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A strategic crossflow filtration methodology for the initial purification of promegapoietin from inclusion bodies

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ABSTRACT

A novel crossflow filtration methodology is demonstrated for the initial purification of the therapeutic protein, promegapoietin-1a (PMP), produced as inclusion bodies (IBs) in a recombinant *Escherichia coli* bioprocess. Two strategic separation steps were performed by utilizing a filtration unit with a 1000 kDa polyethersulphone membrane.

The first step, aiming for separation of soluble contaminants, resulted in a 50% reduction of the host cell proteins, quantified by total amino acid analysis and a 70% reduction of all DNA, quantified by fluorometry, when washing the particulate material with a 10 mM EDTA in 50 mM phosphate buffer, pH 8. The second step, aiming for separation of particulate contaminants from solubilized IBs, resulted in a 97–99.5% reduction of endotoxin, used as a marker for cell debris, and was quantified by the kinetic turbidimetric LAL endotoxin assay.

The overall PMP yield was 58% and 33% respectively for the two solubilizations investigated, guanidine hydrochloride and arginine, as measured by RP-HPLC. The scope was also to investigate the physical characteristics of the intermediate product/s with regard to the choice of IB solvent. Preliminary results from circular dichroism spectroscopy measurements indicate that the protein secondary structure was restored when arginine was used in the second step.

1. Introduction

The recombinant DNA technology has facilitated the production of proteins in different host cells. Many therapeutic proteins are produced in the bacterium *Escherichia coli* (*E. coli*). However, recombinant proteins produced in *E. coli* often result in the formation of insoluble protein inclusion bodies (IBs) and consequently the protein has to be extracted and purified from these aggregates (Graumann and Premstaller, 2006).

The common route for the initial handling when inclusion bodies are produced is by extraction of the IBs by mechanical disruption of the bacterial cells resulting in a lysate/IB slurry followed by purification of the IBs through reduction of the soluble contaminant load by either centrifugation or filtration. These steps are followed by solubilization of the IBs by addition of protein denaturants and finally, renaturation of the product protein into the biologically active conformation through dilution or dialysis (Choe et al., 2006). The primary protein yield after fermentation is often high when IBs are formed, but depending on the outcome of the initial purification steps, the total intermediate yield of the renatured protein often becomes drastically reduced (Choe et al., 2006; De Bernandez Clark, 2001). For example, the extraction step results in a very complex mixture when all the interior cell materials come out from the disruption, i.e. IBs, cell debris, host cell proteins (HCP), host DNA, and endotoxin. It is known that residual contaminants may negatively affect the renaturation performance (Shire et al., 1984; Maachupalli-Reddy et al., 1997; Thatcher and Hitchcock, 1994; Batas et al., 1999; Leong and Middelberg, 2007).

In the study by Patra et al. (2000), IBs of recombinant human growth hormone were washed with 50 mM Tris buffer, pH 8.0 containing 5 mM EDTA and 2% deoxycholate and centrifuged at 8000 × *g* for 30 min. In another study (Valente et al., 2006), IBs of recombinant human interferon α2b were washed either with 1% Triton X-100 (detergent) in 50 mM Tris, pH 8.0 or 20 mM EDTA in 50 mM Tris, pH 8.0 and centrifuged at 5000 × *g* for 10 min. SDS-PAGE was used for the protein purity estimations in both the above studies and resulted in approximately 90% and 24% purity, respectively. In a comparison between the effects of centrifugation and membrane filtration on the purification of recombinant hen egg white lysozyme IBs (Batas et al., 1999), it was found that the most significant purification occurred during removal of cell debris. Additives of EDTA, urea and Triton X-100 gave moderate improvements in the centrifugation washes. In the microfiltration washes, the 0.45 μm cut-off membrane gave purer IBs than the membrane with smaller cut-off size, but none of the membranes gave purer IBs than the centrifugation washes. In this case, the aim was to direct the

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cell debris through the membrane while keeping the IBs on the retentate side.

van Hee et al. (2004) characterized the cell debris after cell disruption. They found that enzymatic treatment gave the highest IB yield, but the smallest cell debris particles, while high pressure homogenization gave larger cell debris particles but poorer IB yields. In this case, when centrifugation was used for separation, the larger cell debris particles were favored. These findings confirm that optimal purification varies from case-to-case depending on the size and density of the cell debris. Thus, a methodology that is different from the conventional methods is needed to compensate for this separation problem.

The use of crossflow microfiltration in the purpose of *E. coli* lysate clarification has been restricted because of protein fouling onto some of the membrane materials used, leading to poor flux and transmission (Venkiteshwaran et al., 2007). Bailey and Meagher (1997a,b) investigated the performance of crossflow membrane filtration of *E. coli* lysates by varying different types of membrane materials, different types of washing additives, and by using different cell disruption conditions. When further investigating the crossflow microfiltration of IB-slurry it was found that the flux remained high throughout the process with minimal fouling when using the 0.1 μm polyethersulphone (PES) membrane (Bailey and Meagher, 2000). Recently, Venkiteshwaran et al. (2007) investigated the optimization of pH and ionic strength of the washing solution on the HCP removal performance using different crossflow filtration membranes. They obtained about 90% removal of soluble HCP in about 5 diafiltration volumes (DVs). In this case the purification was focused on the soluble contaminants.

Rather than aiming to wash out only one of the contaminants (soluble or particulate materials), a methodology that could separate both kinds of materials would be more beneficial.

The rationale behind this study was to look at an alternative approach for the purification and solubilization of IBs that could hasten the commercialization of new biopharmaceuticals. To facilitate this, a novel strategy for the initial purification of the IBs that would benefit the overall process was looked for.

Recently Choe et al. (2006) and Lee et al. (2004) suggested that crossflow microfiltration might be used for the separation of cell debris and the product protein after solubilization of the IBs, but the successful implementation of this methodology has not, to our knowledge until now been reported.

In this study we have used a scalable method, with industrial applicability in mind, for the initial purification of IBs and focused on the reduction of both the soluble host cell materials (HCP and DNA) and the particulate contaminants (cell debris). By utilizing crossflow microfiltration equipment with a 1000 kDa PES membrane, a membrane with known minimal protein fouling (Bailey and Meagher, 2000) and a membrane cartridge with open channel screens developed for viscous solutions, the same processing unit has been used in two processing steps. First, the membrane was used for the purification of IBs and the removal of soluble contaminants and next, it was used for the separation between protein product and cell debris after solubilization of the IBs with guanidine hydrochloride (GdnHCl) or arginine, see Fig. 1. Using this approach, the initial purification process becomes more flexible and robust, since particulate contaminants that stay in the product fraction in the first step may be reduced in the second step, adding value to the whole process. SDS-PAGE and densitometry have in previous studies (Patra et al., 2000; Valente et al., 2006; Batas et al., 1999) been used as purity estimation methods, but since those methods only generate rough estimates we elected to use total amino acid analysis for the total protein measurements and reversed phase high performance liquid chromatography (RP-HPLC) for the product protein measurements.

The protein in this study, PMP, is a human recombinant fusion cytokine protein consisting of engineered interleukin-3 and thrombopoietin receptor agonist moieties linked by a flexible IgG hinge sequence. It has been investigated as a haemopoietic and/or immune recovery therapeutic agent for use in patients undergoing bone-marrow transplantation (Doshi et al., 2001; Farese et al., 2001). PMP contains 306 amino acids with a molecular mass of 33,263 Da. More recently, Boyle et al. (2008) proposed a refolding procedure as part of a production process for this protein. They were able to produce 40–50% refolded protein by optimizing the addition of urea and thiol-group-containing additives and control of dilution, pH, protein concentration and their order of addition.

2. Materials and methods

2.1. E. coli cell growth, harvest and homogenization

E. coli MON105 containing the rpoH358 chromosomal mutation (Obukowicz et al., 1992) with the ATCC number 55204, was kindly provided by Pfizer AB (Sweden). The cultivation medium used has previously been described (Sundström et al., 2004).

E. coli MON105 was inoculated into the shake flask medium and cultivated at 37 °C until the optical density at 550 nm (OD_{550}) reached about 7. Fed-batch cultivation was performed in a 100 l bioreactor (Belach Bioteknik AB, Sweden) with an initial volume of 90 l and an inoculum volume of 2 l. NH_4OH (25%, v/v) was added as required to maintain the pH at 7 and temperature was controlled at 37 °C. A feed of 50% (w/w) glucose solution, was started when the initial concentration of glucose was below 6 g l^{-1}. The feed of glucose was manually regulated to maintain the glucose concentration between 2 and 6 g l^{-1}. When OD_{550} was about 7, approximately 7.5 h after inoculation, the recombinant protein production was induced with nalidixic acid (0.05 g l^{-1}) and the cultivation was terminated 6 h after induction, at OD_{550} about 13.

After cultivation, the bacterial cells were harvested by crossflow filtration with a 1 m^2 1000 kDa PES membrane (Biomax, Millipore) in two steps. First the biomass was concentrated from 92 to 15 l and subsequently diafiltered with 3 DVs of water to wash out the residual growth media.

The washed biomass concentrate was homogenized at 600 bar in a high pressure homogenizer by three homogenization passes. Finally, the lysate/IB-slurry was divided into 50 ml polypropylene tubes and kept frozen at −20 °C until further use.

2.2. Separation of soluble contaminants

Different experiments were conducted for the screening of the optimal wash solution for the removal of soluble contaminants. For each experiment, one 50 ml tube of IB-slurry was thawed and diluted with 450 ml of water. The diluted IB-slurry was diafiltered with different wash solutions in different experiments. The filtration set-up and control system (ProFlux® M12 tangential filtration system, Millipore AB, Sweden) consisted of a 3 l reservoir, a membrane cartridge holder, a peristaltic crossflow pump, in- and outlet manometers, manual retentate- and filtrate valves, a conductivity level sensor attached to the reservoir and an automatically controlled peristaltic pump for the addition of wash solution (diafiltration solution). The membrane used was a 0.1 m^2 1000 kDa PES membrane (same membrane type as used for the harvest but with a smaller membrane area) with open channel screens for high viscous solutions (Biomax, Millipore). By use of this set-up the retentate volume was kept constant at 500 ml throughout each experiment. The crossflow was kept constant at 580 l m^{-2} h^{-1} (lmh) throughout the experiment series (manufacturer's recommendation). The filtrate flux and the inlet pressure to the filter were registered for