Purification of recombinant proteins for pharmaceutical use

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Estimates of the potential market size for genetically engineered pharmaceutical products have varied widely over the past several years. The 1988 market size has been estimated at about $300 million and this is forecast to reach about $4000 million by 1995 [1]. It is difficult to be sure of the accuracy of any of these figures; however, with nine biotechnology-derived products currently on the market, and an apparently endless list of potential products in clinical trials or at earlier stages of development, the future appears very bright indeed [2].

There are many stages involved in preparing a biotechnology-derived pharmaceutical product to the market, these include: (i) identification of a potential product and its biological function; (ii) preparation of recombinant plasmid and introduction into the host cell; (iii) development of fermentation (tissue culture) process; (iv) separation of the protein of interest from contaminating material; (v) preparation of product for clinical testing; (vi) scale-up — design, implementation and validation of the manufacturing processes.

This paper reviews some of the issues which effect large-scale purification of proteins for pharmaceutical use. The development and scale-up of efficient methods for the separation and purification of proteins is a major goal for producers of biotechnology-derived products, including therapeutic drugs. Extensive recovery and purification processes are required to achieve a highly pure protein which is safe for use in humans. The purification process can significantly effect the yield, stability, purity and cost of the final product. At Avondale (Brinny) Chemical Company, the Irish subsidiary of Schering-Plough Corporation, we have during the past 2 years set-up production-scale purification processes for two recombinant-DNA-derived pharmaceuticals, a-2b interferon and GMCSF, both intracellular proteins, which we produce using genetically engineered Escherichia coli. After fermentation and cell harvesting the proteins are released by a process which involves incomplete disruption of the cell wall and membrane, enabling extraction of the protein without full cell disruption.

Cell harvesting/disruption
The first step in downstream processing is the separation of cells from the growth medium or broth. This is achieved by centrifugation (usually continuous flow) or by filtration. Both processes are relatively straightforward; however, it is important that the centrifugation/filtration capacity is matched to the volume of broth to be treated. It is also important that the two major causes of protein inactivation, i.e. high temperature and shear, are avoided [4]. Rapid processing of large volumes can be achieved using high capacity desludgers. For intracellular proteins, harvesting of cells is followed by protein release, which can be carried out by a variety of techniques such as mechanical cell disruption, osmotic shock or treatment with lytic enzymes. The resulting suspension can be further clarified by precipitation, followed by centrifugation or filtration.

Crude extraction/fractionation
The objective of this stage is to remove contaminating proteins present in large quantities such as cell debris or carry over from the growth medium. The procedures used include precipitation, centrifugation and filtration. Precipitation can be achieved by salting out using reagents such as ammonium sulphate or by reducing the dielectric constant using organic solvents such as ethanol. Isoelectric point precipitation can also be used however this must be taken to avoid localized changes in pH which can irreversibly deactivate proteins [4]. After precipitation, the solid and liquid phases are separated by centrifugation or filtration.

Protease inactivation and removal
The problem of inactivation through proteolytic activity is critical during early stages of purification [3]. In large-scale operations, increased processing times and more difficult temperature control amplifies the problem. Rapid removal of proteases or their inactivation is essential and failure to do so can result in product loss and low yields.

Purification
The combination of high-level expression and selective extraction steps summarized above should yield a mixture of product and contaminants that contains a substantial product concentration. Removing the contaminating material, while minimizing loss or damage to the protein of interest is the objective of the subsequent purification steps. Special attention should be directed towards the removal of detectable viral, microbial and nucleic acid contamination and undesirable antigenic materials. Because recombinant-DNA-derived products are produced by living organisms, the removal of nucleic acids from the final product is an essential requirement for product safety. W.H.O. guidelines require less than 100 pg of DNA per dose in the final product. If antibodies are used in affinity-purification steps, then these must also be shown to be free from unwanted substances such as viruses or residual DNA [5].

A typical purification system involves a number of steps such as cation- or anion-exchange chromatography, affinity...
chromatography or gel filtration. The selection of a specific separation system depends on the product concentration and the nature of contaminating proteins to be removed. Optimization of each step in a sequence is usually performed using small-scale equipment. After the process has been optimized, scale-up can be attempted. This requires that column sizes, packing materials, flow rates, buffer pH and ionic strengths be identified. To ensure good resolution, it is essential that columns be packed correctly, inadequate packing results in channelling and poor protein separation. Scale-up of chromatographic procedures is achieved by increasing column diameter and maintaining a constant bed height. Volumetric ratios of feed, wash and elution buffers are also held constant. The main factors which determine the acceptability of a chromatographic procedure are: (i) resolution (selectivity); (ii) recovery and (iii) capacity [3].

Other factors also need to be considered in scaling up chromatographic procedures. These include flow rates, gel cost, number of cycles before gel change, pH, use of hazardous materials, ease of column packing and preparation and regeneration requirements. Most column chromatography operations are carried out at refrigerated temperatures to minimize microbial growth in buffers, product or on the columns. When a procedure has been established, the yield after each process step should be determined and included in the process specifications. The purification process must be carried out under constant process conditions to ensure a final purified product of consistent quality from batch to batch. Specifications for purified material must exist including rejection criteria for batches which do not meet specification.

**Regulatory requirements**

The manufacture and distribution of biological products for human use, including those derived from biotechnology, are regulated by the F.D.A., in the case of the U.S.A., and by equivalent agencies in other countries. Regulations must be complied with through the product development, clinical testing, plant design, scale up, manufacturing, testing and final approval of all pharmaceutical products. A company must obtain a licence for the manufacturing plant and for each product, before its distribution for sale in the U.S.A. Before a licence is issued, the manufacturer must demonstrate that the product is safe and effective and that the manufacturing facility complies with the requirements of GMP (good manufacturing practices) [6]. The GMP requirements cover topics such as personnel, documentation, plant/equipment, material control, storage and testing requirements. The F.D.A. also requires a description of the production process, with full details of each step, including equipment, location, operating parameters, raw material specifications, standard operating procedures and quality control specifications. All of the above information is submitted to the F.D.A. in the form of a product licence application (PLA) [6].

Once a licence has been issued, any change in the production process must be covered by a licence amendment. If a change is significant, such as a switch to a new purification system which results in lower cost and higher yield, or even improved quality, it may be necessary to carry out clinical trials using the product from the new process to demonstrate equivalency. Such trials are extremely expensive and time consuming and may take several years for completion and approval.

**Plant.** The purification facility must be designed and constructed to a high standard. Floors should be finished so as to facilitate cleaning and sanitation and prevent accumulation of potential microbial contaminants. Floors should be sloped to drain and should not have open drains or areas where water can accumulate. Product flow during processing must be arranged in a manner which eliminates risk of mix up. The water system should be designed so that the water used for buffer preparation, equipment washing and rinsing and other production operations is of high quality, preferably meeting U.S. Pharmacopeia specifications for purified water. Water used in the final stages should be of water for injection quality.

The water system must be validated to ensure that the specifications for chemical quality, pyrogen level and microbial content are in-control and meet the required specifications. In addition, the water must be routinely tested to ensure that it meets specification during production operations. Nitrogen and compressed air are the most commonly used gases in biotechnological production processes. Nitrogen must be filtered through a 0.2 μm filter before use, compressed air should be free of oil, dry and filtered through an 0.2 μm filter before use.

**HVAC systems.** It is becoming increasingly necessary to carry out protein purification operations in rooms in which the environment is controlled using HEPA-filtered air. These areas (including cold rooms) should maintain class 100000 conditions during normal operations and should have a minimum of 20 air changes/hour. These requirements are necessary to minimize the risk of microbial contamination of the product during the production operation. The final stages of protein purification, e.g. (sterile filtration) should be carried out under the laminar flow hood.

**Validation.** Validation studies of the purification processes must also be completed. These must confirm the capacity of the purification procedure to remove unwanted host-cell-derived proteins, nucleic acids, carbohydrates, viruses and other impurities [7]. Validation studies must also demonstrate the reproducibility of each stage of the purification process and must show (i) what is filtered at each step of the purification process; (ii) identification of impurities; (iii) removal of endotoxins; and (iv) microbial counts. Column-regeneration procedures must also be established. These involve washing the resins using sodium hydroxide or other reagents between purification runs. The objective is to prevent build-up of lipid or protein residues on the columns and also reduce growth of bacteria and other micro-organisms which may cause product contamination.

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**Personnel.** It has been our experience over the last several years that it is difficult to recruit qualified personnel with experience in
the area of protein purification. This is also the situation in the U.S.A., where the increase in the number of biotechnology firms has occurred at the same time as a reduction in number of graduates in the biological sciences. In addition to the science involved in protein purification procedures, there is also a certain level of art involved in packing and preparation of large-scale columns. Proper column preparation and maintenance is essential if protein separation is to take place correctly. Chromatographic columns are the most important separation tools used to achieve high yield and purity targets in protein purification. Experience in this area is, therefore, extremely valuable for any individual seeking employment. It is likely that over the next several years, many job opportunities will arise for those qualified and experienced in the area of protein purification as the number of firms involved in the research and production of biotechnology-derived proteins increases.


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Large-scale manufacture of monoclonal antibodies for use in humans

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Introduction

In 1975 Kohler & Milstein [1] developed the hybridoma technique which allowed for the first time the production of monoclonal antibodies recognizing specific antigens of choice. The exquisite specificity of monoclonal antibodies, combined with the potential for producing them reproducibly in unlimited quantities, has led to their widespread application in many areas of biological research. This has resulted in the development of a large number of commercial applications with increasing emphasis on the development of monoclonal antibodies for human therapeutic use. This paper will consider some of the issues associated with process development for large-scale production of proteins from mammalian cells. Particular emphasis will be placed on the fermentation process and quality issues for those proteins required for human therapeutic use.

Production of monoclonal antibodies

Laboratory-scale production of monoclonal antibodies is generally carried out by growing hybridoma cells either in small-scale cell-culture systems, such as flasks and roller bottles, or in rodents as ascites tumours. Production in animals becomes less attractive as the scale of production increases. If it is assumed that on average one mouse can produce 50 mg of monoclonal antibody, then it would require 20000 mice to produce 1 kg of antibody.

Various cell culture methods have been proposed for the large-scale production of monoclonal antibodies [2]. These can broadly be defined as those that require immobilization or entrapment of the cells and those in which the cells are grown in homogeneous suspension culture. The selected system must be capable of satisfying a number of criteria. It is imperative that microbial infection is prevented throughout the prolonged fermentation which may extend from 1 or 2 weeks for a batch culture to several months for a continuous process. The method of agitation must be compatible with shear-sensitive mammalian cells and it is important to develop a unit process which can be scaled up as required.

At Celltech we have opted for a homogeneous suspension culture process using airlift reactors as the system which best meets the criteria given above. The growth of animal cells in this type of reactor was first described by Katinger [3], who considered it to have particularly appropriate characteristics for shear-sensitive cells. The principle of the airlift reactor is very simple. Gas mixtures are introduced into the base of a concentric draught