3 Production and Purification of Recombinant Proteins

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INTRODUCTION

The growing therapeutic use of proteins has created an increasing need for practical and economical processing techniques. As a result, biotechnological production methods have advanced significantly over the last decade. Also, single-use production technology which has the potential to mitigate many of the economic and quality issues arising from manufacturing these products has evolved rapidly (Hodge 2004).

When producing proteins for therapeutic use, a number of issues must be considered related to the manufacturing, purification, and characterization of the products. Biotechnological products for therapeutic use have to meet strict specifications especially when used via the parenteral route (Walter and Werner 1993).

In this chapter several aspects of production (upstream processing) and purification (downstream processing) will be dealt with briefly. For further details, the reader is referred to the literature mentioned.

UPSTREAM PROCESSING

■ Expression Systems

General Considerations

Expression systems for proteins of therapeutic interest include both pro- and eukaryotic cells (bacteria, yeast, fungi, plants, insect cells, mammalian cells) and transgenic animals. The choice of a particular system will be determined to a large extent by the nature and origin of the desired protein, the intended use of the product, the amount needed, and the cost.

In principle, any protein can be produced using genetically engineered organisms, but not every type of protein can be produced by every type of cell. In the majority of cases, the protein is foreign to the host cells that have to produce it, and although the translation of the genetic code can be performed by the cells, the posttranslation modifications of the protein might be different as compared to the original product.

About 5 % of the proteome are thought to comprise enzymes performing over 200 types of posttranslation modifications of proteins (Walsh 2006). These modifications are species and/or cell-type specific. The metabolic pathways that lead to these modifications are genetically determined by the host cell. Thus, even if the cells are capable of producing the desired posttranslation modification, like glycosylation, still the resulting glycosylation pattern might be different from that of the native protein. Correct N-linked glycosylation of therapeutically relevant proteins is important for full biological activity, immunogenicity, stability, targeting, and pharmacokinetics. Prokaryotic cells, like bacteria, are sometimes capable of producing N-linked glycoproteins. However, the N-linked structures found differ from the structures found in eukaryotes (Dell et al. 2011). Yeast cells are able to produce recombinant proteins like albumin, and yeast has been engineered to produce glycoproteins with humanlike glycan structures including terminal sialylation (reviewed by Celik and Calik 2011). Still most products on the market and currently in development use cell types that are as closely related to the original protein-producing cell type as possible. Therefore, human-derived proteins, especially mammalian cells, are chosen for production. Further developments in the field of engineering (e.g., glycosylation) may allow bacteria to reproduce some of the posttranslation modification steps common to eukaryotic

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cells (Borman 2006). Although still to be further developed, bacteria and yeast may play a role as future production systems given their ease and low cost of large-scale manufacturing.

Generalized features of proteins expressed in different biological systems are listed in Table 3.1 (see also Walter et al. 1992). However, it should be kept in mind that there are exceptions to this table for specific product/expression systems.

Transgenic Animals

Foreign genes can be introduced into animals like mice, rabbits, pigs, sheep, goats, and cows through nuclear transfer and cloning techniques. Using milk-specific promoters, the desired protein can be expressed in the milk of the female offspring. During lactation the milk is collected, the milk fats are removed, and the skimmed milk is used as the starting material for the purification of the protein.

The advantage of this technology is the relatively cheap method to produce the desired proteins in vast quantities when using larger animals like cows. Disadvantages are the long lead time to generate a herd of transgenic animals and concerns about the health of the animal. Some proteins expressed in the mammary gland leak back into the circulation and cause serious negative health effects. An example is the expression of erythropoietin in cows. Although the protein was well expressed in the milk, it caused severe health effects and these experiments were stopped.

The purification strategies and purity requirements for proteins from milk can be different from those derived from bacterial or mammalian cell systems. Often the transgenic milk containing the recombinant protein also contains significant amounts of the nonrecombinant counterpart. To separate these closely related proteins poses a purification challenge. The "contaminants" in proteins for oral use expressed in milk that is otherwise consumed by humans are known to be safe for consumption.

The transgenic animal technology for the production of pharmaceutical proteins has progressed within the last few years. The US and EU authorities approved recombinant antithrombin III (ATryn®, GTC Biotherapeutics) produced in the milk of transgenic goats. More details about this technology are presented in Chap. 8.

Plants

Therapeutic proteins can also be expressed in plants and plant cell cultures (see also Chap. 1). For instance, human albumin has been expressed in potatoes and tobacco. Whether these production vehicles are economically feasible has yet to be established. The lack of genetic stability of plants was sometimes a drawback. Stable expression of proteins in edible seeds has been obtained. For instance, rice and barley can be harvested and easily kept for a prolonged period of time as raw material sources. Especially for oral therapeutics or vaccines, this might be the ideal solution to produce large amounts of cheap therapeutics, because the "contaminants" are known to be safe for consumption (see also Chap. 21). A better understanding of the plant molecular biology together with more sophisticated genetic engineering techniques and strategies to increase yields and optimize glycan structures resulted in an increase in the number of products in development including late-stage clinical trials (reviewed by Orzaez et al. 2009, and Peters and Stoger 2011). Removal of most early bottlenecks together with the regulatory acceptance of plants as platforms to produce therapeutic proteins has resulted in a renewed interest by the industry.

Protein feature	Prokaryotic bacteria	Eukaryotic yeast	Eukaryotic mammalian cells
Concentration	High	High	High
Molecular weight	Low	High	High
S-S bridges	Limitation	No limitation	No limitation
Secretion	No	Yes/no	Yes
Aggregation state	Inclusion body	Singular, native	Singular, native
Folding	Risk of misfolding	Correct folding	Correct folding
Glycosylation	Limited	Possible	Possible
Impurities: retrovirus	No	No	Possible
Impurities: pyrogen	Possible	No	No
Cost to manufacture	Low	Low	High

Table 3.1 ■ Generalized features of proteins of different biological origin.

More details about the use of plant systems for the production of pharmaceutical proteins are presented in Chap. 7.

■ Cultivation Systems

General

In general, cells can be cultivated in vessels containing an appropriate liquid growth medium in which the cells are either immobilized and grow as a monolayer, attached to microcarriers, free in suspension, or entrapped in matrices (usually solidified with agar). The culture method will determine the scale of the separation and purification methods. Production-scale cultivation is commonly performed in fermentors, used for bacterial and fungal cells, or bioreactors, used for mammalian and insect cells. Bioreactor systems can be classified into four different types:

- Stirred tank (Fig. 3.1a)
- Airlift (Fig. 3.1b)
- Fixed bed (Fig. 3.1c)
- Membrane bioreactors (Fig. 3.1d)

Because of its reliability and experience with the design and scaling up potential, the stirred tank is still the most commonly used bioreactor. This type of bioreactor is not only used for suspension cells like CHO, HEK293, and PER.C6® cells, it is also used for production with adherent cells like Vero and MDCK cells. In the latter case the production is performed on microcarriers (Van Wezel et al. 1985).

Single-Use Systems

In the last decade the development of single-use production systems was boosted. Wave Biotech AG in Switzerland (now Sartorius Stedim Biotech) is regarded as a visionary player in the nurture of single-use technologies for mammalian cell culture. With the development of 3D single-use bags by companies like Hyclone, Xcellerex, and Sartorius, the number of single-use systems used has increased. Single-use bioreactors are nowadays used for the manufacturing of products in development and on the market. Shire (Dublin, Ireland) was the first company that used single-use bioreactors up to 2,000 L for the manufacturing of one of their products. The advantages of the single-use technology are:

- Cost-effective manufacturing technology
  By introducing single-use systems, the design is such that all items not directly related to the process can be removed from the culture system, like clean-in-place (CIP) and steam-in-place (SIP) systems. Furthermore, a reduction in capital costs is achieved by introducing single-use systems.
- Increasing number of batches
  By introduction of single-use systems, it is possible to increase the number of batches that can be produced in 1 year, due to the fact that cleaning and sterilization is not needed anymore. The turnover time needed from batch to batch is shortened.
- Provides flexibility in facility design
  When stainless steel systems are used, changes to the process equipment might impact the design of the stainless steel tanks, piping, etc. These changes will directly influence the CIP and SIP validation status of the facility. By using single-use systems, process changes can easily be incorporated as the setup of the single-use process is flexible, and CIP and SIP validation are not needed. However, in case a change will influence the process, the validated status of the process must be reconsidered and a revalidation might be needed.
- Speedup implementation and time to market
  Due to the great flexibility of the single-use systems, the speed of product to market is not influenced by process changes that might be introduced during the development process. However, the process needs to be validated during Phase 3 development. When changes are introduced after the process is validated, a revalidation might be needed. There is no difference in this respect to the traditional stainless steel setup.
- Reduction in water and wastewater costs
  Due to the fact that the systems are single use, there will be a great reduction in the total costs for cleaning, not only a water reduction but also a reduction in the number of hours needed to clean systems and setup for the next batch of product.
- Reduction in validation costs
  No yearly validation costs for cleaning and sterilization are needed anymore when single-use systems are used.

A disadvantage of the single-use system is that the operational expenses will increase, storage location for single-use bags and tubing will increase, and the dependence of the company to one supplier of single-use systems will increase. Furthermore, there is not yet a standard set in single-use systems like it is with stainless steel bioreactor systems. Suppliers are still developing their own systems.

The advantages of the stainless steel bioreactors are obvious as this traditional technology is well understood and controlled, although the stainless steel pathway had major disadvantages such as expensive design, installation, and maintenance costs combined with significant expenditures of time in facilities and equipment qualification and validation efforts.

Fermentation Protocols

The kinetics of cell growth and product formation will not only dictate the type of bioreactor used but also how the growth process is performed. Three types of fermentation protocols are commonly employed and discussed below:

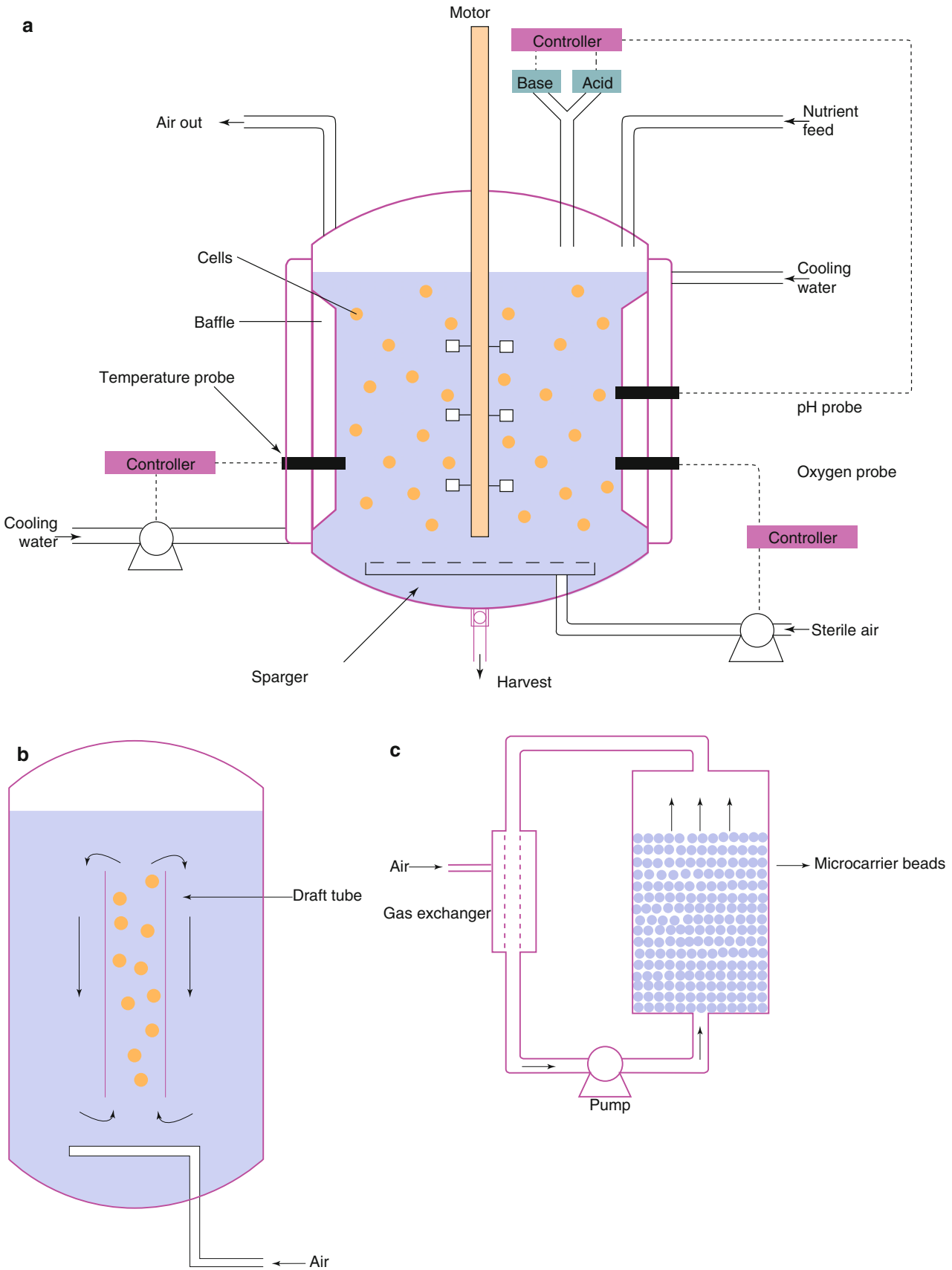

Figure 3.1 ■ (**a**) Schematic representation of stirred-tank bioreactor (Adapted from Klegerman and Groves 1992). (**b**) Schematic representation of airlift bioreactor (Adapted from Klegerman and Groves 1992). (**c**) Schematic representation of fixed-bed stirred-tank bioreactor (Adapted from Klegerman and Groves 1992). (**d**) Schematic representation of hollow fiber perfusion bioreactor (Adapted from Klegerman and Groves 1992)

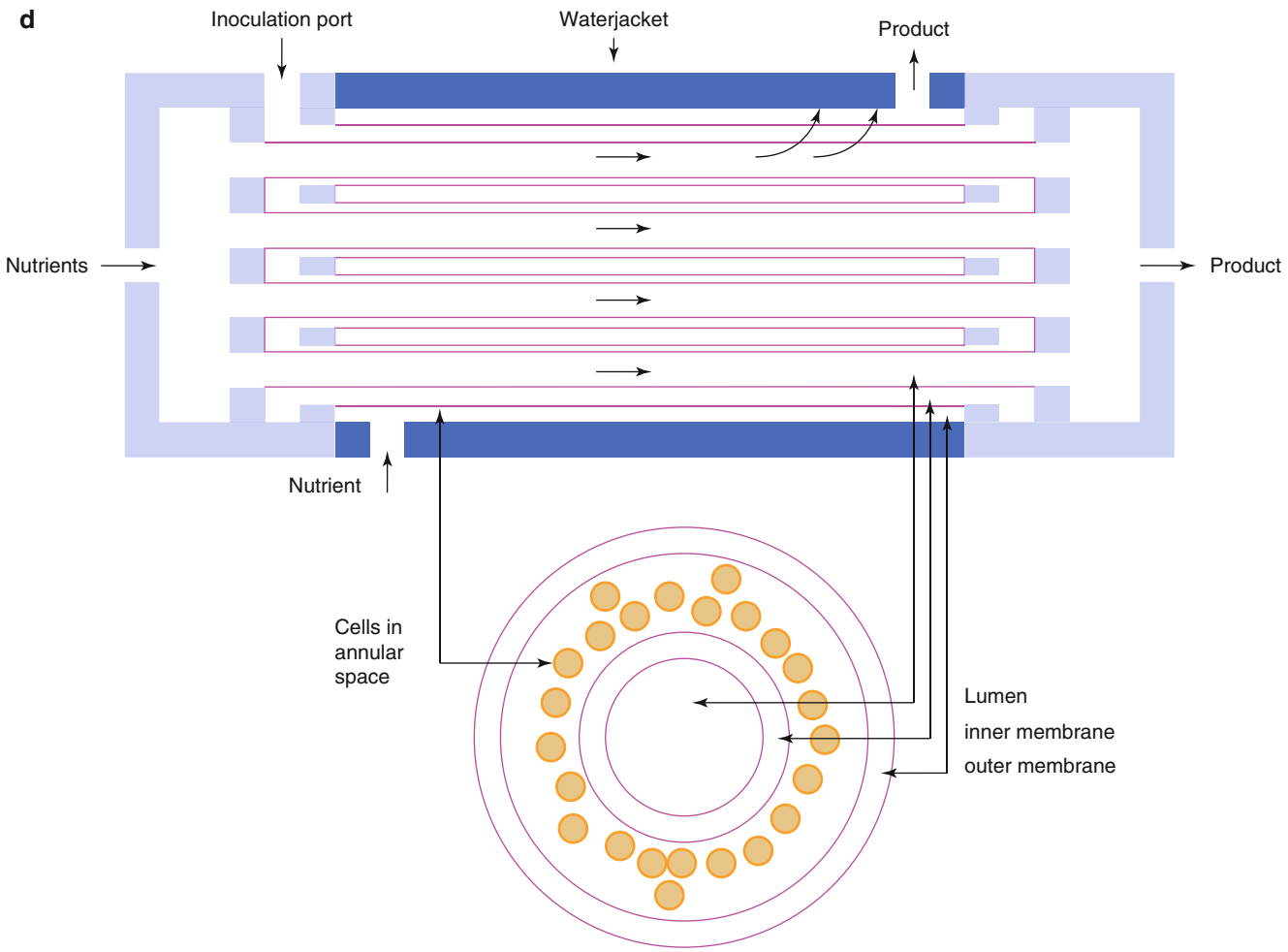

Figure 3.1 ■ (continued)

- Batch
  In a batch process, the bioreactor is filled with the entire volume of medium needed during the cell growth and/or production phase. No additional supplements are added to increase the cell growth or production during the process. Waste products, such as lactate and ammonium, and the product itself accumulate in the bioreactor. The product is harvested at the end of the process. Maximum cell density and product yields will be lower compared to a fed-batch process.
- Fed-batch
  In a fed-batch process, a substrate is supplemented to the bioreactor. The substrate consists of the growth-limiting nutrients that are needed during the cell growth phase and/or during the production phase of the process. Like the batch process, waste products accumulate in the bioreactor. The product is harvested at the end of the process. With the fed-batch process, higher cell densities and product yields can be reached compared to the batch process due to the extension of production time that can be achieved compared to a batch process. The substrate used is highly concentrated and can be added to the bioreactor on a daily basis or as a continuous feed. The fed-batch mode is currently widely used for the production of proteins. The process is well understood and characterized.
- Perfusion
  In a perfusion process, the media and waste products are continuously exchanged and the product is harvested throughout the culture period. A membrane device is used to retain the cells in the bioreactor, and waste medium is removed from the bioreactor by this device (Fig. 3.2). To keep the level medium constant in the bioreactor, fresh medium is supplemented to the bioreactor. By operating in perfusion mode, the level of waste products will be kept constant and generates a stable environment for the cells to grow or to produce. With the perfusion process, much higher cell densities can be reached and therefore higher productivity (Compton and Jensen 2007).

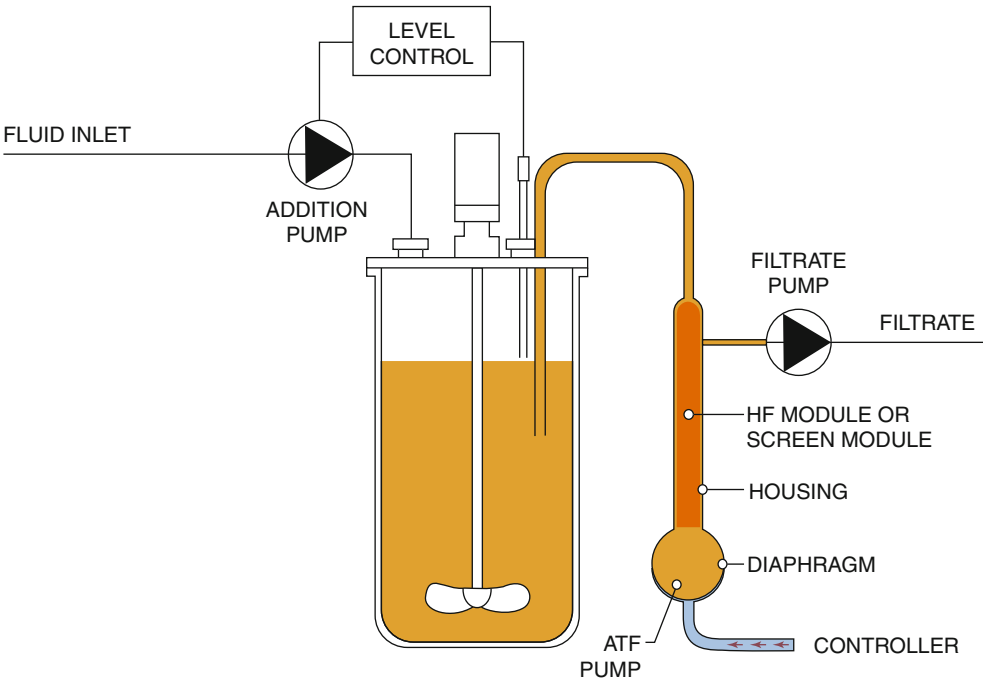

Figure 3.2 ■ Schematic representation of perfusion device coupled to a stirred-tank bioreactor. *ATF* alternating tangential flow.

In all these three protocols, the cells go through four distinctive phases (see also Chap. 1):

1. Lag phase
   In this phase the cells are adapting to the conditions in the bioreactor and do not yet grow.
2. Exponential growth phase
   During this phase, cells grow in a more or less constant doubling time for a fixed period. The mammalian cell doubling time is cell-type dependent and usually varies between 20 and 40 h. Plotting the natural logarithm of cell number against time produces a straight line. Therefore, the exponential growth phase is also called the log phase. The growth phase will be affected by growth conditions like temperature, pH, oxygen pressure, and external forces like stirring and baffles that are inserted into the bioreactor. Furthermore, the growth rate is affected by the supply of sufficient nutrients, buildup of waste nutrients, etc.
3. Stationary phase
   In the stationary phase, the growth rate of the cells slows down due to the fact that nutrients are depleted and/or build up of toxic waste products like lactate and ammonium. In this phase, constant cell numbers are found due to equal cell growth and cell death.
4. Death phase
   Cells die due to depletion of nutrients and/or presence of high concentrations of toxic products like lactate and ammonium.

Examples of animal cells that are commonly used to produce proteins of clinical interest are Chinese hamster ovary cells (CHO), immortalized human embryonic retinal cells (PER.C6® cells), baby hamster kidney cells (BHK), lymphoblastoid tumor cells (interferon production), melanoma cells (plasminogen activator), and hybridized tumor cells (monoclonal antibodies).

The cell culture has to be free from undesired microorganisms that may destroy the cell culture or present hazards to the patient by producing endotoxins. Therefore, strict measures are required for both the production procedures and materials used (WHO 2010; Berthold and Walter 1994) to prevent a possible contamination with extraneous agents like viruses, bacteria, and mycoplasma. Furthermore, strict measures are needed, especially with regard to the raw materials used, to prevent contaminations with transmissible spongiform encephalopathies (TSEs).

■ Cultivation Medium

In order to achieve optimal growth of cells and optimal production of recombinant proteins, it is of great importance not only that conditions such as stirring, pH, oxygen pressure, and temperature are chosen and controlled appropriately but also that a cell growth and protein production medium with the proper nutrients are provided for each stage of the production process.

The media used for mammalian cell culture are complex and consist of a mixture of diverse components, such as sugars, amino acids, electrolytes, vitamins, fetal calf serum, and/or a mixture of peptones,

Type of nutrient	Example(s)
Sugars	Glucose, lactose, sucrose, maltose, dextrins
Fat	Fatty acids, triglycerides
Water (high quality, sterilized)	Water for injection
Amino acids	Glutamine
Electrolytes	Calcium, sodium, potassium, phosphate
Vitamins	Ascorbic acid, -tocopherol, thiamine, riboflavine, folic acid, pyridoxin
Serum (fetal calf serum, synthetic serum)	Albumin, transferrin
Trace minerals	Iron, manganese, copper, cobalt, zinc
Hormones	Growth factors

Table 3.2 ■ Major components of growth media for mammalian cell structures.

growth factors, hormones, and other proteins (see Table 3.2). Many of these ingredients are pre-blended either as concentrate or as homogeneous mixtures of powders. To prepare the final medium, components are dissolved in purified water before filtration. The final medium is filtrated through 0.2 µm filters or through 0.1 µm filters to prevent possible mycoplasma contamination. Some supplements, especially fetal calf serum, contribute considerably to the presence of contaminating proteins and may seriously complicate purification procedures. Moreover, the composition of serum is variable. It depends on the individual animal, season of the year, suppliers' treatment, etc. The use of serum may introduce adventitious material such as viruses, mycoplasma, bacteria, and fungi into the culture system (Berthold and Walter 1994). Furthermore, the possible presence of prions that can cause transmissible spongiform encephalitis almost precludes the use of materials from animal origin. However, if use of this material is inevitable, one must follow the relevant guidelines in which selective sourcing of the material is the key measure to safety (EMA 2011). Many of these potential problems when using serum in cell culture media led to the development of fully defined, free from animal-derived material. These medium formulations were not only developed by the suppliers, there is the trend that the key players in the biotech industry develop their own fully defined medium for their specific production platforms. The advantage of this is that the industry is less dependent on medium suppliers. The fully defined media have been shown to give satisfactory results in large-scale production settings for monoclonal antibody processes. However, hydrolysates from nonanimal origin, like yeast and plant sources, are more and more used for optimal cell growth and product secretion (reviewed by Shukla and Thömmes 2010).

DOWNSTREAM PROCESSING

■ Introduction

Recovering a biological reagent from a cell culture supernatant is one of the critical parts of the manufacturing procedure for biotech products, and purification costs typically outweigh those of the upstream part of the production process. For the production of monoclonal antibodies, protein A resin accounts for some 10 % of the cost, while virus removal by filtration can account for 40 % of the cost (Gottschalk 2006).

More than a decade ago, the protein product was available in a very dilute form, e.g., 10–200 mg/L. At the most concentrations, up to 500–800 mg/L could be reached (Berthold and Walter 1994). Developments in cell culture technology through application of genetics and proteomics resulted in product titers well above 1 g/L. Product titers above 20 g/L are also reported (Monteclaro 2010). These high product titers pose a challenge to the downstream processing unit operations (Shukla and Thömmes 2010). With the low-yield processes, a concentration step is often required to reduce handling volumes for further purification. Usually, the product subsequently undergoes a series of purification steps. The first step in a purification process is to remove cells and cell debris from the process fluids. This process step is normally performed using centrifugation and/or depth filters. Depth filters are often used in combination with filter aid or diatomaceous earth. Often the clarification step is regarded as a part of the upstream process. Therefore, the first actual step in the purification process is a capture step. Subsequent steps remove the residual bulk contaminants, and a final step removes trace contaminants and sometimes variant forms of the molecule. Alternatively, the reverse strategy, where the main contaminants are captured and the product is purified in subsequent steps, might result in a more economic process,

especially if the product is not excreted from the cells. In the case where the product is excreted into the cell culture medium, the product will not represent more than 1–5 % of total cellular protein, and a specific binding of the cellular proteins in a product-specific capture step will have a high impact on the efficiency of that step. If the bulk of the contaminants can be removed first, the specific capture step will be more efficient and smaller in size and therefore more economic. Furthermore, smaller subsequent unit operation steps (e.g., chromatography columns) could be used.

After purification, additional steps are performed to bring the desired product into a formulation buffer in which the product is stabilized and can be stored for the desired time until further process steps are performed. Before storage of the final bulk drug substance, the product will be sterilized. Normally this will be performed by a 0.2 μm filtration step. Formulation aspects will be dealt with in Chap. 4.

When designing an upstream and purification protocol, the possibility for scaling up should be considered carefully. A process that has been designed for small quantities is most often not suitable for large quantities for technical, economic, and safety reasons. Developing a purification process, i.e., the isolation and purification of the desired product also called the downstream process (DSP), to recover a recombinant protein in large quantities occurs in two stages: *design* and *scale-up*.

Separating the impurities from the product protein requires a series of purification steps (*process design*), each removing some of the impurities and bringing the product closer to its final specification. In general, the starting feedstock contains cell debris and/or whole-cell particulate material that must be removed. Defining the major contaminants in the starting material is helpful in the downstream process design. This includes detailed information on the source of the material (e.g., bacterial or mammalian cell culture) and major contaminants that are used or produced in the upstream process (e.g., albumin, serum, or product analogs). Moreover, the physical characteristics of the product versus the known contaminants (thermal stability, isoelectric point, molecular weight, hydrophobicity, density, specific binding properties) largely determine the process design. Processes used for production of therapeutics in humans should be safe, reproducible, robust, and produced at the desired cost of goods. The DSP steps may expose the protein molecules to high physical stress (e.g., high temperatures and extreme pH) which can alter the protein properties possibly leading to loss in efficacy. Any substance that is used by injection must be sterile. Furthermore, the endotoxin concentration must be below a certain level depending on the product. Limits are stated in the individual monographs which are to be consulted (e.g., European Pharmacopoeia: less than 0.2 endotoxin units per kg body mass for intrathecal application). Aseptic techniques have to be used wherever possible and necessitate procedures throughout with clean air and microbial control of all materials and equipment used. During validation of the purification process, one must also demonstrate that potential viral contaminants are inactivated and removed (Walter et al. 1992). The purification matrices should be at least sanitizable or, if possible, steam-sterilizable. For depyrogenation, the purification material must withstand either extended dry heat at ≥180 °C or treatment with 1–2 M sodium hydroxide (for further information, see Chap. 4). If any material in contact with the product inadvertently releases compounds, these leachables must be analyzed and their removal by subsequent purification steps must be demonstrated during process validation, or it must be demonstrated that the leachables are below a toxic level. The increased use of plastic film-based single-use production technology (e.g., sterile single-use bioreactor bags, bags to store liquids and filter housings) has made these aspects more significant in the last decade. Suppliers have reacted by providing a significant body of information regarding leachables and biocompatibility for typical solutions used during processing. The problem of leachables is especially hampering the use of affinity chromatography (see below) in the production of pharmaceuticals for human use. On small-scale affinity, chromatography is an important tool for purification and the resulting product might be used for (animal) toxicity studies, but for human use the removal of any leached ligands below a toxic level has to be demonstrated. Because free affinity ligands will bind to the product, the removal might be cumbersome.

Scale-up is the term used to describe a number of processes employed in converting a laboratory procedure into an economical, industrial process. During the scale-up phase, the process moves from the laboratory scale to the pilot plant and finally to the production plant. The objective of scale-up is to produce a product of high quality at a competitive price. Since the costs of downstream processing can be as high as 50–80 % of the total cost of the bulk product, practical and economical ways of purifying the product should be used. Superior protein purification methods hold the key to a strong market position (Wheelwright 1993).

Basic operations required for a downstream purification process used for macromolecules from biological sources are shown in Fig. 3.3.

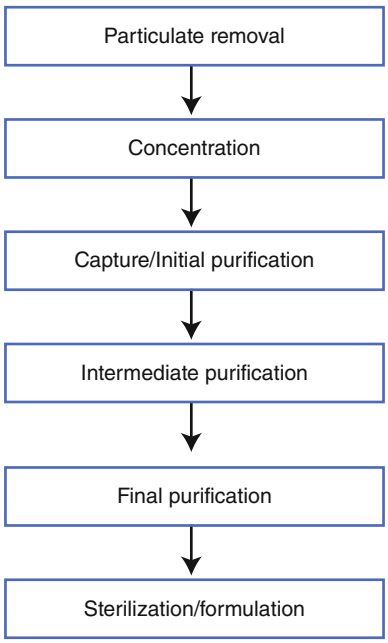

Figure 3.3 ■ Basic operations required for the purification of a biopharmaceutical macromolecule.

As mentioned before, the design of downstream processing is highly product dependent. Therefore, each product requires a specific multistage purification procedure (Sadana 1989). The basic scheme as represented in Fig. 3.3 becomes complex. A typical example of a process flow for the downstream processing is shown in Fig. 3.4. This scheme represents the processing of a glycosylated recombinant interferon (about 28 kDa) produced in mammalian cells. The aims of the individual unit operations are described.

Once the harvest volume and product concentration can be managed, the main purification phase can start. A number of purification methods are available to separate proteins on the basis of a wide variety of different physicochemical criteria such as size, charge, hydrophobicity, and solubility. Detailed information about some separation and purification methods commonly used in purification schemes is provided below.

■ Filtration/Centrifugation

Products from biotechnological industry must be separated from biological systems that contain suspended particulate material, including whole cells, lysed cell material, and fragments of broken cells generated when cell breakage has been necessary to release intracellular products. Most downstream processing flow sheets will, therefore, include at least one unit operation

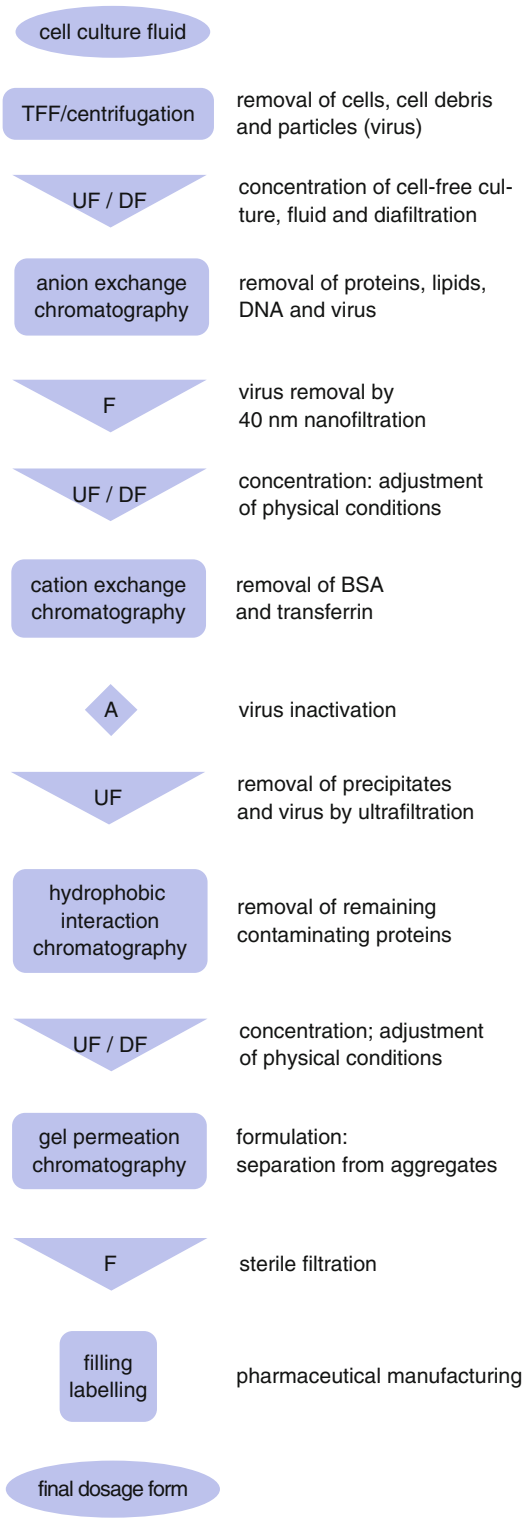

Figure 3.4 ■ Downstream processing of a glycosylated recombinant interferon, describing the purpose of the inclusion of the individual unit operations. *F* filtration, *TFF* tangential flow filtration, *UF* ultrafiltration, *DF* diafiltration, *A* adsorption (Adapted from Berthold and Walter 1994).

for the removal ("clarification") or concentration, just the opposite, of particulates. Most frequently used methods are centrifugation and filtration techniques (e.g., ultrafiltration, diafiltration, and microfiltration). However, the expense and effectiveness of such methods is highly dependent on the physical nature of the particulate material and of the product.

Filtration

Several filtration systems have been developed for separation of cells from media, the most successful being depth filtration and tangential flow systems (also referred to as "cross flow"). In the latter system, high shear across the membrane surface limits fouling, gel layer formation, and concentration polarization. In ultrafiltration, mixtures of molecules of different molecular dimensions are separated by passage of a dispersion under pressure across a membrane with a defined pore size. In general, ultrafiltration achieves little purification of protein product from other molecules with a comparable size, because of the relatively large pore-size distribution of the membranes. However, this technique is widely used to concentrate macromolecules and also to change the aqueous phase in which the particles are dispersed or in which molecules are dissolved (diafiltration) to one required for the subsequent purification steps.

Centrifugation

Subcellular particles and organelles, suspended in a viscous liquid (e.g., the particles produced when cells are disrupted by mechanical procedures), are difficult to separate either by using one fixed centrifugation step or by filtration. But, they can be isolated efficiently by centrifugation at different speeds. For instance, nuclei can be obtained by centrifugation at 400× g for 20 min, while plasma membrane vesicles are pelleted at higher centrifugation rates and longer centrifugation times (fractional centrifugation). In many cases, however, total biomass can easily be separated from the medium by centrifugation (e.g., continuous disc-stack centrifuge). Buoyant density centrifugation can be useful for separation of particles as well. This technique uses a viscous fluid with a continuous gradient of density in a centrifuge tube. Particles and molecules of various densities within the density range in the tube will cease to move when the isopycnic region has been reached. Both techniques of continuous (fluid densities within a range) and discontinuous (blocks of fluid with different density) density gradient centrifugation are used in buoyant density centrifugation on a laboratory scale. However, for application on an industrial scale, continuous centrifuges (e.g., tubular bowl centrifuges) are only used for discontinuous buoyant density centrifugation of protein products. This type of industrial centrifuge is mainly applied to recover precipitated proteins or contaminants. For influenza vaccines, continuous centrifugation is already for decades the workhorse to purify influenza viruses on an industrial scale.

■ Precipitation

The solubility of a particular protein depends on the physicochemical environment, for example, pH, ionic species, and ionic strength of the solution (see also Chap. 4). A slow continuous increase of the ionic strength (of a protein mixture) will selectively drive proteins out of solution. This phenomenon is known as "salting out." A wide variety of agents, with different "salting-out" potencies are available. Chaotropic series with increasing "salting-out" effects of negatively (I) and positively (II) charged molecules are given below:

I. SCN–, I–, CLO4–, NO3–, Br–, Cl–, CH3COO–, PO43–, SO42–
II. Ba2+, Ca2+, Mg2+, Li+, Cs+, Na+, K+, Rb+, NH4+

Ammonium sulfate is highly soluble in cold aqueous solutions and is frequently used in "salting-out" purification.

Another method to precipitate proteins is to use water-miscible organic solvents (change in the dielectric constant). Examples of precipitating agents are polyethylene glycol and trichloroacetic acid. Under certain conditions, chitosan and nonionic polyoxyethylene detergents also induce precipitation (Cartwright 1987; Homma et al. 1993; Terstappen et al. 1993). Cationic detergents have been used to selectively precipitate DNA.

Precipitation is a scalable, simple, and relatively economical procedure for the recovery of a product from a dilute feedstock. It has been widely used for the isolation of proteins from culture supernatants. Unfortunately, with most bulk precipitation methods, the gain in purity is generally limited and product recovery can be low. Moreover, extraneous components are introduced which must be eliminated later. Finally, large quantities of precipitates may be difficult to handle. Despite these limitations, recovery by precipitation has been used with considerable success for some products.

■ Chromatography

Introduction

In preparative chromatography systems, molecular species are primarily separated based on differences in distribution between two phases, one which is the stationary phase (mostly a solid phase) and the other which moves. This mobile phase may be liquid or gaseous (see also Chap. 2). Nowadays, almost all stationary phases (fine particles providing a large surface area) are packed into a column. The mobile phase is passed through by pumps. Downstream protein purification protocols usually have at least two to three chromatography steps.

Chromatographic methods used in purification procedures of biotech products are listed in Table 3.3 and are briefly discussed in the following sections.

Chromatographic Stationary Phases

Chromatographic procedures often represent the rate-limiting step in the overall downstream processing. An important primary factor governing the rate of operation is the mass transport into the pores of conventional packing materials. Adsorbents employed include inorganic materials such as silica gels, glass beads, hydroxyapatite, various metal oxides (alumina), and organic polymers (cross-linked dextrans, cellulose, agarose). Separation occurs by differential interaction of sample components with the chromatographic medium. Ionic groups such as amines and carboxylic acids, dipolar groups such as carbonyl functional groups, and hydrogen bond-donating and bond-accepting groups control the interaction of the sample components with the stationary phase, and these functional groups slow down the elution rate if interaction occurs.

Chromatographic stationary phases for use on a large scale have improved considerably over the last decades. Hjerten et al. (1993) reported on the use of compressed acrylamide-based polymer structures. These materials allow relatively fast separations with good chromatographic performance. Another approach to the problems associated with mass transport in conventional systems is to use chromatographic particles that contain some large "through pores" in addition to conventional pores (see Fig. 3.5). These flow-through or "perfusion chromatography" media enable faster convective mass transport into particles and allow operation at much higher speeds without loss in resolution or binding capacity (Afeyan et al. 1989; Fulton 1994). Another development is the design of spirally wrapped columns containing the adsorption medium. This configuration permits high throughput, high capacity, and good capture efficiency (Cartwright 1987).

The ideal stationary phase for protein separation should possess a number of characteristics, among which are high mechanical strength, high porosity, no nonspecific interaction between protein and the support phase, high capacity, biocompatibility, and high stability of the matrix in a variety of solvents. The latter is especially true for columns used for the production of clinical materials that need to be cleaned, depyrogenized, disinfected, and sterilized at regular intervals. High-performance liquid chromatography (HPLC) systems fulfill many of these criteria. Liquid phases should be carefully chosen to minimize loss of biological activity resulting from the use of some organic solvents. In HPLC small pore-size stationary phases that are incompressible are used. These particles are small, rigid, and regularly sized (to provide a high surface area). The mobile liquid phase is forced under high pressure through the column material. Reversed-phase HPLC systems, using less polar stationary phases than the mobile phases, can in a very few cases be effectively integrated into preparative scale purification schemes of proteins and can serve both as a means of concentration and purification (Benedek and Swadesh 1991).

Separation technique	Mode/principle	Separation based on
Membranes	Microfiltration	Size
	Ultrafiltration	Size
	Nanofiltration	Size
	Dialysis	Size
	Charged membranes	Charge
Centrifugation	Isopycnic banding	Density
	Non-equilibrium setting	Density
Extraction	Fluid extraction	Solubility
	Liquid/liquid extraction	Partition, change in solubility
Precipitation	Fractional precipitation	Change in solubility
Chromatography	Ion exchange	Charge
	Gel filtration	Size
	Affinity	Specific ligand-substrate interaction
	Hydrophobic interaction	Hydrophobicity
	Adsorption	Covalent/non-covalent binding

Table 3.3 ■ Frequently used separation processes and their physical basis.

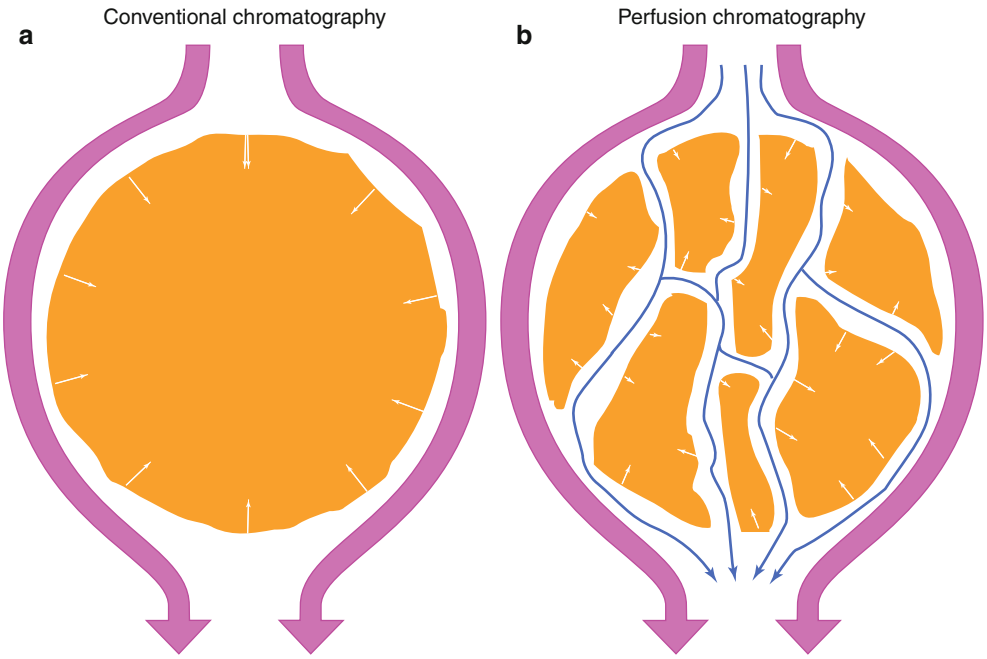

Figure 3.5 ■ The structure of conventional chromatographic particles (**a**) and the perfusion of flow through chromatographic particles (**b**) (Adapted from Fulton 1994).

Unfortunately, HPLC equipment and resin costs are high. Moreover, HPLC is poorly scalable and thus this technology is basically not applied in large-scale purification schemes.

In production environments, columns which operate at relatively low back pressure are often used. They have the advantage that they can be used in equipment constructed from plastics which, unlike conventional stainless steel equipment, resists all buffers likely to be employed in the separation of biomolecules (consider the effect of leachables from plastics). These columns are commercially available and permit the efficient separation of proteins in a single run, making this an attractive unit operation in a manufacturing process. Results can be obtained rapidly and with high resolution. A new development is the use of chromatography equipment with fully disposable flow paths that resists almost all chemicals used in protein purification including disinfection and sterilization media.

Adsorption Chromatography

In adsorption chromatography (also called "normal phase" chromatography), the stationary phase is more polar than the mobile phase. The protein of interest selectively binds to a static matrix under one condition and is released under a different condition. Adsorption chromatography methods enable high ratios of product load to stationary phase volume. Therefore, this principle is economically scalable.

Ion-Exchange Chromatography

Ion-exchange chromatography can be a powerful step early in a purification scheme. It can be easily scaled up. Ion-exchange chromatography can be used in a negative mode, i.e., the product flows through the column under conditions that favor the adsorption of contaminants to the matrix, while the protein of interest does not bind (Tennikova and Svec 1993). The type of the column needed is determined by the properties of the proteins to be purified (e.g., isoelectric point and charge density). Anion exchangers bind negatively charged molecules and cation exchangers bind positively charged molecules. In salt-gradient ion-exchange chromatography, the salt concentration in the perfusing elution buffer is increased continuously or in steps. The stronger the binding of an individual protein to the ion exchanger, the later it will appear in the elution buffer. Likewise, in pH-gradient chromatography, the pH is changed continuously or in steps. Here, the protein binds at 1 pH and is released at a different pH. As a result of the heterogeneity in glycosylation (e.g., a varying number of sialic acid moieties), glycosylated proteins may elute in a relatively broad pH range (up to 2 pH units).

In order to simplify purification, a specific amino acid tail can be added to the protein at the gene level to create a "purification handle." For example, a short tail consisting of arginine residues allows a protein to bind to a cation exchanger under conditions where almost no other cell proteins bind. However, this technique is only useful for laboratory-scale isolation of the product and cannot be used for production scale due to regulatory problems related to the removal of the arginine or other specific tags from the protein.

(Immuno)Affinity Chromatography

Affinity Chromatography

Affinity chromatography is based on highly specific interactions between an immobilized ligand and the protein of interest. Affinity chromatography is a very powerful method for the purification of proteins. Under physiological conditions, the protein binds to the ligand. Extensive washing of this matrix will remove contaminants, and the purified protein can be recovered by the addition of ligands competing for the stationary phase binding sites or by changes in physical conditions (such as low or high pH of the eluent) which greatly reduce the affinity. Examples of affinity chromatography include the purification of glycoproteins, which bind to immobilized lectins, and the purification of serine proteases with lysine binding sites, which bind to immobilized lysine. In these cases, a soluble ligand (sugar or lysine, respectively) can be used to elute the required product under relatively mild conditions. Another example is the use of the affinity of protein A and protein G for antibodies. Protein A and protein G have a high affinity for the Fc portions of many immunoglobulins from various animals. Protein A and G matrices can be commercially obtained with a high degree of purity. For the purification of, e.g., hormones or growth factors, the receptors or short peptide sequence that mimic the binding site of the receptor molecule can be used as affinity ligands. Some proteins show highly selective affinity for certain dyes commercially available as immobilized ligands on purification matrices. When considering the selection of these ligands for pharmaceutical production, one must realize that some of these dyes are carcinogenic and that a fraction may leach out during the process.

An interesting approach to optimize purification is the use of a gene that codes not only for the desired protein but also for an additional sequence that facilitates recovery by affinity chromatography. At a later stage the additional sequence is removed by a specific cleavage reaction. As mentioned before, this is a complex process that needs additional purification steps.

In general, use of affinity chromatography in the production process for therapeutics may lead to complications during validation of the removal of free ligands or protein extensions. Consequently, except for monoclonal antibodies where affinity chromatography is part of the purification platform at large scale, this technology is rarely used in the industry.

Immunoaffinity Chromatography

The specific binding of antibodies to their epitopes is used in immunoaffinity chromatography (Chase and Draeger 1993). This technique can be applied for purification of either the antigen or the antibody. The antibody can be covalently coupled to the stationary phase and act as the "receptor" for the antigen to be purified. Alternatively, the antigen, or parts thereof, can be attached to the stationary phase for the purification of the antibody. Advantages of immunoaffinity chromatography are its high specificity and the combination of concentration and purification in one step.

A disadvantage associated with immunoaffinity methods is the sometimes very strong antibody-antigen binding. This requires harsh conditions during elution of the ligand. Under such conditions, sensitive ligands could be harmed (e.g., by denaturation of the protein to be purified). This can be alleviated by the selection of antibodies and environmental conditions with high specificity and sufficient affinity to induce an antibody-ligand interaction, while the antigen can be released under mild conditions (Jones 1990). Another concern is disruption of the covalent bond linking the "receptor" to the matrix. This would result in elution of the entire complex. Therefore, in practice, a further purification step after affinity chromatography as well as an appropriate detection assay (e.g., ELISA) is almost always necessary. On the other hand, improved coupling chemistry that is less susceptible to hydrolysis has been developed to prevent leaching.

Scale-up of immunoaffinity chromatography is often hampered by the relatively large quantity of the specific "receptor" (either the antigen or the antibody) that is required and the lack of commercially available, ready-to-use matrices. The use of immunoaffinity in pharmaceutical processes will have major regulatory consequences since the immunoaffinity ligand used will be considered by the regulatory bodies as a "second product," thus will be subjected to the nearly the same regulatory scrutiny as the drug substance. Moreover, immunoaffinity ligands can have a significant effect of the final costs of goods.

Examples of proteins of potential therapeutic value that have been purified using immunoaffinity chromatography are interferons, urokinase, erythropoietin, interleukin-2, human factor VIII and X, and recombinant tissue plasminogen activator.

Hydrophobic Interaction Chromatography

Under physiological conditions, most hydrophobic amino acid residues are located inside the protein core, and only a small fraction of hydrophobic amino acids is exposed on the "surface" of a protein. Their exposure is suppressed because of the presence of hydrophilic amino acids that attract large clusters of water molecules and form a "shield." High salt concentrations reduce the hydration of a protein, and the surface-exposed hydrophobic amino acid residues become more accessible. Hydrophobic interaction chromatography (HIC) is based on non-covalent and non-electrostatic interactions between proteins and the stationary phase.

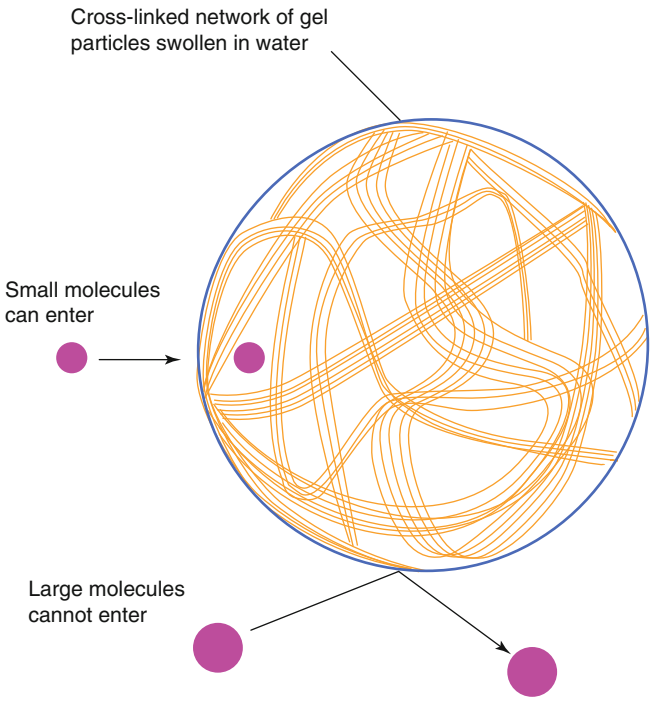

Figure 3.6 ■ Schematic representation of gel filtration (Adapted from James 1992).

HIC is a mild technique, usually yielding high recoveries of proteins that are not damaged, are folded correctly, and are separated from contaminants that are structurally related. HIC is ideally placed in the purification scheme after ion-exchange chromatography, where the protein usually is released in high ionic strength elution media (Heng and Glatz 1993).

Gel-Permeation Chromatography

Gel-permeation or size-exclusion chromatography, also known as gel filtration, separates molecules according to their shape and size (see Fig. 3.6). Inert gels with narrow pore-size distributions in the size range of proteins are available. These gels are packed into a column and the protein mixture is then loaded on top of the column and the proteins diffuse into the gel. The smaller the protein, the more volume it will have available in which to disperse. Molecules that are larger than the largest pores are not able to penetrate the gel beads and will therefore stay in the void volume of the column. When a continuous flow of buffer passes through the column, the larger proteins will elute first and the smallest molecules last. Gel-permeation chromatography is a good alternative to membrane diafiltration for buffer exchange at almost any purification stage, and it is often used in laboratory design. At production scale, the use of this technique is usually limited, because only relatively small sample volumes can be loaded on a large column (up to one-third of the column volume in the case of "buffer exchange"). It is therefore best avoided or used late in the purification process when the protein is available in a highly concentrated form. Gel filtration is commonly used as the final step in the purification to bring proteins in the appropriate buffer used in the final formulation. In this application, its use has little if no effect on the product purity characteristics.

Expanded Beds

As mentioned before, purification schemes are based on multistep protocols. This not only adds greatly to the overall production costs but also can result in significant loss of product. Therefore, there still is an interest in the development of new methods for simplifying the purification process. Adsorption techniques are popular methods for the recovery of proteins, and the conventional operating format for preparative separations is a packed column (or fixed bed) of adsorbent. Particulate material, however, can be trapped near the bed, which results in an increase in the pressure drop across the bed and eventually in clogging of the column. This can be avoided by the use of pre-column filters (0.2 μm) to save the column integrity. Another solution to this problem may be the use of expanded beds (Chase and Draeger 1993; Fulton 1994), also called fluidized beds (see Fig. 3.7). In principle, the use of expanded beds enables clarification, concentration, and purification to be achieved in a single step. The concept is to employ a particulate solid-phase adsorbent in an open bed with upward liquid flow. The hydrodynamic drag around the particles tends to lift them upwards, which is counteracted by gravity because of a density difference between the particles and the liquid phase. The particles remain suspended if particle diameter, particle density, liquid viscosity, and liquid density are properly balanced by choosing the correct flow rate. The expanded bed allows particles (cells) to pass through, whereas molecules in solution are selectively retained (e.g., by the use of ion-exchange or affinity adsorbents) on the adsorbent particles. Feedstocks can be applied to the bed without prior removal of particulate material by centrifugation or filtration, thus reducing process time and costs. Fluidized beds have been used previously for the industrial-scale recovery of antibiotics such as streptomycin and novobiocin (Fulton 1994; Chase 1994). Stable, expanded beds can be obtained using simple equipment adapted from that used for conventional, packed bed adsorption and chromatography processes. Ion-exchange adsorbents are likely to be chosen for such separations.

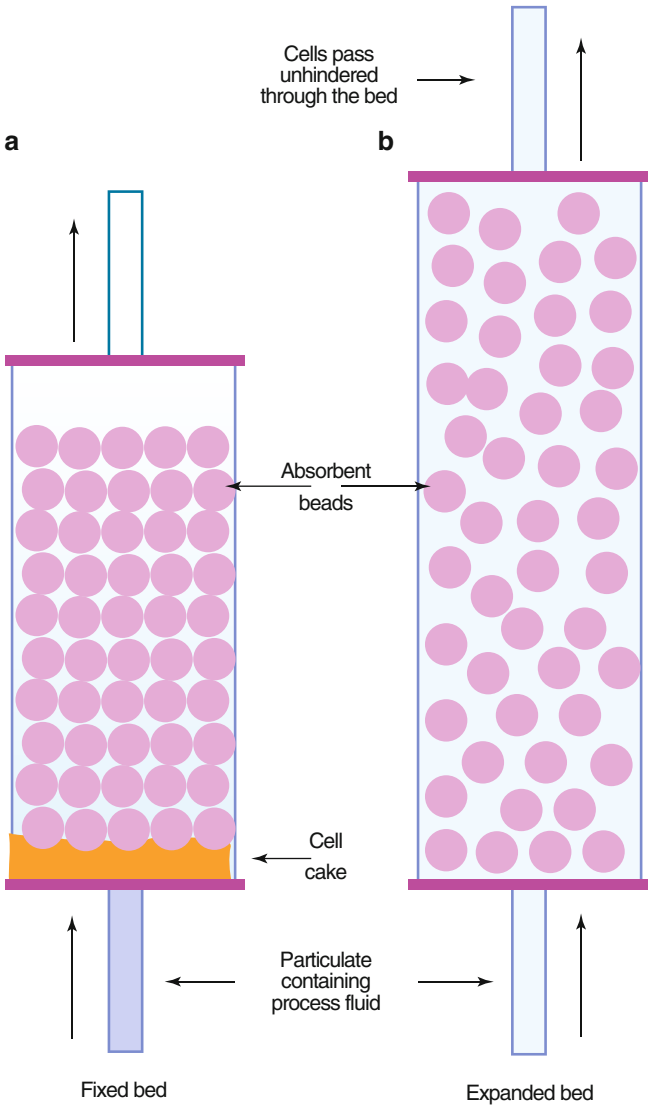

Figure 3.7 ■ Comparison between (**a**) a packed bed and (**b**) an expanded bed (Adapted from Chase and Draeger 1993).

CONTAMINANTS

For pharmaceutical applications, product purity mostly is ≥99 % when used as a parenteral (Berthold and Walter 1994; ICH 1999a). Purification processes should yield potent proteins with well-defined characteristics for human use from which "all" contaminants have been removed to a major extent. The purity of the drug protein in the final product will therefore largely depend upon the purification technology applied.

Table 3.4 lists potential contaminants that may be present in recombinant protein products from bacterial and nonbacterial sources. These contaminants can be host-related, process-related and product-related.

Origin	Contaminant
Host-related	Viruses
	Host-derived proteins acid DNA
	Glycosylation variants
	N- and C-terminal variants
	Endotoxins (from Gram-negative bacterial hosts)
Product-related	Amino acids substitution and deletion
	Denatured protein
	Conformational isomers
	Dimers and aggregates
	Disulfide pairing variants
	Deamidated species
	Protein fragments
Process-related	Growth medium components
	Purification reagents
	Metals
	Column materials

Table 3.4 ■ Potential contaminants in recombinant protein products derived from bacterial and nonbacterial hosts.

In the following sections, special attention is paid to the detection and elimination of contamination by viruses, bacteria, cellular DNA, and undesired proteins.

■ Viruses

Endogenous and adventitious viruses, which require the presence of living cells to propagate, are potential contaminants of animal cell cultures and, therefore, of the final drug product. If present, their concentration in the purified product will be very low and it will be difficult to detect them. Viruses such as retrovirus can be visualized by (nonsensitive) electron microscopy. For retroviruses, a highly sensitive RT-PCR (reverse-transcriptase polymerase chain reaction) assay is available, but for other viruses, a sensitive in vitro assay might be lacking. The risks of some viruses (e.g., hepatitis virus) are known (Walter et al. 1991; Marcus-Sekura 1991), but there are other viruses whose risks cannot be properly judged because of lack of solid experimental data. Some virus infections, such as parvovirus, can have long latent periods before their clinical effects show up. Long-term effects of introducing viruses into a patient treated with a recombinant protein should not be overlooked. Therefore, it is required that products used parenterally are free from viruses. The specific virus testing regime required will depend on the cell type used for production (Löwer 1990; Minor 1994).

Category	Types	Example
Inactivation	Heat treatment	Pasteurization
	Radiation	UV-light
	Dehydration	Lyophilization
	Cross linking agents, denaturating or disrupting agents	β-propiolactone, formaldehyde, NaOH, organic solvents (e.g., chloroform), detergents (e.g., Na-cholate)
	Neutralization	Specific, neutralizing antibodies
Removal	Chromatography	Ion-exchange, immuno-affinity, chromatography
	Filtration	Nanofiltration
	Precipitation	Cyroprecipitation

Table 3.5 ■ Methods for reducing or inactivating viral contaminants.

Viruses can be introduced by nutrients, by an infected production cell line, or they are introduced (by human handling) during the production process. The most frequent source of virus introduction is animal serum. In addition, animal serum can introduce other unwanted agents such as bacteria, mycoplasmas, prions, fungi, and endotoxins. It should be clear that appropriate screening of cell banks and growth medium constituents for viruses and other adventitious agents should be strictly regulated and supervised (Walter et al. 1991; FDA 1993; ICH 1999b; WHO 2010). Validated, orthogonal methods to inactivate and remove possible viral contaminants are mandatory for licensing of therapeutics derived from mammalian cells or transgenic animals (Minor 1994). Viruses can be inactivated by physical and chemical treatment of the product. Heat, irradiation, sonication, extreme pH, detergents, solvents, and certain disinfectants can inactivate viruses. These procedures can be harmful to the product as well and should therefore be carefully evaluated and validated (Walter et al. 1992; Minor 1994; ICH 1999b). Removal of viruses by nanofiltration is an elegant and effective technique and the validation aspects of this technology are well described (PDA 2005). Filtration through 15 nm membranes can remove even the smallest non-enveloped viruses like bovine parvovirus (Maerz et al. 1996). Another common, although less robust, method to remove viruses in antibody processes is by ion-exchange chromatography. A number of methods for reducing or inactivating viral contaminants are mentioned in Table 3.5 (Horowitz et al. 1991).

■ Bacteria

Unwanted bacterial contamination may be a problem for cells in culture or during pharmaceutical purification. Usually the size of bacteria allows simple filtration over 0.2 µm (or smaller) filters for adequate removal. In order to further prevent bacterial contamination during production, the raw materials used have to be sterilized, preferably at 121 °C or higher, and the products are manufactured under strict aseptic conditions wherever possible. Production most often takes place in so-called clean rooms in which the chances of environmental contamination is reduced through careful control of the environment, for example, filtration of air. Additionally, antibiotic agents can be added to the culture media in some cases but have to be removed further downstream in the purification process. However, the use of beta-lactam antibiotics such as penicillin is strictly prohibited due to oversensitivity of some individuals to these compounds. Because of the persistence of antibiotic residues, which are difficult to eliminate from the product, appropriately designed manufacturing plants and extensive quality control systems for added reagents (medium, serum, enzymes, etc.) permitting antibiotic-free operation are preferable.

Pyrogens (usually endotoxins of gram-negative bacteria) are potentially hazardous substances (see Chap. 4). Humans are sensitive to pyrogen contamination at very low concentrations (picograms per mL). Pyrogens may elicit a strong fever response and can even be fatal. Simple 0.2 µm filtration does not remove pyrogens. Removal is complicated further because pyrogens vary in size and chemical composition. However, sensitive tests to detect and quantify pyrogens are commercially available. Purification schemes usually contain at least one step of ion-exchange chromatography (anionic-exchange material) to remove the negatively charged endotoxins (Berthold and Walter 1994). Furthermore, materials used in process such as glassware are typically subjected to a depyrogenation step prior to use often by the use of elevated temperatures (≥180 °C) in a dry heat oven.

■ Cellular DNA

The application of continuous mammalian cell lines for the production of recombinant proteins might result in the presence of oncogene-bearing DNA fragments in the final protein product (Walter and Werner 1993; Löwer 1990). A stringent purification protocol that is capable of reducing the DNA content and fragment size to a safe level is therefore necessary (Berthold and Walter 1994; WHO 2010). There are a number of approaches available to validate that the purification method removes cellular DNA and RNA. One such approach involves incubating the cell line with radiolabeled nucleotides and determining radioactivity in the purified product obtained through the purification protocol. Other methods are dye-binding fluorescence-enhancement assays for nucleotides and PCR-based methods. If the presence of nucleic acids persists in a final preparation, then additional steps must be introduced in the purification process. The question about a safe level of nucleic acids in biotech products is difficult to answer because of minimal relevant know-how. Transfection with so-called naked DNA is very difficult and a high concentration of DNA is needed. Nevertheless, it is agreed for safety reasons that final product contamination by nucleic acids should not exceed 100 pg or 10 ng per dose depending on the type of cells used to produce the pharmaceutical (WHO 2010; European Pharmacopoeia 2011).

■ Protein Contaminants and Product Variants

As mentioned before, minor amounts of host-, process-, and product-related proteins will likely appear in biotech products. These types of contaminants are a potential health hazard because, if present, they may be recognized as antigens by the patient receiving the recombinant protein product. On repeated use the patient may show an immune reaction caused by the contaminant, while the protein of interest is performing its beneficial function. In such cases the immunogenicity may be misinterpreted as being due to the recombinant protein itself. Therefore, one must be very cautious in interpreting safety data of a given recombinant therapeutic protein.

Generally, the sources of host- and process-related protein contaminants are the growth medium used and the host proteins of the cells. Among the host-derived contaminants, the host species' version of the recombinant protein could be present (WHO 2010). As these proteins are similar in structure, it is possible that undesired proteins are co-purified with the desired product. For example, urokinase is known to be present in many continuous cell lines. The synthesis of highly active biological molecules such as cytokines by hybridoma cells might be another concern (FDA 1990). Depending upon their nature and concentration, these cytokines might enhance the antigenicity of the product.

"Known" or expected contaminants should be monitored at the successive stages in a purification process by suitable in-process controls, e.g., sensitive immunoassay(s). Tracing of the many "unknown" cell-derived proteins is more difficult. When developing a purification process, other less-specific analyses such as SDS-PAGE are usually used in combination with various staining techniques.

Product-related contaminants/variants may pose a potential safety issue for patients. These contaminants can be aggregated forms of the product, a product with heterogeneity in the disulfide bridges, N- and C-terminal variants, glycosylation variants, deamidated product, etc. Some of these contaminants/variants are described in the following paragraphs.

N- and C-Terminal Heterogeneity

A major problem connected with the production of biotech products is the problem associated with the amino (NH2)-terminus of the protein, e.g., in E. coli systems, where protein synthesis always starts with methylmethionine. Obviously, it has been of great interest to develop methods that generate proteins with an NH2-terminus as found in the authentic protein. When the proteins are not produced in the correct way, the final product may contain several methionyl variants of the protein in question or even contain proteins lacking one or more residues from the amino terminus. This is called the amino-terminal heterogeneity. This heterogeneity can also occur with recombinant proteins (e.g., α-interferon) susceptible to proteases that are either secreted by the host or introduced by serum-containing media. These proteases can clip off amino acids from the C-terminal and/or N-terminal of the desired product (amino- and/or carboxy-terminal heterogeneity). Amino- and/or carboxy-terminal heterogeneity is not desirable since it may cause difficulties in purification and characterization of the proteins. In case of the presence of an additional methionine at the N-terminal end of the protein, its secondary and tertiary structure can be altered. This could affect the biological activity and stability and may make it immunogenic. Moreover, N-terminal methionine and/or internal methionine is sensitive to oxidation (Sharma 1990).

Conformational Changes/Chemical Modifications

Although mammalian cells are able to produce proteins structurally equal to endogenous proteins, some caution is advisable. Transcripts containing the full-length coding sequence could result in conformational isomers of the protein because of unexpected

secondary structures that affect translational fidelity (Sharma 1990). Another factor to be taken into account is the possible existence of equilibria between the desired form and other forms such as dimers. The correct folding of proteins after biosynthesis is important because it determines the specific activity of the protein (Berthold and Walter 1994). Therefore, it is important to determine if all molecules of a given recombinant protein secreted by a mammalian expression system are folded in their native conformation. In some cases, it may be relatively easy to detect misfolded structures, but in other cases, it may be extremely difficult. Apart from conformational changes, proteins can undergo chemical alterations, such as proteolysis, deamidation, and hydroxyl and sulfhydryl oxidations during the purification process. These alterations can result in (partial) denaturation of the protein. Vice versa, denaturation of the protein may cause chemical modifications as well (e.g., as a result of exposure of sensitive groups).

Glycosylation

Many therapeutic proteins produced by recombinant DNA technology are glycoproteins (Sharma 1990). The presence and nature of oligosaccharide side chains in proteins affect a number of important characteristics, like the proteins' serum half-life, solubility, and stability, and sometimes even the pharmacological function (Cumming 1991). Darbepoetin, a second-generation, genetically modified erythropoietin, has a carbohydrate content of 80 % compared to 40 % for the native molecule, which increases the in vivo half-life after intravenous administration from 8 h for erythropoietin to 25 h for darbepoetin (Sinclair and Elliott 2005). As a result, the therapeutic profile may be "glycosylation" dependent. As mentioned previously, protein glycosylation is not determined by the DNA sequence. It is an enzymatic modification of the protein after translation and can depend on the environment in the cell. Although mammalian cells are very well able to glycosylate proteins, it is hard to fully control glycosylation. Carbohydrate heterogeneity can occur through the size of the chain, type of oligosaccharide, and sequence of the carbohydrates. This has been demonstrated for a number of recombinant products including interleukin-4, chorionic gonadotropin, erythropoietin, and tissue plasminogen activator. Carbohydrate structure and composition in recombinant proteins may differ from their native counterparts, because the enzymes required for synthesis and processing vary among different expression systems (e.g., glycoproteins in insect cells are frequently smaller than the same glycoproteins expressed in mammalian cells) or even from one mammalian system to another.

Proteolytic Processing

Proteases play an important role in processing, maturation, modification, or isolation of recombinant proteins. Proteases from mammalian cells are involved in secreting proteins into the cultivation medium. If secretion of the recombinant protein occurs co-translationally, then the intracellular proteolytic system of the mammalian cell should not be harmful to the recombinant protein. Proteases are released if cells die or break (e.g., during cell break at cell harvest) and undergo lysis. It is therefore important to control growth and harvest conditions in order to minimize this effect. Another source of proteolytic attack is found in the components of the medium in which the cells are grown. For example, serum contains a number of proteases and protease zymogens that may affect the secreted recombinant protein. If present in small amounts and if the nature of the proteolytic attack on the desired protein is identified, appropriate protease inhibitors to control proteolysis could be used. It is best to document the integrity of the recombinant protein after each purification step.

Proteins become much more susceptible to proteases at elevated temperatures. Purification strategies should be designed to carry out all the steps at 2–8 °C (Sharma 1990) if proteolytic degradation occurs. Alternatively, Ca2+ complexing agents (e.g., citrate) can be added as many proteases depend on Ca2+ for their activity. From a manufacturing perspective, however, providing cooling to large-scale chromatographic processes, although not impossible, is a complicating factor in the manufacturing process.

BACTERIA: PROTEIN INCLUSION BODY FORMATION

In bacteria soluble proteins can form dense, finely granular inclusions within the cytoplasm. These "inclusion bodies" often occur in bacterial cells that overproduce proteins by plasmid expression. The protein inclusions appear in electron micrographs as large, dense bodies often spanning the entire diameter of the cell. Protein inclusions are probably formed by a buildup of amorphous protein aggregates held together by covalent and non-covalent bonds. The inability to measure inclusion body proteins directly may lead to the inaccurate assessment of recovery and yield and may cause problems if protein solubility is essential for efficient, large-scale purification (Berthold and Walter 1994). Several schemes for recovery of proteins from inclusion bodies have been described. The recovery of proteins from inclusion bodies requires cell breakage and inclusion body recovery. Dissolution of inclusion proteins is the next step in the purification scheme and typically takes place in extremely dilute solutions which tend to have the effect of increasing the volumes of the unit operations during the manufacturing

phases. This can make process control more difficult if, for example, low temperatures are required during these steps. Generally, inclusion proteins dissolve in denaturing agents such as sodium dodecyl sulfate (SDS), urea, or guanidine hydrochloride. Because bacterial systems generally are incapable of forming disulfide bonds, a protein containing these bonds has to be refolded under oxidizing conditions to restore these bonds and to generate the biologically active protein. This so-called renaturation step is increasingly difficult if more S-S bridges are present in the molecule and the yield of renatured product could be as low as only a few percent. Once the protein is solubilized, conventional chromatographic separations can be used for further purification of the protein.

Aggregate formation at first sight may seem undesirable, but there may also be advantages as long as the protein of interest will unfold and refold properly. Inclusion body proteins can easily be recovered to yield proteins with >50 % purity, a substantial improvement over the purity of soluble proteins (sometimes below 1 % of the total cell protein). Furthermore, the aggregated forms of the proteins are more resistant to proteolysis, because most molecules of an aggregated form are not accessible to proteolytic enzymes. Thus the high yield and relatively cheap production using a bacterial system can offset a low-yield renaturation process. For a non-glycosylated, simple molecule, this still is the production system of choice.

COMMERCIAL-SCALE MANUFACTURING AND INNOVATION

A major part of the recombinant proteins on the market consist of monoclonal antibodies produced in mammalian cells. Pharmaceutical production processes have been set up since the early 1980s of the twentieth century. These processes essentially consist of production in stirred tanks bioreactors, clarification using centrifugation, and membrane technology, followed by protein A capture, low-pH virus inactivation, cation-exchange and anion-exchange chromatography, virus filtration, and UF/DF for product formulation (Shukla and Thömmes 2010). Such platform processes run consistently at very large scale (e.g., multiple 10,000 L bioreactors and higher). Product recovery is generally very high (>70 %). Since product titers in the bioreactors have increased to a level where further increases have no or a minimal effect on the cost of goods, and the cost of goods are a fraction of the sales price, the focus of these large-scale processes is changing from process development to process understanding (Kelley 2009). However, it is also anticipated that the monoclonal antibody demands may decrease due to more efficacious products like antibody-drug conjugates and the competition with generic products. A lower demand together with the increase in recombinant protein yields will lead to a decrease in bioreactor size, an increase in the need for flexible facilities, and faster turnaround times leading to a growth in the use of disposables and other innovative technologies (Shukla and Thömmes 2010).

SELF-ASSESSMENT QUESTIONS

■ Questions

1. Name four types of different bioreactors.
2. Chromatography is an essential step in the purification of biotech products. Name at least five different chromatographic purification methods.
3. What are the major safety concerns in the purification of cell-expressed proteins?
4. What are the critical issues in production and purification that must be addressed in process validation?
5. Mention at least five issues to consider in the cultivation and purification of proteins.
6. True or false: Glycosylation is an important posttranslational change of pharmaceutical proteins. Glycosylation is only possible in mammalian cells.
7. Glycosylation may affect several properties of the protein. Mention at least three possible changes in case of glycosylation.
8. Pharmacologically active biotech protein products have complex three-dimensional structures. Mention two or more important factors that affect these structures.

■ Answers

1. Stirred-tank, airlift, microcarrier, and membrane bioreactors
2. Adsorption chromatography, ion-exchange chromatography, affinity chromatography, hydrophobic interaction chromatography, gel-permeation or size-exclusion chromatography
3. Removal of viruses, bacteria, protein contaminants, and cellular DNA
4. Production: scaling up and design. Purification: procedures should be reliable in potency and quality of the product and in the removal of viral, bacterial, DNA, and protein contaminants
5. Grade of purity, pyrogenicity, N- and C-terminal heterogeneity, chemical modification/conformational changes, glycosylation, and proteolytic processing
6. False; glycosylation is also possible in yeast (and to some degree in bacteria)
7. Solubility, pKa, charge, stability, and biological activity
8. Amino acid structure, hydrogen and sulfide bridging, posttranslational changes, and chemical modification of the amino acid rest groups

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