



Review

# Synthesis and chromatographic purification of recombinant human pituitary hormones

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## Abstract

Recombinant DNA-derived proteins and, in particular, human pituitary hormones, are increasingly used for research, diagnostic and therapeutic purposes. This trend has demanded new synthetic approaches and improved purification techniques. The type and sequence of the purification steps have to be selected in accordance with the cloning and protein expression strategy, the host organism and cellular localization of the protein of interest, with a view to producing the desired product at a required purity, biological activity and acceptable cost. This review article describes and analyzes the main synthetic and purification strategies that have been used for the production of recombinant human growth hormone, prolactin, thyrotropin, luteinizing hormone and follicle-stimulating hormone, giving special consideration to the few published downstream processes utilized by the biotechnology industry. Practically all types of prokaryotic and eukaryotic organisms utilized for this purpose are also reviewed.

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## 1. Introduction

The anterior pituitary gland produces at least six polypeptide hormones which have regulatory roles in the growth, development and function of endocrine tissues such as the thyroid, adrenal cortex, gonads, mammary gland and pancreas, i.e. thyrotropin (TSH), corticotropin (ACTH), luteinizing hormone (LH), follicle-stimulating hormone (FSH), prolactin (PRL) and growth hormone (GH). Some of these hormones also act on peripheral nonendocrine tissues and hence are involved in hundreds of different physiological functions. Since 1886, when the French neurologist, Pierre Marie, first described enlargement of the pituitary in acromegaly, this gland has been used as a source of hormones for research, and later also for therapeutic and diagnostic purposes. Particularly noteworthy are the studies of Vincent du Vigneaud et al., who, in the early 1950s, began the isolation, synthesis and structure studies of the posterior pituitary peptides, oxytocin and vasopressin. The event that most revolutionized endocrinology and possibly physiology in general, was the development in the late 1950s of highly sensitive, specific and quite accurate radioimmunoassays (RIAs) for identification and quantification of the hormones. Specific antibodies against the hormones and highly purified radioiodinated tracers required for the assays, had become available as a result of the relatively high purity that could be achieved for hormone preparations extracted from pituitary glands. Recombinant hormones were not available until 1977 when Itakura et al. [1] produced the first recombinant somatostatin. Of the six anterior pituitary hormones mentioned above, ACTH, a 39-amino-acid peptide, was in general produced by classical chemical synthesis. The other five were always obtained via laborious and inefficient extraction of pituitary glands, using purification processes that gave extremely low yields of products that were never completely free of other pituitary hormones. The content of the five hormones in the pituitary is

of the order of 30–150  $\mu\text{g}$ , with the exception of GH which is present as 5–10 mg/gland. Moreover, the pituitary-extracted material was found to be potentially hazardous for use in vivo because of possible viral and prion contamination [2–4]. These considerations, together with the introduction of recombinant DNA technology more than two decades ago, paved the way for synthesis of recombinant pituitary hormones: i.e. first somatostatin, which was then followed by human growth hormone, insulin and other hormones [5,6].

The structural and chemical characteristics of a protein in its native form clearly define the strategy for its synthesis by recombinant DNA technology, the type and propagation of the host cells involved and the purification process. For the setting up of rationale and profitable bioprocesses it appears advisable to treat cell culture and purification process as a single unit, utilizing coincident designs [7]. It also should be kept in mind that a choice made upstream may positively or negatively influence all downstream processes. Human (h) growth hormone and prolactin are closely-related polypeptide hormones, containing 191 and 199 amino acids, respectively, which share a number of amino acid sequences and have similar properties. Consequently, their synthesis and purification can be approached in a similar manner. However, whereas the major form of hGH is synthesized mainly in the nonglycosylated form (from the hGH-N gene), hPRL, which is present in humans as a single gene, is expressed and secreted by the pituitary in the nonglycosylated and also to a significant degree (20%) in the glycosylated form [8]. The choice of a prokaryotic or eukaryotic expression system therefore provides the option of producing, starting from the same gene, either nonglycosylated, homogeneous hPRL or a heterogeneous product, which is closer to hPRL as it is present in the blood. TSH, LH and FSH are glycoprotein hormones which consist of two noncovalently linked subunits,  $\alpha$  and  $\beta$ . The  $\alpha$  subunit (92 amino acid residues) is common to all three hor-

mones, while the  $\beta$  subunit, consisting of 118 (hTSH), 121 (hLH) or 111 (hFSH) amino acids, is unique for each hormone, determining its biologic specificity. In view of their structural and functional similarity, we regard these hormones as compounds of the same type with respect to the mode of synthesis, cultivation and purification processes.

Recombinant human pituitary protein hormones can be generated using five major expression systems routinely used for protein synthesis: the bacterial, yeast, baculovirus, plant and mammalian cell systems. In selecting an expression system one must first consider what type of post-translational modification is required, or desired, for full activity or, in some cases, even superactivity of the product. The main post-translational modifications encountered with eukaryotic proteins are glycosylation, phosphorylation, sulphation, acylation, myristylation, subunit assembly, isoprenoid and glycosylphosphatidylinositol anchor introduction. Whereas such modifications in general cannot be obtained in bacteria, eukaryotic expression systems can approximate such alterations qualitatively and quantitatively. In insect cells, the oligosaccharide processing pathway diverges from the analogous pathway in mammalian cells. As a consequence, the baculovirus expression system in insect cells is unable to synthesize the same complex carbohydrate moieties characteristic for mammalian glycoproteins, often rendering instead lower-molecular-mass and less bioactive nonsialylated isoforms [9,10]. Even different types of mammalian cells can lead to the synthesis of diverse carbohydrate structures. Chinese hamster ovary (CHO) cells, for example, the most widely used host cells for synthesis of therapeutic glycoproteins, are unable to sulfate oligosaccharides and to add terminal sialic acid residues via an  $\alpha$  2,6-linkage, which is normally performed by many types of human cells, including pituitary thyrotropes and gonadotropes, the specialized cells which synthesize hTSH, hLH and hFSH [11]. From these examples it appears that while a single-chain, unmodified protein such as human growth hormone can in principle be synthesized in any of the above expression systems, bacteria can only be used for the production of prolactin if only its unmodified protein structure is required. Fully biologically active glycoprotein hormones can only be generated using

mammalian cells. Furthermore, while unmodified recombinant proteins, such as human growth hormone, can and must be chemically identical to the native form, this condition can at present not be met for recombinant, therapeutic glycoproteins, which will always be somehow different in their carbohydrate structure when compared to the native, circulating form. In addition, carbohydrate variability cannot only arise from the type of host cell used for the expression of the glycoprotein [9,12–14], but also from different cell cultivation and bioreactor conditions or the presence of specific factors in the culture medium [15–17].

Gene expression, cell cultivation and downstream processing are strictly correlated and constitute the manufacturing process of recombinant proteins in general and of pituitary protein hormones in particular. Together with the selection of the most suitable host cell, the location where the protein will be synthesized or secreted must be defined: DNA recombinant techniques can be used both to direct the protein of interest to a certain location inside or outside the cell, and to control its solubility, accumulation or secretion. This strategy will obviously govern the choice of the cell cultivation conditions and downstream processing. With regard to the location, we have two choices: either intracellular production or excretion into the medium. Intracellular production of the protein in Gram-negative bacteria can occur in three ways: the protein may be generated in a soluble form in the cytoplasm; it may form cytoplasmic aggregations called inclusion bodies; and it may be secreted into the periplasm. Excretion into the medium can be obtained in bacteria by adopting different strategies; it is the common way of obtaining heterologous proteins from yeast, insect and mammalian cells.

Protein purification generates essentially the same problems no matter whether the protein is extracted from natural sources or from an engineered organism [18]. There is general agreement, however, that downstream processing is still the major challenge in the biotechnology industry, representing up to 60–70% of the total bioprocessing operating costs and the most time-consuming part of the undertaking [7,19]. All separation steps are based on four fundamental protein characteristics: size, charge, hydrophobicity and immunobiological activity. On the

basis of these characteristics six major methodologies have been developed: precipitation, membrane filtration, gel filtration, ion exchange, affinity and hydrophobic-interaction chromatography. Their principles have been known for over half a century and have been applied to the purification of all pituitary-derived hormones. With regard to GH, the pioneering work by Li and co-workers is especially memorable [20,21], since by 1944 they had already purified GH from bovine pituitary glands. Other ground-breaking studies were carried out by Raben et al. [22], Wilhelmi et al. [23] and Roos et al. [24]. Human PRL was first purified by Lewis et al. [25] and later also by Hwang et al. [26], Davies and Hartree [27] and Roos et al. [28]. Hartree et al. reported the separation and purification of hTSH, hLH and hFSH in 1964 [29–31]. Important progress in purification of pituitary glycoprotein hormones was also made by Parlow and co-workers [32,33], Trygstad et al. [34], Torjesen et al. [35] and Roos and co-workers [36,37]. All these authors used methodologies that are fundamentally not so different from those later used for the separation and purification of the same hormones obtained via DNA recombinant techniques that we will analyze below. It is noteworthy that the main challenge in the purification of pituitary-derived hormones consisted of the removal of contaminating crossreacting hormones and transmissible degenerative encephalopathy (TDE) agents. In the case of the recombinant hormones, this challenge has been replaced by the equally difficult elimination of DNA, pyrogenic substances and proteins originating from host cells and culture medium. Incidence of TDE, as a result of iatrogenic transmission of a DNA-derived recombinant product, has in fact never been reported.

What has been apparent in recent years is a great emphasis on increased speed and resolution of the purification process and on the purity of the final product. This is due to obvious economical reasons and to the introduction of very stringent purity requirements necessitating the introduction of a battery of control tests. It should be kept in mind that hormone-related forms are probably the most difficult to eliminate contaminants representing in general purification artefacts. Such contaminants have always been present in pituitary-derived or recombinant preparations but, in the early days, regulations

concerning their elimination were not so strict and, in addition, adequate detection and quantifying methods were not available. Examples are sulfoxide and desamido derivatives in growth hormone preparations, whose total limit of acceptability in the final injectable product is 13% [38]. Their elimination during the downstream processing can be very difficult. Methionine sulfoxide-hGH, for example, has practically no mass or charge difference compared to the fundamental form of hGH, but only a slightly lower hydrophobicity. In cases like this one, prevention is more effective than cure. Here again emerges the importance of the speed of the purification process which according to the authors' experience [39], largely determines the yield and quality of the product. The shorter the time the protein of interest is in solution, the lower will be the amount of contaminating hormone-related forms, including aggregates, degraded and cleaved forms, since the negative influence of residual proteases, light, heat, oxygen, pH, microorganisms and agitation will also be reduced. In this respect the use of new types of columns packed with small nonporous particles, highly macroporous particles (flow-through) and more durable polymeric resins has been advantageous for generating fast-flow systems. Affinity chromatography, including use of metal and dye ligands, is notably one of the most efficient chromatographic methodologies, whose application in industrial hormone purification is still relatively limited, especially in view of its high potential [40–44]. Related to these affinity techniques are the attractive recombinant approaches that facilitate purification of proteins by insertion of an affinity tag at the N- or C-terminal of the protein. Such tagging has proven useful for ion-exchange, hydrophobic interaction, affinity, immunoaffinity and immobilized metal ion affinity chromatography (IMAC). Following separation of the protein, the tag can be cleaved out to generate the authentic form of the protein [45]. Some of these approaches have already been used for recombinant pituitary hormone purification, as we will show.

Novel chromatographic and electrophoretic techniques, although not yet applied to pituitary hormone purification, are very promising. Among these are analytical and preparative focusing techniques such as a free-flow protein purification based on isoelec-

tric electrophoresis, which has been applied to fractionation of *Escherichia coli* extracts [46], conductivity gradient focusing [47], recycling isoelectric focusing and isotachopheresis, which offers high resolution and the potential of separating proteins at high concentration [48]. Liquid–liquid extraction of proteins with a reversed micellar phase, which is very effective in selectively solubilizing specific biomolecules from dilute aqueous solutions such as fermentation and cell culture media, shows promising applications in biotechnological processes [49,50]. Micellar liquid chromatography, a technique where a micellar agent is added to a mobile phase containing a buffer and a small amount of organic modifier, is clearly a milder technique than reversed-phase high-performance liquid chromatography (RP-HPLC) and has been used for screening analysis after direct injection of physiological fluids or drugs [51–53]. Finally, preparative continuous elution electrophoresis has been demonstrated to be a very simple and fast tool for high yield purification of proteins from complex biological materials [54,55], as well as for the isolation of gene fragments [56].

Recently, new rational process designs, based on artificial intelligence tools, have been described for setting up optimal recombinant protein separation processes. An example is the approach based on a hybrid expert system of Lienqueo and Asenjo [19] which, when specifically applied to the recovery of growth hormone produced in *E. coli*, proved to be a good starting point for practical industrial application.

A revolutionary approach to the synthesis of recombinant pituitary hormones for therapeutic purposes is the introduction into experimental animals of naked DNA fragments expressing the hormone [57–62]. This promising gene-therapeutic approach has focused on human growth hormone, either because it provides a useful model or in view of potential therapy for GH-deficient patients. To this end, new downstream operations for large-scale purification of plasmid DNA or DNA fragments have recently been set up [63–67]. Considering that regulatory agencies have stringent requirements for large-scale production of all types of biotherapeutics, the total elimination of impurities from pharmaceutical-grade DNA, e.g. host-cell related RNA, will very probably also be required, together with the

introduction of a strict safety control. Although DNA processing is not within the scope of this review, it appears worthwhile to note that purification will remain one of the major challenges of biotechnology.

## 2. Expression systems for pituitary hormone synthesis

### 2.1. Bacterial expression systems

In synthesizing a heterologous protein in prokaryotic organisms there are several options. The protein can be made to accumulate in the cytoplasm either in a soluble form [68], or as aggregates in insoluble inclusion bodies [69]; alternatively, the protein can be exported across the inner plasma membrane to the periplasmic space or excreted into the culture medium [70,71]. All three strategies have been applied by several authors for the synthesis of hGH and hPRL.

#### 2.1.1. Cytoplasmic accumulation

The first, historically important undertaking to synthesize recombinant hGH was carried out by Goeddel et al. [5] who constructed synthetic-natural 'hybrid' DNA, coding for this hormone, by combining cDNA prepared from pituitary mRNA with chemically synthesized DNA. The DNA incorporated in the bacterial vector did not include the leader sequence, because it could have led to the production of unprocessed prehormone containing an extra 26 amino acids, but did code for an N-terminal methionine. Since most bacterial proteins do not contain N-terminal methionine residues, the authors had expected that this terminal amino acid would be efficiently cleaved off in the bacteria. This, however, did not happen. Substantial quantities of soluble Met-hGH (186 000 copies per cell or 2.4 µg per ml extract) were obtained, for the first time. It was subsequently established in vivo that the preparation had biological activity comparable to that of pituitary-derived hGH [72]. Later reports indicated that the presence of a methionyl residue at the N-terminus of the molecule could be responsible for the formation of antibodies against Met-hGH in patients treated with this variant [73]. Although no major drawbacks associated with the use of Met-hGH were

reported in the literature, the attention of research groups turned to methods aimed at producing authentic hGH, such as periplasmic expression systems.

Another fundamental study of the production of hGH in bacteria was published by Ikehara et al. [74]. While the generation of the hormone in this study also involved accumulation of protein in the cytoplasm, it was directed by DNA completely obtained by chemical synthesis. As a first, 78 gene fragments, varying in length from 7 to 26 bases, were synthesized and then ligated into hGH DNA. It was known that there are differences in the levels of the various transfer RNAs in bacteria that can transfer a given amino acid to the site of protein synthesis. To optimize the translational process, the codons in the DNA had been carefully selected to correspond with the codons of the transfer RNAs which, for a particular amino acid, were most frequently observed in *E. coli* [75]. The hGH yield, i.e.  $2.9 \cdot 10^6$  hGH molecules per cell, was much higher than previously reported, presumably a result of the codon selection aimed at optimal translation of the hGH.

In other studies, hGH synthesis, based on accumulation of the protein in the bacterial cytoplasm, was modified by introducing aminoterminal extensions of the hormone aimed at facilitating its subsequent purification. Dalbøge et al. [76], for example, used Met–Ala–Glu which was subsequently removed from the hormone using the exopeptidase DAP-I. This led to high yields of the hormone in the form of a soluble cytoplasmic protein (20% of total cellular protein). Unexpectedly, a bacterial enzyme naturally removed most of the N-terminal methionines before applying the DAP-I treatment. This finding indicated that the action of the bacterial peptidase is probably governed by particular amino acid sequences [77]. Mukija et al. [78] obtained accumulation of the hormone in large inclusion bodies following introduction of a His<sub>6</sub> tag aimed at subsequent purification via single-step metal ion affinity chromatography. It appeared to these researchers that cleavage of the aminoterminal extension was not essential because of its poor immunogenicity and limited interference with authentic protein folding and biological activity. Shin et al. [79] synthesized a more complex fusion protein (T2GH) in which hGH was coupled to amino acid sequences encompassing the N-terminal pentapeptide sequence of tumor necrosis

factor- $\alpha$ , a His<sub>10</sub> tag and the enterokinase cleavage site. As a result, considering also the high cell density culture ( $>85$  g dry cell mass/l) they obtained one of the highest yields ever reported (9 g/l) of T2GH, initially expressed in a soluble form and later, in the fermentation process, as inclusion bodies, which were readily solubilized by increasing the pH to 11.5. More recently, inclusion bodies representing 1.6 g of recombinant hGH/l were obtained by Patra et al. [80] who solubilized them in the presence of low concentrations of urea (2 M) that helped retain native-like secondary structures; refolding of the protein was then possible by simple dilution.

Using *Bacillus subtilis*, a gram-positive bacterium, Franchi et al. [81] directed the synthesis and intracellular accumulation of tag-hGH containing an aminoterminal extension of 7 amino acids, including charged residues (Arg) and a factor X cleavage site, capable not only of facilitating the purification of the hormone, but also of increasing its solubility with respect to Met-hGH, expressed in previous work. The yield of recombinant modified hGH was  $>5\%$  of the total proteins, 70–80% being in the soluble fraction.

Recombinant hPRL was initially also obtained with a methionine attached to the N-terminus and was almost exclusively located in cytoplasmic inclusion bodies. Cunningham et al. [82] synthesized the hormone not for its production per se, but more as a tool to determine if site-directed mutagenesis of hPRL, which is related to hGH, could lead to the development of affinity for the hGH receptor. This was achieved by substituting amino acids of hPRL with those in hGH predicted to be important in binding to the hGH receptor. Thus a variant hPRL containing eight mutations was developed which had a 10 000-fold higher association constant for the hGH receptor. The study showed that it was feasible to develop hormone receptor agonists, or antagonists, by recruiting receptor-binding properties from distantly related and functionally divergent hormones. In contrast, Paris et al. [83], in the same year, described large scale production of rhPRL in *E. coli*, in which the hormone represented about 50% of the total cellular protein. From the parameters provided, we can guesstimate an expression yield of 60  $\mu\text{g/ml}/A_{600}$ , with about 80% of the molecules posses-

sing an N-terminal methionine. Both studies provided evidence that the extra residue has no apparent effect on the structure or function of the hormone. Two fusion proteins containing hPRL were also synthesized in *E. coli* in the form of inclusion bodies and obtained in a partially purified form. Gilbert et al. [84] prepared a  $\beta$ -galactosidase–hPRL fusion protein, a ready-made ‘haptent-carrier’ molecule, and used it successfully as an immunogen to raise antibodies against hPRL. Hiraoka et al. [85] expressed a fusion protein of *S. aureus* protein A, a peptide sensitive to collagenase digestion and hPRL. The protein A segment of the fusion protein facilitated its purification via IgG-affinity chromatography; the collagenase digestion left four additional amino acids at the N-terminus which, however, did not interfere with mitogenic activity in the Nb2 lymphoma cell proliferation assay. Both approaches provided relatively low yields of heterologous protein in the order of 0.2–4.4% of total *E. coli* protein.

### 2.1.2. Periplasmic secretion

The correct processing in *E. coli* bacteria of recombinant rat proinsulin to insulin by a signal peptidase and the transport of the hormone to the periplasm, had already been documented in 1980 by Talmadge et al. [86]. This led Gray et al. [87] to initiate the first studies of the synthesis, secretion and cellular localization of hGH in *Pseudomonas aeruginosa*, another gram-negative bacterium that was transformed with plasmids containing the cDNAs encoding Met-hGH or pre-hGH. The results not only confirmed that such bacteria fail to remove the initiating methionine post-translationally, but also that *P. aeruginosa* has a signal peptidase that efficiently processes the primary hGH translation product in the same way as eukaryotic endoplasmic reticulum. As indicated by radioimmunoassay, more than 80% of the expressed hGH ( $1.49 \mu\text{g/ml}/A_{550}$ ) was found in the periplasm, about 9% in the culture medium, while only 7% was located in the cytoplasm. The most important finding in our opinion, however, was the authors’ observation, that bacterial periplasm provides the proper oxidative environment allowing efficient disulfide bond formation and correct folding of eukaryotic proteins; this was confirmed by other works [88–90]. The subsequent passage of the protein through the outer bacterial

membrane (excretion) is probably determined by another specific mechanism discriminating between periplasmic and extracellular proteins. Gray et al. [70] also demonstrated that the natural and bacterial signal sequences are interchangeable, both providing correctly processed hGH. The highest periplasmic expression level was  $0.45 \mu\text{g/ml}/A_{550}$ , as obtained with the natural signal peptide. The *E. coli* alkaline phosphatase (*phoA*) signal peptide, however, was somehow more efficient in directing a higher percentage of synthesized hGH (82 versus 76%) to the periplasmic space. Since two different promoters (*E. coli phoA* and *E. coli trp*) were used in the two constructions, the exact influence of the two signal peptides is difficult to define.

The above deductions were quite important to the development of the next endeavors, which were more directly concerned with high-level expression of the proteins as secreted into the periplasmic space. Hsiung et al. [89], using a hybrid *lpp*–*lac* promoter and *E. coli amp A* signal peptide [91], obtained an approximately 10–20 fold higher periplasmic secretion than already reported ( $10$ – $15 \mu\text{g/ml}/A_{600}$ ), while Chang et al. [90], using the *phoA* promoter and the *E. coli* heat-stable enterotoxin II (ST II) signal peptide, almost doubled this level, reaching  $15$ – $25 \mu\text{g}$  of hGH/ $\text{ml}/A_{550}$ . This periplasmic secretion should amount to 6–10% of total cellular protein or about 40% of periplasmic protein. The tremendous increase in periplasmic protein production was probably due to the correct matching of strong promoters with efficient signal sequences, all derived from *E. coli*. This development, together with the quality of the protein preparations, made practically all recombinant hGH manufacturers select this strategy. It is also interesting to note that the counterclockwise transcription orientation in the expression vector, used by Chang et al. [90], increased protein synthesis by about 90% when compared to the clockwise orientation. This was probably a result of an effect determined by the strong *phoA* promoter and consequent transcriptional readthrough on the region of the plasmid copy number control located near the origin of replication [90]. The critical influence of the signal peptide has later also been confirmed by Uchida et al. [92] who compared in the same *E. coli* periplasmic expression system *E. coli omp A* and the wild-type neutral

protease (npr) signal peptide from *Bacillus amyloliquefaciens* and obtained a 10-fold higher expression with the latter. These authors also showed that co-expression of the glutathione reductase gene (gor), inserted in the same vector carrying the hGH cDNA, together with a modification of the npr signal to enhance its positive charge and hydrophobicity, can further increase secretion of the hormone more than two-fold, probably by facilitating the transport of the protein across the inner membrane [93,94]. The study by Uchida et al. focuses more specifically on the synthesis in *E. coli* of the  $M_r$  20 000 hGH isoform, but they also report a fairly high secretion level of 10  $\mu\text{g/ml}/A_{600}$  for the regular  $M_r$  22 000 hGH. It is interesting to observe that the W3110 *E. coli* strain, used in this study, was already providing the highest periplasmic secretion level ever reported [90].

It may be noted that all the above mentioned expression/secretion levels of hGH and hPRL have been determined by either RIA or ELISA, whose accuracy is quite limited when applied to a complex mixture like culture broth and crude extracts [39,95]. Moreover, immunoassays cannot discriminate between undesired hGH-related forms and the basic unmodified hormone, as is feasible, for example, using the more recently developed HPLC or freeze-zone capillary electrophoresis (FZCE) methodologies, which are directly applied to *E. coli* fermentation broth for both qualitative and quantitative purposes [95–101].

Still on the subject of periplasmic expression of hGH, it may be noted that Matteucci and Lipetsky [102] could significantly increase the secretion of the hormone in the bacterial periplasm by mutations of its native signal obtained with nitrous acid. Interestingly, the greatest increase was related to a mutation which did not produce a change in the signal peptide, which, among other structural features, is critically important for the synthesis, transport and secretion of the hormone [71]. The authors postulated that the enhanced translation was induced via a favorable modification of the helical regions of the mRNA surrounding the ATG starting codon. Using the same expression system described by Hsiung [89], Igout et al. [103] described for the first time the synthesis, purification and biochemical characterization of human placental growth hormone (hPGH) which,

however, was expressed at a much lower level than hGH. The very low secretion yields (0.1% of total periplasmic proteins) could be increased 20-fold by using a lower activation temperature (30 °C instead of 37 °C) and a low IPTG concentration (50  $\mu\text{M}$ ); these changes also led to increased solubility of the recombinant hormone.

Periplasmic expression of hPRL has, as far as we are aware, only been accomplished in the authors' laboratory [104,105]. Considering, as outlined above, that the type and nature of the signal peptide can critically influence the expression and secretion of the desired protein, we can now conclude that most of our initial unsuccessful attempts were related to the signal peptide used [105]. The natural hPRL signal peptide in our hands never produced significant periplasmic secretion of hPRL in *E. coli*, while the same system led to satisfactory secretion of hGH. A biologically active variant of hPRL, Ala-Ser-(His)<sub>6</sub>-Ile-Glu-Gly-Arg-hPRL (tag hPRL) was for the first time successfully obtained in *E. coli* periplasm (0.7  $\mu\text{g/ml}/A_{600}$ ), using a vector construct based on the signal peptide sequence obtained from the "cex" gene of *Cellulomonas fimi* [104]. However, deleting the "tag sequences of hPRL" from this vector, and using the same expression system, led to decreased periplasmic expression of hPRL, albeit in the authentic form (0.08  $\mu\text{g/ml}/A_{600}$ ) [105]. Recently, utilization of a signal peptide of an *E. coli* periplasmic protein (DsbA) yielded increased hPRL periplasmic expression, >1  $\mu\text{g/ml}/A_{600}$  (unpublished observations). These data illustrate the influence of the signal sequence with regard to the periplasmic secretion of hPRL. In addition, we can speculate that the presence of the "tag" sequences could have a stabilizing effect at the transcriptional, translational or post-translational level [105]. In this connection it may be noted that a His<sub>6</sub> tag, introduced at either the N- or C-terminus, can lead to a more efficient production of heterologous proteins [78,106].

### 2.1.3. Excretion

If we had to indicate the ideal host cells for recombinant, unmodified heterologous protein production, we would without hesitation choose *E. coli* bacteria. If we had to decide on the ideal localization of our protein of interest in *E. coli*, especially with



regard to successful pharmaceutical production, we would definitely point at the bacterial periplasmic space. At the beginning of the recombinant therapeutic protein production era, however, attention was also given to protein secretion into the extracellular compartment or excretion. The reasons for favoring this type of compartmentalization were obvious advantages: (i) low proteolysis of the product, (ii) simpler purification (fewer protein types), (iii) improved folding of the protein, (iv) N-terminus authenticity and (v) applicability even to bacteriotoxic proteins. Unfortunately, there were also considerable disadvantages: (i) very unusual natural excretion of proteins by *E. coli* [71,107] and (ii) a highly diluted product. Regarding hGH synthesis and excretion, however, we can list several interesting reports based either on the use of signal sequences, fusion partners and permeabilizing proteins in *E. coli* or on the utilization of prokaryotic organisms such as *Bacillus subtilis* or *Vibrio cholerae*, that efficiently excrete some of their proteins into the medium. In general, protein yields obtained with this approach have been quite modest.

Three methods utilizing colicin lysis proteins for permeabilization of the *E. coli* envelope and release of soluble cytoplasmic or periplasmic proteins have been described. Kato et al. [108] constructed an excretion vector carrying the *kil* gene, plus a penicillinase promoter and signal sequence. hGH DNA was then coupled to the signal sequence and the resulting plasmid introduced into *E. coli*. The outer membrane was made permeable by the action of the *kil* gene and authentic hGH (11.2 mg/l) was excreted into the extracellular fraction, corresponding to about 55% of the total amount of hGH produced, whereas ~42% remained in the periplasmic fraction. Hsiung et al. [109] used *E. coli* cells which harbored two vectors, the hGH secretion vector already utilized in previous work [89] and a bacteriocin release protein (BRP) expression vector which, under proper culture conditions and a controlled release system, could excrete up to 4.5  $\mu\text{g}/\text{ml}/A_{550}$ . This yield was 2–3 times lower compared to the periplasmic production of hGH previously obtained by the same authors. However, the culture medium could be collected and concentrated without rupturing the cells and hGH could be purified to >98% purity in a single step on RP-HPLC. Lloubès et al. [110] constructed an *E. coli*

expression vector containing the colicin A lysis protein gene coupled to the hGH DNA devoid of its signal sequence. This way, the maturation process of precursor hGH was avoided and Met-hGH was directly released into the culture medium, where the correct disulfide bridges could be formed. Nakayama et al. [111] obtained excretion of hGH by *Bacillus subtilis* cells, transfected with a vector based on the npr signal peptide, and using high-density culture conditions (turbidity up to about 110  $A_{570}$ ). *Vibrio cholerae* cells were also used as a secretory expression system for the efficient expression of hGH, under the control of the heat labile enterotoxin chain B signal sequence [112]. The expressed protein (14  $\mu\text{g}/\text{ml}$ ) was transported to the extracellular environment via an intermediate that was transiently held in the periplasm, indicating the existence, in this organism, of a secretory apparatus which is absent in *E. coli*.

## 2.2. Yeast expression systems

Since yeast cells are eukaryotic, they share the complex cell biology of multicellular organisms such as insects, plants and mammals, including a highly compartmentalized intracellular organization and secretory pathways for proteins. Yeast cells grow rapidly, achieve high cell densities and carry out post-translational modifications such as glycosylation. They are very thoroughly studied organisms and have been used to produce a variety of industrial compounds at concentrations of up to several grams per liter. However, recovering large amounts of the desired product from yeast cells is sometimes very difficult, because the generated proteins may be degraded by several endo- or exoproteases present in yeast. Moreover, because of their different oligosaccharide structures, compared to human glycoproteins, many yeast-produced recombinant proteins are unsuitable for pharmaceutical applications [9,113]. *Saccharomyces cerevisiae* is the most highly characterized yeast for which the most advanced host-vector systems are available [114]. More recently, *Hansenula polymorpha* and especially *Pichia pastoris* have become increasingly popular as hosts for heterologous protein production [115].

The use of yeasts for recombinant human pituitary hormone production has been, up to now, very

limited. *Saccharomyces cerevisiae* was used by Tokunaga et al. [116] for hGH production, but only a hGH with a five-amino acid extension at the N-terminus, and not pre-hGH, was efficiently expressed. The same host organism was also used by Park et al. [117] to obtain methionyl-hGH, which was later processed for methionine removal. Still hGH was recently expressed in *Pichia pastoris* [118]. Its secretion into the growth medium was directed via a tightly-regulated alcohol oxidase promoter and *S. cerevisiae*  $\alpha$ -factor signal peptide, and reached, in high density cultures, a yield of about 49 mg rhGH per liter after 3 days under methanol induction. Advantages for the production in *Pichia pastoris* of hGH and other unmodified proteins can be summarized as (i) high cell density fermentation, (ii) efficient secretion, (iii) a high degree of purity in the culture medium, (iv) fewer purification steps, (v) lack of endotoxins.

We could not find any report describing the synthesis of human PRL, TSH, LH or FSH in yeast, whereas bovine FSH  $\beta$ -subunit [119], ovine FSH [120] and porcine FSH [121,122] all have been synthesized in *Pichia pastoris*. Yeast expression systems are not commonly used for the production of recombinant human therapeutic glycoproteins. This is probably due to the fact that oligosaccharide processing in the Golgi apparatus of these organisms is very different from the processing in mammalian cells [123–125] and sometimes leads to much greater molecular masses, specifically due to elaborate high-mannose (mannan) structures which sometimes contain more than 100 monomers [10,126,127].

### 2.3. Baculovirus expression systems in insect cells

Use of the insect cell/baculovirus expression system has become widespread for producing heterologous proteins of bacterial, plant, insect or mammalian origin [128,129]. Its success is due to many factors: (i) high protein expression levels, (ii) rapid proliferation of the cells, (iii) ease and speed of genetic engineering, (iv) the option of using large DNA inserts, (v) capability of protein folding and post-translational processing (second only to the mammalian cell system). These viruses are non-infectious to vertebrates and, moreover, insect cells can be grown in serum-free medium. This is a great

advantage since the amount of product contamination by, for example, pyrogenic lipopolysaccharides, is greatly reduced, and consequently purification costs are lowered and biosafety of therapeutic preparations is enhanced [7,130]. On the other hand, one of the major disadvantages of products obtained by the baculovirus system is their rapid clearance from the circulation, which is mostly related to the rather poor ability of insect cells to perform sialylation of glycoproteins [9,128]. For these reasons, the suitability of the products for clinical use is highly dependent on attempts and strategies for “mammalianizing” their glycosylation capacity [130,131].

Kadono-Okuda et al. [132] have shown that infection of larvae of the silkworm *Bombyx mori*, with a *B. mori* nuclear polyhedrosis virus vector (BmNPV) containing hGH cDNA, led to proper processing of the hGH signal peptide and to secretion of the hormone into the hemolymph, reaching a level of at least 160  $\mu$ g hGH/ml. In contrast, Sumathy et al. [133] used a BmNPV vector based on the full-length hGH gene. Infection of the silkworm larvae with this vector led to the production of hGH which could be purified by single-step immunoaffinity chromatography.

The baculovirus expression system was also very efficiently and safely applied to the synthesis of hPRL, starting from a cDNA that included hPRL signal sequence, leading to yields of the hormone in its authentic form never reached before (20–40  $\mu$ g/ml), and post-translational modifications similar to that of wild type hPRL [134]. It is notable that the glycosylated hPRL, so generated, accounted for about 7% of the total amount of hormone produced, which is within the range of 5–40% reported for pituitary hPRL [8]. Even though the production of hPRL in the secreted form was lower by 30–40% than when the hormone was generated intracellularly by the same authors using a cDNA lacking the secretory signal sequence, the ease with which the hormone could be purified from serum-free medium offset its lower degree of synthesis. Hexahistidine-tagged hPRL was produced in insect cells by Strokovskaya et al. [135] at about the same yield (20–35  $\mu$ g/ml of medium), but intracellularly, for subsequent purification by metal affinity chromatography. The authors also used milder conditions for solubilization of the cytosolic inclusion bodies aimed

at better preservation of the biological activity of the hormone.

Human TSH was also produced in insect cells. Using recombinant baculovirus containing the human  $\alpha$ -subunit and an hTSH  $\beta$ -minigene, relatively high expression levels (0.8–1  $\mu\text{g}/\text{ml}$ ) were obtained by Grossmann et al. [136] in studies of the role of N-linked oligosaccharides on the biological activity of this glycoprotein hormone. For reasons mentioned above, insect cell-derived hTSH (IC-hTSH) lacked complex-type oligosaccharides terminating with sialic acid, showing a higher *in vitro* activity (5-fold) and a much lower *in vivo* activity when compared with CHO-hTSH. Even though these differences are less dramatic when we compare IC-hTSH and pituitary hTSH, the authors do not consider this product suitable for pharmaceutical use but more for studying structure–function relations of hormone analogs.

Baculovirus-infected insect cells were also used for the expression of hFSH and of human chorionic gonadotropin (hCG). Again, the purpose was not the production of therapeutic proteins, but rather the identification of amino acids involved in structure–function relationships [137–139].

#### 2.4. Mammalian cell expression systems

Although bacterial systems have been shown to be most economical and efficient for expression of heterologous proteins, mammalian expression systems are hard to surpass when production is required of a complex and post-translationally modified protein. This is especially important with regard to the production of pharmaceuticals. Using mammalian cell systems, the generated protein does not require renaturation, its synthesis can be continuous, there are fewer batch to batch variations and, in general, it is possible to obtain, or approximate, the desired post-translational modifications. Drawbacks are (i) a rather low expression level, unless laborious, time-consuming amplification processes are applied, and (ii) frequent production instability. Among the mammalian cell lines applicable, CHO cells are certainly the most productive, readily transfectable, easy to culture cells and hence are widely employed for manufacturing purposes. Gene amplification in these cells has been quite successful, especially when based on the selectable and amplifiable dihydrofolate

reductase (dhfr) or glutamine synthetase marker genes [140,141], and using this strategy, protein expression levels of up to 110  $\mu\text{g}/10^6$  cells per day with concentration-production levels of up to 150  $\mu\text{g}/\text{ml}$  per day [142–144] have been obtained.

Since mammalian expression systems are able to generate glycosylated proteins, they are especially useful for the production of pituitary glycoprotein hormones, including hTSH, hLH and hFSH. Hormones such as hGH and hPRL are in fact more active in the nonglycosylated form and can be produced using cheaper bacterial expression systems. Although there are quite a number of manuscripts describing the synthesis of recombinant pituitary hormones, many of them deal with the production of relatively small amounts of hormone for structure–function studies. Only a small number of papers describe synthetic approaches that are potentially useful for manufacturing purposes and many of them are based on proprietary protocols. The syntheses of hTSH, hFSH and hLH follow a very similar strategy, since these hormones are structurally similar heterodimers containing a common  $\alpha$ -subunit, but different  $\beta$ -subunits which determine their specific biological actions. Their productions, all carried out in CHO cells, are analyzed in the following sections.

Of the three hormones, rhFSH has probably the most extensive clinical application and, consequently, is of major interest to the pharmaceutical industry. The synthesis of rhFSH was first described in 1990 [145], together with its biochemical and biological characteristics [146]. To this end, a “hybrid” hCG- $\alpha$  gene was prepared by combining a cDNA clone encoding the human chorionic gonadotropin (hCG)- $\alpha$  subunit (identical to the FSH  $\alpha$ -subunit) with a genomic clone encoding the same subunit. This hybrid gene was inserted, together with the genomic DNA encoding for hFSH  $\beta$ -subunit, into a vector, which was cotransfected into CHO cells with a selection plasmid containing the neomycin selection gene. FSH-expressing CHO cells could be cultured, in serum-free medium, in large-scale continuous perfusion cultures for prolonged periods (up to 3 months), producing a stable secretion of about 30–60 I.U. (ca. 4–8  $\mu\text{g}$ ) hFSH/ $10^6$  cells per day. Its molecular mass, amino acid and carbohydrate composition and biological properties appeared to be very similar to those of natural hFSH. Moreover, the

first 22 N-terminal amino acid residues of rhFSH  $\alpha$  and  $\beta$ -subunits were identical to those previously reported for pituitary hFSH. Like natural hFSH, rhFSH was obtained in multiple molecular forms, showing an isohormone profile very similar to that of urinary hFSH. In 1998, the Recombinant Human FSH Product Development Group described their biotechnological production process in which two plasmids were used to transform a dhfr-deficient CHO cell line [147]. One plasmid contained the genomic DNA sequences for the  $\alpha$ -subunit as well as the mouse dhfr gene and the other included the FSH  $\beta$ -subunit genomic sequences. After various methotrexate (MTX)-based amplification steps, reaching a maximum MTX concentration of 5.0  $\mu$ M, a clone was obtained which could secrete 15  $\mu$ g/ $10^6$  cells per day. In a continuous perfusion mode bioreactor,  $(2-4) \cdot 10^6$  I.U. of rhFSH could be harvested every 48 h. This represented a production of about 3  $\mu$ g/ml per day, according to our calculations based on the reported data. The carbohydrate structures of this rhFSH were found to be less heterogeneous, and containing less heavily sialylated forms, than those of urinary (u) hFSH, although its *in vivo* bioactivity was essentially equivalent to that of u-hFSH. The preparation showed truncated species lacking two N-terminal amino-acid residues in the  $\beta$ -subunit (for ~50% of the molecules) and in the  $\alpha$ -subunit (for 1–2% of the molecules), changes which, however, are less extensive than those observed for urinary hFSH. The same cassette expression vectors and a similar strategy had been used by Cole et al. [148] for the synthesis of rhTSH. In that case, however,  $\alpha$ -subunit cDNA instead of a genomic fragment was used together with hTSH  $\beta$ -subunit genomic DNA. The dhfr gene amplification protocol also required MTX concentrations up to 5.0  $\mu$ M, producing a clone that was able to synthesize 4.5  $\mu$ g hTSH/ $10^6$  cells per day, equivalent to a daily production of 15–30  $\mu$ g/ml in a microcarrier beads, oxygen-sparged bioreactor. Identity of the recombinant protein with pituitary-derived hTSH was indicated by N-terminal amino acid sequence analysis. In contrast to pituitary hTSH, rhTSH is reported to contain a large proportion of triantennary complex oligosaccharides, with both partial and complete sialylation, and to completely lack terminally sulfated GalNAc residues. This apparently increases its *in vivo* resi-

dence time in different animal models [17,148,149]. In comparing the last two studies on hFSH and hTSH synthesis, it becomes apparent that quite comparable results have been obtained for two structurally similar proteins. This was possible by using (i) analogous strategies (ii) identical starting vectors and (iii) the same dhfr<sup>-</sup> CHO cell line (DXB11). More recently, Hussain et al. [150] reported a production of 100 000  $\mu$ I.U. hTSH/ml, corresponding to approximately 10–14  $\mu$ g of hTSH/ml. They achieved this by using a different dhfr<sup>-</sup> CHO cell line (DG 44), the  $\alpha$ - and  $\beta$ -subunit cDNAs, a vector based on a mutant form of dhfr, amplification with increasing concentrations of MTX up to 10  $\mu$ g/ml and a hollow-fiber perfusion system. Peroni et al. [149], also using the two cDNA sequences, adopted a dual-marker amplification strategy based on the amplifiable gene markers dhfr and adenosine deaminase (ADA) inserted into particularly efficient dicistronic mRNA expression vectors. After a first amplification stage, requiring only a maximum MTX concentration of 1  $\mu$ M, a second amplification protocol, based on 2'-deoxycoformycin, generated a clone expressing about 18  $\mu$ g hTSH/ $10^6$  cells per day and a production level of up to 20  $\mu$ g/ml per day.

Still on the subject of glycoprotein hormone synthesis, one should not forget important contributions made by researchers who were not primarily concerned with production, but rather with molecular expression and assembly mechanisms or structure–function relationships. In studying the secretion of bovine LH in CHO cells and the rate of synthesis of the  $\beta$ -subunit, Kaetzel and co-workers [151,152] obtained remarkable results with MTX-induced gene amplification, reaching secretion levels of up to 20  $\mu$ g bLH/ $10^6$  cells per day. Human CG is a placental hormone similar to hLH, except that the hCG- $\beta$  subunit has 24 additional amino acids and extra carbohydrate residues at the carboxyl end. In studying hCG synthesis in monkey cells, by infecting them with a single simian virus expression vector containing both  $\alpha$ - and  $\beta$ -subunit cDNAs, Reddy et al. [153] obtained expression of hCG in the natural heterodimeric form for the first time. With a similar objective in mind, Chakrabarti et al. [154] constructed two recombinant vaccinia viruses, containing the same  $\alpha$  and  $\beta$ -subunit cDNA sequences, and

obtained expression of the subunit proteins individually as well as in a combined form. Pioneer work on transient and stable expression of biologically active hTSH was also carried out by Watanabe et al. [155] and Wondisford et al. [156], while Matzuk [157] and Keene et al. [158] examined structure–function relationships for hFSH and hTSH to clarify the post-translational processing, subunit assembly and secretory pathway of both hormones. Using  $\alpha$ -,  $\beta$ - or  $\alpha\beta$ -hFSH-subunit expression vectors based on the Harvey murine sarcoma virus long terminal repeat promoter (LTR), they were able to obtain significant secretion of hFSH without the use of an amplifiable marker gene. Also worth mentioning is the contribution by Hakola et al. [159,160] who developed the production by CHO cells of rat FSH and LH, including their purification and characterization. The recombinant hormones so obtained allowed them to carry out physiological studies in the rat, using native and recombinant homologous hormones, as well as biochemical studies comparing the hormones with their human counterparts.

Since the predominant forms of hGH and hPRL are nonglycosylated proteins, there has, in general, been less of an interest in producing these hormones in mammalian cells. The first synthesis of recombinant authentic hGH in mammalian cells was carried out by Pavlakis et al. [161] using primary monkey kidney cells. Infection of these cells by a recombinant simian virus, in which the genomic sequence of hGH including the natural hydrophobic leader sequence had been inserted, produced as much as 2.5  $\mu\text{g}$  hGH/ $10^6$  cells per day. Lupker et al. [162] produced a stable monkey cell line (Vero), which excreted up to 5.6  $\mu\text{g}$  hGH/ $10^6$  cells per day. They used a plasmid vector containing hGH precursor cDNA, the SV40 early promoter and a selectable marker gene, applying a classical transfection by the calcium phosphate precipitation technique. In view of its nontumorigenicity and prospect for large scale production, this cell line was of interest for generating the hormone at an industrial level. Much more recently, Haldankar et al. [163] used dhfr<sup>-</sup> CHO cells to generate a hGH analogue-antagonist via a single amino acid substitution. This procedure has proven to be quite efficient, with high potential for application in industrial production. With such a system, showing an average expression level of 4.3

$\mu\text{g}/10^6$  cells per day, it was possible to markedly increase the production rate of the hormone previously obtained by the authors with a genetically engineered mouse L cell line [164]; at the same time the downstream purification process was improved with regard to both time and efficiency.

The microheterogeneity of recombinant hPRL, consisting of glycosylated (G-hPRL) and nonglycosylated (NG-hPRL) forms, was studied by Price et al. [165]. These workers generated the hormones in murine C127 cells, following the same strategy they had applied earlier to produce recombinant baboon and monkey prolactin [166], i.e. using the same cassette expression vector based only on a selectable gene marker (Neo) without amplification strategy. The expression levels of NG- and G-hPRL ranged from 1 to 2  $\mu\text{g}/\text{ml}$  per day and both forms of the hormone consisted of multiple charge isomers; the heterogeneity of the glycosylated form was primarily due to differences in sialylation. G-hPRL formed approximately 14% of the product generated and was 3–4 times less active than NG-hPRL in the classical Nb2 rat lymphoma cell in vitro bioassay. In analyzing the synthesis and secretion kinetics of hPRL, produced by stably transfected rat pituitary GH3, monkey renal COS-1 and CHO cells, Hirooka et al. [167] found G-hPRL/total hPRL ratios ranging from 17 to 33%. Moreover, the authors observed that the synthesis and release of hPRL were apparently not regulated by its glycosylation. Using CHO cells, Soares et al. [144] obtained the highest hPRL expression level (30  $\mu\text{g}/10^6$  cells per day) thus far reported in mammalian cells and eukaryotic systems, reaching a production of  $\sim 150$   $\mu\text{g}/\text{ml}$  per day. For the production of the hormone they used a cassette expression vector, also used by their group for hTSH expression [149], and an amplification strategy based solely on increasing the MTX concentrations to 100 nM. The percentage of secreted G-hPRL was quite accurately determined (10.3%, RSD=19%, for  $n=9$ ); as expected, the G-hPRL was less active in the Nb2 cell bioassay than NG-hPRL, by a factor of about 2.

It appears that human PRL provides an ideal model for protein glycosylation studies since, in addition to the nonglycosylated form, there is only one glycosylated variant displaying a unique oligosaccharide chain at Asn<sup>31</sup>. This condition has been

exploited by Shelikoff et al. [168] for studying the relationship between the synthesis of rhPRL, as produced in C127 cells, and the degree of occupancy of its N-glycosylation site. By investigating the production of G-hPRL and NG-hPRL in a variety of systems, e.g. insect, CHO, C127 or human pituitary cells, useful information can be obtained with regard to the glycosylation pathways functioning in these systems and the various carbohydrate structures that are generated.

### 2.5. Plants and animals as bioreactors

In a very recent trend to identify alternative systems for large scale and more economical production of therapeutic proteins, attention has focused on use of the most natural of bioreactors: plants and animals [169]. Plants produce a large amount of biomass and pharmaceuticals generated in plants appear to be safer, easier to produce and less expensive than those produced in animals or microbes. The fusion, in the last decade, of molecular medicine and plant biotechnology has created a new field, termed “molecular farming,” for the production of virtually unlimited quantities of recombinant therapeutics, including vaccines, diagnostic compounds such as recombinant antibodies, plasma proteins, cytokines and growth factors [170–173].

It is of interest to note that, although with the use of plants upstream production costs incurred are lower than for other systems, downstream processing is generally thought to be difficult and expensive because of the low ratio of recombinant protein to total biomass of the plant material [174]. Nonetheless, the first commercial pharmaceuticals obtained via plants have already experienced a considerable success, and many of them are currently undergoing clinical trials [169,175]. Although very promising, the use of plants as bioreactors has not yet been explored very extensively for human pituitary hormone production, except in the case of hGH. While application of hGH in plant systems is sometimes aimed at its actual production, the hormone is frequently utilized as a marker for heterologous protein expression, since its expression mechanisms, behaviour and characterization are very well known. A eukaryotic algal species (*Chlorella*) has recently been employed because of its capability of express-

ing protein at high levels, low operational cost, ease of culturing and subsequent product purification, and in particular with regard to safety and effectiveness regulations for use by the consumer. As a result of using a synthetic extracellular secretion signal sequence in the *Chlorella* system, 200–600 ng/ml of hGH could be recovered from the extracellular medium [176]. An identical, correctly folded and biologically active hormone was also obtained in tobacco chloroplasts at a high concentration (>7% on total soluble protein) [177]. Cloning a chimeric ubiquitin–hGH gene, linked to a selectable spectinomycin-resistance gene, into chloroplast transformation vectors, a ubiquitin–hGH fusion protein was synthesized, with the expectation that this type of protein is normally cleaved by ubiquitin protease immediately downstream of the C-terminal glycine residue of ubiquitin. However, the most abundant form of hGH so obtained showed that one additional amino acid had unexpectedly been removed beyond the desired phenylalanine N-terminus, probably a result of secondary protease activity. Correctly processed, authentic hGH was produced by Leite et al. [178] by developing a tissue specific expression system based on promoter and signal sequences from dicot plants that directed the secretion of the hormone into the seed apoplastic space of transgenic tobacco plants, where it accounted for 0.16% of the total soluble protein. rhGH was found remarkably stable in mature seeds for a period of at least 1 year, as determined by immunoblotting analysis.

Transgenic animals are also great candidates for the production of recombinant proteins at an industrial scale [179–181]. The proteins may be generated as products in, for example, milk, egg white, blood, urine, seminal fluid and silk worm cocoon [182]. One of the main advantages using animals is the high concentration of soluble recombinant protein that can be obtained, for example up to 2.6 mg/ml in milk [183–188] and up to 0.5 mg/ml in seminal fluid [189]. However, cost is still one of the main limitations. Thus, even though the development of transgenic farm animals has recently become easier with the introduction of animal cloning based on transfected somatic cells as nuclear donors, the expansion from the founder animal to a useful herd is a long and costly process [7,190]. In view of this, rabbits are relatively good candidates, among the

transgenic species so far employed, for the expression during lactation of tens to hundreds of grams of complex proteins [186,191,192].

With regard to the synthesis of pituitary hormones in transgenic animals, hGH is the only hormone for which several approaches have already been developed. Employing the promoter of the gene encoding the major milk whey protein in mice (mWAP), Reddy et al. [193] expressed 0.4 mg/ml of hGH in the milk of these transgenic animals. Archer et al. [194] could greatly reduce the time of the production of pharmaceuticals in milk from years to weeks through extension of gene therapy techniques, by infusion of replication-defective hGH-encoding retroviral vectors to specifically target mammary glands in goats. The expression levels, however, were quite low, averaging 60 ng of hGH/ml on the first day of lactation and then declining rapidly to a plateau of about 12 ng/ml. Founder transgenic rabbits for the production of hGH were obtained via microinjection of embryos with a chimeric gene encoding 5' sequences from mWAP linked to the hGH gene, using standard procedures [192]. The concentration of hGH produced in the milk was estimated to be approximately 50 µg/ml, which indicates that the rabbit is a good choice as a bioreactor when the required amount of protein is in the range of tens of grams. Cerdán et al. [187] generated transgenic mice carrying a fusion gene containing 3.8 kb of the bovine β-casein gene promoter driving the expression of the hGH gene and directing an extremely high expression level (up to 2.6 mg hGH/ml milk) to the epithelial cells of the lactating mammary gland. In another case, transgenic mice were generated, using a specific promoter from the mouse uroplakin II gene, whose hGH secretion level in urine was in the order of 100–500 ng/ml and remained constant for more than 8 months [195]. It is notable that use of the bladder as a bioreactor offers the advantage of utilizing the animals throughout their lives and greatly facilitates purification of the product, since urine does not contain much protein or lipid. Finally, the mouse P12 gene promoter, specific for the male accessory sex gland, was used to generate transgenic mice that could secrete up to 0.5 mg hGH/ml of seminal fluid [189]. Extrapolating these results to other species, for example the pig, the authors estimated that a single

transgenic boar could produce 22 g/year of a recombinant protein of interest. Due to the prolificacy and relatively short generation period of this species, a herd of transgenic pigs, producing a pharmaceutical protein in their semen, could be generated in a relatively short time. It appears that it would be relatively easy to extract and purify recombinant proteins from seminal fluid which, as distinct from milk, contains casein micelles and fat globules which can interfere with standard separation methods.

### 3. Separation methodologies and downstream processes utilized for recombinant pituitary hormone purification

As already indicated, the most complex and costly step of a recombinant protein production is the purification process [7,18,196,197]. Its efficiency and consistency are of critical importance for the quality of the final product. Effective and economic protein purification strategies must be designed which ensure that the structure and function of the recombinant protein (biological and immunological activity) match those of the native hormone; in addition, high purity and recovery should be attained. Chromatography has been the most widely used purification method, and maximum throughput and high selectivity, requirements of preparative chromatography, are taken into account in schedules described for purification of recombinant pituitary hormones. The selection of chromatographic steps (type, sequence and number of steps) to be followed is governed by the intended use of the final preparations. For example, whereas De Oliveira et al. [39] described a six-step schedule for purification of clinical grade rhGH produced in the periplasmic space of *E. coli*, Ribela et al. [198] reported a single-step purification to obtain this hormone for specific use as a reagent (tracer and standard) in radioimmunoassays. The former strategy applied conventional chromatographic methods, based on ion exchange, size exclusion and hydrophobicity, to obtain a highly purified product for clinical use in compliance with regulatory recommendations. The latter strategy utilized single high-performance liquid chromatography, based on size exclusion, to attain a preparation

which, although not as pure, was still adequate for its intended use as a chemical reagent.

The resolution capability of each of the chromatographic steps depends mainly on the choice of the separation media. Their purity, physicochemical stability, lot-to-lot reproducibility, high tolerance to stringent cleaning and sanitization, mechanical strength to allow high flow-rates, controllable size and pore size distribution, specificity of functional groups and potential for substituting them are crucial factors in the selection of packing material for a particular purification. Important aspects of the performance in preparative chromatography of a number of sorbents, coupled to a variety of polymeric matrices, have been reviewed by Boschetti [199], Leonard [200] and Andersson et al. [201]. Important in a recombinant pituitary hormone purification schedule are sorbent cleaning procedures, in particular when the hormones are intended for therapeutic use. These procedures should include rigorous methods for regeneration of resins used for chromatography of the hormone, such as use of alkaline conditions which are effective for endotoxin breakdown, viruses and DNA removal, and use of detergents which can efficiently remove viruses present in recombinant products generated in mammalian cells [18].

Another important determinant in the development of a purification schedule is the elution mode that can be applied on the material to be purified. Recombinant DNA-derived preparations typically contain impurities, e.g. endotoxins in bacterially expressed products, or potentially oncogenic DNA in preparations produced using transformed mammalian cells. Thus anionic exchange chromatography can be efficient in adsorbing both negatively charged impurities, while cationic exchange chromatography can be useful for washing these contaminants out. In the first case we would be using the bind-elute, while in the second case the flow-through mode. Of course, the choice of the ion exchanger will be mainly governed both by the isoelectric point ( $pI$ ) of the product to be purified and its stability at the pH to be used in the purification. Also very useful in the selection of separation methods for target protein purification are databases of protein components both of culture media and host cells currently used in recombinant DNA technology. A data base con-

structed by Andrews et al. [202] presents a number of physicochemical properties of the major proteins of *E. coli*, *S. cerevisiae* and CHO cells (size, relative surface hydrophobicity and isoelectric point), while a database by Linqueo et al. [203] provides concentrations of the main protein contaminants of products expressed in *E. coli* as well as their charge at various pH values. Such databases have been valuable in the selection of the type, sequence and minimum number of chromatographic steps leading to production of rhGH of a desired purity [19,197,203]. Furthermore, their use led to identification of a marked difference in hydrophobicity between hGH and major contaminating host cell proteins. This differential was used by Dalmora et al. [98] to resolve hGH directly from the bulk of periplasmic proteins via reversed-phase HPLC and by De Oliveira et al. [39] in the removal of contaminating *E. coli* protein by hydrophobic chromatography.

The major problem usually encountered at the start of a downstream process is the concentration of the protein of interest which can be very low especially in the case of extracellular production. The first step of the purification strategy utilized for recombinant pituitary hormones is clarification (separation between solids and soluble components), often carried out by microfiltration and centrifugation of either cultivation broth or cell homogenate. A concentration step, such as ultrafiltration or precipitation, is usually necessary for highly diluted products. A primary adsorption step (frequently ion-exchange chromatography) can also be employed for product concentration, offering the advantage of partial purification as well as concentration. Such steps, however, are time-consuming and may take several hours. Consequently, byproducts such as proteases can affect the target protein, leading to its degradation. Expanded bed adsorption (EBA), a technique which allows direct recovery of the target protein from complex feedstock, and combines particle separation, product concentration and partial purification in a unique procedure, can avoid these problems. EBA is a solid-liquid fluidized-bed technique in which liquid is pumped upwards through a bed of adsorbent beads which is not constrained by the presence of an upper flow adapter. The bed can thus expand and spaces open up between the adsorbent beads such that, when the feedstock is applied, the



particulates can pass freely through these voids in the bed. The bed expansion is controlled by the size and the density of the adsorbent beads, the linear flow velocity and the viscosity of the mobile phase. The principles and application of expanded-bed chromatography, as well as the advantages and problems encountered in its use for the direct extraction of proteins from unclarified feedstock, have recently been addressed [204,205].

Following the clarification and concentration steps, a combination of conventional chromatography steps is often used for the purification of recombinant pituitary hormones. They include gel permeation, ion exchange, affinity and hydrophobic interaction chromatography.

Size-exclusion chromatography (SEC) and ion-exchange chromatography (IEC) are used extensively in the purification of recombinant pituitary hormones. Practically all their purification schemes include at least one of these steps. Separation by SEC is based on the permeability of the matrix rather than on any type of binding, and depends on the relative size or hydrodynamic volume of a protein with respect to the average pore size of the packing material [206]. In the case of recombinant pituitary hormones, this technique has in general been employed as a polishing step or for desalting [81,166,207–212] rather than for the fractionation of proteins [39]. IEC is based on binding of charged solute molecules to oppositely charged moieties covalently linked to a chromatographic matrix [213] and is the technique most frequently used in protocols for purification of recombinant pituitary hormones. Most of these protocols include more than one IEC step, run either in bind-elute or in flow-through mode. These two modes have been utilized by, for example, Cole et al. [148] for the purification of rhTSH.

Affinity chromatography, a method based on highly specific molecular recognition, has been successfully employed in the purification of recombinant pituitary hormones. Its fundamental mechanisms, implications and limitations have been analyzed by Narayanan [40]. This separation technique utilizes a specific ligand, covalently coupled to an insoluble matrix, which will bind to a specific protein. Such affinity ligands can be classified as two major groups: (i) biological and (ii) synthetic or pseudo-

specific ligands. While the first group consists of antibodies, lectins (e.g. concanavalin A), protein A from *S. aureus*, nucleotides, heparin, etc., the second group contains dyes, metals and synthetic peptides as obtained, for example, via combinatorial chemistry. In contrast to biological ligands, which have an inherent natural specificity for a molecule, the affinities of synthetic or pseudo-specific ligands are often made specific for a protein by varying and optimizing the binding and elution conditions. With regard to group-specific natural ligands, an immobilized lectin, such as concanavalin A, has been used in the purification and characterization of recombinant glycoprotein hormones, based on its specificity toward  $\alpha$ -D-mannosyl or glucosyl moieties [136,156]. Another biological ligand, a monoclonal anti-hGH antibody, was used in the purification of rhGH from *E. coli* [70,214]. Synthetic ligands, such as dyes, can mimic natural ligands and bind some protein molecules very specifically at their active sites, achieving tight and specific binding via noncovalent interactions. The interaction between the dye and proteins can be based on a complex combination of electrostatic, hydrophobic, hydrogen-bonding and charge-transfer interactions, all of which are possible considering the structural nature of the dye [44]. Dye ligands such as Cibacron Blue 3G-A, a triazinyl-based reactive dye, have been successfully used for hTSH purification [12,13,148]. Metal ions serving as affinity ligands in immobilized-metal affinity chromatography (IMAC) were successfully utilized in the purification of recombinant hGH [215–217] and hPRL [212]. In IMAC, the adsorption of proteins is based on coordination between metal ions, immobilized on a matrix, and electron donor groups on the protein surface. The adsorption critically depends on the accessibility of certain amino acid residues, histidine in particular. Free cysteines, that could also contribute to binding of protein to chelated metal ions, are rarely available in the appropriate, reduced state. However, aromatic side chains of tryptophan, phenylalanine and tyrosine appear to contribute to the binding if they are in the vicinity of accessible histidine residues [43]. IMAC has also proven to be a powerful tool in the purification of histidine-tagged recombinant proteins. Mukhija et al. [78], Morganti et al. [104], Shin et al. [79] and Strokovskaya et al. [135], for example, carried out metal-based one- or

two-step purifications of recombinant, histidine-tagged hGH or hPRL expressed in *E. coli* or insect cells.

Hydrophobic interaction chromatography (HIC) takes advantage of hydrophobicity of proteins, promoting their separation on the basis of interactions occurring between immobilized hydrophobic ligands and nonpolar regions on the surface of the proteins. These interactions involve Van der Waals forces and the degree of hydrophobicity of a protein is determined by the sum of hydrophobicities of both exposed and buried amino acids [218,219]. HIC has been applied in various rhGH purification schemes. Lefort et al. used octyl-Sepharose and phenyl-Sepharose in their first chromatographic steps leading to 17- and 8-fold purifications of the hormone, respectively [208]; De Oliveira et al. obtained a high reduction in contaminating *E. coli* proteins (ECPs) (676-fold purification) by utilizing Phenyl-Sepharose chromatography as a last step [39].

### 3.1. Human growth hormone

With regard to downstream processing of the recombinant human pituitary hormones covered in this review, and especially of hGH, it is notable that the highest purification levels and most sophisticated methods are undoubtedly associated with the production of pharmaceutical grade proteins intended for clinical and, in particular, parenteral application. At the next level there are purification processes for large scale protein production, which provide highly homogeneous products at the chemical reagent level without necessarily eliminating contaminant host cell proteins, pyrogens, viral particles, hormone-related forms, etc., as is required for the production of therapeutics. A third group of processes could include all those partial purification procedures whose main purpose is to facilitate an initial characterization of the new product and, at the same time, indicate the feasibility of a synthetic approach. Finally, there is a fourth group of procedures that does not really qualify for “downstream processing” and which includes all one-step purifications, designed mostly for testing the efficiency of a novel separation methodology for a particular protein. In analyzing a great variety of published methodologies for the purification of rhGH, we will keep the above

classification in mind, with the notion that the borders between the different groups are not well defined.

In discussing pharmaceutical manufacturing of recombinant therapeutic proteins, we are mainly dealing with publications derived from proprietary protocols in which industrially significant steps often are not fully reported. Moreover, not all companies are willing to publish their data and reports on the subject are therefore relatively scarce.

Olson et al. [207] were first to purify rhGH (i.e. Met-hGH) from *E. coli* cytoplasm, whose synthesis had been described by Goeddel et al. [5]. They obtained a homogeneous preparation which was as active as pituitary-derived hGH in a number of in vivo bioassays. After disruption of the host cells and polyethyleneimine precipitation of nucleic acids and membrane components, proteins were fractionated by precipitation using ammonium sulfate (1.95 M). The desired fraction was then further purified using weakly anionic DEAE-cellulose chromatography, followed by weakly cationic carboxymethyl (CM)-cellulose chromatography and, after a second ammonium sulfate precipitation, concentrated via gel filtration (Sephacryl S-200). No detail is provided on the yields of the individual chromatographic steps used in this study, nor on the overall yields and purity, but Ross [220], from the same group, reported 50% purity after the DEAE and >98% purity after the CM-cellulose step. However, in a previous study [5], in which identical material was processed similarly, but without the use of the two ion exchangers, a purity of only approximately 25% was obtained. Interestingly, Flodh et al. [209] and Fryklund et al. [221], both from the same company, were only able to obtain a pharmaceutical grade preparation of Met-hGH for clinical use (ECP < 7 ppm) after introducing three anion-exchange steps on DEAE-cellulose, followed by a precipitation and the usual final gel filtration step. The authors' comments that much time had been spent in designing purification processes that effectively remove ECP down to a required level (<10 ppm), suggest that 2–3 ion-exchange steps are probably necessary to meet this requirement; this was confirmed by studies in our laboratory [39]. The final product was indeed found to be highly purified with regard to the content of dimers, polymers, deamidated forms, bacterial pyro-

gen, tetracycline, *E. coli* DNA and ECP and, as such, suitable for clinical use. No indications on recovery yields were given by this research group.

In purifying bioactive recombinant Met-hGH for pharmaceutical purposes, Niimi et al. obtained an overall yield of 19.0% [210]. It is of interest that the specific activity of the starting material was 12.3% (based on RIA) and that after cell disruption by sonication, the downstream process consisted of 7 steps: polyethyleneimine precipitation, ammonium sulfate fractionation (50% saturation), anion-exchange chromatography (DE-52), a second ammonium sulfate precipitation, chromatofocusing chromatography (poly buffer exchanger PBE-94), a third ammonium sulfate precipitation and, finally, a gel filtration (Ultragel AcA54). As usually occurs with the development of a therapeutic product for injection, the authors were concerned at each step about eliminating ECP, monitoring the purification by sodium dodecylsulfate–polyacrylamide gel electrophoresis (SDS–PAGE). In our opinion, the struggle to remove ECP was to a large extent responsible for the relatively low yield obtained in this process.

Gu et al. [164] developed an interesting and exceptionally detailed multistep downstream process for purifying multimiligram amounts (50–100 mg) of a recombinant hGH antagonist, G120R, generated from cultured mouse L cells. The flowchart specifies eight steps: cell removal, salt precipitation,  $M_r$  8000 membrane ultrafiltration (UF),  $M_r$  100 000 membrane UF, preparative RP-HPLC, phase separation, buffer exchange and lyophilization. Using this scheme, a 98% purity could be obtained, with an overall recovery yield of 69.0% and an average yield at each step of 95.5%. Since this antagonist preparation was intended for use as an injectable drug and as such required high purity and low pyrogen levels, inclusion of SEC after the  $M_r$  8000 membrane step to remove serum proteins appeared advisable. Production of the rhGH G120R variant by the same group, using a superior eukaryotic CHO system, allowed elimination of the salt precipitation and SEC steps, which reduced the operating time from 122 to 72 h and increased the recovery yield by up to 75% [163]. The unique and highly efficient chromatographic step (RP-HPLC) on which the process is based was then further investigated for upscaling, using a general rate model and parameter estimation protocol [222].

In preparing clinical-grade rhGH from *E. coli* periplasmic fluid, De Oliveira et al. [39] were also particularly concerned with maximizing product yield and purity; elimination of ECP formed a major hurdle in reaching the latter goal. The two objectives were achieved, however, by using osmotic shock in preparing the periplasmic fluid and isolating the hormone using an ammonium sulfate fractionation step, gel filtration (Sephacryl S-100), a weak anion exchanger (DEAE–Sephacryl Fast Flow), a second gel filtration, a stronger anion exchanger (Q–Sephacryl Fast Flow) and finally hydrophobic interaction chromatography (phenyl Sepharose-CL4B). As a result of the five chromatographic steps ECP contaminants could be reduced to <1 ppm. While the starting material had a relatively low specific activity of 7.2%, the overall recovery yield was 43%. If the peak cuts for maximum ECP elimination are not taken into account, the final hGH yield was about 60%, while the average recovery at each purification step amounted to more than 90%. The anion-exchange steps, in particular the one using Q–Sephacryl, run with a unique buffer under conditions of equilibrium [39,223], were also very important for the elimination of hGH-related forms, especially sulfoxides and deamidated forms. The authors gave high priority to accuracy with regard to quantification of total protein and hGH especially on the crude extract and in the early purification steps using a number of analytical methods and not putting too much faith on RIA and western blotting analysis for quantitative purposes.

A fairly complete purification process for *E. coli*-derived authentic hGH [89] has been described by Becker and Hsiung [211]. It reaches a high overall yield (71%) and greater than 90% purity, without facing the problem of ECP or pyrogen elimination. Starting from a periplasmic fraction containing hGH at a specific activity of 30.4% [determined by Mono Q HR 5/5 fast protein liquid chromatography (FPLC)], the downstream process included ion-exchange chromatography (Q–Sephacryl fast flow), gel filtration (Sephacryl S-200) and optative solvent-exchange chromatography (Sephadex G-25). In our opinion, the success in obtaining chemical reagent grade purity, together with one of the highest yields ever reported using just two chromatographic steps, was mostly due to the very high specific activity of

the starting material. Supporting evidence for this observation can be found in a study by Lefort and Ferrara [208] who used large scale procedures to obtain chemical reagent-grade rhGH. In this case, starting from Vero monkey kidney cell culture medium containing rhGH at quite low specific activity (3% by RIA), a homogeneous, 100% pure product was obtained by applying three chromatographic steps: hydrophobic interaction chromatography (octyl- or phenyl-Sepharose), anion-exchange chromatography (DEAE-Sepharose) and gel filtration (Ultragel AcA44), with lower overall yields of either 48% or 28%, depending on the use of either octyl- or phenyl-Sepharose. Thus, starting with low specific activity material, these authors needed at least three chromatographic steps and much lower yields were obtained. This study is especially interesting for introducing and focalizing the use of hydrophobic interaction chromatography as a first step, the strategy of starting with batch adsorption followed by gel transfer to a column for elution and especially the comparison between the performance of octyl-Sepharose CL4B and phenyl-Sepharose CL4B. The latter sorbent was eventually chosen, even though it led to a lower recovery (62 versus 80%), since it could avoid an acetonitrile elution step possibly altering the native conformation of some proteins. It appears that hydrophobic interaction chromatography is certainly useful for eliminating proteolytically nicked rhGH variants (including the “two-chain forms”) whose removal, as well as detection, is a particularly critical issue in the purification of hGH for clinical usage [224–226]. We tend to agree with the authors that hydrophobic gel chromatography also offers several other advantages: (i) relatively mild interaction, (ii) the possibility of applying large volumes obtaining great concentration factors, (iii) the option of repetitive usage over periods of months without any change in adsorption-desorption properties [208].

In purifying their cytoplasmic amino-terminal extended rhGH (Met-Ala-Glu-hGH), Dalbøge et al. [76] also put faith in the performance of phenyl-Sepharose CL4B. This sorbent was used following an anion-exchange chromatographic step (DE52) which exploited the negative charge of the extended peptide and was followed by preparative high-performance ion-exchange chromatography (HPIEC)

(TSK gel DEAE-5PW), carried out according to Nakamura et al. [227]. After the enzymatic conversion of the amino-terminal extended hGH to the mature form using the exopeptidase DAP-I, the product was again chromatographed using gel filtration and HPIEC. This amounted to a total number of four chromatographic steps. The process gave rise to very pure rhGH, especially due to the efficient application of the two preparative HPIEC separations. However, no data were provided on recovery yields, nor on the specific activities of the various intermediate fractions. In a study by Franchi et al. [81] who produced another amino-terminal extended hGH, MRR-IEGR-hGH, in soluble form in *B. subtilis* cytoplasm, the positive charge of the extended peptide was exploited by using cation-exchange chromatography (S-Sepharose Fast Flow) as a first run. After isoelectric point assessment of the partially purified hybrid polypeptide by analytical isoelectrofocusing, this tag-hGH was digested with factor Xa, and the product rechromatographed on the same cation exchanger under identical conditions and applied to a gel filtration column (Sephadex G-100 super-fine). The final product showed 98% homogeneity, while the initial crude extract had a specific activity of 5% (by immuno dot blot analysis), with an overall recovery yield of 34%. More recently Shin et al. [79], using a His<sub>10</sub>-tagged hGH extracted from *E. coli* cytoplasm and applying Ni<sup>2+</sup>-chelated affinity chromatography before and Q-Sepharose Fast Flow anion exchanger after enterokinase digestion, reported a purity for rhGH greater than 99%, as determined by SDS-PAGE and analytical RP-HPLC. The great advantage of this design was especially based on the high expression level (30% of total cell protein, 9 g/l of hGH) that consequently provided a starting material (in inclusion bodies) with the astounding specific activity of 70%. These characteristics, together with the high density cell culture obtained (>85 g/l of dry cell mass) and the highly specific and successful cleavage strategy, rendered this whole synthesis-purification process extremely attractive for the production of this and of other therapeutic proteins.

Interesting small-scale purifications of rhGH have been described by Gray et al. [70] who obtained homogeneous monomeric hGH by subjecting lyophilized *E. coli* osmotic-shock fluids to immuno-

affinity chromatography based on monoclonal anti-hGH antibody (mAB) coupled to Affigel 10. Similarly, Jonsdottir et al. [214] purified hGH using an analogous, rapid and gentle immunoabsorbent purification on anti-hGH mAB coupled to Sepharose 4B followed by gel filtration (AcA54 Ultrogel), to remove mouse immunoglobulins leaking from the gel, and HPLC anion exchange (Mono Q) to obtain an extremely pure preparation of the major  $M_r$  22 000 form of hGH. The study of Nakamura et al. [227] has already been mentioned, in which two different HPIEC columns were scaled up for preparative purposes without loss of resolution. Furthermore, Llobès et al. [110] based their purification methodology totally on FPLC (Mono Q anion exchanger) and RP-HPLC, mostly aiming at quantification and characterization of excreted Met-hGH. Igout et al. [103] based their biochemical and immunochemical characterization of recombinant human placental GH on two ammonium sulfate precipitations, a Q-Sepharose Fast Flow and a Sephacryl-S200 HR, while Maisano et al. [215] worked out the most suitable adsorbent (iminodiacetate- $\text{Ni}^{2+}$ -agarose) testing the performance of different metals for a two-step purification of rhGH via IMAC and DEAE-cellulose; this study was based on pioneer work by Porath and Olin [228]. The production of hGH in tobacco chloroplasts by Staub et al. deserves special mentioning [177]. In this process the acidified and clarified soluble protein extract obtained from green house-grown leaves, containing 7% of hGH, was passed through an anion-exchange column, and following elution with high salt acidic buffer, the purified product was characterized on a  $\text{C}_{18}$  RP-HPLC column and eluted using a linear acetonitrile gradient.

In finally dealing with one-step purification methodologies, we should mention the 98% purity (as estimated by densitometric scanning) obtained via simple RP-HPLC gradient purification (Aquapore RP-300) of authentic hGH excreted in *E. coli* culture medium [109] and the virtual homogeneity obtained by purifying tag( $\text{His}_6$ )-hGH from inclusion bodies on  $\text{Ni}^{2+}$ -nitrilotriacetic acid (NTA)-agarose [78]. Also exploiting metal-protein affinity is a one-step IMAC purification based on utilization of cellulose-based chelating supports, charged with  $\text{Cu}^{2+}$  after optimization of their density. This procedure was

able to enrich the specific activity of rhGH in a crude bacterial extract from 10% to about 80% [217]. We would finally like to recall the previously mentioned mild and fast single-step purification of rhGH from *E. coli* periplasmic fluid on high-performance size-exclusion chromatography (HPSEC) leading to a specific activity of about 64%. Such a purification procedure appears to be particularly useful for the economic production of freshly prepared radioiodination grade, recombinant protein [198].

Basically, we have described a great number of purification methods which in the last 2 decades have been applied to preparation of rhGH in the laboratory and at an industrial scale: they are practically all based on classical techniques and conventional heuristic approaches. It is therefore of interest that the purification of *E.-coli*-derived rhGH has been chosen as an example for testing mathematical models aimed at studying how a protein of interest can be purified from a hypothetical mixture containing 13 protein contaminants [203,229]. The main objective of this approach is to determine the optimal selection and sequencing of purification steps that are necessary to achieve a 98% purity for the protein [19,197]. The models have been tested in several examples containing up to 22 candidate high-resolution separation steps and four chromatographic techniques (anion and cation exchange, hydrophobic interaction and gel filtration). Sequences of six operations so generated were then compared to current downstream processing. The results obtained deserve the attention of all purification chemists, since the proposed methods have been found to be more reliable than those reported in the literature and theoretically are able to attain higher purity levels.

### 3.2. Human prolactin

As far as we know, routine therapeutic applications of hPRL have not yet been established. Recent studies, however, indicate possible use of this hormone in the treatment of women with poor lactation associated with temporary pituitary ischemia, in the restoration of normal sperm characteristics in hypoprolactinaemic, infertile men and in the treatment of infections and immunosuppression. Clinical use of hPRL or its variants in the treatment of patients suffering from such diseases as AIDS and cancer has

also been proposed [144,230–232]. Thus, although hPRL even a few years ago was only considered for in vitro diagnostic applications, it now is presenting several potential therapeutic applications. In discussing purification processes of this hormone we will also roughly follow the classifications applied to hGH.

Only one laboratory, indeed the first that submitted recombinant hPRL to clinical trials, has in our opinion set up downstream processing that comes close to pharmaceutical development of a therapeutic protein [165]. The published study, does not only deal with the microheterogeneity and bioactivity of glycosylated (G-hPRL) and nonglycosylated (NG-hPRL) human prolactin, but also describes the development of a novel purification process. This process starts with clarification of medium, conditioned by transfected murine C127 cells, in which the hPRL mass fraction is quite high (about 0.32). It then continues with cationic exchange (S-Sepharose Fast Flow) and anion-exchange (Q-Sepharose Fast Flow) chromatography, producing at this point a heterogeneous G-hPRL+NG-hPRL preparation with a purity exceeding 97% and a cumulative recovery of 53%. This product, which was resolved by isoelectric focusing (IEF) gel electrophoresis into six distinct bands, mostly due to multiple combinations of terminal sialylation, is probably not so different from the natural product, which is also glycosylated in a similar proportion. In any case, the purification process was continued and aimed at completely resolving the two homogeneous isoforms of G- and NG-hPRL, exploiting one of the main advantages of a recombinant product: the prospect of always obtaining purified isoforms due to the unlimited amounts that can be synthesized. This last separation was achieved utilizing again a cationic exchanger (this time high-performance S-Sepharose) and buffer supplemented with 5% ethylene glycol and 5% *n*-butanol, to help dissociate the two isoforms. The latter were perfectly resolved and their purity, determined by laser densitometry, was >99% and >95% for NG-hPRL and G-hPRL, respectively.

It is worth recalling previous work carried out by the same group on baboon and monkey PRL synthesis, purification and characterization [166]. In that case the two isoforms (G-PRL and NG-PRL), even if detected, were not preparatively resolved, while the

purification process was very similar to the one described above, but with the addition of gel filtration (Sephacryl S-200) after S- and Q-Sepharose Fast Flow chromatography, reaching a cumulative yield of 40%. It is of interest that in this development an accuracy problem arose that always has been a concern to us: RIA greatly underestimated the concentration of PRL (by approximately 3-fold). According to the authors, the mass fraction that had been estimated in the final pool as 0.31 by RIA, was in fact judged to be >0.90 by immunoblotting analysis in conjunction with laser densitometry of Comassie blue stained gels. We believe that even the mass fraction of 0.01 in the starting material could have been underestimated by RIA. Using SDS-PAGE under reducing conditions, three major bands were identified with apparent  $M_r$  values of 27 000, 23 000 and 16 000 corresponding to G-hPRL, NG-hPRL and to a cleaved hPRL form at position 133, respectively. The latter was later identified as a physiologically relevant hPRL form exhibiting antiangiogenic activity. Another interesting monkey and baboon PRL variant was also identified by amino-terminal sequence analysis: a PRL<sub>11–191</sub> isoform of probably 22000 starting with a Cys at position 11. An analogous variant has been reported by Shelikoff et al. [168] and by Soares et al. [144], who obtained it in murine C127 and in CHO cells, respectively. A physiological role for this truncated form of hPRL needs further investigation.

The research of Hiraoka et al. [85], even if intended more for elucidation of hPRL structure–function relationships, can still be considered useful for large-scale production of hPRL in the laboratory. These workers started with a relatively high culture broth volume (*E. coli*) containing a fusion protein of hPRL, a collagenase-sensitive peptide and *S. aureus* protein A. This was subjected to a complex separation procedure involving four columns and a collagenase digestion of the fusion protein. A cleared cell homogenate with a fusion protein mass fraction of only 0.03%, was applied to an IgG-linked Sepharose affinity column, which made purification easy, leading to a specific activity of about 13% and a 90% recovery yield. Following dialysis, anion-exchange (DEAE-5PW) chromatography increased the specific activity to 43% and, after hydrophobic interaction chromatography (Phenyl-5P), this rose to 70–80%

with an overall recovery yield of 44%. Collagenase digestion was then carried out and followed by Phenyl-5P chromatography. The two operations provided a 20% yield of 95% pure hormone. Of the two collagenase cleavage sites present in the intermediate peptide linker, only the site proximal to the N-terminus was cleaved by the enzyme, thus leaving four amino acids tagged at the N-terminus of hPRL. The problems encountered with this extraction–purification process somehow are characteristic of the difficulties encountered, even in later studies, with the extraction of soluble hPRL from bacterial cytoplasm or periplasm and are probably due to the relatively low stability of hPRL when compared, for example, to hGH. In view of that, our laboratory started production of hPRL from CHO cells [144], also to circumvent the difficulties already faced in obtaining hPRL expression and its secretion into the *E. coli* periplasmic space [104,105]; furthermore, we were interested in the glycosylated form of hPRL which cannot be obtained in bacteria. A primary focus of the research was to identify a powerful eukaryotic expression vector and develop an efficient selection–amplification strategy capable of rapidly generating highly productive, rhPRL-secreting CHO cell lines. Purification of the hormone was carried out using Price's methodology, but with Sephacryl S-100 taking the place of Q-Sepharose in the second step. After this step, the overall recovery was 86%. The last step to resolve the hPRL isoforms, however, gave quite low recoveries (<18%): a sacrifice required to achieve relatively high purities, i.e. 95% and 99.5% for G-hPRL and NG-hPRL, respectively. The starting material had a much lower specific activity (10–15%) compared to that of Price et al. Furthermore, most of the products obtained, i.e. G-hPRL and NG-hPRL, were frequently contaminated by the previously mentioned truncated form of the hormone (hPRL<sub>11–191</sub>), especially when they were generated in a hollow fiber bioreactor; this greatly decreased the overall yields.

The purification strategy developed by Cunningham et al. [90] for the production of wild type hPRL and hPRL variants required for hPRL structure–function analysis, typically started with the hormone/variant contained in *E. coli* cytoplasmic inclusion bodies. After solubilization, refolding and ammonium sulfate precipitation (45% saturation), an 85%

pure product was subjected to two chromatographic steps: FPLC and DEAE–Sepharose Fast Flow, leading to a purity >95%. Even though it was the first described process for purification of hPRL, it was treated as a simple tool required for the structure–function studies.

A two-step preparative technique for the purification of authentic rhPRL secreted into *E. coli* periplasm, developed in the authors' laboratory and described by Ueda et al. [212], involves a novel application of Ni(II)-based IMAC as a substitute for the cation-exchange step utilized by several other workers in the purification of the hormone [144,165,166]. The presented data demonstrate a clear advantage for the IMAC-based purification. Starting from a mass fraction of 0.08, almost complete purity (97.7%) was obtained already after the first chromatographic step, coupled to a 84% yield. In contrast, the older process after the first chromatographic step, led to a mass fraction of only 0.56 and a 61% yield. After a second chromatographic step (Sephacryl S-100) in the new procedure, the purity of the compound was increased to 99.5%, with a higher overall yield of 77% (versus 46% for the older procedure). These higher recovery values, coupled to a much lower protein contamination, are basically related to the relatively high and specific adsorptive capacity of the IMAC column for hPRL; the low contamination by proteins also reduced the chances of proteolysis of the hormone. It is likely that this method will also be valuable for the purification of a number of hPRL variants, including glycosylated hPRL isoforms which, according to preliminary data, also showed affinity toward the Ni(II)-charged matrix. Accurate determinations of the hPRL content in all chromatographic pools, as well as the crude starting material, were made possible by application of novel RP-HPLC methodology, specifically developed and validated for the qualitative and quantitative analysis of PRL [101]. In the described purification process this method was always applied together with RIA.

Several single-step purification methods have been described for hPRL, whether derived from *E. coli* cytoplasm inclusion bodies [83], from the periplasm [104,105] or from baculovirus-infected insect cells [134,135]. Paris et al. [83] probably achieved the highest bacterial production of Met-hPRL in cyto-

plasmic inclusion bodies, as already mentioned in the section on synthesis. It is of interest that these inclusion bodies, after low-speed centrifugation of disrupted cells, contained >90% hPRL. Therefore, after solubilization, renaturation, lyophilization and gel-permeation chromatography by FPLC (Protein Pak 300SW column), it was not unexpected that a 100% pure product was obtained. Conventional Sephadex G-100 gel filtration was used instead of FPLC, but was later abandoned because it produced a certain amount of reduced prolactin molecules. The approach of Morganti and co-workers [104,105] was specifically directed at acquiring correctly folded hPRL molecules in *E. coli* periplasm. In their first study [104], hPRL with a peptide tag containing 6 histidines and the factor Xa cleavage site, and a mass fraction in the periplasmic fraction of <0.01, was purified via metal-chelate affinity chromatography, using trinitrilotriacetic acid (Ni-NTA)-agarose, to 99% purity with a yield of about 40%. Unfortunately, the factor Xa digestion to remove the tag was only partially successful, and this form of perfectly bio- and immunoreactive hPRL was therefore only considered for use in vitro. The second study, however, was successful in obtaining fully bioactive, authentic hPRL. This was probably the first time that authentic rhPRL had been produced by secretion into *E. coli* periplasm [105]. On the other hand, the hPRL mass fraction of the starting material was this time even lower (<0.002) and the single-step immunoaffinity chromatography (anti-hPRL IgG coupled to CNBr-activated Sepharose 4B) with a recovery yield of about 20%, was only used for a first characterization of the product.

Recent studies of rhPRL synthesis in insect cells have indicated that monolayer cultures of High Five cells are better for the production of the hormone than *Spodoptera frugiperda* cells. Das et al. [134] produced serum-free culture medium in which the amount of insect cell-secreted prolactin was about 18–20 µg/ml, i.e. ideal for purification purposes. After dialysis and concentration of the sample, a single Mono-Q FPLC run already provided a 90% pure authentic prolactin preparation. The method can be used for large scale preparation of bioactive hPRL and hPRL variants and production can be increased several fold by using a suspension culture in a bioreactor instead of a monolayer culture. Strokov-

skaya et al. [135] generated a His<sub>6</sub>-tagged prolactin, accumulated inside insect cells in the form of cytosolic inclusion bodies, as in *E. coli* cells. Since solubilization under denaturing conditions was found to cause loss of immuno- and bioactivity, the authors developed a milder solubilization and purification method. Starting from quite a high mass fraction (0.35–0.40), the crude extract was purified by affinity chromatography on a Co(II)-IMAC column; a yield was obtained of about 20 µg of product/ml of medium. In addition to the main protein of  $M_r$  28 000–29 000 (95% of total), two other proteins were identified by immunoblotting: an  $M_r$  30 000 glycosylated protein and an  $M_r$  55 000 dimer.

### 3.3. Glycoprotein hormones: thyrotropin, luteinizing hormone and follicle-stimulating hormone

Only two complete biotechnological processes have been described for the production of these recombinant glycoprotein hormones for clinical use: one for hTSH and the other for hFSH. In both cases they were developed by pharmaceutical companies that are now manufacturing and commercializing the products.

Cole et al. [148] started their process for CHO cell-derived hTSH production with pilot scale bioreactor-conditioned, serum-free medium (160 l) containing about 25 µg hTSH/ml with a mass fraction of 0.26. After clarification by filtration, a 10-fold concentration on a spiral-wound cartridge system (10 000  $M_r$  cut-off) and pH adjustment to 5.0, the product was first chromatographed through a cation exchanger (S-Sepharose Fast Flow) and then, at pH 7.0, through a dye affinity (Trisacryl Blue) column, followed by an anionic exchanger (Q-Sepharose Fast Flow). Finally, after a 10- to 20-fold concentration to approximately 1.5 mg protein/ml, the preparation was subjected to gel filtration (Sephacryl S-200 R) chromatography. It is of interest that the first two chromatographic runs (with both sorbents packed into acrylic or stainless steel radial flow columns) were carried out in the bind-elute mode, while the third one was performed in the flow-through mode. After the first two columns the mass fraction was already 0.96, indicating that the last two chromatographic runs were only providing the final polishing;



an overall yield of 54% was obtained, together with a purity >99%, as determined by SDS–PAGE, RP-HPLC and HPSEC. It may be noted that the bioactivity of the rhTSH, measured via the *in vitro* bovine thyroid membrane bioassay, did not change throughout all the purification steps. This control is quite important in view of the lability of the hormone and the tendency of the noncovalently bound heterodimer to dissociate. The specific biological activity of the final purified material was 8.2 I.U./mg, determined against the World Health Organization (WHO) Human Pituitary Research Standard B whose specific activity was 4.93 I.U./mg. The overall process had been very efficiently designed; the individual step recoveries were >90% with the exception of the recovery of the final gel filtration which, due to quite conservative pooling, amounted to only 78%.

The study on the synthesis and purification of CHO cell-derived rhFSH as a therapeutic protein has been described in two publications, first by Howles [233] and then by Loumaye et al. [147] of the same group. The first paper merely provides the general strategy of the manufacturing process, divided in three phases, without going into details. It starts with the cell culture process or scale-up phase dealing with an expansion of the cultures from flasks to roller bottles and then to a bioreactor vessel. Then we have the hFSH production phase involving a bioreactor, consisting of cell attachment and growth, hFSH secretion and harvesting of the conditioned medium. Finally comes the downstream purification process, composed of an initial ultrafiltration step and five chromatographic steps followed by a second ultrafiltration. Of the chromatographic steps, only the second one, immunoaffinity chromatography based on murine-derived anti-hFSH monoclonal antibody, is defined, while it is mentioned that one of the final steps is RP-HPLC. It is worth noting that the potency (10 000 I.U./mg) of the purified product (>97% purity), determined via the rat ovarian weight gain assay, was indistinguishable from that of highly purified hFSH extracted from urine. The complete description of the downstream purification process, however, was 2 years later provided by Loumaye et al. [147]. The design is basically the same, starting with an 11.5-l bioreactor in continuous perfusion mode. After a concentration/diafiltration step (10 000  $M_r$  cut-off) there was a gross purification

phase involving three columns: an anion exchanger (DEAE–Sephacryl CL6B), the immunoaffinity column previously mentioned and a second, stronger anion-exchange (Q–Sephacryl Fast Flow) column. Only the first column was run in the flow-through mode; furthermore, each run was followed by a concentration/diafiltration step. According to our calculations based on the reported data, the starting material had an hFSH mass fraction of 0.02 and an overall purification factor of 41 had already been reached after the first phase. The last two columns, a  $C_{18}$  RP-HPLC containing 11% isopropyl alcohol in the mobile phase and a size-exclusion (Sephacryl S-200) column provided the final polishing effect, removing trace amounts of contaminant host cell proteins. After a last concentration/diafiltration and a microfiltration (0.2  $\mu$ m membrane), the pharmaceutical grade product was stored at  $-20^\circ\text{C}$ . The final purity was  $\geq 95\%$ , the overall recovery 36%, while the average recovery of all columns was about 85%. All batches of clinical grade hFSH had <25 ppm of cell culture-derived proteins, <10  $\mu$ g DNA per dose (150 I.U.), <1% aggregated or dissociated hFSH and less than 8 endotoxin units (EU)/vial. Interestingly, the mature protein sequence deduced from the nucleotide sequence of the gene indicated that the hFSH  $\beta$ -subunit was 111 residues long, confirming previous literature data [158].

In comparing hTSH and hFSH downstream purification processes and trying to find some general rules, it can be noticed that the latter started with the disadvantage of a  $\sim 10$ -fold lower mass fraction. Probably for this reason, the purification of the hFSH required an extra chromatographic step to obtain a 10-fold higher purification factor (41 for hFSH versus 4 for hTSH) and hence led to a 20% lower overall recovery yield. Both processes used affinity column chromatography as a second step which provided a purity close to or well above 95% for hFSH and hTSH, respectively. Both procedures used two ion-exchange columns, one in flow-through and one in bind-elute mode and a gel filtration as a final polishing/buffer exchange step. The basic difference between the two processes therefore appears to lie in the utilization of the preparative (and costly)  $C_{18}$  RP-HPLC step for the purification of hFSH. This step also involved use of an organic solvent in its mobile phase and was probably required to remove

contaminants which were present in greater abundance in the starting hFSH material.

A first approach to the purification of rhTSH reported by Cole et al. [148], has previously been described by Thotakura et al. [12]. These workers examined the carbohydrate composition, *in vitro* biological activities and *in vivo* clearance rates of rhTSH obtained from one of the several CHO cell lines investigated by the two collaborating research groups. An initial, simpler purification scheme was developed, based only on dye affinity (Blue Trisacryl), anion-exchange (Q-Sepharose FF) and cation-exchange (S-Sepharose FF) chromatography. The total purification yield was lower (32%) and the final purity greater than 97%. Evidently this purification process was used more as a tool for the development of the later studies, and not yet aimed at clinical applications. Subsequently, this same group described a slightly different purification scheme for rhTSH based on cationic exchange (S-Sepharose FF), dye affinity (Blue Sepharose Fast Flow) and optative anion-exchange chromatography as a third step [13]. Purity was judged to be greater than 99% (by SDS-PAGE analysis). Of interest about this process is that a large amount (more than 200 mg) of rhTSH was produced using two small hollow-fiber bioreactor systems (Cellmax 100 and 200) and that these conditions, quite different from those typical of a large scale oxygen-sparged bioreactor-based process using microcarrier beads [148], had a profound effect on the carbohydrate structure of the hormone, leading to a lower sialylation and reduced galactose content.

Still dealing with the second category of purification processes not aimed at the production of therapeutics, we would like to mention the important work by Hakola and co-workers [159,160] in the large-scale production and purification of rat LH and rat FSH. In the present review we have restricted our analysis to human pituitary hormones, but in these two studies the authors' objectives were both to obtain highly purified homologous hormone preparations and study their actions in an important animal model. It may be noted that these studies were not only important with regard to gonadotropic action, but that the purification processes employed showed essentially the same manufacturing quality typical of a pharmaceutical production. In the first study [159],

a large-scale rat LH production is described, starting from 23.6 l of CHO cell-conditioned medium produced in a bioreactor and based on gelatin microcarriers. After concentration by ultrafiltration the product was applied batchwise to an S-Sepharose cationic sorbent, loaded on a column and, after elution and dialysis, subjected to dye affinity (Red-1 agarose A6XL) chromatography, followed by gel filtration (Sephacryl 300 HR). After 0.5 M ammonium sulfate treatment and filtration, the product was submitted to hydrophobic interaction chromatography on Phenyl agarose 6XL, dialyzed and lyophilized. Having started from a total of 25 mg recombinant rat-LH, finally 13 mg of purified product (ca. 95% purity) was obtained with an overall recovery yield of 56%. In the case of rat FSH [160], 8 l of CHO culture supernatant obtained in spinner flasks were treated with ammonium sulfate (1.3 M), filtered and submitted to hydrophobic interaction (phenyl agarose A6XL) chromatography, followed by dye affinity (Blue-2 agarose A6XL) and anionic (Q-Sepharose FF) chromatography, with a dialysis step between each of these runs. The last chromatographic step was gel filtration on Sephacryl S-100 HR. The final purity obtained was 98% with an overall recovery yield of 48%. The analogy between the purification processes of the two hormones is apparent: both use four chromatographic steps and start from rather dilute medium ( $\sim 0.3\text{--}0.7 \mu\text{g/ml}$ ). It may be noted that the dye affinity chromatography was in general the most efficient of the purification steps.

Single-step purifications merely for an initial characterization of a new product have been described by Wondisford et al. [156] who for the first time synthesized hTSH in human embryonic kidney cells (293 cells) and by Grossmann et al. [136] who generated the same hormone in insect cells. Both groups applied lectin affinity (concanavalin A Sepharose) chromatography for studying the glycosylation pattern of the hormone. Chromatofocusing was also used as a single-step for characterization of different preparations of CHO cell-derived hFSH. It separated the different isoforms of the hormone on the basis of differences in isoelectric properties, and allowed determination of their charge profiles and bioactivities [158,234]. To study the effect of changes in environmental conditions (e.g. oxygen levels) on

hFSH expression and glycoform patterns, single-step immunopurification on a Protein-G affinity column was used [235]. Finally, purification by C4 RP-HPLC, using linear gradient elution, was used for structural characterization, both at the protein and carbohydrate levels, of  $\alpha$  and  $\beta$  subunits of the three human recombinant gonadotropins (hFSH, hLH, hCG), all obtained in CHO cells [236].

#### 4. Conclusions and perspectives

The synthesis and production of human hormones is probably the most traditional field of recombinant DNA-derived pharmaceutical technology. During more than 2 decades we have witnessed a real explosion of information on the molecular mechanisms involved in protein synthesis. This information has, most of the time, immediately been applied to the laboratory and within a very short time, also to the industrial production of pharmaceuticals. Genetic approaches directed at tailoring the protein or modifying the organism of interest for the purpose of increasing product yields, purity and of creating humanized or more potent agonists or antagonists, will probably continue to be developed in an accelerated way using microorganisms, plants and animals. New revolutionary applications are being developed in DNA technology, in connection with gene therapy, which probably will greatly influence the future of the clinical and pharmaceutical fields. As we can perceive from this review, the actually applied purification techniques are, at least in the case of human pituitary hormones, mostly conventional and there is a certain resistance toward the industrial introduction of revolutionary approaches. An example is the utilization by major manufacturers of essentially the same 3–5 classical chromatographic steps (not including filtration or precipitation) to obtain a final product. Cost-effective and new rational process designs based on artificial intelligence tools have, however, been developed and are being tested right in this biotechnological field. Automated purification systems, new types of sorbents and columns, new polymeric membrane filters and, in particular, industrial preparative HPLC systems, have already conquered a large territory.

In general we may conclude that this rapidly

growing development in the pharmaceutical area has never been more attracted to the natural bioprocessing mechanisms of living organisms. The latter are now not only considered as an unlimited source of scientific knowledge, but also as real production tools that can provide a great contribution toward improving the quality, purity, safety and yields of therapeutic proteins at reduced costs.

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