use of a 10 ml urea gradient, which gave the highest refolding yield for single load SEC refolding, was clearly unsuitable for PF-SEC applications.

Figure 6.10: The effect of urea gradient length on final urea concentration during PF-SEC refolding, at a constant mobile phase flow rate of 0.5 ml/min.

The effect of protein load on rhAFP refolding yield in SEC was also determined, and the results are shown in Figure 6.11. PF-SEC refolding was clearly effective in facilitating rhAFP refolding even at a high protein load of 18 mg.

Figure 6.11: The effect of sample load on SEC rhAFP refolding yield. rhAFP was refolded by single load SEC for rhAFP load less than 6 mg, while multiple load SEC was used when rhAFP load was higher than 6 mg.

The best rhAFP refolding productivity using the Superdex 200 10/300 GL column was obtained with three feed pulses using a 5 ml urea gradient. Under this condition, rhAFP could be refolded at 0.9 mg/ml rhAFP concentration (or total rhAFP load of 17.7 mg) in a single SEC step, to achieve a refolding yield of 53%, as determined by RP-HPLC analysis. Figure 6.12 shows the FPLC chromatogram of PF-SEC (triple pulse feeds) refolding of rhAFP at 5 ml urea gradient. Peaks 1 to 3 contained refolded rhAFP from the first, second and third pulse feed respectively, as confirmed by RP-HPLC analysis (Figure 6.13), while peaks 4 to 6 contained only DTT.

Figure 6.12: FPLC chromatogram of the elution peaks following SEC refolding of rhAFP with three pulse feeds. The inset provides a 10 times magnified view of peaks 1 to 3. Peak 1-3 and peaks 4-6 are refolded rhAFP and DTT elution peaks from the first, second and third pulse feed respectively.

Figure 6.13: RP-HPLC analysis of PF-SEC-refolded rhAFP. A, native std-AFP (0.1 mg/ml, 10 μ l); B, denatured-reduced std-AFP (0.1 mg/ml, 20 μ l); C, denatured-reduced rhAFP (SEC load, 0.25 mg/ml, 5 μ l); D to F, refolded rhAFP fractions from FPLC peaks 1, 2 and 3 (Figure 6.12), respectively (5 μ l each).

6.3.4 Comparison of PF-SEC and pulse dilution refolding

The rhAFP refolding performance of PF-SEC was compared with pulse dilution refolding at a fixed refolding concentration. In pulse dilution refolding, denatured rhAFP (after DTT removal step) was intermittently introduced into the refolding buffer over three loads at 1.58 mg rhAFP per load, to achieve a final rhAFP refolding concentration of 0.95 mg/ml. Triple pulse feed mode was used in SEC refolding to achieve a final refolding concentration of 0.89 mg/ml. The refolding outcome using these two methods were significantly different, where pulse dilution achieved a refolding yield of 7%, while PF-SEC refolding achieved a 53% rhAFP refolding yield and improved refolding productivity by 64-fold (Table 6.2). In pulse dilution refolding, the rapid accumulation of protein refolding intermediates over short pulse feeding intervals and the inability to separate correctly refolded protein from refolding intermediates in subsequent feed loads will increase protein aggregation (Buswell and

Middelberg, 2002). In PF-SEC refolding, the availability of matrix pores to separate aggregates from refolding intermediates reduces off-pathway aggregation, even at high feed loads. However, increasing protein load *via* pulse feeding did compromise the refolding yield and refolded protein purity of rhAFP to a small extent (Table 6.3).

Table 6.2: rhAFP refolding experimental parameters and performance indicators using PF-SEC and pulse dilution refolding methods.

	PF-SEC refolding	Pulse dilution refolding
rhAFP per load (mg)	5.9	1.58
Number of loads	3	3
Mass of total rhAFP load (mg)	17.7	4.7
rhAFP load purity (%)	74	74
SEC elution volume (ml)/ Dilution refolding buffer (ml)	20	5
Refolding volume (ml)	20	5
Refolding concentration (mg/ml)	0.89	0.95
Refolding incubation time (h)	0.67	6
Refolding yield determined RP-HPLC analysis (%)	53	7
Refolding yield determined by ELISA (%)	55	5
Refolded rhAFP purity (%) ^a	40	5
Refolded rhAFP concentration (mg/ml) ^b	0.67	0.084
Refolding process productivity (mg/ml/h)	0.704	0.011

^a determined by Bradford assay, densitometry and RP-HPLC analyses.

^b determined by RP-HPLC analysis.

Table 6.3: rhAFP refolding yield, purity and recovery following triple pulse SEC refolding.

rhAFP load (mg)	Total rhAFP in SEC elution fraction ^a	Refolded rhAFP in SEC elution fraction	Refolding yield (%) ^b	Refolded rhAFP purity ^c (%)
3 (1 mg per load)	2.9	1.76	59	44
17.7 (5.9 mg per load)	17.1	9.38	53	40

^a determined by Bradford assay and SDS-PAGE analysis.

^b determined by RP-HPLC analysis.

^c determined by Bradford assay, SDS-PAGE and RP-HPLC analysis.

6.3.5 Comparison of PF-SEC and on-column AEC refolding

A parallel comparison study of PF-SEC with on-column AEC at a fixed refolding concentration of ~ 1 mg/ml was also performed (Table 6.4). The highest achievable rhAFP refolding yield by on-column AEC refolding at 1 mg/ml rhAFP concentration was 28% using a 1 ml DEAE-FF column under optimised refolding conditions (i.e. 3 h incubation in refolding-favouring environment with 50 mM NaCl), as reported in Chapter 5. rhAFP refolding using PF-SEC at three pulse feeds readily achieved a 53% refolding yield at 0.9 mg/ml rhAFP refolding concentration. The 8-fold increase in rhAFP refolding productivity achieved by PF-SEC compared to on-column AEC refolding was attributed to the reduced refolding time, higher allowable protein load, and higher refolding yields achieved by PF-SEC. Refolding incubation time was significantly reduced for SEC refolding as no adsorption was required, where refolding proceeded simultaneously with protein elution. Additionally, the protein molecules were also free from matrix-induced steric constraints when refolded in the SEC column. These factors contribute to a higher rate of refolding in SEC compared to on-column AEC. However, AEC refolding could easily separate correctly refolded rhAFP from misfolded species due to the difference in charge between the two species. Additionally, it was also observed that majority of the misfolded rhAFP was precipitated and/or physically trapped on the column. The purity of refolded rhAFP was, significantly compromised due to the inability of SEC to separate correctly refolded rhAFP from the misfolded species. The majority of the misfolded rhAFP from SEC refolding were found to be monomeric, as determined by RP-HPLC, Bradford assay and SDS-PAGE analyses, as discussed in Section 6.3.1. The simultaneous purification and refolding capability of SEC could not be exploited in this case, rendering ‘on-column AEC refolding’ superior to PF-SEC in terms of refolded product purity.

Table 6.4: Comparison of rhAFP refolding parameters and performance indicators using PF-SEC and on-column AEC methods.

	PF-SEC refolding	AEC refolding on 1 ml DEAE-FF column
Mass of rhAFP load (mg)	17.7	1
rhAFP load purity (%)	74	74
Column volume (ml)	24	1
SEC elution volume (ml)/AEC column volume (ml)	20	1
Refolding concentration (mg/ml)	0.89	1
Refolding time (h)	0.67	3
Refolding yield (%) ^a	53	28
Refolded rhAFP purity (%) ^b	40	95
Refolded rhAFP concentration (mg/ml)	0.67	0.13
Productivity (mg/ml/h)	0.704	0.093

^a determined by RP-HPLC analysis.

^b determined by Bradford assay, densitometry and RP-HPLC analyses.

6.4 Conclusion

A notable number of studies to enhance SEC refolding performance have been reported (Batas and Chaudhuri, 1996; Gu *et al.*, 2001; Gu *et al.*, 2002; Schlegl *et al.*, 2003; Park *et al.*, 2005) but the low productivity bottleneck remains unresolved to date. In this study, the development of a novel and robust PF-SEC refolding platform addresses this problem by exploiting the SEC column length for high productivity refolding of rhAFP *via* multiple pulse feeding. An optimised physicochemical environment in the column was established with the development of an easily tunable urea gradient in the axial direction of the SEC column to facilitate rhAFP refolding.

rhAFP refolding by PF-SEC was clearly superior to batch dilution, pulse dilution and on-column AEC refolding platforms with respect to refolding yield and productivity, at normalised refolding concentrations. At a rhAFP refolding concentration of 1 mg/ml, PF-SEC with triple pulse feeds enhanced the refolding productivity of rhAFP by 8- and 64-fold compared with on-column AEC refolding and pulse dilution refolding, respectively. The PF-SEC refolding platform clearly resolved the low concentration and productivity bottlenecks inherent in SEC refolding applications. Importantly, this study shows that PF-SEC facilitated rhAFP refolding at protein concentrations that were not practically attainable by dilution refolding of comparable scale, where a rhAFP refolding yield of 53% was readily achieved at 1 mg/ml rhAFP refolding concentration. The co-elution of misfolded rhAFP monomers with the correctly refolded rhAFP fraction, however, necessitates a final polishing to purify the refolded protein.

The outcome of this chapter demonstrates the feasibility of an 'SEC refolding'-based process for rhAFP, which has a clear advantage in refolding productivity enhancement. A new milestone for high throughput SEC refolding with potential applications in industrial refolding processes is demonstrated through this study.

CHAPTER 7 : CONCLUSIONS AND RECOMMENDATIONS

This chapter presents the overall conclusions of the rhAFP chromatography refolding and bioprocess research reported in this thesis. Recommendations for future work of this study are also presented.

7.1 Conclusions

The positive clinical trial indications shown by rhAFP in modulating the immune system imply that transgenic-derived rhAFP produced by Merrimack Pharmaceuticals is fast approaching commercial reality. The high over-expression of rhAFP as IBs in *E. coli* renders *E. coli* an economically attractive host system if high refolding yields can be achieved. Although two *E. coli* rhAFP bioprocesses have been reported (Boismenu *et al.*, 1997; Leong and Middelberg, 2007), the sub-optimal refolding outcomes associated with the use of batch dilution refolding have kept overall process yield and productivity low. Confronted by this problem, the aim of this Ph.D. project was to research alternative refolding strategies to improve the overall refolding yield and productivity of rhAFP, leading to the development of a streamlined ‘chromatography refolding’-based *E. coli* bioprocessing route for rhAFP production. Chromatography column refolding was chosen to be studied because it offers the possibility to (i) perform refolding at high concentrations, (ii) decrease protein aggregation due to matrix-induced spatial isolation, (iii) simultaneously achieve protein purification and refolding, and (iv) implement bioprocess intensification, leading to improved process economics. This Ph.D. thesis reports the studies undertaken to deliver this vision.

The first part of this project which was reported in Chapter 3, focused on the development of a simple laboratory process that could efficiently deliver high purity rhAFP for chromatography column refolding studies. Parallel comparison of mechanical disruption and chemical extraction processing for pre-refolding rhAFP recovery and purification showed that the chemical extraction route was unsuitable

due to low protein purity and contaminant-induced viscosity problems. The sonication-based mechanical disruption route, on the other hand, yielded high product recovery (83%) and purity (74%), as well as excellent cellular DNA removal ability, which opened the way for the direct use of solubilised rhAFP IBs in chromatography refolding studies. The inclusion of a Triton X-100 wash step after sonication was effective in integrating protein release and purification, and hence eliminates the need for further conditioning steps prior to refolding. Following the successful development of a mechanical disruption laboratory process for rhAFP isolation and removal of insolubles, chromatography column refolding of the protein was subsequently studied.

Chapter 4 reports the successful development of an adsorption-based refolding strategy, using anion exchange chromatography for rhAFP refolding. AEC on-column refolding readily facilitated the refolding of rhAFP at 1 mg/ml, to give a refolding yield of 20% and refolded protein purity of 95% in 3 h. Optimisation of resin chemistry and refolding physicochemical environment was important to maximise on-column rhAFP refolding yields. Compared to dilution refolding, on-column AEC refolding improved rhAFP refolding productivity by 6-fold. Off-column did not show any significant advantage over on-column refolding AEC refolding, where comparable refolding yields and productivity were obtained. Although the subsequent addition of L-Arginine in off-column refolding was found to significantly improve refolding yield, it is expensive to scale-up and is hence unpractical to be further utilised and improved for large scale rhAFP refolding. Concurrent purification of the on-column refolded rhAFP was also achieved, where the refolded protein was recovered at a high purity of 95%. Such purity could only be achieved following chromatography polishing steps for dilution-refolded rhAFP. The results from Chapter 4 show the superiority of an on-column AEC refolding platform over conventional refolding strategies in improving refolding yield, purity and productivity.

The role of NaCl in on-column AEC refolding of rhAFP was studied in Chapter 5, with the aim to weaken protein-resin association for improved refolding efficiency. Zeta potential analysis showed that NaCl had a charge shielding effect on the AEC resins and rhAFP protein, which will influence the Coloumbic protein-resin interaction during on-column refolding. At optimised NaCl concentrations, the

reduction in protein-matrix interaction strength increases the flexibility of the adsorbed rhAFP molecule to refold and perform oxidative shuffling, without compromising protein binding capacity in the denatured-reduced conformation. NaCl-assisted on-column refolding improved rhAFP refolding yield by 1.5-fold, compared to that in the absence of salt. By determining the elution volumes required to desorb the protein from the matrix under varying NaCl concentrations, the role of NaCl in weakening protein-resin binding strength was confirmed. The outcome from this study provides a new strategy to control surface refolding on AEC columns, and contributes to an increased understanding of AFP refolding at a biomolecular level.

The development of an alternative non-adsorptive chromatography refolding strategy for rhAFP using size exclusion chromatography to exploit the advantages of chromatography refolding whilst removing surface adsorption constraints was researched in Chapter 6. A new pulse-fed SEC refolding strategy was developed which facilitated multiple pulse feeding in a SEC column with a linearly reducing urea gradient in the axial direction. This novel refolding platform proved effective in significantly improving rhAFP refolding yields and productivity. At a normalised refolding concentration of 1 mg/ml, rhAFP refolding productivity achieved by PF-SEC was 8- and 64-fold higher compared to on-column AEC and pulse dilution refolding, respectively. Due to the predominantly monomeric nature of misfolded rhAFP species obtained, a separate post-refolding purification step will be required to further purify the SEC-refolded rhAFP. The pulse feeding component in PF-SEC was very effective in overcoming low load and low product concentration bottlenecks inherent in SEC operations. The feasibility of a PF-SEC refolding platform to increase overall process yield and productivity for rhAFP bioprocessing is clearly demonstrated from the results of this study.

The basis for a 'chromatography refolding'-based rhAFP process has been provided through Chapters 4 to 6 of this thesis. Although the successful refolding of rhAFP using dilution refolding has been previously reported in the literature (Boismenu *et al.*, 1997; Leong and Middelberg, 2007), the low refolding concentration necessitated to minimise off-pathway aggregation will inevitably increase scale-up operation and capital costs of the rhAFP bioprocess. The need for subsequent post-refolding purification and concentration steps following dilution refolding also increased the

total number of unit operations in the bioprocess, resulting in increased product loss. These limitations were overcome in the development of two chromatography refolding strategies for rhAFP in this thesis; (a) on-column AEC refolding, and (b) non-adsorptive SEC refolding. Both column refolding methods were superior to dilution refolding, where at a fixed rhAFP refolding concentration of 1 mg/ml:

- (i) a 9- and 64-fold improvement in rhAFP refolding productivity was readily attained with (a) and (b), respectively,
- (ii) the refolded rhAFP was recovered at concentrations that were 2- and 8-fold higher for (a) and (b), respectively,
- (iii) simultaneous protein purification was apparent with method (a), where a refolded rhAFP purity of 95% was readily achieved in a single on-column refolding step; although method (b) was only effective in concurrent purification of contaminants and aggregates having different molecular sizes, the refolded rhAFP purity of 40% obtained was still higher than that achieved by pulse dilution refolding (i.e. 5%).

Due to the generic method of refolding, the IEC and SEC refolding platforms will not be limited to rhAFP but will be readily extended for refolding other types of protein. However, due to the different structure and behavior of different proteins, which are determined by their amino acid sequences, detailed and systematic optimization of refolding conditions (e.g., physicochemical conditions, protein concentration and refolding additives) for each protein will need to be studied on a case by case basis.

In summary, the results presented in this thesis show that chromatography column refolding using both adsorptive and non-adsorptive chromatography modes, is not limited to pure and/or simple proteins, but is also readily applicable for high-throughput refolding of IB-derived proteins with complex molecular structures such as rhAFP. The high protein refolding concentration, concurrent purification capability and high refolding yields and productivities demonstrate incomparable superiorities over conventional dilution refolding. These results open the way for the development of a rhAFP bioprocess route using chromatography-based refolding, where the

remarkably improved refolding productivity will translate into more efficient product delivery to market. The groundwork for the development of an improved rhAFP process that addresses existing process roadblocks pertaining to sub-optimal refolding yield and low overall process productivity have now been laid, which will open the way to generic competition, and/or enable the rapid and cost-effective commercialisation of rhAFP and new variants.

7.2 Recommendations for future research

7.2.1 ‘On-column AEC refolding’- versus ‘SEC refolding’-based rhAFP bioprocessing

In this study, two refolding strategies were successfully established using AEC and SEC. Table 7.1 shows the overall mass balance for the production of rhAFP using ‘on-column AEC refolding’- and ‘SEC refolding’-based bioprocessing, while the final bioprocess flowsheets for ‘on-column AEC refolding’- and ‘SEC refolding’-based rhAFP production are presented in Figure 7.1. Both chromatography refolding methods have their advantages and disadvantages. The ‘PF-SEC refolding’-based process achieved a 2-fold higher final rhAFP recovery compared to the ‘on-column AEC refolding’-based process. However, the low refolded rhAFP purity from PF-SEC necessitates an additional post-refolding purification step. The ‘on-column AEC refolding’-based process, on the other hand, demonstrated superior simultaneous purification ability over PF-SEC refolding, which will largely simplify the overall bioprocess. Further studies to develop a purification strategy for the SEC-refolded rhAFP must be performed. Chromatography purification is considered more suitable than a solvent or acid precipitation step due to the high disulfide bond content of the AFP molecule. The misfolded protein molecules are hypothesised to have different three dimensional conformations from the correctly refolded AFP species and hence different hydrophobicity and/or net charge. This difference in molecular attribute between the misfolded and correctly refolded rhAFP will be exploited for purification of the molecules by hydrophobic interaction or ion exchange chromatography. The final rhAFP recovery and purity after this purification step will then be compared with those of the ‘on-column AEC refolding’-based process, to guide the choice of the most optimum chromatography refolding strategy for the new rhAFP bioprocess.

Table 7.1: Overall mass balance for rhAFP production using on-column AEC and PF-SEC refolding strategies.

Refolding Mode	Total rhAFP expressed	IB isolation using MD (with Triton washing step)		Refolding process		
		Denatured rhAFP recovered (% recovery)	Denatured rhAFP purity	Refolding yield	Refolded rhAFP purity	Overall rhAFP recovered (% recovery)
On-column AEC refolding (with 20 mM NaCl)	55 mg	46 mg (83%)	74%	28%	95%	12.9 mg (23%)
SEC refolding (triple pulse feeds)	55 mg	46 mg (83%)	74%	53%	40%	24.2 mg (44%)

This study is based on a cell pellet originating from a 1 L cell culture.

Figure 7.1: rhAFP bioprocess flowsheets showing (A) ‘on-column AEC’- and (B) ‘SEC refolding’-based processing.

7.2.2 Bioprocessing and refolding scale-up studies for rhAFP production

Considering the commercial potential of rhAFP, it is important that the immediate future work is focused on scale-up studies and process intensification to facilitate process validation, and further improve process economics for pilot-scale manufacture.

In this study, a laboratory process comprising a sonication-centrifugation step followed by a Triton washing, was effective in purifying rhAFP from contaminant proteins and cellular DNA to obtain a high pre-refolding protein purity of 74%. To facilitate bioprocess scale-up, the sonication step will be substituted with high pressure homogenization, and optimised to minimise repetitive centrifugation and wash steps. Scale up studies of the on-column AEC or SEC refolding processes (depending on the outcome from the study proposed in Section 7.2.1.) will also be performed using well-established chromatography scale-up rules reported in earlier

studies (Li *et al.*, 1998; Rathore and Velayudhan, 2003; Al-Jibbouri, 2006). The effect of increasing column diameter on rhAFP load, adsorption behaviours, refolding efficiency and simultaneous purification capability will be investigated. The performance criteria to be assessed for scale-up feasibility will be refolding throughput (i.e. mass of refolded rhAFP per unit time) for specified product purity.

7.2.3 Cost analysis of the ‘chromatography refolding’-based rhAFP process

Process simulation using commercially available software such as SuperPro Designer Simulator (Intelligen, Inc.) to obtain cost analyses of the ‘AEC’- or ‘SEC refolding’-based rhAFP processes will be performed. Economic evaluation of the process will include (i) equipment purchase cost based on size and construction material, and (ii) operating costs including labour costs, consumable costs (which covers periodic replacement of chromatography resins and membranes), waste treatment and disposal as well as utilities. The results from this exercise will provide an estimate of the extent of economic requirement of column refolding processes compared to the previously reported ‘dilution refolding’-based rhAFP processes (Boismenu *et al.*, 1997; Leong and Middelberg, 2007). Based on the outcome of this study, the economic feasibility of a ‘chromatography-refolding’-oriented *E. coli* production route for rhAFP compared to conventional processing routes can thus be assessed to guide second-generation production of this important biopharmaceutical.

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APPENDIX

Appendix 1: RP-HPLC standard curve attained by calibration using native and denatured-reduced std-AFP

1 mg/ml native std-AFP was diluted in deionised water to different final AFP concentrations ranging from 0.05 to 0.4 mg/ml to generate the native RP-HPLC standard curve for rhAFP quantitation. 1 mg/ml denatured-reduced std-AFP was diluted in Denaturation Buffer to different final AFP concentrations ranging from 0.05 to 0.5 mg/ml, and incubated for 5 h at room temperature to generate the denatured-reduced RP-HPLC standard curve for rhAFP quantitation. 10 µl of each native and denatured-reduced std-AFP sample was analysed by RP-HPLC using the method reported in Section 3.2.7.1 in Chapter 3. Standard native or denatured-reduced AFP concentration (x-axis in Figure A.1) was determined from the number of dilution folds made from the 1 mg/ml stock std-AFP solution, whilst peak area (y-axis in Figure A.1) was determined by peak integration of the chromatogram peaks using the Shimadzu LC-20AVP software. The calibration curves used for quantitation of native or denatured-reduced AFP is shown in Figure A.1.

Figure A.1: RP-HPLC standard curve attained by calibration using native and denatured-reduced std-AFP.

Appendix 2: Bioanalyzer calibration curve of std-AFP

std-AFP at 1 mg/ml was diluted in deionised water to different final AFP concentrations ranging from 100 to 800 µg/ml, prior to Bioanalyzer analysis. Figure A.2 shows the calibration curve used for absolute quantitation of AFP. Nominal AFP concentration (x-axis in Figure A.2) was determined from the number of dilution folds made from the 1 mg/ml std-AFP solution, whilst relative AFP concentration (y-axis in Figure A.2) was determined by the Agilent 2100 Bioanalyzer software. Relative protein concentration is based on a one-point calibration with the Protein 230 upper marker (myosin), used as an internal standard (by the Bioanalyzer software) for quantitation in each sample, where the peak area of the unknown sample is compared to the peak area of the upper marker, which has a known concentration. The inclusion of the upper marker in each sample also corrects for differences in sample injection into the separation channel and allows for reproducible quantitation. A calibration curve generated from both the nominal and relative AFP concentrations was then established for absolute quantitation of rhAFP (Figure A.2). Table A.1 compares the

nominal and relative AFP concentration, against the absolute AFP concentration, obtained from the calibration curve.

Figure A.2: Bioanalyzer calibration curve used for absolute quantitation of AFP.

Table A.1: Nominal, relative and absolute AFP concentrations for Bioanalyzer analysis.

Nominal AFP concentration (µg/ml)	Relative AFP concentration (µg/ml)	Absolute AFP concentration (from calibration) (µg/ml)
100	75	91
200	150	183
400	330	402
600	475	578
800	675	821

Appendix 3: ELISA calibration curve of native std-AFP.

Native std-AFP at 1 mg/ml was diluted with bovine serum to concentrations ranging from 10 to 350 ng/ml. Figure A.3 shows the calibration curve used for the quantitation of native AFP. Standard AFP concentration (x-axis in Figure A.3) was determined

from the number of dilution folds made from the 1 mg/ml std-AFP solution, whilst UV absorbance at 450 nm (y-axis in Figure A.3) was determined by the microplate reader. A calibration curve generated from both the standard AFP concentrations and UV absorbance at 450 nm was then established for the quantitation of native rhAFP (Figure A.3).

Figure A.3: ELISA calibration curve of native std-AFP.

To determine rhAFP bioactivity or refolding yield, native std-AFP at 1 mg/ml, denatured-reduced rhAFP (sample load), AEC-, and SEC-refolded rhAFP was diluted with bovine serum to concentrations within the range from 10 to 350 ng/ml. Figure A.4 shows the sample wells after an ELISA assay for the study of rhAFP refolding by AEC and SEC.

Figure A.4: ELISA assay to determine rhAFP refolding yields after AEC and SEC refolding.

(a): Wells 1-6, ELISA calibrator (containing 0 to 350 ng/ml AFP); 7 and 8, denatured-reduced and native std-AFP (100 ng/ml).

(b): Well 1, negative control (AEC Refolding Buffer); 2 and 3, denatured-reduced rhAFP (sample load, 100 ng/ml and 300 ng/ml, respectively); 4 and 5, refolded rhAFP by AEC refolding using Q-FF and DEAE-FF columns, respectively (300 ng/ml and 170 ng/ml, respectively); 6, refolded rhAFP by SEC refolding (150 ng/ml); 7, refolded rhAFP by dilution refolding (65 ng/ml); 8, native std-AFP (100 ng/ml).

PUBLICATIONS

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