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Review

In vitro protein refolding by chromatographic procedures

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Abstract

In vitro protein refolding is still a bottleneck in both structural biology and in the development of new biopharmaceuticals, especially for commercially important polypeptides that are overexpressed in *Escherichia coli*. This review focuses on protein refolding methods based on column procedures because recent advances in chromatographic refolding have shown promising results.

Keywords: In vitro protein refolding; Inclusion bodies; Chromatographic techniques; Gradient techniques

Expression of valuable proteins in bacterial systems, especially in *Escherichia coli*, plays a major role for the efficient production of genetically engineered proteins when their biological function does not depend on post-translational modifications such as glycosylations. However, the high expression rates of these proteins in *E. coli* frequently lead to the accumulation of insoluble polypeptide aggregates, often termed "inclusion bodies." Expression as inclusion bodies has certain advantages [1,2]:

1. The expression levels are often very high, up to 30% of the total cell protein or around 8.5 g protein per liter *E. coli* culture has been reported [3] as a consequence of transient overexpression at high-culture densities.
2. The proteins are largely protected from proteolytic degradation by host cell enzymes.
3. During host cell disruption and initial purification one does not have to take the risk of denaturation of the target proteins into consideration since the aggregated inclusion body proteins have no biological activity.
4. The inclusion body proteins can easily be separated from the soluble proteins of the host cells by centrifugation, filtration or size-exclusion chromatography. Thus, reducing the number of separation steps required and increasing the yield of purified product.
5. If the expression product is toxic to the host, the formation of inactive inclusion bodies might increase the viability of the cells and the yield of the target protein.
6. The expression of the target protein as inclusion bodies can be directly observed by phase contrast microscopy avoiding the need for initial identification by electrophoresis after cell disruption.

The challenge is to convert the inactive and insoluble inclusion body protein aggregates into soluble, correctly folded biologically active products [4].

After the inclusion bodies have been solubilized by high concentrations of denaturing agents, refolding is then accomplished by the controlled removal of excess denaturant. In most cases, this is allowed to occur in the presence of a suitable redox system and of other folding promotion agents according to one of the three principally different models [5]:

1. *Dilution*. Dilution of the solubilized protein directly into the renaturation buffer is the most commonly used method in small-scale refolding studies because of its simplicity. However, the protein concentration has to be carefully controlled to prevent aggregation [6]. Also, dilution is time-consuming and buffer-consuming, thus, not optimal for large-scale production as extensive concentration is required after renaturation.

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Recently, a method called "pulse renaturation" was introduced that gave about 10% higher yield compared with batch dilution [7].

2. *Solvent exchange by dialysis, diafiltration or size-exclusion chromatography*. Diafiltration [8] and dialysis [9] using ultra-filtration membranes have been used to reduce high denaturant concentrations. However, fouling can impair refolding yields and clog the membranes, reducing their lifetime. Some unfolded polypeptides can escape by permeating the membrane. This is why refolding by size-exclusion chromatography has gained increasing interest in recent years.
3. *Reversible adsorption of the denatured proteins onto a solid support*. Proteins bound to solid supports, such as IEC or IMAC adsorbents, are spatially constrained during the refolding process, thus, preventing them from diffusing toward each other and aggregating when they are in a partially refolded, sticky state. Fusion partners, such as His-tags [10] or the cellulose-binding domain [11] retains their binding capabilities also in the presence of denaturing agents. Proteins in general can be adsorbed to ion exchangers at low ionic strength and suitable pH in the presence of high concentrations of urea.

Recent literature data have provided information aimed at enhancing the refolding yield of inclusion body proteins by reducing the causes of aggregation and misfolded configurations, respectively. For example, certain low molecular weight additives are known to inhibit the intermolecular interactions that cause aggregation [12]. Commonly used additives are L-arginine (0.4–1 M), low concentration of denaturants such as urea (1–2 M) and Gu–HCl (0.5–1.5 M), and detergents (CHAPS, SDS, and Triton X-100). High hydrostatic pressures (1–2 kbar) in combination with low concentrations of denaturants have been used for the simultaneous solubilization and refolding of inclusion body proteins [13–15].

Refolding using a chromatographic process is attractive because it is easily automated using commercially available preparative chromatography systems and can often be combined with simultaneous partial purification. There are three principally different approaches to chromatographic refolding [16]:

1. Solvent-exchange size-exclusion chromatography (SEC).
2. Reversible adsorption of the denatured protein onto a matrix and subsequent denaturant removal to promote refolding.
3. Immobilization of a folding catalyst onto a chromatographic support causing the column to behave like a catalytic folding reactor.

This review will focus on recently developed chromatographic refolding processes primarily based on the use of decreasing concentration gradients of the denaturing agents.

Size-exclusion chromatography techniques

One of the major causes of low refolding yields is aggregate formation due to too high protein concentrations during the refolding process. SEC restricts the available pore volume for various protein forms in the gel matrix, thus, facilitating the separation of correctly folded and aggregated species. Although only marginally related to refolding of proteins, it is appropriate to mention that in 1981 Amons and Schrier [17] used SEC to remove SDS from denatured protein after dissociation using propionic acid. The first report on protein refolding using SEC is probably that by Werner et al. [18] in 1994. A Superdex 75 HR 10/30 column was equilibrated with the following refolding buffer: 20 mM HEPES, pH 6.8, containing 150 mM NaCl, 3.3 mM Na_2EDTA, and 0.1% Tween 20. The rETS-1 isoform proteins (1–10 mg) were dissolved in 1–2 ml of 50 mM Tris–HCl, pH 8.5, containing 50 mM DTT, 200–500 mM NaCl, and 6–8 M Gu–HCl and directly loaded to the column and eluted, resulting in $71 \pm 15\%$ active protein. Refolding of hen egg-white lysozyme and bovine carbonic anhydrase was achieved by Batas et al. [19] using a Sephacryl S-100 column loaded at a very high initial protein concentration (up to 80 mg/ml). The average recovery of lysozyme was 63% with an average-specific activity of 104%, while 56% mass recovery and 81% specific activity recovery achieved for carbonic anhydrase. The refolding of secretory leukocyte protease inhibitor has been achieved by Hamaker et al. [20] using a rolled stationary phase of DEAE-cellulose in a chromatography column using a similar principle to the SEC-based method, resulting in 46% activity yield and 96% mass recovery. Müller et al. [21] used SEC on Superdex 75 prep grade to refold heterodimeric platelet-derived growth factor (PDGF) from *E. coli* inclusion bodies. The denatured protein was first applied to the column under denaturing conditions and then the buffer was changed to renaturation conditions to refold the monomer. Finally, prolonged incubation under renaturation conditions promoted the dimerization of the refolded monomer and gave an overall 75% yield of active PDGF-AB.

A successful SEC refolding process depends on two key factors as discussed by Batas et al. [22]. The first is the loading of the protein to the column in the presence of a denaturant solution. The second factor is the change in protein size that occurs as it renatures during elution with the refolding buffer. In another paper, Batas et al. [23] made a quantitative study of the changes in Stokes radius, hydrodynamic volume, and partition coefficient that occur when lysozyme is refolded from urea in a SEC column. In 8 M urea, partially folded and unfolded lysozymes were resolved using Superdex 75 HR. As the urea concentration was reduced, the amount of unfolded species gradually decreased until at 4 M

urea only partially folded lysozyme species remained, which continued to fold on further reduction of the urea concentration. Fahey et al. [24,25] examined the effect of gel type on renaturation yields. They demonstrated that as the fractionation range of the gel matrix increased from Sephacryl S-100 to S-400, aggregation decreased but the resolution between protein and denaturant decreased as well. Optimum renaturation yields, amounting to higher than 60% for a urokinase plasminogen activator fragment (M_r 45,000), were obtained using a Sephacryl S-300 column.

The sample application conditions have been shown to affect the SEC refolding yield, as a partial structural collapse might occur at the sample front due to low solubility or to intermolecular interaction of the folding intermediates. As a consequence, precipitation on the gel bed surface or unspecific binding to the gel particles might occur resulting in an uneven flow profile or even total column clogging [18].

To increase the folding yield of concentrated reduced lysozyme, gentle removal of urea from denatured protein by means of dialysis was investigated by Maeda et al. [26]. The urea concentration in the dialyzing vessel was gradually reduced by pumping in refolding buffer without urea. A concentration as high as 5 mg/ml of reduced lysozyme could be renatured in 80% yield, while the folding yield was <5% at a concentration of 1 mg/ml using a conventional rapid dilution method as reported by Goldberg et al. [27].

Gu et al. [28] introduced a new SEC refolding concept based on a decreasing urea gradient, thus, providing a gentle and easily controllable environment for protein renaturation. In this process, a quick change in urea concentration is avoided. The procedure is illustrated in Fig. 1, while Fig. 2 shows the elution curve of lysozyme by urea gradient SEC.

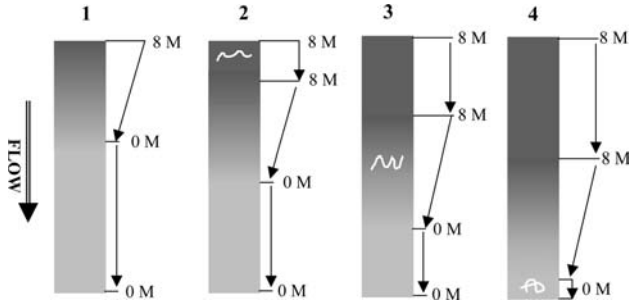

Fig. 1. Flow scheme of gradient size-exclusion chromatography for refolding. The graph marked 1 shows a column equilibrated in refolding buffer. In the upper part of the column, there is a urea gradient area. In graph 2 is shown the loading of the sample dissolved in 8 M urea. When the sample is eluted, the gradient moves downwards as well. However, the protein moves faster than the urea gradient, as shown in graph 3. At the end of the column, the protein has passed through the whole urea gradient, refolded, and left the column. The key design parameters for this refolding procedure are the shape of the denaturant concentration gradient and the flow-rate, respectively, both affecting the kinetics of the refolding process.

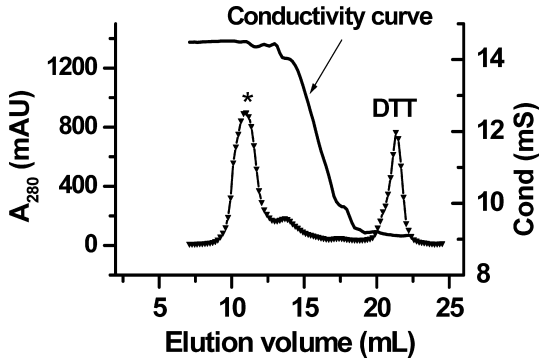

Fig. 2. Urea gradient size-exclusion chromatography refolding of lysozyme. Denatured and reduced lysozyme was loaded into a Superdex 75 HR (10/30) column, supplied with a urea gradient at the top. The curve describing the decrease in conductivity reflects the increase of urea concentration. From Gu et al. [27].

In another study by Gu et al. [29], a recombinant scFv fusion protein expressed as inclusion bodies in *E. coli*, was refolded on a HiLoad 16/60 Superdex 30 prep-grade column in which a dual-gradient of decreasing denaturant concentration, combined with an increasing pH-gradient, was introduced. A 25% activity yield was reported. In comparison, yields of 17.3% with only an urea gradient and 14.5% without any gradient demonstrated the advantage of a dual-gradient for this protein. The principle of this method is that before sample application the column is equilibrated with the refolding buffer, followed by the introduction of a descending gradient of denaturant (e.g., from 6 M Gu–HCl or 8 M urea down to a predetermined concentration in the refolding buffer), sometimes combined with an increasing pH-gradient. The gradient is allowed to occupy the upper 60% of the column. The sample in the highest denaturant concentration is then added followed by a small volume of the same denaturant concentration to avoid uncontrolled dilution of the protein in the rear part of the sample zone. During the elution the proteins will be restricted to the void volume only, meaning that they will pass through regions with gradually decreasing denaturant concentration reaching the final refolding buffer concentration just before leaving the column. By controlling the gradient shape and flow-rate, the refolding process can be carefully kinetically controlled.

A linear gradient is probably adequate in most applications. However, some proteins might be unstable at a certain denaturant concentration. So, the protein should pass that denaturant concentration quickly. In other cases, the protein might need more time to pass through an intermediate conformation step at a certain denaturant concentration or between two different denaturant concentrations. This is why different gradient profiles should be tested. Fig. 3 shows two extreme alternatives to the linear gradient type.

The denaturant gradient SEC process seems to meet the challenge of refolding at high protein concentration

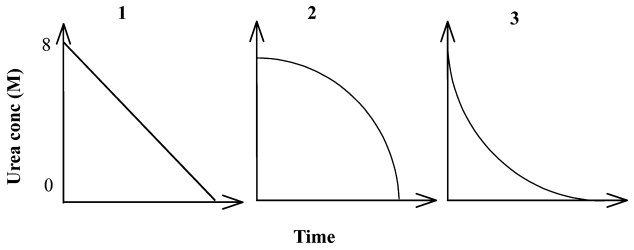

Fig. 3. Examples of urea concentration gradient profiles.

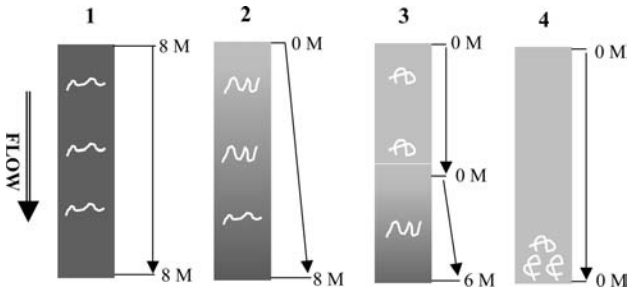

Fig. 4. Flow schedule of the three-buffer system for refolding by ion-exchange chromatography. In addition to ion exchange chromatography, also immobilized metal ion affinity chromatography, hydrophobic interaction chromatography, and affinity chromatography techniques are applicable to this refolding procedure. In the graph marked 1, the denatured protein has been adsorbed in the chromatographic medium, pre-equilibrated with denaturation buffer containing 8 M urea. In graph 2, the protein refolding is induced by a decrease in urea concentration caused by the gradual introduction of refolding buffer lacking urea. In graph 3, the still adsorbed protein continues to refold as the gradient of decreasing urea concentration moves down the column. In the upper part of the column the protein molecules are already refolded. In graph 4, the refolded proteins are eluted from the column as a consequence of the addition of a high ionic strength buffer.

with high recovery. However, some proteins could not refold or have low solubility at some point of denaturant concentration. For example, chymotrypsinogen A has low solubility at Gu–HCl concentrations above 3 M or below 0.5 M [30]. Thus, dilution refolding experiments should be carried out to get preliminary information about appropriate refolding conditions that would influence the target protein refolding before being applied to a SEC refolding process.

Adsorption refolding

A very efficient strategy to prevent aggregation is to minimize the risk of intermolecular interactions by adsorbing the denatured protein molecules to a solid support, thus, effectively separating the individual protein molecules from each other during refolding.

The first studies on the refolding of denatured proteins after adsorption to a solid matrix surface were carried out many years ago. Thus, Epstein et al. already in 1962 adsorbed trypsin and ribonuclease to carboxymethyl cellulose for the purpose of studying the reversible reduction of their disulfide bonds [31]. Studies on the refolding of chymotrypsinogen and trypsin after covalent attachment to agarose gel media were performed in 1975 by Light et al. [32,33], resulting in 50–70% refolding yield. In 1979, Mozhaev et al. [34] covalently immobilized trypsin for the same reason.

Following the development of new protein adsorbent media during the last couple of decades, various chromatographic methods were developed to improve refolding yield.

Non-covalent (reversible) adsorption of denatured proteins to ion-exchange media was used by Creighton [35] for the purpose of refolding using a three-buffer system. The procedure is described in Fig. 4. The column is equilibrated with 8 M urea. The sample dissolved in 8 M urea is adsorbed to the ion exchanger. The urea concentration in the column is gradually decreased by the introduction of refolding buffer leading to gradual refolding of the adsorbed protein molecules. After all the denaturant is washed out of the column, the refolded and still adsorbed protein molecules are released by the addition of salt or other additives to the refolding buffer. Prochymosin, tissue inhibitor of metalloproteinases (TIMP), and porcine growth hormone expressed as inclusion bodies were also refolded with high activity yields using a three-buffer ion-exchange chromatography (IEC) refolding system [36,37]. Fig. 5 shows a model elution curve using the three-buffer IEC refolding system.

In another approach, the inclusion bodies were dissolved in 0.01 M NaOH, 8 M urea, and 1% SDS and loaded to a column packed with Mono Q, a strong anion exchanger, equilibrated with 20 mM Tris–HCl, pH 8.0. The inclusion body protein could be eluted with a gradient of NaCl [38]. The author did not calculate the activity yield, but the refolded protein was reportedly capable of binding to antibodies against the "native" virus.

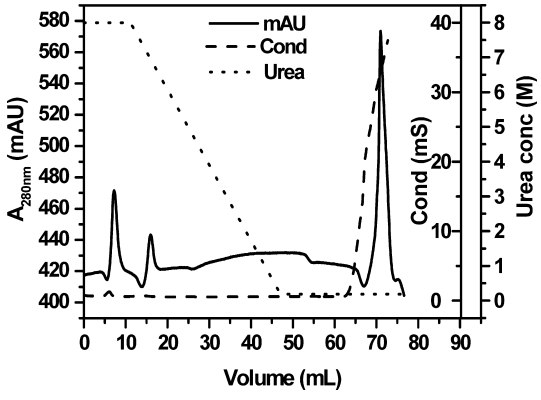

Fig. 5. Chromatogram of an ion-exchange chromatography refolding procedure using a three-buffer system. Refolding of recombinant full-length NS3 protease-helicase using a 7 ml DEAE Sepharose Fast Flow column [75].

The denaturant gradient procedure is, however, not always successful. Thus, in one report reduced bovine α-lactalbumin yielded less than 10% protein eluted from the column and the best yields with reduced hen lysozyme were only about 10% [35]. A tentative interpretation is that refolding intermediates, or possible protein aggregates, bound tightly to the medium and were very difficult to elute from the column. To prevent the accumulation of non-eluted protein during refolding of matrix adsorbed protein molecules, a new process using a two-buffer system to improve activity yield and mass recovery has been developed [39] and is described in Fig. 6. The sample is loaded on to the ion exchanger and adsorbed in the presence of 8 M urea. A descending urea concentration gradient is introduced in parallel with an increasing ionic strength gradient allowing the protein to simultaneously structurally rearrange and elute during its migration down through the column. When it enters regions where the salt concentration is low, the protein will adsorb to the ion exchanger again. However, the salt concentration increases and the urea concentration decreases gradually, leading to desorption of the protein. Finally, at the column outlet, the urea concentration is 1 M and the salt concentration is high enough for the protein to be eluted. A tentative interpretation of the positive results obtained in this type of "on/off" cascade process is that it would allow a single protein molecule to refold gradually in each protein optimal urea concentration in the gradient. This optimal urea concentration reduces the number of intermolecular interactions reducing the risk of aggregate formation and facilitates the refolding of the protein to a native, biologically active conformation.

Another factor to be considered in optimizing a refolding process, especially in the formation of disulfide bonds, is the pH of the refolding buffer [40]. The most favorable pH value varies from protein to protein [3]. Usually, aggregation decreases when the pH of the medium is far away from the protein's isoelectric point [41]. The effect of nearby charged residues on the oxidation potential also makes a difference [42]. In addition to the effect of pH, protein folding and protein aggregation are also strongly influenced by the temperature [3].

To accelerate the thiol-disulfide exchange, the pH of the renaturation buffer should be at the upper limit that still allows the protein to form its native structure. However, it may be difficult to optimize denaturant concentration and pH simultaneously in a refolding process, especially in a large-scale production environment. Considering the importance of both denaturant concentration and pH in refolding, a dual-gradient IEC process was introduced to enhance the refolding recovery at high protein concentration [43]. After the dissolved human lysozyme expressed as inclusion bodies was loaded onto the column, elution was started by gradually decreasing the urea concentration, combined with a gradual increase of pH of the elution buffer. The dual-gradient provides an incremental change of the solution environment for the protein refolding and for the formation of disulfide bonds.

Fe-SOD, that is lacking disulfide bonds, showed an increased refolding yield when a dual-gradient IEC refolding process was applied [44]. At high pH, far away from the protein's isoelectric point, aggregate formation was prevented, while at low pH near the isoelectric point the establishment of a biologically active conformation was facilitated.

Fig. 7 shows a schematic of a dual-gradient ion-exchange chromatography refolding process and Fig. 8 shows the chromatographic curve obtained during

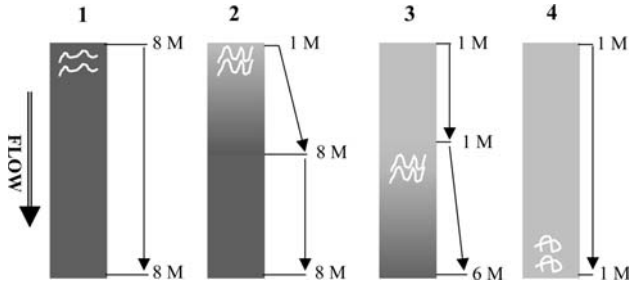

Fig. 6. Flow schedule of a two-buffer ion-exchange chromatography refolding procedure. In graph 1, the denatured and unfolded protein is adsorbed to the chromatographic medium. The column is pre-equilibrated with buffer containing 8 M urea. In graph 2, a combination of a descending urea concentration gradient down to 1 M and an ascending salt concentration gradient (not shown) is introduced to the column. The adsorbed protein starts refolding as the urea concentration decreases. In graph 3, the already refolded protein is desorbed from the ion exchanger as a consequence of the gradual increase in salt concentration. In graph 4, the protein is completely refolded and elutes from the column at the end of the gradient.

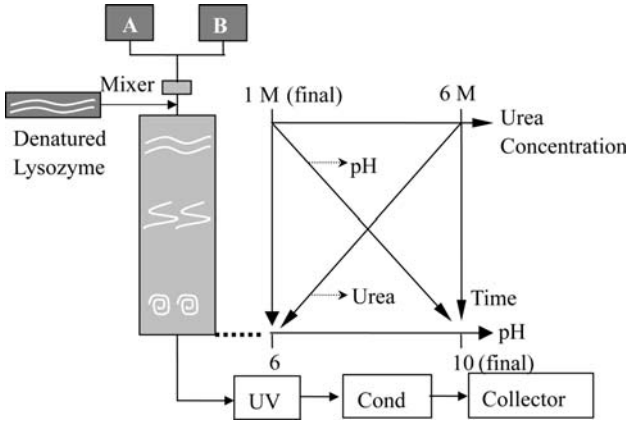

Fig. 7. Schematic representation of a dual-gradient ion-exchange chromatography refolding procedure for lysozyme with a descending urea concentration gradient combined with an ascending pH gradient. The protein is adsorbed to the ion exchanger in the presence of buffer A at pH 6, containing 6 M urea. Buffer B at pH 10, containing 1 M urea is gradually introduced into the column simultaneously inducing protein refolding and desorption from the ion exchanger [39].

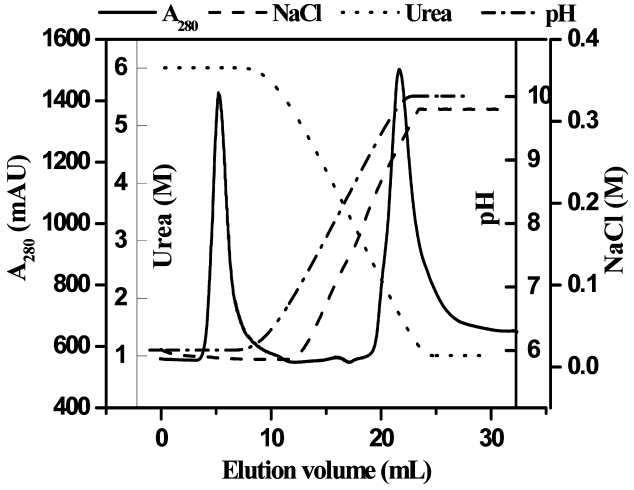

Fig. 8. Example of a dual-gradient ion-exchange refolding procedure. Eight milligrams of lysozyme dissolved in denaturing buffer 0.05 M Tris–HCl at pH 6.2, containing 6 M urea, 3 mM GSH, and 0.3 mM GSSG was adsorbed to a 5 ml HiTrap SP Sepharose HP column equilibrated in the same buffer. Refolding buffer 0.1 M Tris–HCl at pH 10, containing 1 M urea, 0.3 M NaCI, 3 mM GSH, and 0.3 mM GSSG was allowed to gradually replace the denaturing buffer in the column in a total gradient volume of 5 ml. The flow-rate was controlled a 0.4 ml/min. Refolding of the adsorbed protein occurred as a consequence of the decrease in urea concentration and increase in pH. At some point in the gradient the protein was desorbed from the ion exchanger as a consequence of the increase in salt concentration. The left peak in the chromatogram is DTT and the right peak the refolded lysozyme [39].

lysozyme refolding and elution. Here, a linearly decreasing urea concentration gradient, in combination with a gradual increase in pH, proved effective in refolding as well as in the formation of disulfide bonds. Recovery of activity and mass of refolded protein were higher than in processes without gradient, or with only one gradient, for the refolding of denatured lysozyme.

Immobilized metal ion affinity chromatography (IMAC) has opened up new prospects for efficient simultaneous purification and refolding of proteins equipped with engineered polyhistidine tags. Polyhistidine tags form high-affinity complexes with immobilized divalent metal ions even in the presence of high concentrations of chaotropic agents, thereby allowing isolation and refolding of tagged protein. Thus, one-step on-column affinity refolding and purification processes have become quite popular [45–48]. As in the three-buffer IEC refolding process, a gradual decrease of denaturant concentration induces protein refolding and elution is achieved by increasing the imidazole concentration or by using a decreasing pH-gradient [10,49]. Thus, the fusion protein His-TNF expressed in *E. coli* as inclusion bodies was refolded after adsorption to a Ni^{2+}-Sepharose 6B column, resulting in a higher than 90% refolding yield [50]. However, if the concentration of the adsorbed proteins is too high when applying the sample from the top of the column, there is a risk of aggregate formation and low elution yields. This is why it is more favorable to bind the proteins to the adsorbent batchwise in the presence of 8 M urea followed by column packing, washing out of non-adsorbed protein, refolding by reducing the urea concentration, and finally elution of the adsorbed and refolded target protein.

The primary requirement is that the polyhistidine tag must neither block the protein's folding capability nor have any other effect on its native structure. Some metal ions, such as Cu^{2+}, might catalyze the oxidation of cysteine to form mis-paired disulfide bonds [51]. In another example, the strongly negatively charged Heparin–Sepharose was used for the binding of a denatured protein containing a polyarginine fusion tag. Renaturation could be achieved under conditions allowing the protein to remain bound to the matrix and resulted in high yields of active protein [51].

High performance hydrophobic interaction chromatography (HIC) was used to refold recombinant human interferon-α. Refolding and purification could be achieved in one step. The refolding yield was twice as high as that obtained using dilution or dialysis [52]. Reverse phase high performance liquid chromatography (RPC) as a refolding tool could refold recombinant human interleukin-2 expressed as inclusion bodies. The total activity recovery and specific activity were increased 9- and 14-fold, respectively [53].

However, successful polypeptide folding is also dependent on undisturbed hydrophobic interaction forces. This is why HIC or RPC interactions should not be as strong as to prevent proper protein refolding. Some binding strength modifying agents added to the refolding buffer might reduce the hydrophobic interaction and improve the refolding. The additives may influence both the solubility and the stability of the native, denatured or intermediate states. They may act by changing the ratio of the rates of proper folding and aggregate formation or they might simply act by solubilizing aggregates already formed.

In addition to improving refolding yields, reversible adsorption could be a means to refold difficult proteins. The technique enables extensive searches for appropriate refolding conditions without kinetic restrictions or limitations. Since in vitro refolding of pepsin has long been attempted without success, it has been suspected that pepsin has no intrinsic in vitro refolding ability. Renaturation of adsorbed pepsin was observed exclusively at pH 3–5 [54]. The process was extremely slow and reached equilibrium after 300 h. Sixty percent of the proteolytic activity was recovered at pH 5. This is the first report on the successful in vitro refolding of pepsin.

Refolding using immobilized folding catalysts

Alternatives to the reversible adsorption refolding processes involve the use of covalently attached proteins

that help protein refolding such as chaperones and antibodies.

The in vivo competition between folding and aggregation is modulated by chaperones and foldases [55]. The *E. coli* chaperones GroEL and GroES can bind to nascent or unfolded polypeptides and/or their folding intermediates, preventing improper polypeptide chain interactions that lead to aggregation. It is not surprising that these proteins can also affect the in vitro competition between folding and aggregation [56]. Because chaperones and foldases are proteins that need to be removed from the renaturation solution at the end of the refolding process and as they may be costly to produce, their commercial use will require a recovery–reuse scheme [4]. GroEL immobilized to agarose gel has been utilized in a lysozyme refolding study [57]. Also, immobilized molecular chaperones could be used for the refolding of difficult proteins [58]. The three components of the chaperone system: GroEL mini-chaperone (that can prevent protein aggregation), DsbA (that catalyzes the shuffling and oxidative formation of disulfide bonds), and peptidyl-prolyl isomerase were immobilized on an agarose gel for oxidative refolding of the scorpion toxin Cn5 [59]. The immobilized chaperones could be re-used and thus, the cost of the process be lowered. Recently, Kohler et al. [60] developed a chaperone-assisted refolding bioreactor that uses a stirred-cell membrane system to immobilize the GroEL–GroES complex. In its current design, the bioreactor could only be used for three cycles of refolding, which is less than that achieved in chromatographic processes.

Although not based on any theory of its mechanism, it is believed that cyclodextrins form weak and reversible non-covalent complexes with hydrophobic sites present in partially refolded protein intermediates. The relatively polar cyclodextrin molecules that are weakly bound to hydrophobic sites in the folding intermediates are gradually removed as the protein becomes increasingly polar during the refolding process. In addition, the low molecular weight of cyclodextrins allows them ready access to and from the interior of the protein during refolding [61]. A linear dextrin could also inhibit the self-aggregation and assist in the refolding of proteins [62]. It is believed that cyclodextrin or linear dextrin immobilized on a matrix could help protein refolding when this protein is passed through a column packed with particles made from this matrix.

Since lipid bilayer membranes can selectively bind to conformational variants of proteins recognizing changes in local hydrophobicity, liposomes may selectively recognize the intermediate states and prevent them from forming intermolecular inactive aggregates, thus acting as artificial chaperones [63]. This is why immobilized liposome chromatography could improve the refolding yield and simultaneously purify the target protein [64]. The process does not require the separation of liposomes from the refolded proteins.

The yield of correctly refolded protein is markedly increased in the presence of high concentrations of L-arginine. The beneficial effect of L-arginine on protein refolding probably originates from increased solubilization of folding intermediates [65] or from the suppression of aggregation [66]. Covalently immobilized L-arginine might be re-used for enhancing the refolding yield.

Polyethylene glycol (PEG) added to the renaturation buffer inhibited aggregation during refolding of bovine carbonic anhydrase B through the formation of a non-associating PEG-intermediate complex [67]. Although weak, the amphiphilic interaction between PEG and polypeptides requires one more purification step. However, in one weak HIC variant using immobilized PEG the polymer removal step could be ignored leading to improved refolding yield [68].

Antibodies have been shown to facilitate the refolding of the target protein antigen. The antibody may function as a refolding mold by recognizing the motif of denatured antigen and facilitating the correct refolding. It was shown that only the antibody towards the target native antigen had the refolding improvement ability [69]. If the antibody could retain or recover its binding to the antigen at reasonably high denaturant concentrations, immobilized antibody would be a useful tool to assist in the refolding of the antigen, especially from an industrial point of view.

In some cases, other proteins added to the refolding buffer as co-refolding components have been shown to improve the renaturation yield. Thus, basic proteins added as co-refolding aids could improve the refolding of a basic protein, while acidic proteins decreased the yield, demonstrating that hetero-interchain interactions might occur when basic protein is refolding together with acidic protein [70]. Soto et al. [71] postulated that short synthetic peptides containing the self-recognition motif of the protein and engineered to destabilize the abnormal conformation might be useful to correct the structure of mis-folded proteins. These peptides, called synthetic mini-chaperones, are designed to be similar to the sequence of the protein region responsible for self-association and contain residues that specifically favor or disfavor a particular structural motif [72]. Such peptides, covalently immobilized to a suitable gel medium, might facilitate refolding of the target protein.

Integration of processes

The key to a commercially viable renaturation process lies in minimizing the number of steps (to increase the overall yield) and reducing the amount and cost of chemicals required. Future developments in protein

Table 1
Examples of representative chromatographic refolding processes

Mode of refolding	Mode of chromatography	Mode of elution	Protein	Recovery (%)	Reference
Solvent-exchange by SEC	Normal SEC	Normal SEC	RETS-1 isoform PDGF	75	[17]
				75	[20]
	Gradient SEC	No gradient	ScFv	14.5	[29]
		Urea gradient		17.3	
		Urea and pH gradients		25	
Solvent exchange during reversible adsorption	IEC	Three-buffer system	Some inclusion bodies	ND	[34–36]
		Dual-gradient system	Human lysozyme SOD	50	[43]
				40	[44]
	IMAC	Three-buffer system	His-TNF	90	[50]
	HIC	Normal HIC	Human interferon-α	ND	[52]
	RPC	Normal RPC	Human interleukin-2	ND	[53]
Use of an immobilized folding catalyst	GroEL, GroES	Mixture of denatured protein and medium	Lysozyme	85	[58,59]
	Liposomes	Normal chromatographic elution	Lysozyme	90	[63,64]
	PEG	Normal chromatographic elution	Lysozyme	90	[68]

ND = not determined.

refolding will benefit from a more fundamental understanding of inclusion body solubilization methods and on the role that additives play in the inhibition of aggregation [4]. Moreover, refolding processes when integrated with other processes would reduce the costs of chemicals. Thus, the washing step after protein binding to the matrix could be integrated with the collection of reducing agents and denaturing agents. Protein aggregates could be collected, dissolved, and re-cycled into the column in high denaturant concentrations for repeated refolding.

A rigid gel matrix surface might hamper the folding of polypeptides. To solve this problem, inert, flexible spacer arms between the matrix and the ligand might be useful during refolding. Such supports, also called ''tentacle supports'' [73], might provide a more flexible three-dimensional surface for refolding.

Chromatographic refolding processes have got the potential to achieve purification of the target protein in situ. To this end, Cho et al. [74] dissolved a whole crude *E. coli* homogenate in a denaturant and fed this directly into an expanded bed ion-exchange chromatography column. As the denaturant was slowly washed out, the bound proteins were refolded while attached to the solid matrix. Two proteins, hGH-GST and rIFN-α-2a, could be refolded with 3-fold improved yield and purity. The process is very robust, reproducible, reduces the number of renaturation steps, and allows high-concentration refolding.

Conclusion

In Table 1 are summarized the main types of chromatographic refolding techniques. There is no single refolding technique or method that satisfies all protein refolding requirements. The proper chemical conditions also vary from protein to protein. Being a typical empirical science, several experiments are required in the search for the optimum refolding process. Chromatographic refolding processes have demonstrated their advantages for different proteins, whether denatured native protein or polypeptides expressed as inclusion bodies. The basic rules for protein refolding such as to prevent the formation of aggregates, to provide a compatible environment for the renatured protein, and to minimize the amount of chemicals used, are easily kept in chromatographic refolding processes. Also, the colloid osmotic protective environment induced in a hydrophilic gel column is close to the condition in a cell, resulting in more effective refolding with higher yields.

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