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Review

Separation used for purification of recombinant proteins

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Abstract

The purification of molecules from recombinant cells may be strongly influenced by the molecular biology of gene isolation and expression. At the beginning of the process there may be a demand for information on the minute amounts of proteins and thus for ever increasingly sensitive techniques. Purification of recombinant proteins can differ from conventional purifications in several ways, depending on the solubility of the protein, occurrence in inclusion bodies, creation of fusion proteins with tags that enable simpler purification. Sometimes a (re)naturation step is required to get a bioactive protein. On the other hand, the techniques used in separation are essentially the same as for purification from the natural source and environment. © 1997 Elsevier Science B.V.

Keywords: Reviews; Recombinant proteins; Proteins

Contents

1. Introduction

The design of effective methods for purification of recombinant proteins from complex biological mixtures at all levels from laboratory bench to large scale persists as a major challenge in the biotechnology industry, despite the availability of a wide range of ingenious approaches to the problems. There is a demand for increased speed, economy and precision that leads to new technologies and methodologies.

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The driving force for improving protein purification at biopharmaceutical companies is increased product purity and decreased cost. Very often, however, in order to enter the market rapidly, manufacturers prefer what has been proven rather than what is optimal. There is a continuous conflict between resolution, capacity and speed. The refinement of existing technology takes place or even the resurrection of old methods in a new guise.

Purification, more than almost any other activity, defines the profitability of a bioprocess. Purification represents about 60–70% of the operating costs of the entire bioprocessing. The best way to simplify the purification and to design a rationale procedure is not to treat the cell culture and the purification processes as separate and distinct entities. The process should be optimised and considered as a single unit, rather than two, and some recovery steps should take place before, during and after the traditional cell culture steps. The purification process design should be coincident with the cell culture process design, not following it. The techniques that retain cells can produce cultures with higher densities and product concentrations but can also increase the amount of cell debris and the release of enzymes that can damage the product.

Proteins have diverse characteristics with respect to size, charge and hydrophobicity, among other characteristics, that complicate the purification process. Generally, six major methodologies in combination for protein purification are being used: precipitation, membrane filtration and affinity chromatographies, ion exchange, reversed-phase and hydrophobic interaction chromatographies.

When purifying a recombinant product, there is a need to remove cellular debris, perhaps some precursor proteins, and finally cell culture-derived contaminants that typically differ significantly from the desired product. The first opportunity for product recovery and purification-process enhancement is in the selection of the expression system which also defines future possible contaminants – cell culture nutrients and process additives. Many cell-culture processes, however, are still being designed with little consideration regarding the final product purification. For all practical purpose, many purification starting materials are too expensive to purify. The consumable costs in the steps required, and the product losses inherent in these steps, should be unacceptable.

The requirements for purifying recombinant proteins are similar for all usual source materials such as bacterial culture media, mammalian cells, or the milk of transgenic animals. There is, of course, one major difference resulting from the choice of the expression system. The starting material may be insoluble in the form of an aggregate (produced as inclusion bodies) where a refolding step is usually necessary, or soluble (usually at much lower available concentration). A sequence of six major processing tasks is usually required: solids removal, volume reduction, pre-purification, viral inactivation, high resolution purification, and a final low resolution clean-up. In addition, the protein chemist must be able to follow accurately the concentration of the protein through all the steps in the process as well as have reliable assays for the contaminants.

The recombinant protein must be in a reasonable concentration, proteolytic degradation must be controlled, and the process must produce the protein in a biologically active form. These requirements are not easy to accomplish in any system, but it is obviously easier in some than in others. With bacterial expression systems the recombinant protein may constitute 25% or even more of the total protein in the starting material; however, this protein may need to be solubilized, denatured, and refolded. The main problem with bacterial systems is that procaryotes cannot do the post-translational modifications which are frequently required for the biological activity of many eukaryotic proteins. Although recombinant proteins produced by eukaryotic expression systems may be made in a sufficiently native and soluble form, the recombinant protein is usually at very low concentrations. Bioprocessors acknowledge that the most difficult and expensive separation step for the products of biotechnology involves column chromatography. Scientific studies have shown that polymeric membrane filters modified with ion exchange, affinity or other active groups typical in purification offer the potential for significant improvements in the convenience and speed of bioseparations as compared with traditional resins. No single method, however, provides the one process that universally solves all of the industry's requirements. A trend that has become particularly apparent in recent years is

application of a number of methods, electing to use 'the right combinations for the particular protein'.

2. Expression systems

The principal differences in isolation of recombinant and other 'natural' proteins result from various expression systems: bacterial and mammalian expression systems, filamentous fungi. Heterologous proteins produced in microbial systems are either secreted into the periplasmic space and/or into the external conditioned medium, in soluble form or in aggregates requiring one additional step – refolding.

So far, current popular choices of expression systems have been yeast (*S. cerevisiae*), bacteria (*E. coli*), and Chinese hamster ovary (CHO) mammalian cells. This is unlikely to change significantly in industry in the near future, at least in the US, because of the extensive background data already submitted on these host organisms to the US Food and Drug Administration [1] and a number of approved protein products secreted from yeast and CHO cells or refolded from *E. coli*.

The choice of expression system depends upon many factors, including the desirability of post-translational modification and secretion, the solubility, stability and refoldability of the protein in question, and the projected amount of produced protein.

For proteins with a simple structure and no secondary modification, prokaryotic expression systems are of long-standing availability and may be faster and more straightforward than other, more elaborate expression systems. However, many proteins of interest are complex in structure, requiring extensive secondary modifications. Eukaryotic proteins are post-translationally modified (signal-sequence trimming, glycosylation, phosphorylation, sulphation, acylation, myristylation, isoprenoid and glycosylphosphatidylinositol [GPI] anchors, etc.). The products must be passed through the labyrinth of cellular structures where folding, disulphide bond formation, and the post-translational changes are made.

One of the limiting factors in the production of soluble functional heterologous proteins in *E. coli* is the aggregation tendency during folding. Recombinant proteins are often expressed at levels that are orders of magnitude higher than their normal expression levels. It is immediately obvious that the propensity to aggregate and the precise mechanistic reasons for aggregation will be strongly dependent on the particular recombinant protein being expressed, and that universally useful solutions are thus unlikely to exist [2,3]. With a better understanding of the protein export process and a greater awareness of the conditions necessary for correct folding of proteins in the periplasm, serious efforts are now being made to manipulate this system to achieve substantial increases in the yield of authentically folded proteins. Periplasm is a gel-like region between the outer surface of the cytoplasmic membrane and the inner surface of the lipopolysaccharide layer of Gram-negative cells where, because of its non-reducing environment disulfide-containing heterologous proteins must generally be expressed. The folding and subunit assembly of eukaryotic proteins requires chaperoning activity that is not available in prokaryotic organisms. To date, no general molecular chaperones that prevent non-productive folding reactions have been identified in the periplasm, though SecD and SecF, transmembrane proteins with extended periplasmic domains, might help to prevent aggregation of newly translocated proteins during the initial stages of folding [4]. Although the mechanisms by which chaperones and foldases facilitate productive protein folding are becoming clearer, as yet, no universal strategy is available to unerringly pick a successful over-expression or refolding procedure for a given protein. The process is still largely one of trial and error. The reasons for this may be beginning to emerge. For high-level expression in vivo, specific proteins may require a combination of multiple chaperones and foldases acting in a defined sequence. Mimicking this sequence of events may be difficult with heterologous expression systems or in vitro; however, it may be possible to develop and employ effective assisted-folding systems in individual cases [5]. The effects of folding catalyst over-expression [6] quite clearly demonstrate the potential of this approach in partially solving at least some of the problems in the folding of several heterologous proteins in *E. coli*. Certain recombinant proteins are actually recovered as soluble folded products after expression intra-cellularly in *E. coli*. Some proteins that are normally produced in inclu-

sion bodies may be rendered soluble and active by expressing them intracellularly in *E. coli* engineered to over-express bacterial chaperones [7–10]. For each success story, however, there are many more (often unpublished) failures at present.

A further, albeit related, problem in the expression of heterologous proteins in the *E. coli* periplasm is that of degradation by host proteases. When manipulating host cell proteases, however, one must bear in mind that proteolytic degradation may be a symptom, rather than a cause, of folding problems, serving to remove misfolded material [11] and already formed aggregates whose accumulation in the absence of proteases might prove to be toxic. Thus, the possibility exists that a primary effect of protease depletion may be to actually increase toxic effects of heterologous protein expression on the cell.

Future exploitation of *E. coli* export and secretion systems is likely to focus on the achievement of greater yields by manipulation of the levels of components of the protein-folding machinery and the levels of particular membrane translocators. The large number of different secretion systems now available in *E. coli* by transplantation from other Gram-negative bacteria will also provide much greater choice than before for the selection of the optimum process for a given heterologous protein [12,13]. The simultaneous overproduction in *E. coli* of the chaperones of the Hsp70 (DnaK, DnaJ and GrpE) or Hsp60 (GroEL and GroES) and the human non-receptor protein tyrosine kinases Csk, Fyn or Lck was reported to increase solubility of the recombinant kinases. This provides the basis for future successful production and purification of large quantities of soluble proteins from *E. coli* expression systems. The results suggest that the approach may be generally applicable to improve the solubility of recombinant proteins which otherwise are produced in an insoluble form in *E. coli* [14].

The possibility of suppressing aggregation through genetic engineering can also be envisaged. With rational selection of the parameters necessary for correct maturation (temperature, ionic conditions, cofactors etc.), one could influence and perhaps control the aggregation and the fate of the folding intermediates towards the native outcome. Alternatively, it may be possible to suppress aggregation by specifically changing amino acid positions that play a critical role in the folding process. To address the very low solubility of a protein, two independent approaches: (1) mutagenesis of the β-sheet framework residues and (2) addition of a solubilizing motif, made of three lysine residues, at the N or C termini were used. Engineering and production of mutants was facilitated by the achievement of high level expression of the protein in *E. coli*. Both approaches led to protein variants with a solubility ranging up to ten-fold higher [15]. An authoritative review on theoretical, structural, and mutational studies of the effects of amino acid substitutions in protein cores has been published [16].

In order to direct the recombinant protein towards the periplasmic space, the coding sequence of mature pallidipin was fused with that of the leader peptide of alkaline phosphatase, a secreted bacterial protein. The recombinant protein was purified in three chromatographic steps including cation exchange, anion exchange and size exclusion gel chromatography [17].

Procaryotes that have legitimate potential as alternative hosts for heterologous protein production include the Gram-positive bacteria *Bacillus subtilis* and *Streptomyces lividans* [18–20].

The ability of *Streptomyces* species to express eukaryotic gene products in a secreted and biologically active form has been actively investigated over the past 5–10 years [21,22]. Interest in *Streptomyces* expression systems has been fueled by the natural ability of these bacteria to secrete active and properly folded proteins directly into the culture supernatant, the extensive fermentation experience gained from their long use in antibiotic production, and the availability of a variety of well characterized *Streptomyces* transcription initiation signals.

In recent years the baculovirus system has become the premier means for the expression of a wide range of proteins. Baculoviruses (family *Baculoviridae*) belong to a diverse group of large, double-stranded DNA viruses, infecting many different insect species as their natural hosts [23–27]. These organisms are attractive because they lack lipopolysaccharide, which can be a toxic contaminant (particularly for human therapeutic products), and they have the ability to secrete large quantities of proteins into the growth medium, which allows easier purification in

the downstream processing. Moreover, a wealth of historical fermentation data has been obtained with these organisms because they produce a variety of industrial compounds, such as antibiotics or extracellular hydrolytic enzymes, at high concentrations (up to several grams per liter [28]. A major area that must be addressed before *B. subtilis* and *S. lividans* can gain a wider acceptance as recombinant hosts are the elimination of their extracellular proteases (which can degrade the recombinant products).

Yeasts other than *S. cerevisiae* (e.g. *Hansenula polymorpha* and *Pichia pastoris*) are becoming more popular as hosts for heterologous protein production [29]. High productivities recently achieved using yeasts such as *H. polymorpha* and *P. pastoris* [30] indicate that these organisms still have excellent potential as recombinant host organisms.

The potential of the filamentous fungus *Trichoderma reesei* for producing heterologous proteins has recently been demonstrated with a number of secreted proteins. Versatile molecular systems have been developed for *Trichoderma*, allowing different strategies for the production of heterologous proteins. Fusion strategies seem to work well, not only in *Trichoderma*, but also in *Aspergillus* [31,32]. Because of its good performance in large-scale production conditions (up to 360 m^2 fermenters), *Trichoderma* is especially suited for production of proteins needed in large amounts [33].

Dictyostellium discoideum (a well known amoeboid organism) is being used to express heterologous proteins that are difficult to study in other systems, and its unique cell biology is being exploited to facilitate a wide range of protein modifications [34].

The expression of recombinant protein in mammalian cell lines is a fundamental technique in modern biology. The main advantages of mammalian cell expression are that the protein product does not have to be refolded, the synthesis can be continuous, and the desired post-translational modifications occur. Recombinant proteins secreted from CHO cells (and yeast) do not require refolding, and CHO cells and transgenic mice were found to produce unique, complex sets of sugars, whereas insect cells did not carry out complex glycosylations. Such differences could have dramatic effects on the circulating half-life of an administered glycoprotein [35]. Previous drawbacks have been that the level of expression is fairly low without time-consuming amplification or that production is not very stable. As a remedy, mammalian cells have been engineered, primarily BHK cells, but also CHO cells to express the herpesvirus VP16 protein. Expression of VP16 has given mammalian cell expression a much needed boost in terms of yield and has resulted in long-term stable expression of a variety of gene products [36]. An improved vector for the stable expression of recombinant protein in mammalian cells have been developed. In this vector, designated pCIN, both the recombinant cDNA and the neomycin phosphotransferase selection marker are transcribed from a single promoter element. The vector predisposes all antibiotic-resistant cells to express recombinant protein at apparently high levels [37].

The use of milk as a vehicle for the expression of a recombinant protein has the advantage of high concentration of soluble recombinant protein which has been post-translationally modified [38]. In certain cases, expression of recombinant proteins in the milk of transgenic animals may solve both the refolding and cost-of-drug issues. One drawback to all transgenic animal expression systems is the number of years it takes to expand from the founder animal to a herd of useful size. Milk is a complex bio-colloid and resembles blood plasma more than it resembles most other proteinaceous media. Caseins constitute over 50% of the protein in various milks, but there is some variation in the proportion of total caseins in milks. In addition, casein micelles cannot be separated from milk by industrial scale centrifugation and require ultracentrifugation. Because of this, the casein micelles usually are precipitated before centrifugation. Serum albumin is a major constituent of milk from healthy mammary glands and thus appears to be actively transported into milk from plasma. The immunoglobulins also are thought to originate in the plasma and then are specifically transported into milk. They occur at very high concentration in the early days of lactation (colostrum). Most plasma proteins are found in milk in minor amounts. The method of purification must address the likelihood that many slightly different forms of the recombinant protein will be present. Milk presents some unique problems for the protein isolation chemist, but the majority of the processing

criteria for purifying recombinant proteins are the same as with any complex biological mixture. The casein micelles and fat globules behave as separate phases; they prevent filtration of the milk and interfere with the usual separation methods. The usual first step is to centrifuge the milk to remove the fat and precipitate the casein micelles with low pH or precipitating agents. Some recombinant proteins may associate to some degree with the micelles which may necessitate solubilizing them with chelating agents. If the majority of the product protein associates with either the fat or micelles, this can be used to advantage. Once the casein micelles have been removed or disrupted, the clarified milk can be processed by the usual separation methods. There also are proteases in milk which can degrade recombinant proteins. The greatest advantage of producing recombinant proteins in milk is the high concentration which can be obtained. The high levels of product protein can alleviate many problems associated with the application of classical purification strategies to transgenic milk proteins [38].

2.1. Advances in protein refolding

E. coli is often used for the over-expression of heterologous genes because many mutant *E. coli* strains and vector systems have been constructed for gene cloning and expression. Expression of recombinant proteins in *E. coli* is also convenient due to its low cost and easy handling. However, over-expression yields polypeptide chains that are often produced as insoluble and biologically inactive, with incorrectly formed intra- and inter-molecular disulphide bonds especially when expressed with strong promoters. The insoluble aggregates are often called inclusion bodies [39]. The proteins must be renatured in the correct folded form before a useful product is obtained. It requires solubilization, denaturation and reduction, with a subsequent renaturation procedure to promote formation of the correct intra-molecular disulphide bonds and a native conformation. Refolding of the solubilized protein into active material can be challenging and expensive, depending upon whether or not the protein of interest has a tendency to aggregate during refolding. The presence of a high number of non-native disulfide bridges usually represents a major problem. In vitro folding of denatured proteins has remained an inefficient, time-consuming, and empirical process that has limited the use of bacterially expressed recombinant proteins.

A common and often successful method of refolding proteins produced in bacteria consists of the treatment of inclusion bodies with a weak denaturant to remove soluble contaminants. The refolding procedure then usually requires solubilizing the protein in denaturing chemicals, such as guanidine hydrochloride and urea, or alkaline pH [40]. The conditions used are highly dependent upon the specific protein being refolded and must largely be determined in an empirical, case-by-case manner.

Inclusion body formation during the recombinant production of proteins has been known for some time but it is only recently that its mechanisms of formation are being looked at carefully. Controlling the aggregation phenomena requires understanding the stabilities of folding intermediates and factors interacting with them. New strategies and protocols have been presented that lead to the active and stable protein conformational state. The techniques include chaperonin-assisted refolding, amino acid substitution, non-ionic detergents, polyoxyethylene and polyethylene glycol-assisted refolding, protein refolding in reverse micelles, and antibody-assisted refolding of proteins [41–45].

A common observation has been that the final yield of renatured protein decreases, sometimes drastically, with increasing concentration of the solubilized protein undergoing renaturation, caused mainly by hydrophobic interactions of the denatured peptide chains. If the rate of addition of denatured protein to the refolding solution is equal to or less than its rate of renaturation, the actual concentration of denatured protein in solution can be maintained at low levels. In this way the re-aggregation during renaturation caused by hydrophobic interactions between partially folded intermediates is reduced, allowing higher concentrations of refolded material to be obtained after renaturation.

A sufficient success in refolding, however, has been achieved to date with individual proteins to encourage any researcher to use a battery of folding modulators to attempt to solve folding problems. It has been observed that aggregation can be greatly suppressed if protein molecules are separated from each other during refolding. Polyoxyethylene and

polyethylene glycol detergents enhance refolding [42]. It seems that the detergents bind specifically to the refolding intermediates and form non-aggregating complexes. They are only weekly attached which permits the refolding intermediates to displace them and to fold to their native protein conformation [20,41–50].

A method to improve refolding yields and to increase the concentration of refolded proteins in a single operation has been developed. The method uses size-exclusion chromatography matrices to perform buffer exchange, aggregate removal, and the folding reaction. The reduced diffusion of proteins in gel-filtration media has been shown to suppress the non-specific interactions of partially folded molecules, thus reducing aggregation [41]. Another example of gel filtration is the use of Superdex recently utilised for the refolding of high concentrations of extracted recombinant proteins (human recombinant protein with tumor suppressant potential (ETS1) and bovine ribonuclease A). These were simply dissolved in buffer and run through a Superdex column. The gel filtration permitted the preparation of a large quantity of protein. The success has been attributed to the conditions of gel filtration, where refolding takes place without the occurrence of re-aggregation. The procedure seems to be generally applicable to refolding problems associated with macromolecular complexes treated in high concentration [51]. Other methods recently used, based on previous studies on denaturation phenomena, for separating protein molecules during refolding, include immobilisation on gel matrices [52], binding to an ion-exchange matrix [53] and the use of reverse micelles [54].

Refolding of the recombinant protein with a fusion partner – a tag (very often His6-tag at its C-terminus), that enables immobilisation to the affinity resin may enhance effective refolding by preventing undesirable folding from the C-terminus [50]. An iterative method for the refolding of recombinant proteins, that exploits the kinetic control of both folding and unfolding of proteins has been developed. This method has allowed refolding over 20 different, mostly disulfide-bridge containing, eukaryotic proteins or protein domains produced as fusion proteins in *E. coli*. The fusion proteins, all containing six histidine residues were expressed in *E. coli* and applied to Ni^{2+}-activated nitrilotriacetic acid coupled agarose columns. The purified fusion proteins were not eluted from the affinity column. While immobilised on the affinity column the fusion proteins were subjected to more than 20 cycles of renaturation and denaturation. Each cycle comprised a renaturation step where the column was exposed to a non-denaturing buffer followed by a pulse of denaturing buffer. A mixture of reduced and oxidised glutathione was included in the renaturing step to allow disulfide-bridge reshuffling and reduced glutathione was added in the denaturing pulse if the recombinant protein contained disulfide-bridges. In the overall process the level of denaturant was successively lowered from cycle to cycle [55].

Optimization of the protein refolding by the use of a micro-renaturation assay in 96-well microplates has been described. Microplate wells were filled with buffers varying in pH and urea and substrate concentration. Denatured and reduced protein was then rapidly diluted and allowed to refold for a variable time at different temperatures. The extent of renaturation was measured by a sandwich enzyme-linked immunosorbent assay (ELISA), based on the use of two monoclonal antibodies. Among about 100 different combinations tested, a maximum refolding yield of 21.5% was obtained [56].

In another interesting and reportedly efficient inclusion body refolding method from recombinant *E. coli*, the protein was refolded when a heat treatment was applied to a protein denaturing solution containing guanidine hydrochloride. The total enzyme activity and the specific activity in response to the 50°C heat treatment compared to normal method (25°C) were enhanced by about 10 and 25%, respectively. Correct protein folding was closely related to the protein concentration in the refolding solution [57].

A major problem related to production of gene products in heterologous hosts is the proteolytic degradation of recombinant proteins. Production by the inclusion body strategy has the expected advantage that the recombinant product is protected from proteolysis. If an intra-cellularly produced recombinant products exists as partly soluble and partly precipitated material, and the soluble material is significantly degraded, it is obviously of interest to increase the amount of precipitated material. In contrast, if no problems with proteolysis exist during

intra-cellular production, it might be desired to obtain the recombinant protein in a soluble form. Increased solubility might be achieved by lowering the growth temperature [58]. Recombinant DNA technology offers several alternative strategies for stabilisation of expressed gene products. These strategies can often give dramatic stabilisation effects and can be combined with strategies involving optimisation of fermentation conditions or down-stream processing schemes. Various genetic approaches to improve the stability of recombinant proteins include (i) choice of host cell strain, (ii) product localisation, (iii) use of gene fusion partners and (iv) product engineering. In addition, the solubility of the gene product can be influenced by factors such as growth temperature, promoter strength, fusion partners, and site-directed changes. Altogether, a battery of approaches can be used to obtain stable gene products. To make things even more complicated, however, truncated fragments of several recombinant proteins were found only in the insoluble fraction of *E. coli*, in inclusion bodies [59].

3. Major separation methodologies

3.1. Sample preparation

Advances in the field of sample preparation have been relatively modest in comparison with the development of sophisticated methodologies and instruments dedicated to the separation and detection of recombinant proteins. However, sample preparation is the most tedious and time-consuming step and the source of much of the imprecision and inaccuracy of the overall analysis.

The principal objectives of sample preparation for further purification and analysis are dissolution of the analytes in a suitable solvent, removal from the solution of as many interfering compounds as possible, and, if necessary, viral inactivation.

The preparation may considerably differ if the desired recombinant protein is in a soluble form or as an aggregate. The first step in the recovery of protein from inclusion bodies involves cell disruption. Cell disruption is usually done using sonication or on a large scale, either with a high-pressure homogenizer or a bead mill. In addition to higher energy consumption and increased process times, increasing the number of passes causes a reduction in the size of the cell debris. To reduce the amount of small cell debris, different pre-treatments have been tried in combination with mechanical disruption to increase the disruption of cells in fewer numbers of passes. Combinations of heat and enzyme pretreatment were followed by high-pressure homogenization for the release of a heat-stable enzyme from *Bacillus cereus* [60]. Enzymatic pretreatment of *Candida utilis* and *Saccharomyces cerevisiae* improved cell disruption relative to that of untreated [61]. One pass through the homogenizer at a low operating pressure allows for shorter process times, lower energy consumption, and less reduction in the size of the cell debris as compared to that of multiple passes at higher pressures. Integration of cell disruption and inclusion body washing could also reduce the number of down-stream processing steps involved in the recovery of the target protein from the inclusion bodies. To take advantage of the integration of cell disruption and the removal of inclusion body contaminants, the effect of combinations of various guanidine HCl and Triton X-100 pre-treatments and high-pressure homogenizer operating pressures on the release of protein from recombinant *E. coli.* was investigated. Pretreatment of recombinant *E. coli* with 1.5 *M* guanidine HCl and 1.5% triton X-100 prior to high-pressure homogenization does not have an adverse effect on the physical characteristics of the cell homogenate. Pre-treatment with this combination of guanidine HCl and Triton X-100 not only allows the use of one pass through the homogenizer at a low operating pressure but also removes the need for an additional inclusion body washing step during downstream processing since the inclusion bodies are released and washed during the disruption step. Soluble recombinant proteins that may be sensitive to guanidine HCl can be released from cells without pretreatment by using one pass through the high-pressure homogenizer at 83 Mpa [62,63].

In the last ten years a great increase in the use of solid-phase extraction (SPE, or its synonyms, liquid–solid extraction (LSE), and sorbent extraction) as a preparation step in the overall process has occurred. Before the mid 1970s the term solid-phase extraction did not appear and did not start to be commonly used until 1985. The principles of SPE or LSE are similar

to that of liquid–liquid extraction, involving a partitioning of compounds between two phases. Nowadays, SPE is a well-established technique due to its advantages over other sample preparation techniques. It has been used for the preparation of numerous different classes of compounds in a variety of matrices. SPE is used extensively in biomedical and pharmaceutical analysis. A review with discussion on new solid phases, chromatographic modes, experimental configurations and off-line and on-line automated devices and on the basic principles and recent developments in the solid phase extraction has been published [64].

It is often necessary to purify the sample prior to any high-performance chromatography. Although the proteins of interest might tolerate chromatographic conditions, the sample matrix may not, resulting in column fouling. In addition, many samples are so complex that the resolution power of a single chromatographic separation is not sufficient [65], and also the load capacity may be insufficient. There are no 'standard conditions', there is an almost endless number of options. Ion-exchange chromatography (IEC) and reversed-phase chromatography (RPC) or affinity chromatography are recommended as a general set-up for a two-dimensional separation. This should allow a mild and efficient separation, starting very close to the crude material [66].

Many pharmaceutical products and raw materials are potentially virus contaminated. For some of these products elementary heat treatments achieve satisfactory viral clearance. Many others, however, cannot withstand elevated temperatures for the time periods normally associated with pasteurisation. A number of methods have been developed for inactivating viruses in labile materials, most of them have, however, significant weaknesses that have compromised their applicability for most products. Filtration is costly and limited by protein content and throughput restrictions. Steam heating is too slow, resulting in both protein and virus destruction. Radiation often destroys an unacceptable fraction of the product and has small batch limitations. UV systems, ineffective against certain types of virus, often rely on the activation of a contaminating photosensitive chemical, yet uniform light penetration of the product is often difficult to achieve. Solvent detergent systems are widely used, but are limited because non-lipid enveloped viruses are unaffected by these processes. Ozone systems are difficult to control and often result in unacceptable product destruction as well as creating chemical contaminants. Microwave viral inactivation, however, seems to have all of the benefits of steam – no contaminants, large scale, low cost and broad spectrum viral inactivation – with minimal product destruction [67].

3.2. Chromatographic purification

Slow and steady improvement marks biopurification methods development [68]. Most of the purification schemes of proteins are based on multi-step sequences of chromatographic methods. These procedures are sometimes troublesome and time-consuming, usually resulting in low final yields. Rational design of purification for recombinant proteins have been suggested including integrated expert systems [69]. Peptides and proteins are molecules with multiple functionalities and flexible structures, their chromatographic behaviour is often far from ideal. Consequently, the separation of these molecules is regarded as one of the most challenging areas of chromatography [70]. Developing a separation scheme can be the most time-consuming part of a research project. The past years have witnessed continued development and application of columns packed with materials that differ in size and porosity. The innovative use of columns packed with either small non-porous particles or extreme macroporous (flow-through) particles has led to dramatic increases in the speed and resolution of many bioseparations. Particles formed from polymeric resins are generally more durable than in the past, and in comparison to silica are offered in a diverse array of matrices and functionalities. This will be most evident in affinity chromatography, where the trend continues to be away from the classical supports, such as agarose, and toward supports that will truly be high-performance in nature.

Biological samples consist of innumerable compounds, many of them present in minute amounts. Good separation systems reduce the amount of impurities, but there will always be some remaining that will co-elute with the desired compound. The critical point is to decide when small peaks should be regarded as significant.

An account of HPLC separation methodologies, specifically reviewing the topic of direct injection techniques and advances in packing materials has been published [71].

Fundamental development in the field of column liquid chromatography (LC) equipment and instrumentation between October 1993 and October 1995 have been covered [72].

One of the more novel separation techniques being evaluated for protein purification employs large diameter particles (100–300 mm) that are suspended in a column as the product passes through them. Such 'expanded bed' chromatography typically involves affinity ligands and is used in early steps to capture either the product or a major contaminant [73].

For downstream processing, a new development is ceramic hydroxyapatite (CHT), a calcium phosphate-based chromatography support that exhibits unique separation properties and provides an alternative to ion-exchange (IEX) and hydrophobic interaction (HIC). Hydroxyapatite was one of the very first materials used for protein separations. In its fragile, crystalline form, the media came to be known as 'concrete chromatography' because columns would collapse under their own weight. Eventually, bioprocess engineers spurned hydroxyapatite in favor of synthetic ion-exchange supports that provide higher flow-rates and higher resolution. Now hydroxyapatite has returned in a mechanically rigid, chemically stable, ceramic form. The new media is available in particle sizes for analytical, preparative and process chromatography.

3.2.1. Affinity chromatography

Affinity techniques belong among the most efficient methods available for the isolation and purification of recombinant proteins. A recombinant protein can either possess a property that can be exploited for affinity purification or a peptide (polypeptide) 'tag' can be added as a fusion partner.

Many conventional affinity protein purification processes have used monoclonal antibodies (or polyclonal antibody) as affinity ligands. However, monoclonal antibodies are normally produced by animal cells in complex media or expressed on the phage. Therefore, the process of obtaining FDA (Food and Drug Administration) approval is lengthy. At present, monoclonal antibodies must be purified extensively prior to use as affinity ligands. In addition, immobilized antibodies are sensitive to operating conditions.

Metal and dye affinity ligands have lower specificity for proteins in comparison with antibody ligands and that is often rather an advantage. Metal ions such as zinc and copper have been found to bind favorably with histidine residues in a protein. Proteins with different total numbers of histidine residues are separable by metal affinity processes. To increase the specificity, an artificial tail consisting of multiple histidine residues can be genetically engineered to the C-terminal of a protein. This genetically engineered protein will have much higher specificity for metal binding than other proteins in the mixture. The tail can be cleaved after the protein has been purified. This extra step of tail cleavage is, however, not the only disadvantage of metal affinity separation. The metal ions are chelated to the affinity matrices and severe leakage can occur during operation.

Proteins containing vicinal dithiols were purified by affinity chromatography using Sepharose 4B linked to aminohexanoyl-4-aminophenylarsineoxide (As-Sepharose). The protein vicinal dithiols form stable dithioarsine derivatives with the arsine oxide moieties of the gel. The procedure allows purification of proteins inhibited by phenylarsine oxide such as phosphotyrosine phosphatases, proteins that are subject to redox regulation, and dithiol proteins that are targets of oxidative stress [74].

Dye ligands can be coupled to affinity matrices firmly via covalent bonds. They may be toxic, however, and their interactions with proteins are non-specific. It is possible to screen one dye ligand that binds favorably to a specific target protein, but the purity of recovered product is generally not high.

It has been demonstrated that for the purification of proteins peptides can be used as ligands after immobilization to an affinity matrix. Peptides may be much better ligands than antibodies because they consist of only a few amino acids, which are not likely to cause an immune response in case of a leakage into the product. Peptide ligands are also much more stable in comparison with antibody ligands. They can be manufactured aseptically in large quantities under GMP (good manufacturing

practices) conditions which can significantly reduce cost. When compared with metal and dye ligands, peptides are much more specific. As opposed to dyes, peptides are generally non-toxic. The interactions between peptides and proteins are generally moderate, and this can result in mild elution conditions for separation. For the choice of the right peptide, peptide libraries provide a rational technique for screening tens of millions of peptides. Peptide libraries can be constructed on bacterial phages or can be obtained from direct chemical synthesis. In the phage peptide libraries, a random gene of a given length is synthesized and inserted into the bacterial phage gene. Once the peptide sequences are identified, they can be chemically synthesized and immobilized to affinity supports. This concept has been demonstrated by Baumbach and Hammond [75]. In their article, several peptides containing the consensus sequence of His–Pro–Gln were immobilized on Affi-Gel (BioRad, Richmond, CA, USA) and streptavidin in an artificial mixture with human plasma was specifically retained.

Good fusion partner proteins must possess properties that can be exploited for easy generic protein purifications [76]. Fusion tags that have been used in protein purification include polyhistidine (binding nickel ions as a ligand), in vivo biotinylated peptide (avidin–streptavidin) FLAG peptide (anti-FLAG antibody), strep-tag (streptavidin), polyaspartic acid (anionic resins), polyarginine (cationic resins), polyphenylalanine (HIC resins), polycysteine (thiols), calmodulin-binding peptides (calmodulin). As protein fusion partners were used for affinity purification (among others): Protein A, glutathione-S-transferase, maltose-binding protein, galactose-binding protein, β-galactosidase, chloramphenicol acetyl transferase, lac repressor, and cyclomaltodextrin glucanotransferase (rewieved in [76]).

The biotin–streptavidin interaction is used ubiquitously in modern biology and has been recently adapted for use by recombinant fusion systems in two innovative ways. The first uses a ten amino acid carboxy-terminal peptide extension ('Strep-tag') selected from a random peptide library for its ability to bind to streptavidin [77]. This peptide mimics the structure of biotin and binds to streptavidin with good affinity via protein–protein interactions. In a different approach, a 13-residue peptide was selected from a random *lac*I library for its ability to be biotinylated in vivo [78]. When this tag was placed at either the amino or carboxyl terminus, the fusions were actually biotinylated and available for high-affinity binding to streptavidin.

Methods have been developed for expression and purification of eukaryotic proteins by creating fusions with the carbohydrate-recognition domain of the galactose-specific rat hepatic lectin. The feasibility of using this approach as an aid to protein purification has been demonstrated using human placental alkaline phosphatase [79].

A modified pGEX expression system producing a fusion product with two tags that eliminates degradation products and thrombin from the recombinant protein has been reported. The dual-tagged fusion protein was purified to homogeneity with two consecutive affinity chromatography steps [80].

3.3. Other methods

Repeated cycles of freezing and thawing may be sufficient to separate highly expressed recombinant proteins away from the bacterial cytoplasm of *E. coli* [81]. Precipitation processes are receiving renewed attention as means of reducing downstream bioprocessing and purification costs. Though generally non-specific, precipitation as an initial purification step can minimize the number of subsequent processing steps before final purification by, for example, chromatography. In addition to the target molecule, impurities can also be removed and the supernatant solution containing the target processed further. Numerous methods are available for precipitating proteins, including pH and temperature changes, and the use of halide and other inexpensive salts, organic solvents, metal ions, polyelectrolytes, affinity ligands and dyes, and thermosensitive and reversibly soluble polymers. With high concentrations of salt in supersaturated solutions, precipitation is instantaneous and the result is an amorphous precipitate, that usually contains a combination of many different proteins. Protein precipitation is hoped to reduce downstream processing costs.

Crystallisation operates much closer to the solubility limit than precipitation, uses much smaller amounts of salt, and is a much slower process which may provide a pure form of the crystalline protein.

Crystallisation as a tool for bioseparation has so far primarily been in a basic research setting. Crystallisation provides, however, an efficient means of purifying commercial quantities of enzymes from recombinant fermentation. The wide variety of reported effectors for crystallisation include various salts, of which ammonium sulfate is widely used; non-ionic polymers such as dextran and polyethylene glycol; weakly polar solvents such as ethanol; metal ions such as copper; and ionic polyelectrolytes such as carboxymethycellulose and polyethyleneimines.

To add further specificity to precipitation processes, several variations on affinity precipitation schemes have been developed. A variation of affinity precipitation involves coupling thermoreactive polymers with affinity ligands and precipitating the affinity complex by small changes in the temperature of the solution [82].

Among the new techniques for bioseparations are solute focusing techniques including recycle isoelectric focusing, counteracting chromatographic electrophoresis, and countercurrent gradient chromatography. In these systems, multicomponent separations can be performed and the solute peaks produced are both highly resolved and concentrated [83].

Reversed micellar systems can be used as a first step of purification procedures because of their simplicity and ease of scaling-up. Therefore, similar recovery yields and purification factors may be achieved with a fewer number of steps when combining this novel methodology with chromatographic processes. The recovery of proteins using reversed micelles in a liquid–liquid extraction process has received increasing attention since proteins were shown to be solubilized in organic solvents with surfactants, maintaining their functional properties, and to be transferred between an aqueous solution and a reversed micellar organic phase. As a first step, after cell lysis, if the recombinant protein is not secreted, the cellular material is separated from the liquid medium, using a centrifugation or a filtration step, followed by chromatographic steps. It has been demonstrated that it is possible to use liquid–liquid extraction technology to integrate the first two steps into one to obtain separation of the product from the cellular material with simultaneous concentration of the protein. Two different systems were proposed. The first one uses two immiscible aqueous phases of simple electrolytes and water soluble polymers (like polyethylene) or of incompatible water soluble polymers (e.g., dextran–polyethylene). The second one uses a water in oil microemulsion with an excess of the aqueous phase to achieve the desired separation/concentration of the product. In both cases, it has been claimed that the ease of scale up and the high partition coefficients obtained allow its application in the downstream processing of proteins produced by fermentation. Significant enhancements in the selectivity of the protein extraction process with reversed micellar systems can be achieved by introducing affinity ligands in the organic phase. The liquid–liquid extraction of proteins with reversed micelles has been studied with more than 30 proteins. In most cases it is accepted that the driving force for this process is an electrostatic interaction between the charged polar heads of the surfactant and opposite charged groups in the protein. However, there are studies indicating that other forces may also play an important role in the solubilization mechanism, such as hydrophobic interactions and solubilization by an ion-pairing mechanism. The selective solubilization of proteins in reversed micelles aims the development and application of this technique to extract the protein of interest from the production medium without loss of its activity. Significant applications of reversed micellar systems as a bioseparation technique for recovery and purification of recombinant proteins are still at the experimental level [84].

Micellar liquid chromatography (MLC) is a technique where a micellar agent is added to a mobile phase that contains a buffer and a small amount of organic modifier. Several advantages are apparent with MLC when compared to reversed-phase liquid chromatography. MLC uses a much smaller amount of organic modifier and is therefore less toxic, MLC does not denature peptides and proteins as does RPLC, and gradient MLC is done without the need for long column re-equilibration. It was found that the use of a micellar mobile phase for the separation of short to medium chain peptides is possible [85].

It has been shown that a single-step process using an aqueous two-phase partitioning system can replace a multi-step process that includes (a) osmotic shock, (b) acid precipitation and chromatography. Aqueous biphasic systems show considerable versatility, allowing protein separation to be based on

molecular weight, conformation, hydrophobicity, and/or electrostatic properties [46]. Such systems are possible to scale up and are also suited for automated continuous operations.

Purification of membrane proteins is generally more difficult than that of soluble proteins, because of the presence of detergents which may interfere with any chromatographic step, including immunoaffinity columns. It has been shown that continuous elution electrophoresis could be a useful tool for the purification of membrane proteins. Compared to the traditional approach, which included affinity chromatography, the continuous elution electrophoresis showed the same purification and had a higher yield [86].

The high purity requirements for both natural and recombinant DNA-based therapeutic proteins have also stimulated developments in advanced electrophoretic techniques. For example, sodium dodecyl sulfate polyacrylamide gel electrophoresis and isoelectric focusing, as well as Western blots, have become indispensable tools for identifying proteins. Capillary electrophoresis has made great advances in recent years and is rapidly gaining acceptance as a powerful technique for protein analysis [87].

The use of conventional preparative chromatography for large-scale preparation of proteins has been limited by decreased resolution upon increase of flow. This serious drawback is hoped to be diminished by the use of membrane adsorbers for rapid and scaleable protein separation. It claims to provide preparative separations with high capacity, low pressure operation and short cycle times. Applications include concentration and purification of proteins in adsorption–desorption mode, selective removal of contaminants such as DNA endotoxin from targeted proteins, screening for optimal purification conditions for uncharacterised proteins, and protein purification and characterization in conjunction with chromatography [88].

4. Detection and analysis

Detection, purity evaluation, and identification of recombinant proteins during purification is a very important aspect of protein engineering and especially recombinant DNA technology. It has stimulated developments in advanced chromatographic and electrophoretic techniques but new approaches are also reported.

There has been a rapid development of biospecific detection in column liquid chromatography and also in related flow systems such as flow injection, sequential injection analysis (SIA) and capillary zone electrophoresis. The term biospecific detection can include any type of biochemical recognition reaction which can be transformed by an appropriate physical transducer to an electrical signal of some sort. These biochemical components can be of enzyme-, whole cell-, whole tissue-, nucleic acid-, receptor- or antibody origin. The main ones are enzyme and antibody–antigen based systems that have been used to measure a large number of analytes in relation to these different flow techniques [89]. Immunosensors are important analytical tools for monitoring antibody–antigen reactions in real time. Depending on the transducer technology employed, immunosensors can be divided into three principal classes – optical, piezoelectrical and electrochemical [90].

Ion-exchange chromatography has been used to fractionate glycoprotein glycoforms into classes that differ in their degree of sialylation. Glycoproteins typically exist as a family of glycoforms in which the protein backbone is the same but the attached oligosaccharides differ in structure and degree of sialiation. Since differences in glycosylation can influence therapeutic efficacy, it is important to monitor microheterogeniety during product development. On an anion-exchange column the greater the degree of sialylation, the longer the glycoforms are retained on the column [91].

Experimentation with cultured cells often requires analysis of cellular protein extracts by gel electrophoresis and immunoblotting. Analytical applications of liquid phase traditional methods for extracting cellular proteins by homogenisation or detergent solubilization usually produce protein samples that are viscous (due to the presence of DNA) and prone to degradation due to presence of endogenous protease activity. A method has been reported that involves solubilization of cells with sodium dodecyl sulfate, precipitation of proteins with trichloroacetic acid with special physical exclusion of DNA aggregate and reconstruction of precipitated proteins with Tris base. Protein samples prepared by this method

contain little DNA, marking them ideal for long-term storage [92].

For the analysis of individual steps during the purification an application of the methodology of surface plasmon resistance (SPR) is very promising. It can specifically determine a recombinant protein in a complex mixture and in a real time. An SPR-based binding assay is generally rapid compared with an activity assay (for example, the measurement of cell activation), and unlike DNA-homology-based cloning approaches it identifies proteins involved in specific interactions, not merely proteins related by sequence [93]. Surface plasmon resonance sensor for use as an liquid chromatography detector has been described [94]. Non-specific adsorption onto the biorecognition surface remains a major problem for direct systems.

There is often a need to detect a recombinant protein without detailed knowledge about its individual biochemical properties. The construction of affinity tags may solve this problem. The use of a novel kind of random peptide library for the stepwise engineering of a C-terminal fusion peptide which confers binding activity towards streptavidin has been described. Because of its widespread use as part of a variety of conjugates and other affinity reagents, streptavidin constitutes the binding partner of choice for detection as well as for purification. The optimized version of the affinity peptide termed 'Strep-tag' allowed the detection of the Fv fragment both on Western blots and in ELISAs by a streptavidin–alkaline phosphatase conjugate [95].

A trend that has become particularly apparent in recent years is an increase number of papers in which two or more complementary spectroscopic techniques are used in combination to characterize the conformational or structural features of the peptide or protein under investigation.

Mass spectrometry (MS) is at present one of the most important tools for characterization of recombinant proteins and for comparing the recombinant and the natural protein. It is anticipated that the rapid development of these tools will extend the field of application to the earliest stages of protein studies to provide sufficient information to select and clone the corresponding gene. Two techniques in particular, electrospray ionization (ESI) and matrix-assisted laser desorption ionization (MALDI) MS, have provided simple and rapid strategies to establish the chemical integrity of recombinant protein with respect to amino acid sequence, disulphide bonds and post-translational processing and modification. MS is now regarded as the method of choice for the characterization of post-translational modifications because the M_r can be measured with sufficient resolution and accuracy to reveal even very small modifications. Detection and characterization of post-translational modifications at the pre-cloning stage have the advantage that appropriate expression systems can be selected. Different strategies for recombinant protein characterization use MS [96].

The advantages of chemiluminiscence (CL, the emission of electromagnetic radiation, ultra-violet, visible or infra-red, produced in a chemical reaction from the decay of an excited species to the electronic ground state) for quantitative analytical applications include low detection limits, wide linear dynamic ranges and speed of response. Flow injection is ideally suited to monitoring liquid phase CL reactions because it provides rapid and reproducible mixing of sample and reagent(s) in close proximity to the detector in a flow-through manner. It also permits in-line chemical and physical treatment of the sample to produce species amenable to CL detection and to remove matrix interferences. In situations where high efficiency separations are required prior to detection, liquid chromatography presents a suitable alternative although the instrumentation is more complex than flow injection and the reaction conditions must be compatible with mobile phase composition [97].

5. Large-scale separation

There is a strong, still unmet need for better large-scale separation and purification methods for the proteins that are being made by the genetic engineering companies. There already exist several successful applications of recombinant protein renaturation at manufacturing scale, involving proteins such as tissue-type plasminogen activator, somatotropin, human and bovine growth hormones, interferon, urokinase, etc. Refolding is, however, both a materials- and labor-intensive manufacturing step. Protein refolding steps contribute significantly to the

cost of downstream processing. The need to develop refolding strategies that are specific for each protein further increases the cost. Solubilization of inclusion bodies in weakly denaturing solutions was shown not to be practical. High concentrations of urea, guanidine HCl and sodium dodecylsulfate have been mainly used for solubilization of inclusion bodies. Both air oxidation, renaturation using the glutathione system, and renaturation starting from mixed disulfides of proteins–S–sulfonate and protein–S–glutathione were employed to generate active proteins and resulted in similar yields. These results show that renaturation of proteins at an industrial scale employs similar methods and strategies as those developed for low-scale preparations [98]. The costs were a major driving force for the development of mammalian cell culture manufacturing technology. However, even with the advent of this upstream technology, expression levels in mammalian cells still do not approach those seen in bacteria. A number of changes and advances in protein folding technology are therefore being worked out [99].

Chromatography, the primary separation technology, has improved in the last several years to accommodate large-scale processes. Bioprocess companies are developing innovative techniques to address the biotech industry's concern with the cost and throughput of chromatographic purifications. Innovations are aimed at an increase of the throughput of chromatographic purifications. These approaches include new media and column technologies, on-line monitoring systems, an economic model and contract services. Small-bead technology was developed for analytical applications and presents significant drawbacks in the realm of large-scale protein manufacturing. The theory has been that to get high productivity from chromatographic media you need to maximise surface area within a given matrix by using smaller and smaller beads. The smaller the beads, the greater the surface area. However, small beads also compact more readily, creating greater back pressure and necessitating greater external pressures to move the fluid stream through the column. It seriously limits the applicable flow-rates. In addition, running a column under high pressure requires hard, sturdy beads, powerful pumps and thick-walled stainless steel columns, all of which affect operating costs and performance, especially in production.

Large-bead matrix hydrogel matrices composed of 1–7% lignocellulose and/or cellulose and 93–99% water have been developed by LigoChem. The hydrogels range in size from 0.2–3.0 mm diameter (50–500 fold larger than standard smallbead chromatographic media). They are spherical and resemble translucent pearls. LigoChem's IOLA technology (inside-out ligand attachment) ligand-binding technology causes ligands to attach preferentially to the internal surfaces of porous membranes or the cellulose hydrogel. Selective protein adsorption is determined by size exclusion and by specific association with the covalently bound ligand within the beads. Larger, unwanted molecules, including virus particles, remain outside the beads and pass through the column with the process stream. Selective desorption then allows for recovery of the purified protein. IOLA is a two-step process: first, saturation of the hydrogel with the ligand and suppression of reaction conditions; and then changing of the pH and other conditions to stimulate covalent binding of the ligand. The combination of large beads and IOLA technology results in a ligand-binding efficiency of about 60–70%. LigoChem has filed world-wide patent applications on IOLA. Large-bead bioseparation technology and a ligand attachment process was discovered at Virginia Polytechnic Institute.

Several innovative methods have been developed and are available in recombinant protein purification. Some of them are profiled here: perfusion chromatography; aqueous two-phase extraction, liquid–liquid extraction or microemulsion, displacement chromatography, UpFront chromatography and hyperdiffusion chromatography. Perfusion chromatography is being widely used in the biotechnology industry. Perfusion chromatography, launched in 1990 by PerSeptive Biosystems, allows liquid to actually flow directly across, as well as around, particles. Perfusion chromatography combines a highly reactive surface per bed volume with extremely high elution flow-rates. It may be used advantageously for very rapid purification of labile proteins or for isolation under denaturing conditions. As an example, perfusion chromatography opens up new possibilities and strategies for membrane protein purification. The isolation of hydrophobic membrane proteins or protein complexes require solubilization by detergents. The very short time of purification would increase

the possibilities of obtaining membrane proteins in a more native and functional state including the maintenance of ligands. Separation can be completed within minutes, there is a possibility of chromatographic screening in order to find optimal subfractionation conditions. An overview of this chromatographic method has been published [100].

Aqueous two-phase extraction is a system whose 'medium behaves as water' [101]. The process requires that two polymers are mixed together; they then become two phases. The upper phase has a water maturity which is good for extracting protein. A major advantage of this system is that it is possible to discard the polymers or salt that are part of the water base because it is non-toxic. The implications for a quicker validation by the Federal Drug Administration due to such non-toxicity is evident. With good selectivity, the process can be run in 1–2 h. All materials in the process are relatively inexpensive compared to other chromatographic processes.

In the liquid–liquid extraction (reverse micelle systems) process, instead of using a simple organic solvent, microemulsion can be used [102]. Microemulsion provides properties similar to molecular clusters with respect to shape and characteristics; it can selectively take up proteins. The system discriminates based on size, charge and distribution of charge of the protein molecules. There are two steps to this process. That is why it is called, redundantly, liquid–liquid. In the first step, proteins must be selectively solubilized into the microemulsion. In the second step, a more difficult phase to accomplish, the enzyme that has been solubilized in the microemulsion must be released. By adding small amounts of isopropyl alcohol to the microemulsion, enzyme release is facilitated without affecting microemulsion structure. By adding 10–15% isopropyl alcohol to the aqueous phase full release of pepsin and 70% recovery of chymosin was demonstrated.

In conventional elution chromatography purifications is achieved through selective desorption of adsorbed molecules by changing equilibrium conditions, such as pH, salt concentration and organic content. These changes significantly reduce binding affinity and result in the sequential elution of components from the column. By contrast, displacement chromatography, a preparative technique new to the biotechnology industry, essentially leaves the binding affinity of the adsorbed molecule unchanged. Instead, the displacer processes a higher affinity for the stationary phase than any of the other feed components, thus competing effectively for the binding sites and producing a wide displacement zone rather than a narrow peak. This advantage makes displacement chromatography an extremely attractive preparative technique for the isolation of biomolecules from the dilute solutions often resulting from biotechnology production. It is hoped that displacement chromatography will purify biotherapeutic compounds where conventional elution chromatography fails to provide efficient and cost-effective separations [102].

With the UpFront chromatography (UFC) technology for protein purification, steps of centrifugation, filtration and precipitation are circumvented; instead, the unclarified feed-stock is directly subjected to the chromatography. A new type of chromatographic matrix (patent pending) is the central feature in the UFC technology. Industrial application of protein purification in either fluidized beds or batch adsorption mode from crude feedstock requires a chromatographic matrix that is capable of fast and efficient separations. The optimal matrix must have a sedimentation velocity in water that is higher than traditional chromatographic matrices. The UFC matrices are based on agarose but have an increased density as the result of incorporation of dense inert particles within the polymeric agarose solid phase. This increased density ensures a rapid separation of the matrix from the surrounding liquid phase. The manufacture of the UFC matrices is carefully controlled to generate matrix particles with a close tolerance on density and size distribution, which results in optimal behaviour in a fluidized bed. After the application of the feedstock the remaining particulates and unbound protein are removed by washing in the fluidized state with a suitable buffer. For elution, the flow is reversed and the matrix particles are packed against a coarse filter in the bottom of the column before the application of the elution buffer, giving a more concentrated eluate than if this process were carried out in the fluidized state [103].

HyperDiffusion chromatography is differentiated from other technologies on several levels. HyperD medium is composed of a porous, rigid support filled with a hydrogel. The beads can be described as a

'gel-in-a-shell.' Because of its rigidity, the media can be operated at high linear velocities, providing beneficial for both high speed-methods development and gram-quantity purification [104].

The capture of product from unclarified feedstocks represents one of the problems that are open to innovative technologies [51]. SepraSorb Biospecific Cartridges are such a product based on monolithic cellulose, cross-linked and surface-modified with ion-exchange groups. Cartridges accept unclarified feeds from a variety of sources common to the biotech industry and specifically bind target proteins with high capacity, good kinetics and excellent physical and chemical stability [68]. SepraSorb, which Sepragen acquired in 1994 from BPS Separations, is now in field trials. A European patent was awarded late in 1995 and a US patent is pending. The major benefit of SepraSorb is the potential to eliminate several clean-up steps early in a bioseparation, thereby saving time and improving yield, and its ability to lower manufacturing costs. SepraSorb works because it is not a bead but rather a foam-like material with large channels that allow particles to pass through and has much smaller pores with binding sites for molecules. Thus the cellular debris passes through while proteins are retained, all without clogging [59].

6. Validation

One of the important aspects in any purification procedure is the long-term consistency and validation of its operation and materials used. Purification of recombinant proteins to achieve homogeneity, purity, consistency and potency as required for therapeutic proteins and in vivo diagnostics should be performed under stringent and validated conditions.

The term validation is relative in the sense that it implies an activity of demonstrating that the process or procedure under examination accomplishes what is claimed or intended. Validation of analytical methodologies is an important aspect of their development/utilisation and is widely required in support of product registration applications. Definitions, procedures and acceptance criteria which appear in the pharmaceutical literature have been summarised for the more commonly encountered validation parameters. Parameters examined include accuracy, precision, specificity, linearity and sensitivity limits [105–107]. The reader is strongly encouraged to examine applicable primary references in greater detail than can be presented here.

As liquid chromatography in one of the major technologies used for the purification of recombinant proteins, it has to be carried out according to special regulatory guidelines [108].

7. Perspectives and conclusions

Technological developments have brought an explosion of information on the mechanisms that control mRNA production, protein synthesis and folding, and secretion. As a consequence, the way is opening for further developments and applications of expression technologies. Thus, in the future, it seems to be possible to specifically design the host cell to optimize protein production and purification.

Cost-effective strategies for bioprocessing with integrated process design will be developed. Innovative techniques will address the concern with the throughput of the purification. These approaches will include automated protein purification systems, new media and column technologies, on-line monitoring systems, and economic model and contract services. HPLC will be increasingly utilised in medium and large columns as a preparative procedure.

Polymeric membrane filters modified with ion-exchange, affinity or other active groups typical in purification offer the potential for significant improvements in the convenience and speed of bioseparations as compared with traditional resins. One of the most rapidly growing applications for membrane adsorbers is the removal of contaminants during processing.

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