DEPARTMENT OF CHEMICAL ENGINEERING

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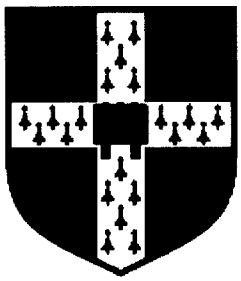

Intensification of Inclusion Body Purification and Protein Refolding

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Preface

This dissertation describes work carried out at the Department of Chemical Engineering of the University of Cambridge, for the degree of Doctor of Philosophy. It is my original work except where stated in the text and includes nothing that has been submitted for any degree, diploma or other qualification at any other university. This dissertation contains approximately 46800 words and 62 figures in 206 pages. Parts of this work have been presented as follows:

Refereed Journal Articles

1. Lee CT, Mackley MR, Stonestreet P, Middelberg APJ. 2001. Protein refolding in an oscillatory flow reactor. Biotechnol Lett 1899-1901.
2. Lee CT, Buswell AM, Middelberg APJ. 2002. The influence of mixing on lysozyme renaturation during refolding in an oscillatory-flow and stirred-tank reactor. Chem Eng Sci 57(10):1679-1684.
3. Lee CT, Morreale G, Middelberg APJ. 2003. Combined in-fermenter extraction and cross-flow microfiltration for improved inclusion body processing. Biotechnol Bioeng. Submitted.

Conference, Oral and Poster Presentations

1. Lee CT, Middelberg APJ. 2001. Protein refolding in an oscillatory-flow-mixing reactor. The proceeding of the 10th European Congress on Biotechnology. Madrid, Spain. p180.
2. Lee CT, Stonestreet P, Malcolm MR, Middelberg APJ. 2001. Protein refolding in an oscillatory-flow-mixing reactor. 4th National Meeting on Oscillatory Flow Mixing. Cambridge, UK.
3. Lee CT, Middelberg APJ. 2002. An integrated refolding and extraction system for the production of recombinant protein from inclusion bodies. The Proceedings of the ESBES-4 Symposium Life Science and Technology. Delft, The Netherlands. p28.
4. Lee CT. 2002. The Best Oral Presentation Prize: Intensification of inclusion body purification and protein refolding. The IChemE Biochemical Engineering Subject Group Postgraduate Research Event. Cambridge, UK.
5. Lee CT, Middelberg APJ. 2003. Intensification of inclusion body purification and protein refolding. 6th Interlaken Conference on Advances in Production of Biologicals: From Gene to Market. Interlaken, Switzerland. p13.

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Summary

There is an increasing need to translate genomic information into commercial products through protein expression and production. Formation of the protein as solid inclusion bodies in *E. coli* is advantageous as it enables good initial purification, provided that acceptable refolding yields can be achieved. However, the recovery of active recombinant protein from complex biological mixtures via the route of IB-formation involves a series of complicated recovery steps, each of which can compromise the purity and yield of the desired product. Purification of IBs using traditional methods of homogenisation and centrifugation are difficult to automate for high-throughput applications, and are costly to scale.

Process intensification by minimising the number of unit operations without a loss of product purity is desirable to improve yield and reduce production cost. Such a process should ideally be generic, scalable, easily automated to facilitate continuous processing, and should result in an improved refolding yield. Based on these criteria, an intensified unit operation termed an integrated Membrane Oscillatory Refolding (MOR) is introduced. The integrated MOR unit consists of an oscillatory flow reactor (OFR) that is integrated with a hollow ceramic membrane for cross-flow microfiltration operation. The key is the use of innovative chemical extraction technology in a non-solubilising mode for IB release, coupled with the MOR unit that acts as a novel microfiltration unit for IB purification and protein refolding. The technologies rely only on chemical reagents and on microfiltration that can be easily scaled and automated.

The objective of this study is to establish and research the *three key process steps* essential to realise the integrated MOR unit: a non-solubilising chemical extraction method; a cross-flow microfiltration for IB recovery; and protein refolding via the hollow ceramic membrane in a novel reactor. Three of these process steps, validated using granulocyte macrophage-colony stimulating factor (GM-CSF) IBs, are successfully demonstrated and ready for integration into a single unit of MOR.

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2-HEDS	2-hydroxyethyl disulfide
B1 to B9	First baffle to the 9th baffle
BME	β-mercaptoethanol
CMC	Critical Micelle Concentration
CWF	Clean water flux
CSTRs	Continuous stirred-tank reactors
D or d	Diameter (m or mm)
D_e	Equivalent diameter (m)
DEAE	Diethylaminoethyl
D_m	Inner diameter of a tubular ceramic membrane (m)
DO	Dissolved oxygen
DTT	Dithiothreitol
EBA	Expanded bed adsorption
EDTA	Ethylenediaminetetraacetic acid
f	Oscillation frequency (Hz)
GM-CSF	Granulocyte macrophage-colony stimulating factor
GSH	Reduced glutathione
GSSG	Oxidised glutathione
GuHCl	Guanidine hydrochloride
HGMS	High gradient magnetic separation
HR	High resolution
h	Baffle spacing (m)
IB	Inclusion body
i. d.	Internal diameter (mm)
IGF	Insulin-like growth factor
IPTG	Isopropyl β-D-1-thiogalactopyranoside
LMH	L m^{-2} h^{-1}
LPS	Lipopolysaccharide
MOR	Membrane oscillatory reactor
n	Number of unit operations (Figure 2-1, Chapter 2) or number of orifices (Section 2.6.4, Chapter 2)
NaOH	Sodium hydroxide
o. d.	Outer diameter, mm

OD	Optical density
OD_{600}	Optical density measured at 600 nm
OFR	Oscillatory flow reactor
P1	Pressure gauge 1 (for inlet)
P2	Pressure gauge 2 (for outlet)
PES	Polyethersulfone
PVDF	Polyvinylidene fluoride
Re	Reynolds number
Re_T	Reynolds number for stirred-tank
Re_o	Oscillatory Reynolds number
r-IL-2	Recombinant interleukin-2
r-HPI	Recombinant human proinsulin
RP-HPLC	Reversed-phase high performance liquid chromatography
SEC	Size-exclusion chromatography
-SH	Cysteine residue or thiol group
SDS	Sodium dodecyl sulfate
PAGE	Polyacrylamide gel electrophoresis
STR	Stirred-tank reactor
TB	Terrific broth
TEM	Transmission electron microscopy
TFA	Trifluoroacetic acid
%T	Transmission level for microfiltration
TMP	Transmembrane pressure (bar)
tPA	Tissue plasminogen activator
Triton X-100	t-octylphenoxypolyethoxyethanol
UV_{280}	Measurement of UV absorbance at 280 nm
v	Linear velocity ($m\ s^{-1}$)
ν	Fluid kinematic viscosity ($m^2\ s^{-1}$)
x_o	Oscillatory amplitude (centre-to-peak, m)
ω	Angular frequency ($rad\ s^{-1}$)

CHAPTER 1

INTRODUCTION

There is an increasing need to express and purify proteins to add value to the human genome sequencing effort, and to speed up the commercialisation of new biopharmaceutical products. The bacterium *E. coli* is a widely used expression system as it offers advantages including high-level expression, known molecular biology and simple culturing procedures (Cleland, 1993). It therefore has a key role in supporting post-genomic efforts in both academic and industrial laboratories. However protein over-expressed in *E. coli* is often sequestered into biologically inactive and insoluble aggregates, known as inclusion bodies (IBs) (Marston, 1986; Mitraki et al., 1991). Despite the disadvantage of expression in an inactive form, which requires efficient *in vitro* refolding to give the bioactive product, the insoluble state facilitates primary recovery and enrichment using solid/liquid separation unit operations (e.g., centrifugation/filtration), as the target protein usually represents more than 50% of the total polypeptide content in IBs (Clark and Georgiou, 1991). Expression as an IB offers other advantages including protection against proteolytic degradation (Valax and Georgiou, 1993; Marston, 1986; Shortle and Meeker, 1989) and prevention against host cell toxicity (Clark and Georgiou, 1991).

Production of recombinant protein as an IB represents a competitive way to introduce new commercial products compared with other expression routes (e.g., soluble protein expression using mammalian cells) that are inherently more complex in terms of product expression and recovery. The 'first to market' priority means that unoptimised moderate refolding yields may be tolerated (Middelberg, 2002), although a very low refolding yield can render this expression route economically infeasible (for instance, refolding yield of < 3% reported for tissue plasminogen activator (tPA) at 2.5 mg L^{-1}) (Datar et al., 1993). Nevertheless, production via IBs becomes very competitive whenever reasonable refolding yields can be achieved. For instance, a

range of modified tPAs are now expressed as IBs in *E. coli*, e.g., Retaplase.

Once an adequate refolding method has been established, the immediate focus becomes rapid scale-up to commercial production (Middelberg, 2002). Ideally, the process should use technology that is approximately scale-invariant, easily automated for high-throughput processing, generic for a broad range of proteins, and economical (Middelberg, 2002). However, the conventional IB processing strategy involves a series of processing steps which, for tPA, accounts for 50-70% of production cost (Datar et al., 1993). Additionally the steps may compromise product yield and thus economic feasibility (Datar, 1986). For example, a large number of downstream processing steps coupled with poor refolding yield in the tPA IB flowsheet accounted for 88% of the entire production costs (Datar et al., 1993), thereby making the IB route unfavourable.

Research is clearly needed to generate economic generic processes for protein production via the IB route. However, the generation of an optimal process is challenging as the number of alternatives and parameters that can be investigated are large (Petrides et al., 1989). A range of research strategies is available, including intensification of the downstream process to improve yield (Fish and Lilly, 1984; Koltermann et al., 1997), molecular manipulation such as fusion technology to simplify downstream processing (Steffens, 2000), use of bioprocess simulation tools to evaluate process economics based on laboratory and pilot plant data (Petrides et al., 1989), and optimisation of key unit operations (e.g., centrifugation optimisation as reported by Wong et al. (1996), and improved refolding reactor strategies as reported by Kotlarski et al. (1997)).

In this study, process intensification is employed as a key research strategy to improve current technology for IB purification and protein refolding.

1.1 Process Intensification

Process intensification by minimising the number of unit operations without a loss of product purity is desirable to improve yield and reduce production cost. Such a process should ideally be generic, scalable, easily automated to facilitate continuous processing, and should result in an improved refolding yield. Based on these criteria, a novel unit operation, termed an integrated Membrane Oscillatory Reactor (MOR) is introduced in this thesis (Figure 1-1).

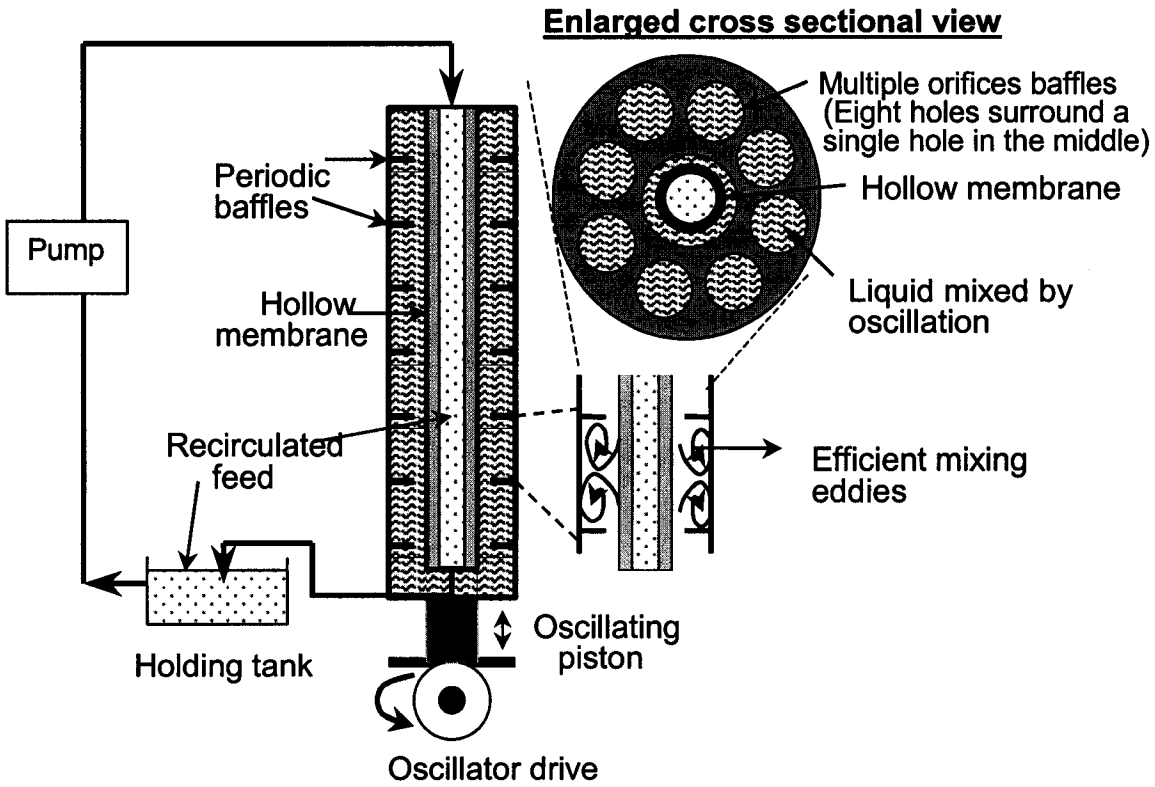

Figure 1-1 The novel Membrane Oscillatory Reactor (MOR) developed based on integration of an oscillatory flow reactor and a hollow ceramic membrane. The enlarged cross sectional view of the reactor and the efficient mixing pattern generated within the reactor are also illustrated.

The MOR unit consists of an oscillatory flow reactor (OFR) that is integrated with a hollow ceramic membrane for microfiltration and perfusive refolding. For IB intensification, the key is the use of innovative chemical extraction technology in a non-solubilising mode for IB release, coupled with a MOR unit that acts as a novel

microfiltration unit, initially for IB purification and then protein refolding. The technology relies only on the use of chemical reagents and microfiltration, and can therefore be easily scaled and automated.

The concept of the MOR system is simple. Fermentation broth is first added to the holding-tank of the unit, and chemical extraction reagents are then added to disrupt the cells and release the insoluble IBs. Alternatively, chemical extraction can also be conducted in the fermenter (as described in this study), and then charged into the holding-tank for subsequent microfiltration. In both cases, soluble contaminants are then removed by operating the unit as a microfiltration unit in diafiltration mode, giving cleaned insoluble IB suspension as the retentate. The retentate is collected in the holding tank, and chemical reagents are then added to solubilise the IBs. The denatured protein from the solubilised IBs is subsequently perfused through the ceramic membrane into refolding buffer that is mixed efficiently by intense oscillation; concomitant removal of the gel-like contaminant (in the retentate), formed from residual cell debris, is achieved. By using the same membrane for microfiltration and protein perfusion, only two unit operations are required to produce substantially pure refolded protein: a fermenter and the MOR system.

The potential advantages of the MOR unit include:

i) process intensification, as the cumbersome steps of repetitive homogenisation and centrifugation are eliminated. IB loss, due to repetitive washing steps, can be minimised;

ii) reduced validation costs due to fewer process steps;

iii) a potentially efficient and scalable mixing device for protein refolding;

iv) easy scale-up, to facilitate continuous and automated operation at both laboratory and process scale, as these technologies rely only on chemical reagents and liquid-handling;

v) minimum risk of proteolytic degradation, as the protective IB state is preserved until most of the soluble contaminants have been removed.

1.2 Research Objective and Scope

To enable the development of the integrated unit, three enabling process steps are identified and developed individually. The steps are:

(i) an efficient non-solubilising chemical extraction method suitable for interfacing with microfiltration;

(ii) an efficient microfiltration method to recover IBs from the chemical extract in a commercial cross-flow microfiltration unit;

(iii) a novel refolding step in the MOR unit. Provided that the membrane used for refolding in the MOR is the same as that selected for microfiltration, process steps(ii) and (iii) can be integrated in a single MOR unit.

The key objective of this study is *to research and establish these three enabling process steps* for subsequent integration into a single MOR unit operation for IB purification and protein refolding.

An investigation into the potential of the MOR to act as an efficient dispersion device for protein refolding represents a secondary objective of the study. This is done by proving that efficient dispersion is necessary for efficient refolding, using lysozyme as a model protein in an ordinary oscillatory flow reactor (without integration of the central membrane). Experiments are conducted in parallel with control experiments using a conventional fed-batch mode stirred-tank reactor, and show that efficient dispersion is necessary. The OFR characteristic of the MOR system may thus offer significant process advantage, but this remains to be proven.

1.3 Thesis Outline

This dissertation consists of seven chapters. Chapter 2 reviews background knowledge and case studies pertinent to the development of the *three enabling process steps* for the integrated unit. The conventional process for IB recovery and protein refolding, and the corresponding key issues, are described. The review facilitates selection of experimental design for each process step while minimising the need for extensive optimisation.

Chapter 3 reports the development of a non-solubilising chemical extraction method. Extraction protocols are first screened and developed in a small-scale experiment and then validated at larger scale in a fermenter.

The successful chemical extraction method developed in Chapter 3 is coupled with a microfiltration unit for IB purification. Microfiltration protocols are developed using a commercial cross-flow microfiltration unit fitted with a hollow ceramic membrane. The microfiltration tests are reported in Chapter 4.

Prior to characterisation of the MOR unit for protein refolding in Chapter 6, Chapter 5 characterises the OFR basic reactor for protein refolding. To investigate the MOR as an improved mixing reactor in Chapter 6, the effect of mixing on refolding is also investigated in the OFR and in a stirred-tank reactor.

Chapter 6 validates the MOR as a refolding reactor using lysozyme and granulocyte macrophage-colony stimulating factor as model proteins. The potential of the MOR to constitute an improved mixing reactor for refolding is also investigated.

Chapter 7 discusses the integration of the three enabling process steps into a single MOR unit, with special focus on the *potential* of the integrated unit. Future work and overall conclusions for this study are then presented.

CHAPTER 2

INTENSIFICATION OF INCLUSION BODY PURIFICATION AND PROTEIN REFOLDING

2.1 Introduction

This chapter reviews background knowledge and case studies pertinent to the development of the *three key process steps* needed for an integrated unit: non-solubilising chemical extraction, cross-flow microfiltration, and protein refolding in a novel reactor.

The conventional process for recombinant protein production via the route of inclusion bodies (IBs) is first described. Due to the inefficiency of inclusion body (IB) recovery steps in the conventional approach, a combined chemical extraction and microfiltration method is presented to intensify the process steps. Common chemical reagents used for IB washing and cell permeabilisation (non-denaturing condition) are reviewed for their potential to constitute a disruptive but non-solubilising extraction protocol to release IBs.

Various key parameters, advantages and issues for microfiltration, and previous microfiltration studies for IB recovery, are then reviewed. The review facilitates the selection of key parameters applicable for current microfiltration operations.

Protein refolding and the key issues pertinent to this study are then reviewed. The key issue of protein aggregation during refolding and the likelihood of mixing effects on protein refolding are discussed. Several workers have proposed different reactor designs to improve mixing characteristics with the aim of increasing the refolding yield. In all cases the exact mechanism leading to mixing effects being observed are not clearly elucidated. Nevertheless, these studies confirm reactor mixing efficiency to be an important parameter requiring further research, especially upon scale-up.

A novel refolding reactor, the Membrane Oscillatory Reactor (Section 1.1, Chpater 1), potentially having effective and scalable mixing characteristics, is introduced. Background studies on protein refolding, including protein denaturation and refolding protocols and analytical methods, are reviewed to facilitate the selection of the corresponding protocols for validating the novel refolding reactor.

2.2 The Conventional Process for Inclusion Body Processing

Figure 2-1 shows the general process route to obtain active purified protein from IBs.

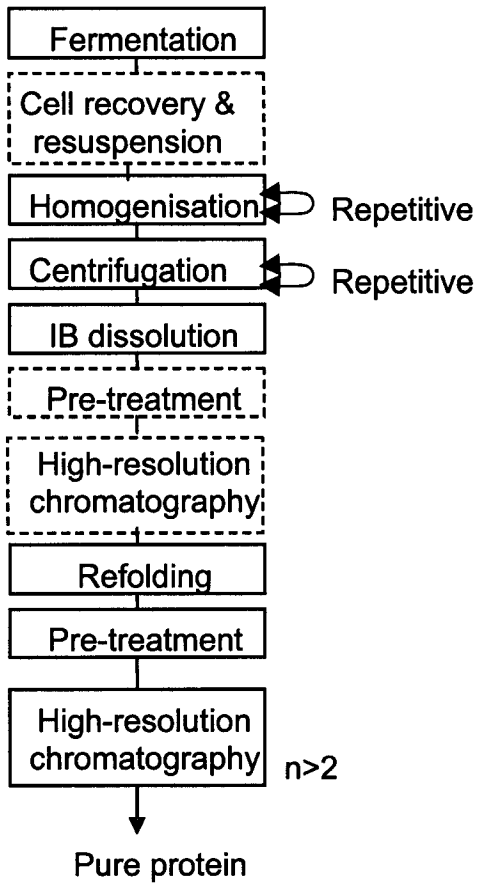

Figure 2-1 The conventional approach for producing recombinant protein following expression as an IB in *E. coli* (optional unit operations are enclosed by dotted lines) (Datar et al., 1993).

Figure 2-1 captures mainly the laboratory approach for IB processing, refolding and purification to produce pure bioactive protein. Several modifications are usually introduced at process scale due to economic considerations (Middelberg and O'Neill, 1998). For a given protein, variation in unit operation optimisation is likely to occur and is a function of the composition of the starting material and the characteristics of the IB (Clark and Georgiou, 1991).

Initial IB recovery

Cells containing the desired IB product are first recovered or concentrated following fermentation by means of a centrifugation or filtration step, though this step may be optional. The primary recovery of IBs employs repeated cycles of mechanical disruption, followed by repeated cycles of enzymatic and chemical treatment interspersed with centrifugal washing (Clark, 2001; Middelberg, 2002).

Cell disruption is usually repeated to micronise cell debris so that separation from the denser IBs can be facilitated using differential centrifugation (Fischer et al., 1993; Thatcher and Hitchcock, 1994; Middelberg and O'Neill, 1998). Repetitive centrifugal washing, usually with low concentrations of chaotropic reagents (e.g., 2 M urea, 1 M guanidine hydrochloride) or detergent (e.g., 0.1-5% Triton X-100) (Fischer et al., 1993), aim to remove most of the soluble contaminants (soluble cell proteins, nucleic acids, lipids and membrane vesicles) and insoluble fragments of the cell wall (e.g., peptidoglycan and associated cell-wall proteins and lipids) to provide substantially clean IB paste (Middelberg and O'Neill, 1998; Clark, 2001).

IB washing with intensive use of chemical reagents is costly both in terms of reagent and waste disposal at process-scale. Therefore, optimisation of centrifugation parameters such as feed-rate and multiple centrifuge passes without intensive use of chemical reagents will be a better strategy to improve IB purity (Wong et al., 1996). The strategy successfully improved the purity of Gly-IGF-II IBs, that were susceptible to proteolytic degradation during IB dissolution, and resulted in a net improvement in the overall protein yield following dissolution (Wong et al., 1996).

However, good fractionation of cell debris from IB sediment in the laboratory is often difficult to achieve, depending on the ease of pellet re-suspension (Middelberg, 2002). At process scale, the cumbersome and costly IB washing steps are usually simplified, resulting in reduced IB purity (Middelberg, 2002).

Dissolution and refolding

Dissolution of IBs is typically achieved using high concentrations of denaturing reagents such as urea or guanidine hydrochloride. Addition of reducing agents such as dithiothreitol or β-mercaptoethanol may be required if IB contains disulphide bonds (Rudolph and Lilie, 1996; Thatcher and Hitchcock, 1994) though reduction may not be essential for some disulphide-bonded IBs (e.g., recombinant bovine growth hormone stored in a completely reduced form (Langley et al., 1987)). The solubilised protein is then refolded to gain the native 3D structure, by lowering the denaturant concentration. Refolding can be achieved using different methods such as dilution of the protein into a large volume of refolding buffer (Rudolph, 1990; Galliher, 1991; Fischer et al, 1993) or by buffer exchange using dialysis (Kelly and Price, 1992; Fischer et al., 1993).

High-resolution purification and pre-treatment

A single step of high-resolution purification prior to refolding may be necessary to remove residual contaminants (from the denatured protein) that can induce aggregation during refolding (Maachupalli-Reddy et al., 1997). Typically this step can be achieved using high-resolution (HR) separation methods such as ion exchange, size-exclusion, metal affinity, hydrophobic or reversed-phase chromatography (Clark and Georgiou, 1991).

A pre-treatment step prior to HR purification is also generally essential to avoid excessive fouling of column resin due to the presence of residual insoluble contaminants (Clark, 1998; Middelberg and O'Neill, 1998). Separation of these impurities from the target protein dissolved in highly concentrated and viscous solution of chaotrope can be tedious (Middelberg, 2002). This can be achieved using ultracentrifugation or filtration at