The clinical pharmacology of biotechnology products.
M.M. Reidenberg, editor


# PURIFICATION OF PROTEINS: CONVERTING A CULTURE BROTH INTO A MEDICINE

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## SUMMARY

In living organisms proteins maintain functions important to life. Faulty functioning or deficiency of proteins give rise to pathological reactions. Such physiologically occurring proteins can now be produced, using the methods of recombinant DNA technology and administered to patients for replacement therapy. A number of proteins as active ingredients of pharmaceutical preparations are already available for therapeutic use as immunomodulators, agents for tumour treatment, plasma proteins and hormones. They are in various stages of development, ranging from cloning of the producing cells to marketing of the finished products. Since the active substances are proteins synthesized by recombinant cells, their purification presents a particular challenge to protein chemists.

After fermentation of the appropriate production organism, hosting the gene for the protein synthesis, the purification of recombinant DNA-derived proteins intended for human use is an essential part of the biotechnical process. The characteristics of the protein determine if microorganisms or cell cultures are used and this in turn defines the first purification step. The microorganisms are disrupted, and the insoluble protein, deposited in insoluble inclusion bodies, has to be renatured, or the proteins secreted by mammalian cells have to be concentrated. The subsequent strategy for purification of the protein does not depend on the fermentation process but is entirely determined by the physicochemical properties of the proteins. The goal of the first purification step is to isolate as fast and quantiatively as possible the recombinant DNA derived protein from the culture filtrate, in order to minimize potential changes brought about by proteases or glycosidases. Immunoaffinity or ligand-affinity chromatography is used preferentially for this purpose.

The concentration of protein and buffer changes are carried out by preci-

pitation followed by reconstitution or, preferably, by dialysis and ultrafiltration/diafiltration. The methods used for the separation of cellular proteins from recombinant DNA derived proteins are combinations of anion and cation exchange chromatography and gel permeation. Nucleic acids are removed efficiently by anion exchange chromatography on DEAE-columns or by degradation with nucleases.

Potential viral contaminants which might be associated with mammalian cell culture are preferably removed by ultrafiltration or inactivated by detergents. The purification process, which is carried out under aseptic conditions, is completed by sterile filtration, followed by freeze-drying thereby stabilizing the product. The efficiency of the purification process and the quality of the final product depend on the selection and sequence of the purification steps.

For therapeutic use in patients, active proteins must be free of microbial and viral contaminants. The content of protein impurities must be below 0.1%. According to international guidelines the limit for contamination by nucleic acids may not exceed 100 pg per dose. Biological contaminants, which are difficult to detect at low concentrations in the final product, must be removed by a validated method of processing, and the purification factors must be sufficiently effective.

## 1. INTRUDUCTION

Proteins are essential for the maintenance of bodily functions. Structural proteins contribute to the construction of cell membranes, intracellular microtubuli and microfilaments. The porins transport substrates and ions through the cell membrane, which controls the energy supply and osmotic pressure in the cell.

Enzymes catalyze chemical transformations taking place in the body, with substrates undergoing anabolism or catabolism to supply energy, detoxification, or metabolism for the de novo synthesis of essential compounds. Proteineous hormones regulate the body's metabolism and maintain the balance of synthetic activity. Protein receptors transmit signals which, in turn, initiate metabolic reactions. The antibodies are part of the immune response of the body, defending it against microbial or viral infections.

Generally, proteins act to maintain the state of physiological equilibrium in the body. Malfunctioning of these proteins can result in reactions which cause pathological states at the level of the protein molecules. This situation has led to the logical strategy of restoring the physiological equilibrium by providing the body with sufficient quantities of the proteins involved.

The low concentrations of functional proteins in human organs or body fluids, and the risk of transmission of viral infections, such as hepatitis and AIDS, or Creutzfeld-Jakob disease, make it either impossible or too risky to obtain proteins from human tissue or body fluids for therapeutic purposes. Chemical synthesis is no alternative because of the high molecular weight and the complex structure of most of the medically relevant proteins or glycoproteins. At present, chemical synthesis of proteins is possible only up to a molecular weight of about 15.000 Daltons, although not on an economically basis. The only possibility of producing such proteins or glycoproteins for therapeutic purposes is by biotechnology based on recombinant DNA technology. This paper deals exclusively with the purification and quality control of recombinant DNA-derived proteins which have been obtained by fermentation of microorganisms or cell cultures for use as active ingredient in pharmaceuticals.

## 2. PROTEINS AND GLYCOPROTEINS OF MEDICAL RELEVANCE

Basic medical research has identified a large number of proteins whose faulty activity in the body leads to disease symptoms. According to their profile of action these proteins can be classified as immunomodulators, antitumour agents, plasma proteins and hormones (1). Producing active proteins by biotechnological methods is a relatively new but promising technology, that potential protein-drugs are found in various stages of development.

Human insulin, interferon alpha, interferon beta, interferon gamma, human growth hormone, tissue plasminogen activator and erythropoietin are already commercially available. Tumour necrosis factor, interleukins, coagulation factor VIII, cardiactin, calcitonin, epidermal growth factor, relaxin, renin, oncostatin, protein A and other products are undergoing intensive clinical trials.

In addition, many more proteins are still in the stage of cloning, fermentation scale-up, purification or preclinical evaluation.

Thus, the production of biologically active proteins on the basis of recombinant DNA technology presents a challenge to develop effective and economic processes for producing proteins of high purity and their characterization (2, 3). All these proteins are used for life saving applications where no other pharmaceuticals are available or not satisfactory in patient benefit.

## 3. OUTLINE OF THE PROCESSING AND PURIFICATION METHODS

The first step in the processing of proteins produced biotechnologically is determined by the producing organisms chosen. Proteins with relatively low molecular weights, few disulfide bridges and no glycosylation of relevance to the biological or immunological action, can be produced economically by microorganisms. In contrast, proteins with high molecular weights, numerous disulfide bridges and glycosylation which is relevant for the action, can be produced only by cell cultures, since only they have a synthetic apparatus which guarantees correct folding of the protein via the disulfide bridges, and glycosylation comparable to that in the natural glycoprotein.

In the case of microorganisms the protein is found in insoluble form as an inclusion body in the cell, in the case of cell cultures it is secreted in soluble form (4). The consequence is that, with microorganisms the first processing step is to disrupt the cells and to renature the protein which is in the form of an insoluble inclusion body. Although this results in enrichment of the recombinant protein, it also causes contamination with intracellular proteins and components of the medium, as well as incorrectly folded recombinant proteins. This problem can be alleviated to a certain extent, by the use of an appropriate expression system, if the proteins can be secreted into the periplasmic space of Escherichia coli, instead of being concentrated in inclusion bodies (5, 6). The goal of this first processing step is to obtain a homogeneous solution of the recombinant DNA derived protein.

In contrast to this problem associated with microbial fermentation, which adds to the difficulties of purifying the recombinant DNA derived proteins, the proteins secreted by cell cultures are present in their natural dissolved form (7).

For the efficiency and economy of the separation of potential contaminants it is of decisive importance that these contaminants are reduced to a minimum already in the cell culture medium. Measures, which significantly facilitate a proteinchemical purification due to reduced protein contaminants, are serum-free or preferentially protein-free cell culture media and an efficient removal of the production cells without cell lysis from the culture medium containing the active protein.

Because of the low concentration of the recombinant DNA derived protein in the cell culture filtrate, the first processing step in this case, after removal of the cells, consists of concentration of the protein, usually by affinity chromatography or ultrafiltration.

From this stage of recovery onwards, further purification of the recombinant DNA derived protein can be designed irrespective of the producing organism - microorganism or cell culture - and is chiefly determined by the physicochemical properties of the protein. The most important of these are: the molecular weight, the shape, the charge - determined by the amino acid composition and the glycosylation - and the hydrophobicity of the protein. These properties result in the following methods being used to purify the proteins: based on the shape and size of the molecule - ultra-filtration and gel permeation; based on the charge - ion exchange chromatography; based on the hydrophobicity - precipitation or affinity chromatography (8); and based on the specific affinities of active centres of the protein - chromatography using corresponding ligands or immobilized metal ions. Although this is a sufficient number of separation methods, the skill is to select the optimal combination of purification steps. The design of the purification process determines the yield, and thus the cost of the process, and often also the quality of the product.

In the first purification step as much as possible of the recombinant protein should be concentrated from the culture filtrate, in order to

minimize any breakdown by proteases or glycosidases during the downstream processing. Monoclonal antibodies are particularly suitable since they are covalently bound to chromatography materials and recognize specific epitopes of the recombinant protein (9, 10). Thus, immunoaffinity chromatography is frequently used for the quantitative isolation of recombinant DNA derived proteins such as interferons, tumour necrosis factor or tissue plasminogen activator. However, despite the selective advantages of this method, it is relatively cumbersome: hybridoma techniques must be used to produce large amounts of monoclonal antibodies which, after appropriate purification, are then coupled to suitable chromatography materials, such as cyanogen bromide activated agarose. The useful lifespan of these immunoaffinity columns are relatively short. In addition, antibodies may become detached from the matrix during elution of the recombinant DNA derived protein. Moreover, because transformed cells have been used to produce the monoclonal antibodies, their freedom from oncogenic material must be demonstrated.

GMP-Guidelines for the pharmaceutical production of proteins for parenteral use require for all reagents which will be in contact with the product during the manufacturing process, that they have to be produced, analyzed and certified according to GMP-Guidelines. In accordance to these guidelines, the FDA requires for the production of monoclonal antibodies used as ligands for immunoaffinity chromatography that they are produced comparable to a therapeutic agent. This implies an expensive GMP-production for the monoclonal antibody itself in order to obtain the ligand. When using immunoaffinity chromatography, additional purification steps must be validated to remove any antibody contamination by column leakage, analytical assays must be established respectively.

Thus, a common alternative to immunoaffinity chromatography for the first purification step is bioaffinity chromatography. This method makes use of the specific binding of the protein, such as substrate specificity or specific affinity for metal ions (11, 12). The recombinant DNA derived protein is selectively purified by adsorption onto appropriate ligands coupled to the chromatography material. The ligands often used for this purpose are peptides or aminoacids which correspond to a specific centre of the enzyme. Thus, efficient purification of urokinase is possible with

benzamidine-Sepharose (13) or pyroglutamyl-lysyl-leucyl-arginal-Agarose (14), and of tissue plasminogen activator with Lysine-Sepharose (15).

Basic matrices which allow an application of this technology at a technical scale are preferably Sepharose FF, Affi Gel or Eupergit because of their rigidity. Ligands, which specifically interact with the recombinant DNA derived protein, are linked to these matrices via appropriate spacers to avoid steric hindrance.

Affinity chromatography is specially designated for quantitative purification of proteins present in small concentrations in large volumes and with a high degree of contaminating proteins. The separating performance and capacity correlates with the specificity of the ligand. A rapid and economic purification of monoclonal antibodies for therapeutic use can be achieved by using affinity chromatography on protein A- or protein G-substituted matrices such as Repligen protein A-agarose or Genex Gammabind Plus.

For subsequent purification steps such as precipitation, followed by centrifugation or filtration, the protein solutions can be concentrated and the buffer be changed. Reconstitution of the precipitate results in transfer of the protein into a suitable buffer solution. However, recent developments in membrane technology have lead to an increase in the use of ultrafiltration and diafiltration techniques for concentrating and exchanging the buffer in protein solutions. In this method the protein remains in its natural, dissolved form (16, 17).

Electrical charge of proteins resulting from basic or acid amino acids are suitable for chromatographic separations on ion exchangers. This separation procedure is suited for the chromatography of large volumes during the initial procesing steps, also providing a good separating performance at a high capacity (up to 15 mg/ml gel) and a high linear flowrate (up to 1,500 cm/h). Chromatography materials preferably used in this aspect are ion exchange resins based on highly cross-linked agarose or equivalent matrices concerning rigidity and sanitization. There is no obvious preference for weak or strong ion exchangers as both types have

their advantages: strong ion exchangers allow very robust process steps because of their relative insensitivity over a broad pH-range, which becomes important for elimination of potential hazardous contaminants, e.g. DNA removal by anion exchange chromatography. On the other hand, weak exchangers are more accessible to fine tuning of physical conditions regarding pH-value and ionic strength of the sample, but for this the corresponding purification step is somewhat sensitive to any deviation of physical conditions.

Combinations of anion and cation exchange chromatography are suitable for subsequent removal of cellular proteins or of proteins from the nutrient medium. A separation according to the molecular weight, by gel permeation, is also suitable for this purpose.

The hydrophobicity of proteins as a selective parameter can be exploited to full advantage on particularly substituted matrices of Sepharose, Fractogel TSK, Eupergit as well as CPG (Controlled Pore Glass) and Bioran. These procedures are appropriate primarily for materials with a high ionic strength following precipitation or ion exchange chromatography. Hydrophobic matrices can be utilized in two modes: for hydrophobic interaction chromatography, using buffers of high and low salt molarities and for low pressure reversed phase chromatography, using buffers which contain organic solvents such as ethanol, 1-propanol or 2-propanol as an eluting agent. By this, the reversed phase mode predestinates such a purification step to be validated to inactivate potential viral contaminants.

Gelpermeation chromatography with high resolving matrices such as Sephacryl HR or Superdex allows separations according to molecular sizes of proteins. The efficiency of separation depends extremely on the volume of the sample to be separated, therefore gelpermeation is designated to be one of the latest steps in protein purification. Gelfiltration with conventional dextran gels is primarily suited for buffer exchange or formulation.

For economic reasons chromatographic materials for large scale purification of recombinant DNA derived proteins intended for therapeutic use must

fulfill some basic requirements: high separating performance and capacity (> 10 mg product/ml gel) at a constant quality, pressure stability at high flow rates (> 200 cm/h), possibility of scaling-up from laboratory conditions to production scale, and long life time (> 100 cycles). In addition, the materials must be suited for sanitization and the initial costs should be low. In this respect the sanitization has for the production of biotechnically synthesized pharmaceuticals the goal to achieve sterility of the column materials by treatment with 0.1 - 1 M NaOH, 0.01 % Merthiolate/ Thimerosal or 25 % ethanol. It is also used for the removal of pyrogens by 0.5 - 1.0 M NaOH and the elimination of ionically bound proteins by 2 M NaCl or that of hydrophobically bound proteins by 1 M NaOH, 3 M Na-Thiorodanite or organic solvents like 75% propanol. The materials used for chromatography must resist these sanitization procedures without impairment of separating performance or life time.

Since recombinant proteins of these types are used almost entirely for parenteral administration, processing and purification are carried out under aseptic conditions, with the buffer solutions and final product being sterilized by filtration and stored at low temperatures. These steps minimize the risk of microbial contamination or protein denaturation, and the pyrogen content of such parenteral products can be controlled.

In most cases, after downstream processing the recombinant DNA derived protein has been obtained in a purity greater than 99 % and is subjected to freeze-drying. The freeze-dried product is very stable over years, even at room temperature.

For economic reasons the design of the purification process is of decisive importance because the sequence and number of process steps determines yield, purity and costs of downstream processing. However, the selection of appropiate purification schemes is determined by physicochemical protein parameters and the particular contaminants (18).

## 4. GUIDELINES FOR THE PURIFICATION OF RECOMBINANT DNA-DERIVED PROTEINS

The production of recombinant DNA derived proteins, which are

predominantly administered intravenously or subcutaneously, repeatedly or in high, non-physiological doses, must be reproducible and provide adequate purity (19, 20, 21, 22, 23, 24, 25). This is the only way to guarantee effects comparable with the natural product and to prevent immunological or toxicological reactions due to contaminants. Therefore, it is necessary to validate the purification process for removal of antigenic material originating from the producer cell or the culture medium. The content of protein impurities must be below 0.1%.

Concerning contamination with potentially infectious material, such as viruses or microorganisms which may be associated with the producer strain, it is necessary to demonstrate that these can be removed entirely by a number of purification steps. The final product must be free of viral and microbial contamination.

## 5. REMOVAL OF POTENTIAL HAZARDOUS CONTAMINANTS

### 5.1 Potential viral contaminants

Measures for the removal of potential viral contaminants can be taken all the more reliably, if only cell cultures essentially free of endogenous viruses are employed for the expression of recombinant DNA derived proteins. For the fermentation only batches of fetal calf serum tested for absence of viruses should be used. For the proteins which are added to the medium, validated purification schemes suited for the removal of potential viral contaminants should be employed.

In most cases of cell culture fermentation processes viral contaminants are not detectable. Therefore, spiking experiments in which distinct viruses are added the cell culture fluid must be performed for the validation of the purification procedure. As not all viruses can be ascertained and examined with these spiking experiments, a selection should cover all types of viruses if possible (26).

ß-Propiolactone, UV-light, gamma-irradiation, extreme pH-values, high temperatures or high concentrations of chaotropic salts such as urea or

guanidine are suited for virus inactivation. With the use of these methods it must be ensured that the active protein is not influenced negatively by the treatment. In addition, an efficient removal of viruses by means of ultrafiltration through 300 KD membranes is feasible. The virus retention with Sindbis virus being a 6 log reduction starting from a virus titer of $5.7 \times 10^9$ PFU (plaque forming units), the murine xenotropic virus, a 40 nm retrovirus, could be retented to a full 4 log reduction starting from a virus titer of $1 - 5 \times 10^4$ FFU (focus forming units).

During the fermentation process virus tests concerning endogenous and adventitious virus must be performed in order to guarantee that the validated purification scheme eliminates potential contaminants. Purification factors for the inactivation and removal of potential viral contaminants of individual elimination steps are in the range of $10^3 - 10^{10}$, depending on the types of virus to be eliminated. A definite recovery process should contain different steps which account to a 12 log reduction of retrovirus like the murine xenotropic virus.

## 5.2 Contaminating nucleic acids

Based on tumorigenicity studies with intact viral nucleic acid with oncogenic potential the WHO (27) defined the maximum concentration of DNA for reasons of product safety to 100 pg DNA per dose. This concentration remains under the tumour inducing dose by a factor of $10^{10}$. Thus in respect of drug safety the purification procedure must be validated accordingly.

The content of nucleic acid essentially is minimized by a high degree of viable cells in the fermentation broth and by efficient removal of the cell mass from the cell culture fluid without cell lysis and thus the protein-chemical purification is facilitated. In most cases the nucleic acid concentration can be quantitatively determined only in the cell culture medium or after the inital process step. Therefore, in particular spiking experiments radioactive labelled DNA is added to the cell culture fluid in such an amount that the desired purification factor can be quantitatively established. Procedures of choice for nucleic acid removal are affinity chromatography which selectively binds the active protein molecule leaving

nucleic acids in the flowthrough volume, or ion exchange chromatography using anion exchangers like DE52 or DEAE-Sepharose-FF, to which nucleic acids bind quantitatively under appropriate physical conditions. By combination of different steps purification factors up to $10^{14}$ can be achieved.

### 5.3 Pyrogens

Aseptic processing procedures including methods such as "cleaning-in-place and sterilization-in-place for devices, sanitization of column materials and the use of pyrogen-free buffers considerably contribute to a pyrogen-free final product. While buffer solutions can be depyrogenized very easily and also efficiently by means of ultrafiltration with an exclusion limit of 10KD, apart from the above described preventive measures affinity chromatography is the best choice for the selective binding of the active protein and thus for the removal of pyrogens. Ion exchange chromatography methods, deep filtration or alternatively filtration with nylon filter material have been used for the removal of pyrogens. The pyrogen content in the final product should be below 1 endotoxin unit per dose to prevent a pyrogenic effect.

## 6. VALIDATION OF PROTEIN RECOVERY

Separation procedures must be validated such that they are robust against variations of the fermentation process and do not negatively influence the native structure of the product. This emphasize primarily the shifting of the quantitave relationship of the active protein to potential contaminants in the cell culture medium and protein stability during processing, whereby capacity and life cycles for filters and chromatographic material must guarantee identical conditions for each use, exhibiting reproducibility of elution profiles and meeting of in-process-control specifications (28).

## 7. QUALITY CONTROL OF THE PURIFIED PRODUCT

The development of an efficient purification procedure is only possible if expressive protein-analytical methods for the determination of the

purity, identity and activity of the product are available. Due to the complex structure of the proteins to be examined, numerous analytical methods must be applied whose results as a whole testify the quality of the product.

## 7.1 Determination of purity

For the determination of the purity the following methods are available:

SDS-PAGE for the determination of the relative molecular weight, detection of oligomeric structures and contaminants above 200 ppm of single contaminating proteins. Contaminants below this detection limit or possessing electrophoretic properties comparable to those of the active ingredient are lost for detection.

Isoelectric focussing as a guarantee for the constancy of complex proteins, for the determination of the microheterogenicity of glycoproteins, detection of deamidation of glutamine or asparagine.

HPLC-size exclusion chromatography for the determination of oligomeric structures and proteolytic cleavages.

Multiantigen-ELISA for the recovery of low concentrations (1 - 100 ppm) of contaminating proteins from the production cell or culture medium.

DNA-hybridization test for the determination of contaminations with nucleic acid with a detection limit in the nanogram range.

## 7.2 Determination of identity

For the determination of the identity the following protein-analytical methods are useful:

Amino acid analysis for smaller proteins (< 15,000 Dalton) and for the determination of norleucine substitution of methionine.

Partial sequencing of 8 - 10 amino acids at the N-terminal end of the protein and for the determination of potential proteolytic cleavages in this section.

Peptide mapping by HPLC separation of peptides following proteolytic cleavage of the active protein for the determination of the primary protein structure including the microheterogenicity of carbohydrate containing peptides, N- and C-terminal peptides, point mutation in the section of the cleavage sites and methionylated proteins with an additonal N-terminal amino acid methionin.

Immunoassay for epitope mapping of the active protein.

Carbohydrate analysis via determination of the neutral sugars, the sialic acid content and the sequence of monosaccharides as well as the enantiomeric configuration of glycoside links.

Circular dichroism for the determination of secondary and tertiary structures, conformation changes and structural equivalence.

### 7.3 Determination of activity

Analytical methods establishing the biological activity of the active protein are primarily determined by the mode of action of the protein. However, for the pursuit of process steps during purification and for the determination of the content of the active ingredient in the final product only those methods are appropriate which provide a respective accuracy. These are preferably enzymatic tests or biomimetic assays which allow the active principle to be reproduced (26).

## 8. FUTURE PROSPECTS FOR THE PRODUCTION OF RECOMBINANT DNA DERIVED PHARMACEUTICALS

Fundamental medical research, has helped to explain pathological states at the level of the protein molecule and molecular biology, has demonstrated the relationship between the nucleotide sequence in the genetic code

and the amino acid sequence in the protein. This has provided the foundation for the new biotechnology, based on recombinant DNA technology, to produce endogenous proteins for replacement therapy in patients (29, 30). On the one hand, this is a challenge to protein chemists to prepare such recombinant DNA derived proteins of human origin in a pure state and to characterize them, and, on the other hand, it is an exceptional opportunity to provide new and promising approaches to treatment of patients. This applies particularly where chemically synthesized alternatives are not available or not satisfactory.

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Discussion - PURIFICATION OF PROTEINS CONVERTING A CULTURE BROTH INTO A MEDICINE

P. Juul

I have two questions. If you could choose between E. coli and yeast what would you prefer or, to put it into different words, what is the reason that different companies chose either E. coli or yeast? The second question concerns stability and storage problems. We accept a higher extent of degradation of some of the biological products. I am not afraid of a loss of potency of 10% but I'm afraid of the 10% which has gone to something which I don't know what it is, and might possibly constitute neoantigens.

R.G. Werner

Concerning the choice between E. coli and yeast, by using E. coli, one has a very cheap fermentation process, and the yield can be up to 3 g per liter. If one chooses the yeast, the fermentation costs are about the same, but the yield is far below, in the range of 500 - 800 mg/l. The advantage of choosing the yeast is that the molecule is secreted into the medium. It is there in its native form, so there is no need for refolding compared to E. coli. One has to take into account the cost of the recovery process. If one chooses E. coli for the production of an interferon molecule, for example, one needs, additional purification steps to separate uncorrectly folded molecules from the correctly folded molecules, and this is very difficult because the molecular weight and the charge of the molecule is identical. As far as the second question is concerned, an uncorrectly folded protein in a pharmaceutical preparation carries the risk that the immune response recognizes it as an antigen. If during the shelf life proteins are degradated and still used for therapy, antibodies against these fragments can be raised, which also can react against the endogenous protein.

A.J.H. Gearing

You said earlier that those molecules that you described were not too amenable to chemical synthesis. It's not actually quite true in that several of the cytokines, like interleukin 3, have been chemically-synthesized in gram quantities and seem to be fully active.

R.G. Werner

There is a possibility to chemical synthesis protein up to 15,000 molecular weight. Human growth hormone can be chemically synthesized. And, of course, insulin, with a

molecular weight of 6000 was one of the first therapeutically useful proteins to be synthesized. However, the process is not economic because one loses too much at each step, even with a 99% yield in each chemical peptide bound synthesis.