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Combined In-Fermenter Extraction and Cross-Flow Microfiltration for Improved Inclusion Body Processing

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Abstract: In this study we demonstrate a new in-fermenter chemical extraction procedure that degrades the cell wall of *Escherichia coli* and releases inclusion bodies (IBs) into the fermentation medium. We then prove that cross-flow microfiltration can be used to remove 91% of soluble contaminants from the released IBs. The extraction protocol, based on a combination of Triton X-100, EDTA, and intracellular T7 lysozyme, effectively released most of the intracellular soluble content without solubilising the IBs. Cross-flow microfiltration using a 0.2 μm ceramic membrane successfully recovered the granulocyte macrophage-colony stimulating factor (GM-CSF) IBs with removal of 91% of the soluble contaminants and virtually no loss of IBs to the permeate. The filtration efficiency, in terms of both flux and transmission, was significantly enhanced by in-fermenter Benzonase® digestion of nucleic acids following chemical extraction. Both the extraction and filtration methods exerted their efficacy directly on a crude fermentation broth, eliminating the need for cell recovery and resuspension in buffer. The processes demonstrated here can all be performed using just a fermenter and a single cross-flow filtration unit, demonstrating a high level of process intensification. Furthermore, there is considerable scope to also use the microfiltration system to subsequently solubilise the IBs, to separate the denatured protein from cell debris, and to refold the protein using diafiltration. In this way refolded protein can potentially be obtained, in a relatively pure state, using only two unit operations. © 2004 Wiley Periodicals Inc.

Keywords: Inclusion Body; Protein; Refolding; Microfiltration; Chemical Extraction

INTRODUCTION

There is an increasing need to express and purify proteins to add value to the human genome sequencing effort, and to speed the commercialisation of new biopharmaceutical products. The bacterium *Escherichia coli* is a widely used expression system as it offers advantages including high expression yield, known molecular biology, and simple culturing procedures. However, overexpressed protein in *E. coli* is often sequestered into biologically inactive and insoluble aggregates, known as inclusion bodies (IBs) (Marston, 1986; Mitraki et al., 1991). Efficient ways of processing IBs at both industrial and laboratory scale are required. Ideally, such methods should use technology that is approximately scale-invariant, is easily automated for high-throughput processing, is generic for a broad range of similar proteins, and is economical (Middelberg, 2002). The conventional laboratory process for IBs employs mechanical disruption of the cells, usually by sonication, followed by repeated cycles of enzymatic and chemical treatment interspersed with centrifugal washing (Clark et al., 1999). This process is both time-consuming and labour-intensive, and is difficult to economically scale. The procedures are often simplified on scale-up by reducing the rigour of treatment and washing, leading to inefficiencies in subsequent downstream processing operations including centrifugation (Middelberg, 2002; Choe et al., 2002).

It is apparent that conventional methods for IB processing do not satisfy the criteria specified earlier. To address this, we have recently developed chemical methods that extract intracellular insoluble protein at high efficiency using chaotrope-based solutions (Falconer et al., 1998; Choe and Middelberg, 2001a). We have also demonstrated a method for selectively removing DNA from the chemical extraction mixture using spermine (Choe and Middelberg, 2001b). These extraction methods have been successfully coupled with primary capture methods including expanded bed chromatography (Choe et al., 2002) and high-gradient magnetic separation (Choe, 2002). However, this chemical extraction technique is suitable only for those proteins that are not degraded by proteases associated with the cell wall. Such proteases can rapidly degrade protein product, even in concentrated chaotrope, and can lead to significant product loss (Babbitt et al., 1991; Wong et al., 1996). For such proteins, complete chemical extraction will prove suboptimal. The aim of the research reported here was to develop an

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approach for IB processing that meets the criteria specified earlier. A primary objective was to maintain the protective IB state until contaminating proteins had been removed. A secondary objective was to minimise the number of unit operations involved in the process, thus minimising process and validation costs (Gehmlich et al., 1997) while also simplifying the ease and economy of laboratory automation. Our concept is to couple nonsolubilising chemical extraction with cross-flow microfiltration. Other researchers have developed chemical extraction techniques for *E. coli* that selectively disrupt the cell envelopes under nonsolubilising conditions (Hettwer and Wang, 1989; Naglak et al., 1990; Middelberg, 1995). The combined use of guanidine hydrochloride (GuHCl, 0.1 M) and Triton X-100 (0.5% v/v) permeabilised *E. coli* cells giving 50–60% protein release (Hettwer and Wang, 1989). The use of chaotropic agents, such as GuHCl or urea, to solubilise protein from *E. coli* membrane fragments, and use of Triton X-100 as a nonionic detergent to solubilise the *E. coli* inner membrane, has also been reported (Novella et al., 1994). While these techniques are good for differentially releasing soluble target protein, they are inefficient if the product is an IB, as more than 50–60% removal of contaminating proteins is required. A method to recover IGF IBs by means of reversible oxidation of the IB surface has been developed by Falconer et al. (1999). Initial permeabilisation using a combination of chaotrope, EDTA, and oxidising agent rendered the IB refractory to dissolution, and this process could be reversed by the use of DTT in a second step (after removal of the soluble contaminating protein). The general applicability of this method has not, however, been demonstrated. A nonsolubilising commercial bacterial-extraction kit for IB processing has been recently introduced (Pierce Biotechnology, Rockford, IL). The package makes use of the existing Pierce B-PER Bacterial Protein Extraction Product (Pierce, Product 78243, which contains a nonionic detergent in 20 mM Tris) to disrupt the bacterial cell membrane, supplemented with lysozyme (Pierce, Product 89833). However, the cost of lysozyme can be prohibitive at large scale. Studier (1991) developed a system for the intracellular expression of T7 lysozyme expressed intracellularly by host cells carrying pLysS or pLysL plasmids, which might be useful to overcome lysozyme cost limitations. It was reported that thorough bacterial cell lysis can be achieved when cells resuspended in 50 mM Tris-HCl, 2 mM sodium EDTA (pH 8.0) were treated with detergent (e.g., 0.1% (v/v) Triton X-100 or 0.2% (w/v) deoxycholate) or 1% (v/v) chloroform. This technique is cost-efficient in terms of lysozyme use, but the procedure assumes that cells are collected and resuspended in buffer prior to chemical treatment. From the above studies, we conclude that a combination of mild chaotrope, EDTA, lysozyme, and nonionic detergent (Triton X-100) would seem appropriate for further investigation.

Several studies have reported the feasibility of using microfiltration in cross-flow diafiltration mode for IB recovery from cell lysate. Meagher et al. (1994) recovered rIL-2 IBs from an *E. coli* cell lysate using a 0.1 μm Durapore hydrophilic membrane to remove 85–90% of the UV 280 nm absorbing compounds to the permeate. Forman et al. (1990) recovered IBs of the gp41 transmembrane protein of the HTLV-III virus from an *E. coli* lysate using a 0.45 μm Durapore membrane. Up to 87% of the soluble protein was removed using three volumes of buffer exchange. In a microfiltration study to purify human growth hormone IBs from a homogenised *E. coli* cell lysate, removal of greater than 90% of soluble protein using a 0.15 μm cellulose acetate membrane and a 0.1 μm polyether-sulfone membrane has also been reported (Bailey and Meagher, 1997, 2000). However, these studies have only considered the recovery of IBs from cells following mechanical disruption, and have not considered recovery from complex chemical extraction mixtures. In this work we extend these previous studies by coupling nonsolubilising chemical extraction with cross-flow microfiltration for the preparation of clean IBs of granulocyte macrophage-colony stimulating factor (GM-CSF). Importantly, we perform nonsolubilising extraction directly in fermentation media using a mixture of Triton X-100 and EDTA, coupled with enzymatic attack of the cell wall using constitutively expressed intracellular T7 lysozyme encoded by the pLysS plasmid in *E. coli* BL21(DE3)-pLysS (Studier, 1991). By performing the extraction directly in fermentation media we minimise the number of unit operations: only a fermenter and a cross-flow microfiltration system are required. We also demonstrate that extracted IBs can be efficiently separated from contaminating proteins using nuclease treatment and cross-flow microfiltration, despite the presence of Triton X-100, yielding a high-purity product having good refolding characteristics.

MATERIALS AND METHODS

Materials

Native granulocyte macrophage-colony stimulating factor (GM-CSF) and GM-CSF IBs purified by preparative reversed-phase chromatography were kindly supplied by Novartis Pharma AG (Basel, Switzerland). Guanidine hydrochloride (GuHCl), oxidised and reduced glutathione (GSSG and GSH), Tris-HCl, cupric chloride, and ethylenediaminetetraacetic acid (EDTA) were purchased from Sigma-Aldrich (Poole, Dorset, UK) and were ACS reagent grade. Terrific broth (modified powder), carbenicillin disodium salt, chloramphenicol, magnesium chloride, and Benzonase were from Sigma-Aldrich. Dithiothreitol (DTT) and isopropyl β-D-1-thiogalactopyranoside (IPTG) were from Melford Laboratories (Chelsworth, UK). Triton X-100 (t-octylphenoxypolyethoxyethanol, 98%) was purchased from BDH Chemicals (Poole, UK). Sodium hydroxide pellet, HPLC-grade acetonitrile, and trifluoroacetic acid (TFA) were purchased from Fisher Scientific (Loughborough, UK). Sodium hypochlorite solution (~13% active chlorine) was purchased from Fluka (Poole, Dorset, UK).

Analytical Methods

Extraction Efficiency Using SDS-PAGE Analysis

Extraction efficiency was evaluated qualitatively using SDS-PAGE analysis. One mL of extraction broth was centrifuged at 10,000*g* for 15 min at 4°C. The supernatant was collected and the pellet was washed 1× with PBS buffer by ~30 sec of vortexing. The pellet was resuspended in 1 mL of solubilising buffer by 1 min of vortexing (50 mM Tris-HCl, 1 mM EDTA, 8 M urea, 100 mM DTT, pH 8). Fifty μL of the sample was mixed with 50 μL of sample buffer (Laemmli 2× concentrate, Sigma-Aldrich), boiled for 4 min, and centrifuged (14,000*g*, 1 min). Thirty μL of sample was loaded on a SDS-PAGE gel (8–16% Ready Precast Gel, 50 μL well, Bio-Rad Laboratories, Hempsted, UK) prior to electrophoresis (run for 2 h at a fixed voltage of 80 V and an initial current of 25 mA).

Total Protein Assay for Evaluating Microfiltration Efficiency

Microfiltration efficiency was assessed by comparing protein concentration in the final retentate with that in the feed. Total protein was measured using Bio-Rad protein assay dye reagent, 5× concentrate (Catalog no. 500-0006, Bio-Rad Laboratories) based on the Bradford assay (Bradford, 1976) using a bovine serum albumin (BSA) standard.

Agarose Gel Electrophoresis

DNA molecular weight in samples from chemical extraction was estimated on a 1% (w/v) agarose gel using a Sub-Cell GT Agarose Gel Electrophoresis System (Bio-Rad, 170-4402) with a molecular marker (Invitrogen Life Technologies, 15615-016, Paisley, UK). Two μL of sample was mixed with 2 μL of gel loading solution (Sigma, G2526), 2 μL of the mixture was loaded onto the 1% agarose gel.

RP-HPLC Analysis

The concentrations of native GM-CSF, denatured GM-CSF from solubilised IBs, and refolded GM-CSF were measured using a C_5 Jupiter reversed-phase column (5 μm, 300 Å, 150 × 4.6 mm, Phenomenex, Macclesfield, UK) on a high-performance liquid chromotography (HPLC) system comprising a X-Act 4-Channel Degassing Unit (Jour Research, Sweden), a 7725I Injection Valve (Rheodyne, Cotati, CA, USA), two HPLC 422 Pumps (Kontron Instruments, UK), a C030 HPLC column chiller/heater (Torrey Pines Scientific, San Marcos, CA, USA), a 2151 Variable Wavelength Detector (LKB, Sweden), and Chromeleon HPLC Management Software (Dionex, Sunnyvale, CA, USA). The denatured and refolded samples were acidified to 0.4 and 0.2% (v/v) TFA, respectively, prior to sample injection. An acetonitrile-water gradient (from 26–38% (v/v) acetonitrile over 3.5 min, followed by 38–42% over 0.5 min and then 42–60.25% over 15 min) with 0.1% (v/v) TFA counterion was used to elute samples, at a total solvent flow-rate of 0.5 mL min^{-1}. Absorbance was measured at 214 nm. Native GM-CSF and GM-CSF IBs purified by preparative reversed-phase chromatography were kindly supplied by Novartis Pharma AG (Basel, Switzerland) to enable system calibration. Denatured GM-CSF was prepared by solubilising these IBs in GuHCl-buffer (50 mM Tris-HCl, 7M guanidine hydrochloride (GuHCl), 50 mM DTT, pH 8.0) overnight at 4°C. Native GM-CSF was analysed following resuspension of the freeze-dried powder in TE buffer (50 mM Tris, 1 mM EDTA) pH 8.

Molecular Biology and Cell Suspension Preparation

Molecular Biology

Granulocyte macrophage-colony stimulating factor (GM-CSF)/pET17b plasmid was kindly provided by Novartis Pharma. Competent *E. coli* cell strain BL21(DE3)pLysS (Novagen, 70236-3, Madison, WI, USA) were transformed with this plasmid. Strains having the designation (DE3) are lysogenic for a λ prophage that contains an IPTG-inducible T7 RNA polymerase. Strains having the pLysS designation carry a pACYC184-derived plasmid that encodes T7 lysozyme, which is a natural inhibitor of T7 RNA polymerase that serves to repress basal expression of target genes under the control of the T7 promoter. The existence of cytoplasmic T7 lysozyme has been reported to facilitate the production of cell extracts for purification of target protein (Studier, 1991). Transformed cells were grown on 1.5% agarose gel (Bacto agar, Difco, Detroit, MI, US) with Luria Broth, LB (Miller's modification, Sigma L3397) and incubated overnight at 37°C. The growth medium was supplemented with 50 μg mL^{-1} of carbenicillin and 34 μg mL^{-1} of chloramphenicol. A single colony of the transformed cells was inoculated in a 250 mL baffled Erlenmeyer flask containing 26 mL of TB medium. The culture was harvested at OD 18 measured at 600 nm, centrifuged at 8,000*g* for 15 min, and transferred into cryotubes (Technical Service Consultants, Protect Bacterial Preservative System TS70-AP, UK) for storage at −80°C.

Shake Flask Cultures

To prepare the inoculum for the shake-flask culture, a bead coated with *E. coli* BL21(DE3)pLysS was picked from a cryotube using a sterile plastic inoculating loop and added in a 250 mL baffled Erlenmeyer flask containing 26 mL of TB medium. The culture was mixed by agitation for 18–24 h using a platform shaker set at 200 rpm and 37°C. Two mL of the inoculum was inoculated into a 250 mL baffled Erlenmeyer flask containing 26 mL of TB medium. In all cases, the TB medium was supplemented with 50 μg mL^{-1} of sterilised carbenicillin and 34 μg $mL^{-1}$of chloramphenicol in ethanol immediately before inoculation. The culture

was incubated in a horizontal shaker at 200 rpm and 37°C. When the optical density, measured at 600 nm (OD_{600}) reached 5.5 ± 0.5, the culture was induced with 1 mM IPTG. Following confirmation that the culture was in its stationary phase by measuring the optical density of culture broth at 600 nm using a spectrophotometer (OD_{600} of 16 ± 2, culture time of 4 ± 0.5 h), the culture was terminated. In the case where pellet collection was necessary (e.g., for B-PER control extractions), culture was centrifuged at 8,000*g* for 15 min. For chemical extraction without removal of the growth medium, the cell culture was pipetted directly into 50 mL Falcon tubes containing preweighed chemical extraction reagents (in powder form).

Fermentations

To prepare the inoculum for fermentation, a bead coated with *E. coli* BL21(DE3)pLysS was picked from a cryotube using a sterile inoculate loop and added in a 250 mL baffled Erlenmeyer flask containing 100 mL of TB medium. Cultures were prepared with agitation using a platform shaker set at 200 rpm and 37°C (18–24 h). TB with the addition of 10 g L^{-1} glycerol and 0.25 mL L^{-1} polypropylene glycol (PPG; BDH, 297676Y, Poole, UK) was used as fermentation medium. Carbenicillin and chloramphenicol were added to a final concentration of 50 and 34 μg mL^{-1}, respectively, immediately before inoculation. All fermentation work was conducted using a 5 L fermentor (4 L working volume, New Brunswick Scientific, Edison, NJ, USA, BioFlo 3000). Agitation-DO cascade control was applied to maintain a DO level above 20% and temperature was controlled at 37°C. Pure oxygen supply was mixed with the air inlet when agitation control alone was not adequate to keep the DO at its set point. Induction of GM-CSF expression was done by adding IPTG to a final concentration of 1 mM when OD_{600} reached 5.5–6.0. Following confirmation that the culture was in its stationary phase by measuring the optical density of culture broth at 600 nm using a spectrophotometer (OD_{600} of 16 ± 2, induction time of 4 ± 0.5 h), fermentation was terminated. Chemical extraction reagents in powder form were then added directly into the fermenter and agitated at 800 rpm for 40 min. The pH and DO sensor probes were removed from the fermenter during this chemical extraction procedure.

Chemical Extraction

Chemical Extraction Benchmarking Using B-PER® Reagent

B-PER Bacterial Protein Extraction Reagent (Pierce, 78243) was used as described by the manufacturer, without the addition of extracellular lysozyme, as a control for the extraction methods screened in this study. Supernatant and pellet samples were analysed by SDS-PAGE.

Evaluation of Chemical Extraction Methods: Denaturing Selective Extraction

An extraction procedure reported by Falconer et al. (1999) achieved the selective extraction of an insoluble variant of insulin-like growth factor by using reversible oxidation. In this work, we examine the effectiveness of the first-stage extraction in removing the intracellular soluble contaminants without solubilising the GM-CSF IBs. One mL of culture broth in a 2 mL microcentrifuge tube was first centrifuged (8,000*g*, 4°C, 15 min). Cell pellets were resuspended in 1 mL of first-stage extraction buffer (0.1 M Tris, 3 mM EDTA, 20 mM 2-hydroxyethyl disulphide (2-HEDS), pH 9.0 containing either 4, 6, or 8 M urea). The extraction broth was stirred at 200 rpm and 37°C for various times (30 min, 2 h, and overnight). Extraction efficiency was evaluated using SDS-PAGE by analysing the soluble and insoluble fractions.

Evaluation of Chemical Extraction Methods: Nonsolubilising Extraction

Ten mL of shake-flask cell suspension at an OD_{600} of 16 ± 2 were aliquoted into 50 mL Falcon tubes without separating the growth medium. The tubes were filled with different extraction reagents (all in powder form except for Triton X-100 which was in concentrated liquid form, 98% purity) to give the extraction conditions listed in Table I. Upon dissolution of the reagents, the pH of the extraction broth was adjusted to 9.0 using 8 M sodium hydroxide (NaOH). All samples were agitated on a horizontal shaker at 200 rpm and 37°C for 30 or 60 min. Extraction efficiency was evaluated using SDS-PAGE.

Larger Scale In Situ Chemical Extraction

Chemical extraction was performed in the 5-L fermenter by the direct addition of concentrated extraction chemicals (powdered EDTA and Tris and concentrated 98% purity Triton X-100) to the fermentation suspension, without prior removal of fermentation media. Chemicals were added to give a final extraction environment of 0.05% (v/v) Triton X-100, 5 mM EDTA, 0.1 M Tris, pH 9 by addition of 8 M NaOH (plus undefined media components). Extraction was

Table I. Summary of screened extraction protocols.

Protocol	Chemical	Triton X-100, %(v/v)*	EDTA, mM	Extraction time min
1	B-PER	Unknown	Unknown	5
2	2M Urea	—	5	30 and 60
3	—	0.1	—	30 and 60
4	—	0.1	5	30 and 60
5	2M Urea	0.1	5	30 and 60
6	—	0.1	10	30 and 60
7	—	0.05	5	30

*Assuming the stock solution of Trion X-100 has ~100% purity instead of ~98%.

performed for 30 min at an impeller speed of 300 rpm and a temperature of 37°C. Agitation speed was increased to 800 rpm for 40 min to counter the increase in medium viscosity caused by the release of the intracellular nucleic acids. Extraction efficiency was evaluated using SDS-PAGE.

Reduction of Nucleic Acid Fragment Size Using Benzonase

A significant increase in viscosity and non-Newtonian behaviour was observed during *in situ* chemical extraction. Benzonase (Sigma, E1014) treatment was employed to provide a suspension suitable for further processing (i.e., prior to microfiltration), as it is effective over a wide range of operating conditions (Table II). Tests were conducted to assess the effect of chemical extraction conditions on Benzonase efficiency. Initial experiments investigated the impact of broth dilution, as TB medium contains at least 54 mM K_2HPO_4 and 16 mM of KH_2PO_4. The need for $MgCl_2$ addition to neutralise excess EDTA was also investigated. Test samples of chemical extraction medium diluted and/or supplemented with appropriate concentrations of $MgCl_2$ were treated with 50 and 100 U mL^{-1} of Benzonase and were incubated at room temperature for 2 h. Efficiency was significantly improved by dilution. Further tests aimed to reduce the concentration of Benzonase. Two-fold diluted extracts were incubated in 50, 30, 20, and 10 U mL^{-1} of Benzonase, in the presence of 6 mM $MgCl_2$, for 30 min and overnight (14–18 h). In all cases, digestion efficiency was analysed by agarose gel electrophoresis.

Cross-Flow Microfiltration

Following chemical extraction to release intracellular soluble contaminants, a cross-flow microfiltration unit (Pilot Unit X-LAB 3, Exekia, Bazet, France) was used to remove the soluble contaminants (as permeate) from the IBs. This unit comprises a 3 L feed tank with a double jacket for temperature control, a Membralox component holding a single cylindrical ceramic filtration ''monotube'' and a cross-flow volumetric pump providing a fixed cross-flow rate of ~1 m^3 h^{-1}. Transmembrane-pressure was generated by pressurising the feed tank. The ceramic monotube (T1-70 Membralox, Exekia) has a filter area of 50 cm^2, 7 mm i.d., 10 mm o.d., is 25 cm in length, and is available in various microfiltration pore sizes (0.1, 0.2, 0.5 μm). Initial microfiltration tests using a 0.1 μm pore size and a transmembrane-pressure (TMP) of 0.5 bar gave very poor protein transmission based on Bradford protein assay and SDS-PAGE of the permeate (data not shown). A small-scale test using manual filtration with a 0.1 μm filter (Whatman 10 mm syringe filter, Anotop™, Maidstone, UK) confirmed poor transmission for this complex extraction mixture. Similar tests using a 0.2 μm filter gave good transmission, so a 0.2 μm monotube was selected for further investigation.

Table II. Optimal and effective operating conditions for Benzonase nuclease.

Condition	Optimal[a]	Effective[b]
Mg^{2+} concentration	1–2 mM	1–10 mM
pH	8.0–9.2	6.0–10.0
Temperature	37°C	0–42°C
Dithiothreitol (DTT)	0–100 mM	>100 mM
β-Mercaptoethanol	0–100 mM	>100 mM
Monovalent cation concentration (Na^+, K^+, etc.)	0–20 mM	0–150 mM
PO_4^{3-} concentration	0–10 mM	0–100 mM

[a]''Optimal'' is defined as the operating range in which Benzonase retains 90% or more of its activity.

[b]''Effective'' is defined as the operating range in which Benzonase retains at least 15% of its activity.

Microfiltration of Untreated Chemical Extraction Broth

Chemical extraction broth (750 mL) was diluted with 750 mL of TE buffer (50 mM Tris and 1 mM EDTA, pH 8). A two-volumes buffer exchange (3,000 mL TE buffer) was carried out at a constant TMP of 0.5 bar for 0 to 5 h, and at 0.75 bar from 5 to ~12 h. Pulsed backwashing was employed every 5 min throughout the filtration process and the diafiltration operation worked incrementally by adding 150 mL of TE buffer every time 150 mL of permeate had been removed. Permeate samples were collected at variable time intervals throughout the filtration, while duplicated 1 mL retentate samples were withdrawn after each volume (1500 mL) of buffer exchange. A new 0.2 μm ceramic monotube was used for this experiment.

Microfiltration of Sheared Chemical Extraction Broth

Chemical extraction broth (750 mL) was sheared for 20 min using a hand-mixer device (Multipimer MR400, Braun, UK). Samples were taken at 10, 15, and 20 min and analysed for DNA size using agarose gel electrophoresis (see Analytical Methods, above). The sheared extract was diluted 2-fold with 750 mL of TE buffer, pH 8. A 1.5 volumes buffer exchange (2,250 mL TE buffer) was carried out at a constant TMP of 0.5 bar for 0 to 5 h, and at 0.75 bar from 5 to ~12 h. Pulsed backwashing was employed every 5 min throughout the filtration process, and the diafiltration operation worked incrementally by adding 150 mL of TE buffer every time 150 mL of permeate had been removed. Permeate samples were collected at variable time intervals throughout the filtration, while duplicated 1 mL retentate samples were withdrawn at one volume (1,500 mL) and 1.5 volumes (2,250 mL) of buffer exchange. A new 0.2 μm ceramic monotube was used for this experiment.

Microfiltration of Benzonase-Treated Chemical Extraction Broth

Chemical extraction broth (750 mL) was diluted with 750 mL of TE buffer (pH 8) to reduce ionic strength and

improve Benzonase activity (see Table II). The diluted broth was supplemented with 6 mM $MgCl_2$ and 8.33 U mL^{-1} of Benzonase before overnight (12–14 h) incubation at room temperature. A 3-volumes buffer exchange (4,500 mL TE buffer) was carried out at a constant TMP of 0.7 bar with pulsed backwashing every 5 min throughout the filtration process. Sampling and diafiltration procedures were as for the preceding tests, except that retentate samples were withdrawn at each half volume (750 mL) of buffer exchange. After diafiltration, the retentate was concentrated 2-fold to 750 mL over 82 min, using the same membrane without cleaning. This facilitated a comparison of IB yields following centrifugation or microfiltration and Benzonase treatment. A one-time-recycled 0.2 μm monotube (used in the microfiltration of the sheared chemical extraction broth) was used. The recycled monotube was restored to 95% of the original clean water flux prior to this filtration using the cleaning procedures in the next section.

Clean Water Flux Measurement and Membrane Regeneration

Clean water flux (CWF) was measured by recirculating deionised and 0.2 μm-filtered water at a cross flow-rate of 1 $m^3\ h^{-1}$ at a TMP of 1 bar and a temperature of 20°C. The 0.2 μm ceramic monotube was regenerated within the cross-flow microfiltration unit based on the Cleaning-In-Place procedures recommended by the unit supplier.

Refolding

GM-CSF IBs in the retentate from the Benzonase-treated microfiltration experiment were collected by centrifugation (10,000*g*, 25 min, 4°C) and were solubilised overnight at 4°C in GuHCl buffer (see Analytical Methods, above). The denatured GM-CSF concentration was quantified by RP-HPLC analysis and was adjusted to 0.7 ± 0.1 mg mL^{-1} using GuHCl buffer. This denatured GM-CSF solution (100 μL) was renatured by 7-fold rapid dilution into 600 μL of refolding buffer (50 mM Tris-HCl, 3 mM GSH, 0.3 mM GSSG, 0.067 mg mL^{-1} $CuCl_2$, pH 8.0) for 48 h at 4°C to give a final protein concentration of ~0.1 mg mL^{-1}. A control experiment was run to test whether the microfiltration and Benzonase treatment had reduced refolding yield. IBs in the original chemical extraction mixture (without Benzonase treatment) were recovered by centrifugation, as above. The IB pellet was solubilised and refolded using the same procedures as described above at a similar refolding concentration (0.08 mg mL^{-1}).

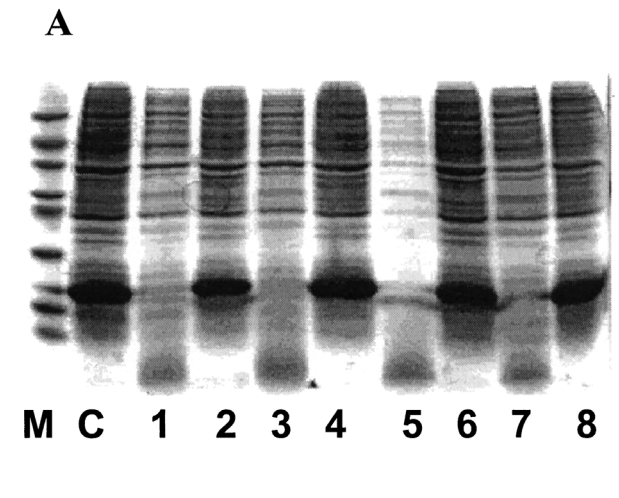
RESULTS AND DISCUSSION

Chemical Extraction

A primary objective of this work was to develop a non-denaturing chemical extraction method for IBs that would be suitable for subsequent microfiltration purification. Non-denaturing extraction achieves a higher initial purity of inclusion body (IB) material and is therefore useful when an affinity system is not available to purify the denatured protein, or when there is advantage in maintaining a protective IB state until contaminating proteins have been removed.

As a first step, we examined a selective chemical extraction method developed previously (Falconer et al., 1999). The method has been successfully used to recover ~80% (w/w) of Long-R^3-IGF-I from cytoplasmic IBs at a purity of 46% (w/w). The method first employed a permeabilising buffer containing 6 M urea, 15 mM 2-hydroxyethyl disulphide, 3 mM ethylenediaminetetraacetic acid (EDTA), 0.1 M Tris pH 9, to release contaminating proteins. It was hypothesised that 2-HEDS stabilised the IB against solubilisation, through surface oxidation of the disulphide bonds. The IBs were then solubilised using the same buffer, but with a reducing agent (DTT) replacing the oxidising agent. In this work we attempted to use this same approach for GM-CSF IBs. The addition of 20 mM 2-HEDS into the permeabilising buffer used by Falconer et al. (1999) failed to prevent GM-CSF IB solubilisation for all urea concentrations tested (4 M, 6 M, and 8 M). IBs were found to be solubilised within 30 min of incubation at 37°C. Falconer et al. (1999) claimed that their method has higher likelihood of success for recombinant proteins having a high proportion of cysteine residues. Although the method was successful for Long-R^3-IGF-I, having three disulphide bonds in a protein of 83 amino acid residues, it failed for GM-CSF, which has two disulphide bonds and 124 amino acid residues.

We therefore sought an alternative chemical extraction system. We decided to employ a combination of Triton X-100, urea, and lysozyme, as these agents act complementarily on different components of the cell wall (Middelberg, 1995). To avoid the cost of adding lysozyme we used a host-vector system, *E. coli* BL21(DE3)pLysS (Novagen), which constitutively expresses a small amount of lysozyme intracellularly. This system is sold commercially and it is well documented that cells will be easily lysed in the presence of a small amount of mild detergent (e.g., 0.1% Triton X-100) (Studier, 1991). A control system using the commercial B-PER reagent (Pierce, 78243) was also investigated. The control used cells that had been separated from fermentation media according to an established method (see Materials and Methods), whereas other protocols used cells suspended directly in fermentation media. Figure 1A,B are SDS-PAGE gels of pellet and supernatant samples for extraction protocols 2–5 summarised in Table I. A comparison of Figures 1A and B shows that the cleanest IB pellets were obtained by a combination of Triton X-100 and EDTA (protocols 4 and 5), whereas protocols 2 and 3 gave poor product purity, as the pellet samples were quite dirty. A combination of both Triton X-100 and EDTA, along with constitutive lysozyme expression, is necessary to ensure good purification. Efficient lysis by protocols 4 and 5 was confirmed by the observation that considerable release of nucleic acid occurred. From Figure 1B, the addition of 2 M



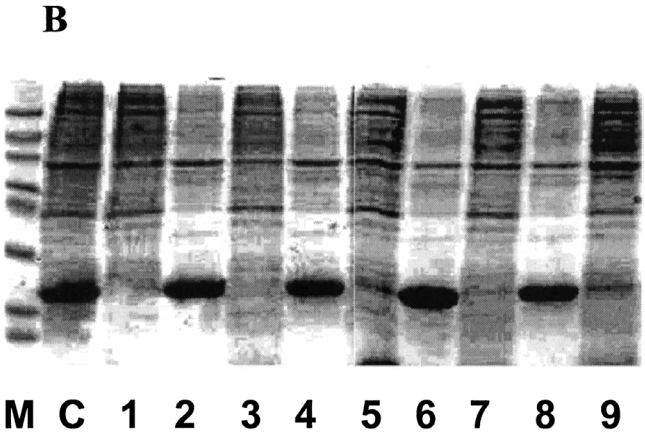


Figure 1. **A:** SDS-PAGE analysis for extraction protocols 2 and 3. Samples 1 and 2 are the supernatant and pellet samples from protocol 2 (2 M urea, 5 mM EDTA) following 30 min extraction; samples 3 and 4 are similar to 1 and 2, but following an extraction time of 60 min; samples 5 and 6 are the supernatant and pellet samples from protocol 3 (0.1% Triton X-100), following 30 min of extraction time; samples 7 and 8 are similar to 5 and 6, but following an extraction time of 60 min. **B:** A comparison of protocols 4 and 5. M, Marker; C, whole cell extract; samples 1 and 2 are the supernatant and pellet samples from protocol 4 (0.1% Triton X-100, 5 mM EDTA) following 30 min extraction; samples 3 and 4 are similar to samples 1 and 2, but following an extraction time of 60 min; samples 5 and 6 are the supernatant and pellet samples from protocol 5 (0.1% Triton X-100, 5 mM EDTA, 2 M urea) following 30 min extraction; samples 7 and 8 are similar to 5 and 6, but following an extraction time of 60 min.

urea did not improve extraction efficiency. Further tests on protocol 4 also indicated that an increase in EDTA concentration to 10 mM (protocol 6 in Table I) did not further improve extraction efficiency (data not shown). To reduce downstream impact and cost, we also sought to reduce Triton X-100 concentration. A reduced Triton X-100 concentration (0.05% v/v) was screened in the extraction buffer containing 5 mM EDTA, 0.1 M Tris, pH 9 (protocol 7 in Table I). The result, as shown in Figure 2, was comparable to that achieved using 0.1% Triton X-100 (protocol 4, Fig. 1B). This modified extraction protocol gave an IB pellet of comparable purity to that obtained using B-PER reagents (as shown in Fig. 2), and was the

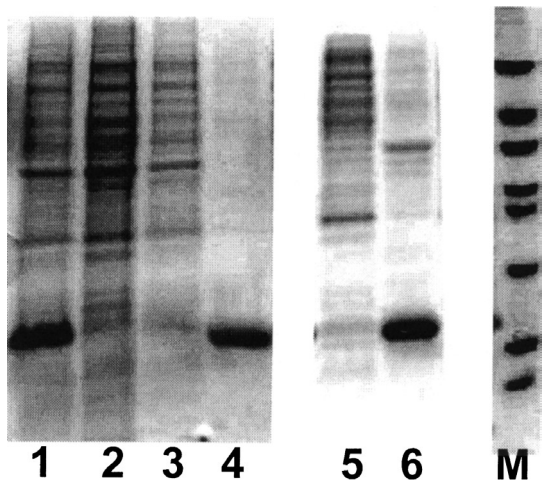


Figure 2. SDS-PAGE showing that the developed chemical extraction method using protocol 7 (0.05% Triton X-100 and 5 mM EDTA, 0.1 M Tris, pH 9) gave a comparable results to protocol 4 (shown in Fig. 1B) and also the control experiment using a commercial extraction reagent, B-PER. (1, induced whole cells; 2, uninduced whole cells; 3, supernatant sample using B-PER; 4, pellet sample using B-PER; 5, supernatant sample of the developed chemical extraction; 6, pellet sample of the developed chemical extraction; M, marker.)

method chosen for further investigation in a larger-scale extraction test.

A larger scale extraction was conducted in 4-L of fermentation broth, directly in the fermenter. Figure 3 is an SDS-PAGE gel comparing the result for large-scale extraction with that from the optimised small-scale protocol (protocol 7, Table I). The protocols are comparable to that achieved by shake-flask extraction, and the purity of the IB pellet obtained by centrifugation is very good. This in-fermenter extraction method was therefore selected for further microfiltration-based studies.

Cross-Flow Microfiltration

Having developed a successful nonsolubilising in-fermenter extraction protocol, the next step was to develop a separation method based on microfiltration. As stated in the Introduction, a key objective is to minimise the number of process unit operations and eliminate the complexity associated with repeated mechanical disruption and centrifugation.

In the first microfiltration test using an IB suspension generated by in-fermenter extraction, a two-volumes buffer exchange diafiltration was conducted at a low permeate flux ranging from 50–75 L m^{-2} h^{-1} (Fig. 4). This low flux necessitated an extended filtration time of ~12 h. Approximately 30% of soluble contaminating protein was removed. It was hypothesised that filtration efficiency would be improved by reducing nucleic acid (NA) size, thus lowering both viscosity and the extent of gel-layer formation. A reduction in NA size can be achieved using either shear or enzymatic degradation (e.g., Benzonase). A hand-held mixer was employed to assess the effect of shear on filtration

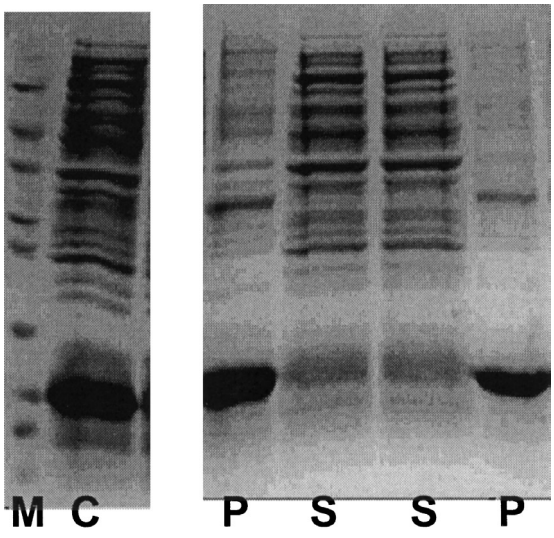


Figure 3. SDS-PAGE of the pellet and supernatant samples following larger scale chemical extraction using protocol 7 (0.05% Triton X-100, 5 mM EDTA, 0.1 M Tris, pH 9) performed in 4 L of fermentation broth. The extraction result was comparable to results for smaller scale extraction performed in a 10-mL shake flask as shown in Figure 2. (M, marker; C, whole cell extract; P, pellet sample of extract; S, supernatant sample of extract.)

performance (see Materials and Methods). Protein removal for sheared material increased to 58% after a 1.5 volume diafiltration. A low average flux rate ($\sim$40 L m^{-2} h^{-1}) was still observed, leading to an extended diafiltration time (12 h) (see Fig. 4). The system was not considered acceptable for practical use.

To test the effect of enzymatic degradation, a large number of tests were conducted with Benzonase. The aim of Benzonase treatment was to create an extract having a maximum NA size of $\sim$500 bp. Phosphate and EDTA concentrations in the extraction mixture were high because of the fermentation and extraction conditions, respectively. These concentrations exceeded the recommended limits for optimal Benzonase activity (see Table II), and dilution and Mg^{2+} supplementation were required to achieve good NA degradation with minimal Benzonase use. The final conditions involved diluting the in-fermenter extract 2-fold with TE buffer and the addition of Benzonase (8.33 U mL^{-1} final concentration) and $MgCl_2$ (6 mM), to give 1.5 L of feed material at pH 8. This mixture was incubated overnight (12–14 h) at room temperature prior to diafiltration. As shown in Figure 4, a microfiltration run using this extract successfully removed 91% of soluble contaminant proteins after three volumes of buffer exchange in $\sim$6 h. Microfiltration efficiency was greatly improved both in terms of protein removal and flux. The filtration flux remained above $\sim$100 L m^{-2} h^{-1}, which will be acceptable for large-scale economic microfiltration (Kroner et al., 1984). Following diafiltration, the retentate was concentrated 2-fold to restore the IB concentration in the original chemical extract (i.e., to compensate for the 2-fold dilution prior to Benzonase treatment). The diafiltration retentate and the 2-fold concentrated retentate samples were sampled and sedimented by centrifugation to give a pellet and supernatant sample suitable for SDS-PAGE analysis. A sample of the feed to the microfiltration system (i.e., the in-fermenter extract without 2-fold dilution) was also retained and analysed in parallel. Figure 5 gives a comparison of these samples. It is clear that microfiltration gives an IB product (Fig. 5, sample 4) that is of similar purity to that obtainable by centrifugation (Fig. 5, sample 2). The retentates also contain very little soluble contaminating protein (as evidenced in Fig. 5, samples 5 and 7), and the IB yield following filtration and 2x concentration is comparable to that obtained by centrifugation of the undiluted feed material (as evidenced by comparable GM-CSF pellet bands in both sample 4 (microfiltration) and sample 2 (centrifugation)). SDS-PAGE analysis of permeate samples confirmed no loss of IBs through the microfiltration membrane (data not shown).

Denaturation and Refolding of GM-CSF

Two samples of cleaned IBs were tested for their refolding ability. The first sample was a control using IBs collected by centrifugation following extraction using the in-fermenter protocol (protocol 7 in Table I, no Benzonase treatment). The second sample consisted of IBs pelleted from the concentrated retentate (i.e., the final microfiltration sample generated above and analysed in Fig. 5, sample 4). Both samples were solubilised in denaturing buffer (7 M GuHCl, 50 mM DTT, 50 mM Tris, 1 mM EDTA, pH 8, overnight at 4°C). Analysis by RP-HPLC showed that the denatured samples were similar. Both samples also gave comparable refolding yields (23% for the concentrated retentate refolded

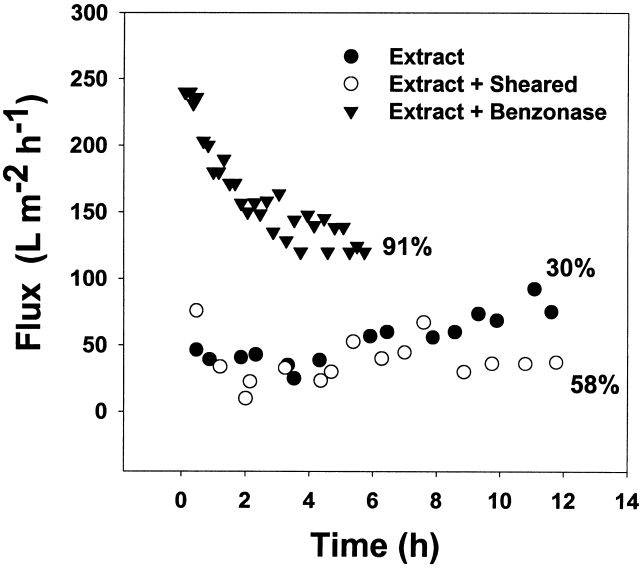


Figure 4. Flux versus time for cross-flow microfiltration runs using 2-fold diluted extract (closed circles), 2-fold diluted extract sheared with a hand-mixer device (open circles), and 2-fold diluted extract treated with Benzonase (closed triangles).

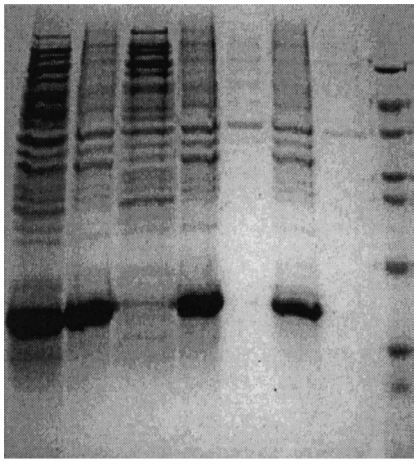


Figure 5. SDS-PAGE showing that the purity of IBs obtained by diafiltration were comparable to the pellet via centrifugation. (1, whole cell chemical extract; 2, pellet sample of the chemical extracts; 3, supernatant sample of the chemical extracts; 4, pellet sample of the 2× concentrated final retentate after 3 volumes of buffer exchange; 5, supernatant sample of the 2× concentrated final retentate after 3 volumes of buffer exchange; 6, pellet sample of the final retentate (the initial extract was diluted 1:1 with TE buffer); 7, supernatant sample of the final retentate.)

at a refolding protein concentration of 0.11 mg mL^{-1}, and 24% for the pelleted extract refolded at 0.08 mg mL^{-1}). This result confirms that the IBs were not affected by the extended Benzonase and diafiltration treatments, as the folding characteristics remained similar. Moreover, independent tests on IBs prepared using conventional methods suggest a refolding yield of 20–30% is reasonable for GM-CSF for the refolding buffer conditions employed in this work (Ho, 2003).

Further Discussion

The preceding results confirm that an in-fermenter extraction protocol for *E. coli* cells containing cytoplasmic IBs has been successfully developed, and that the extract can be processed by microfiltration to give a relatively pure IB suspension. Addition of powdered chaotrope to the final retentate will yield a solubilised IB mixture suitable for subsequent refolding. In this way a denatured protein ready for refolding can be obtained in a relatively pure form using only two unit operations: a fermenter and a microfiltration system. This process eliminates the need for repeated mechanical disruption and centrifugation and, importantly, allows the definition of a single integrate fermenter-filtration domain for validation purposes. Figure 6 provides a direct comparison of the conventional process with our modified process. The integrated process also offers scope for further intensification, as the IBs may be solubilised and refolded directly in the diafiltration system (Vicik and Clark, 1991). The current extraction protocol using a combination of cytoplasmic lysozyme, EDTA, and 0.05% (v/v) Triton X-100 effectively disrupts the *E. coli* wall and maintains the protective IB state during initial purification. The technique is effective even in the presence of fermentation media, eliminating the need for a cell recovery and resuspension in buffer. It is expected to be reasonably universal for disrupting *E. coli* cells; in the case where lysozyme is not constitutively expressed within the cell, the in vitro addition of lysozyme may prove to be an acceptable alternative.

Suboptimality remains in the current process because of the need to use costly Benzonase prior to microfiltration. For many products this cost will be minimal when offset against the savings achieved by the elimination of two major unit operations (e.g., homogenisation and centrifugation). However, Benzonase cost may present a barrier for some commodity protein products. For such products it may be possible to genetically modify the cells to express, toward the end of the fermentation cycle, enzymes that degrade nucleic acids. Such a strategy would mimic the approach used in this study to avoid in vitro lysozyme addition. A genetically modified *E. coli* strain capable of degrading nucleic acids has recently been developed by a team of academic and industrial researchers for use in large-scale bioprocessing (Cooke et al., 2003).

The viscosity increase caused by the release of nucleic acids proved to be the major problem that had to be overcome in this work. In previous filtration studies reporting the high removal of soluble protein (80–90%) (Meagher et al., 1994; Forman et al., 1990; Bailey and Meagher, 2000), the feed material for microfiltration was a cell suspension that had been mechanically disrupted. Homogenate produced by repeated mechanical treatment has a significantly reduced NA fragment size, giving a viscosity suitable for filtration. In another successful microfiltration study using 0.65 μm Nylon 66 membranes to recover a small soluble protein, Staphylokinase (SAK), from the permeabilised cell extract, cell permeability was controlled by adequate chemical selection to limit the release of DNA (Gehmlich et al., 1997).

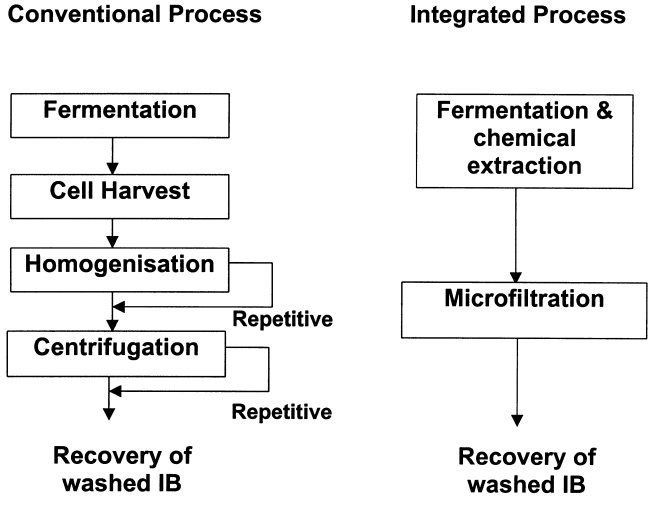


Figure 6. A comparison of the current integrated system with the conventional approach for IB processing.

In that study, only 25% of the total SAK was released in the supernatant by using the combination of 0.1% Triton X-100 and 0.1 M GuHCl to permeabilise the *E. coli* cells. Microfiltration yield and initial flux both dropped by about 33% when DNA release was enhanced from 5 to 10% due to higher degree of cell permeabilisation, confirming the significant deleterious effect that DNA has on filtration performance. However, partial permeabilisation will not be suitable for the current study, where the product is an insoluble IB. To investigate the viscosity problem further, DNA size was analysed using agarose gel electrophoresis. Figure 7 shows the results for samples following various treatments. Following chemical extraction and incubation, DNA in the suspension had a size greater than 12 kbp. The use of high-power of a hand-mixer to shear the DNA fragments reduced the fragment size to below ~10 kbp, a small improvement. This size range is still considerably larger than the 500 bp limit observed following repeated mechanical disruption (Choe, 2002). Initial experiments with Benzonase treatment showed that DNA molecules greater than 10 kbp could be degraded to <76 bp using 50 U mL^{-1} Benzonase following a 2-fold dilution of the in-fermenter extract (see Fig. 7). A slower digestion rate was observed under similar conditions when the extract was not diluted, presumably because of the high phosphate concentrations (at least 54 mM K_2HPO_4 and 16 mM of KH_2PO_4, exceeding the optimal limits defined in Table II). Also, no digestion was observed in either case when $MgCl_2$ was not added to the extract to bind EDTA used in the extraction procedure. Tests showed that 6 mM $MgCl_2$ was sufficient to bind the 2.5 mM of EDTA in the diluted extract while also providing sufficient enzyme cofactor. Dilution of the extract, though not critical for DNA degradation, was anticipated to significantly improve the digestion rate, allowing a significant reduction in Benzonase concentration. In subsequent experiments aimed at reducing Benzonase concentration, 2-fold diluted extract was treated at room temperature with 6 mM $MgCl_2$ at 10–50 U mL^{-1} of Benzonase. Figure 7 shows that DNA fragment size can be easily reduced below 220 bp in 30 min using >20 U mL^{-1} Benzonase, with a significant improvement in performance following overnight incubation. Even a low concentration of 10 U mL^{-1} of Benzonase could achieve an acceptable DNA size following overnight incubation.

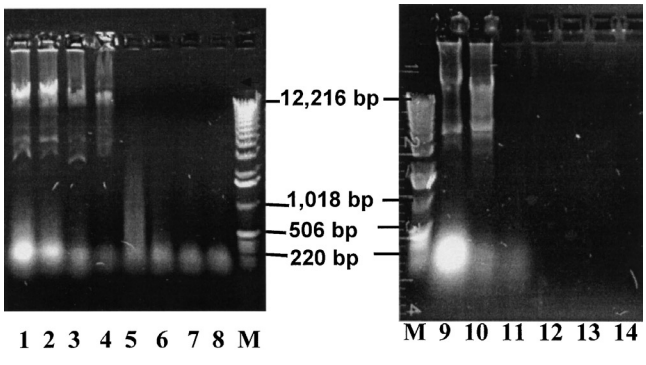


Figure 7. Agarose electrophoresis results for optimizing the chemical environment for Benzonase treatment. (1, original extract without Benzonase, no dilution and no $MgCl_2$; 2, similar to 1, but with addition of 12 mM $MgCl_2$; 3, similar to 1, but extract was 2-fold diluted; 4, similar to 1 but with addition of 6 mM of $MgCl_2$ and 2-fold diluted; 5: 2-fold diluted extract, with 10 U mL^{-1} of Benzonase, in the presence of 6 mM $MgCl_2$; 6, 2-fold diluted extract, with 20 U mL^{-1} of Benzonase, in the presence of 6 mM $MgCl_2$; 7, 2-fold diluted extract, with 30 U mL^{-1} of Benzonase, in the presence of 6 mM $MgCl_2$; 8, 2-fold diluted extract, with 50 U mL^{-1} of Benzonase, in the presence of 6 mM $MgCl_2$. 1–8 were incubated for 30 min; 9, overnight sample of 2; 10, overnight sample of 4; 11, overnight sample of 5; 12, overnight sample of 6; 13, overnight sample of 7; 14, overnight sample of 8. M, DNA Marker.

CONCLUSIONS

The viability of recovering GM-CSF IBs expressed within *E. coli* using nonsolubilising chemical extraction and cross-flow microfiltration was demonstrated. The extraction protocol, based on a combination of Triton X-100, EDTA, and intracellular T7 lysozyme, effectively released most of the intracellular soluble content without solubilising the GM-CSF IBs. A cross-flow microfiltration using a 0.2 μm ceramic membrane successfully recovered the GM-CSF IBs with removal of 91% of the soluble contaminants and virtually no loss of IBs to the permeate. The filtration efficiency both in terms of flux and transmission level was enhanced significantly by reducing the DNA fragment size with Benzonase digestion. Both the extraction and filtration methods exerted their efficacy directly on a crude fermentation broth, eliminating a step for medium removal. The recovered IBs had purity comparable to that obtained via centrifugation following similar chemical extraction. We also demonstrated that, following extraction and Benzonase treatment, the IBs recovered from the retentate of a microfiltration run exhibited similar refolding characteristic to those recovered by centrifugation. Importantly, the processes demonstrated here can all be performed using just a fermenter and a single unit of cross-flow filtration unit, demonstrating a high level of IB process intensification. Furthermore, there is considerable scope to also use a microfiltration system to subsequently solubilise the IBs, to separate the denatured protein from cell debris, and to refold the protein using diafiltration. In this way refolded protein can potentially be obtained, in a relatively pure state, using only two unit operations.

References

Babbitt PC, West BL, Buechter DD, Chen IH, Kuntz ID, Kenyon GL. 1991. Active reatine-kinase refolded from inclusion-bodies in *Escherichia coli* — improved recovery by removal of contaminating protease. ACS Symp Ser 470:153–168.

Bailey SM, Meagher MM. 1997. Crossflow microfiltration of recombinant *Escherichia coli* lysates after high pressure homogenisation. Biotechnol Bioeng 56:304–310.

Bailey SM, Meagher MM. 2000. Separation of soluble protein from

inclusion bodies in *Escherichia coli* lysate using crossflow microfiltration. J Membr Sci 166:137–146.

Bradford MM. 1976. A rapid and sensitive method for quantification of microquantities of protein utilizing the principle of protein-dye binding. Anal Biochem 72:248–254.

Choe WS. 2002. The intensification of inclusion bodies processing. PhD Thesis, University of Cambridge, UK.

Choe WS, Middelberg APJ. 2001a. Direct chemical extraction of a recombinant viral coat protein from *Escherichia coli* at high cell density. Biotechnol Bioeng 75:451–455.

Choe WS, Middelberg APJ. 2001b. Selective precipitation of DNA by spermine during the chemical extraction of insoluble cytoplasmic protein. Biotechnol Prog 17:1107–1113.

Choe WS, Clemmitt RH, Chase HA, Middelberg APJ. 2002. Comparison of histidine-tag capture chemistries for purification following chemical extraction. J Chromatogr A 953:111–121.

Clark ED, Schwarz E, Rudolph R. 1999. Inhibition of aggregation side reactions during in vitro protein folding. Methods Enzymol 309: 217–236.

Cooke GD, Cranenburgh RM, Hanak JAJ, Ward JM. 2003. A modified *Escherichia coli* protein production strain expressing staphylococcal nuclease, capable of autohydrolysing host nucleic acid. J Biotechnol 101:229–239.

Falconer RJ, O'Neill BK, Middelberg APJ. 1998. Chemical treatment of *Escherichia coli*. II. Direct extraction of recombinant protein from cytoplasmic inclusion bodies in intact cells. Biotechnol Bioeng 57:381–386.

Falconer RJ, O'Neill BK, Middelberg APJ. 1999. Chemical treatment of *Escherichia coli*. III. Selective extraction of a recombinant protein from cytoplasmic inclusion bodies in intact cells. Biotechnol Bioeng 62:455–460.

Forman SM, Debernardez ER, Feldberg RS, Swartz RW. 1990. Crossflow filtration for the separation of inclusion bodies from soluble-proteins in recombinant *Escherichia coli* cell lysate. J Membr Sci 48:263–279.

Gehmlich I, Pohl HD, Knorre WA. 1997. Laboratory-scale permeabilization of *Escherichia coli* cells for recovery of a small recombinant protein — Staphylokinase. Bioprocess Eng 17:35–38.

Hettwer D, Wang H. 1989. Protein release from *Escherichia coli* cells permeabilized with guanidine-HCl and Triton X-100. Biotechnol Bioeng 33:886–895.

Ho JGSH. 2003. An investigation of protein-protein interactions during renaturation. Ph.D. Thesis, University of Cambridge, UK.

Kroner KH, Schutte H, Hustedt H, Kula MR. 1984. Cross-flow filtration in the downstream processing of enzymes. Process Biochem 19:67–74.

Marston FAO. 1986. The purification of eukaryotic polypeptides synthesized in *Echerichia coli*. Biochem J 240:1–12.

Meagher MM, Barlett RT, Rai VR, Khan Fr. 1994. Extraction of rIL-2 inclusion bodies from *Escherichia coli* using cross-flow filtration. Biotechnol Bioeng 43:969–977.

Middelberg APJ. 1995. Process-scale disruption of microorganisms. Biotechnol Adv 13:491–551.

Middelberg APJ. 2002. Preparative protein refolding. Trends Biotechnol 20:437–443.

Mitraki A, Haasepettingell C, King J. 1991. Mechanisms of inclusion body formation. ACS Symp Ser 470:35–49.

Naglak TJ, Hettwer DJ, Wang HY. 1990. Chemical permeabilization of cells for intracellular product release. In: Asenjo JA, editor. Separation processes in biotechnology. New York: Marcel Dekker. p 177–205.

Novella IS, Fargues C, Grévillot G. 1994. Improvement of the extraction of penicillin acylase from *Escherichia coli* cells by a combined use of chemical methods. Biotechnol Bioeng 44:379–382.

Studier FW. 1991. Use of bacteriophage T7 lysozyme to improve an inducible T7 expression system. J Mol Biol 219:37–44.

Vicik S, Clark DE. 1991. An engineering approach to achieving high-protein refolding yield. In: Protein refolding. ACS Symp Ser 470:180–196.

Wong HH, ONeill BK, Middelberg APJ. 1996. Centrifugal recovery and dissolution of recombinant Gly-IGF-II inclusion-bodies: the impact of feedrate and re-centrifugation on protein yield. Bioseparation 6: 185–192.