



Review

Protein folding liquid chromatography and its recent developments[☆]Xindu Geng^{*}, Chaozhan Wang*Institute of Modern Separation Science, Key Laboratory of Separation Science in Shaanxi Province,
Northwest University, Xi'an 710069, PR China*Received 16 June 2006; accepted 27 October 2006
Available online 20 November 2006**Abstract**

The ultimate goal of proteomics is to identify biologically active proteins and to produce them using biotechnology tools such as bacterial hosts. However, proteins produced by *Escherichia coli* must be refolded to their native state. Protein folding liquid chromatography (PFLC) is a new method developed in recent years, and it is widely used in molecular biology and biotechnology. In this paper, the new method, PFLC is introduced and its recent development is reviewed. In addition the paper includes definitions, advantages, principles, applications for both laboratory and large scales, apparatus, and effecting factors of PFLC. In addition, the role of this method in the future is examined.

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1. Introduction

One of the purposes of proteomics is to identify unknown biologically active proteins and use this information to develop novel drugs. Some active proteins occur at very low levels in the human body and thus have to be produced by biotechnology. *Escherichia coli* is one of the mostly used host cell in biotechnology. But when proteins are expressed in *E. coli*, they often form inactive protein aggregates called inclusion bodies. A step necessary in recovering active proteins from *E. coli* is protein refolding (it is simply called protein folding here) or protein renaturation; it is usually the key step during the production of therapeutic proteins by biotechnology, especially at the industrial scale. The yields from refolding by traditional methods are usually very low, typically 5–20%. Application of liquid chromatography (LC) to protein folding is one of the most interesting and exciting methods to develop in recent years. When it is used in protein folding, the bioactivity recovery increases, the folded protein can be easily separated from misfolded forms, protein concentration after refolding is relatively high, and it is easy to scale up and automate, therefore it is regarded as an efficient, and close to ideal refolding method [1,2]. Additionally, it has the potential to be used at an industrial or large scale, today it has become a very popular technique for protein folding.

Protein refolding by liquid chromatography can be simply named as “protein folding liquid chromatography”. It is defined as “a kind of liquid chromatography, with various kinds of biochemical and/or physicochemical processes originally accomplished in solution, which can result in either raising the efficiency, or shortening the time of protein folding” [3].

An ideal PFLC should have the following four functions depicted simultaneously in Fig. 1 [3]. They are the removal of denaturants, refolding of target proteins, separation from contaminant proteins including misfolded intermediates of the

target protein, and easy recovery of denaturants. It usually takes 20–40 min to complete a chromatographic run with simultaneous protein folding. In addition, by continuously changing the components of the mobile phase, different proteins can be separated with suitable folding conditions to refold and simultaneously purify in only one chromatographic run.

By using the normal dilution method for protein folding, denaturants and contaminant proteins cannot be removed. Some precipitates of target proteins will form during dilution; this not only results in a low recovery, but also requires centrifugation after an overnight incubation. Therefore, the target protein must be further processed using coarse fractionation and fine fractionation. In addition, using the usual dialysis method for protein folding, it typically takes 24 h to refold a protein, with numerous changes of buffer during dialysis. This method can remove most of the denaturants, but cannot completely remove them, and cannot separate the target protein from contaminant proteins.

In the past years, Guo and Geng [4], Li et al. [5], Jungbauer et al. [6], Middelberg [2] and two books [3,7] separately introduced PFLC and reviewed its development from different aspects. A comprehensive review of this field is presented in this paper, including their principles, recent developments and applications, apparatus, recent developments for PFLC at large scale, and effecting factors were summarized and discussed.

2. Principles

From a scientific point of view, PFLC includes size exclusion chromatography (SEC), hydrophobic interaction chromatography (HIC), ion exchange chromatography (IEC), and affinity chromatography (AFC). As it is well known that there is no obvious interaction between denatured protein and the stationary phase of SEC, but there are strong interactions between denatured proteins and the stationary phase in the other three chromatographic methods, therefore for convenience, the latter are referred to as adsorption liquid chromatography. The mechanisms for protein folding are discussed for the two kinds of LC.

2.1. SEC

The principle of protein folding by SEC was proposed by Batas and Chaudhuri [8]. They reported that denatured proteins have a much larger Stokes radius than denaturants, so the former move much faster than the latter and result in a gradual decrease in denaturant concentration around denatured protein molecules, causing protein folding step by step, and as this happens their Stokes radius decreases gradually. When protein folding is accomplished, their Stokes radius is constant, and the protein is eluted out in its native state. They thought that their

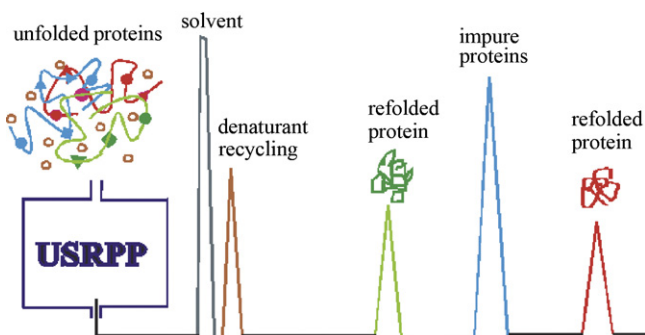


Fig. 1. Scheme of an ideal protein folding liquid chromatography having four functions simultaneously [3]. Four functions: removal of denaturants, refolding of target proteins, separation from contaminant proteins including misfolded intermediates of the target protein, and easy recovery of denaturants.

dispersion rate decreased when the denatured protein molecules entered gels, which reduced aggregation. They further tested the above mechanism using high performance SEC [9].

2.2. Adsorption chromatography

2.2.1. Thermodynamic equilibria [3,7]

The key point here is that the chromatographic process for any kind of adsorption LC can be elucidated by thermodynamic equilibrium, providing a basis to describe the principle of protein folding for this kind of LC. In regular LC, only adsorption ($P_{(N,mo,a)}$) and desorption ($P_{(N,mo,d)}$) of the native protein in a monomeric state with the stationary phase involving the usual LC and any separation depends on the partition coefficient of proteins between these two phases, while protein folding in the usual buffer is mainly controlled by the primary structure of the protein. During protein folding in buffer, on the one hand, a series of aggregation processes, such as unfolding of the monomer, $P_{(U,mo,d)}$ to dimer, trimer, multimer, until protein precipitates may form, being unfavorable protein folding. On the other hand, the denatured state $P_{(U,mo,d)}$, can also be converted to its native state, $P_{(N,mo,d)}$ under a suitable conditions, being favorable protein folding. However, with PFLC, the stationary phase (see Section 2.2.2) makes chemical equilibrium move from precipitate and/or its polymers to its monomer in the unfolded state, $P_{(U,mo,d)}$ to fold to its monomeric native state, $P_{(N,mo,d)}$, until finally it elutes out of the column.

In summary, PFLC is the favorable chemical equilibrium to convert from aggregate to $P_{(N,mo,d)}$, resulting in either an increase in the protein folding efficiency, or an acceleration in the folding process. As long as the chromatographic process can be described by thermodynamic equilibrium, the reported principle is suitable for each kind of LC listed above, IEC, HIC, and AFC.

2.2.2. Molecular mechanism

From the standpoint of molecular interactions, the mechanism of protein folding for each kind of adsorption chromatography mentioned above should be different from each other. Because the molecular mechanism of protein folding by IEC and AFC has not yet been reported, HIC is taken as an example to demonstrate it.

The mechanism of protein folding by HIC was presented in 1992 [10] and reported in detail in 2002 [3,11,12]. The unfolded protein molecules are pushed by hydrophobic interaction forces from the mobile phase at a high salt concentration to move forward to stationary phase of HIC (STHIC) and tightly contact the STHIC with apolar region of amino acid sequence residues to form a stable complex and the hydrophilic parts of the unfolded protein molecules face to the mobile phase. Thus, the unfolded protein molecules cannot aggregate in this circumstance. The unfolded protein molecules take enough energy at the molecule level from the STHIC and simultaneously carry out three functions: (i) The STHIC can recognize a specific hydrophobic region of a polypeptide. (ii) Squeezing out water molecules in a hydrated state from both the hydrated unfolded protein and the STHIC. (iii) The microdomains of the protein molecules

on the STHIC are formed. The unfolded protein molecules desorb from the STHIC as the salt concentration decreases, or as the water concentration in the mobile phase increases. Protein molecules with incorrect microdomains would be corrected by the microdomains spontaneously disappearing in the mobile phase due to their unstable thermodynamics. After many rounds of adsorption and desorption of the protein during gradient elution, the incorrect microdomains would decrease, while the protein molecules with correct microdomains would increase, resulting in the protein attaining complete refolding. The complete refolded protein can be separated from its stable intermediates, or its incompletely refolded forms. Several unfolded proteins can be simultaneously refolded and, at the same time, separated from each other.

3. Applications

3.1. SEC

SEC is most often applied to PFLC due to its ease of operation, easy scale up and suitability to the refolding of a wide range of proteins. In 1992, one of the authors [10] used SEC to refold three kinds of standard proteins. In 1994, Werner et al. [13] prepared recombinant human ETS-1, bovine ribonuclease A and integration host factor (IHF), and increase the capacity of the technique to the mg scale. In 1996, Batas and Chaudhuri [8] used Sephacryl S 100 as SEC gel, and refolded hen egg white lysozyme and bovine carbonate anhydrase (CAB) at an initial protein concentration of 80 mg/mL, their bioactivity recovery was 63% (a specific bioactivity recovery of 104%) and 56% (a specific bioactivity recovery of 81%), respectively, they investigated the SEC refolding method in detail. In order to provide a wild environment for protein folding, Gu et al. [14] reported urea gradient SEC, in which a gradually decreasing gradient of urea concentration from top to bottom was initially formed in the SEC column, denaturants were removed linearly by this method and good results were obtained for denatured proteins with a high protein concentration. For denatured/reduced lysozyme at an initial concentration of 17 mg/mL, a bioactivity recovery as high as 90% could be obtained in 40 min. In addition, they developed a SEC refolding method with dual gradients of pH and urea concentration, the results were relatively good. Dong et al. [15] combined SEC and the artificial molecular chaperone, they used cetyltrimethylammonium bromide (CTAB) as a detergent and β -cyclodextrin as a stripping agent (i.e., eluent), denatured/reduced lysozyme with an initial concentration of 80 mg/mL was refolded using this method. The results showed that this method is favorable for protein folding under conditions of high flow rate of the mobile phase. Chaperone solvent plug SEC proposed by Liu and Chang [16] could obviously reduce precipitates formed before denatured protein entered the top of the column, and relatively high mass recovery was obtained. Schlegl et al. [17] reported a continuous matrix assisted protein folding system based on SEC refolding and continuous annular chromatography (CAC), it forces denatured proteins to refold to their native states quantitatively and continuously. Recently, simulated moving bed chromatography (SMB) in SEC mode

Table 1
Examples of refolding of proteins by SEC

Refolding proteins	Type of gel	Results of refolding	Years	Reference
Bovine serum albumin ribonuclease A lysozyme	Shimadzu diol 150		1992	[10]
<i>E. coli</i> integration host factor	Superdex 75	60%	1994	[13]
rhETS-1	Sephacryl S 100	71%	1994	[13]
RNase	Sephacryl S 100	>90%	1994	[13]
Bovine carbonic anhydrase	Sephacryl S 100 HR	56%	1996	[8]
Lysozyme	Sephacryl S 100 HR	Activity recovery was 46% at a protein concentration of 80 mg/mL	1996	[8]
Recombinant interleukin-6	Superdex G-25	Activity recovery was 17%	1999	[20]
Recombinant lysozyme	Sephacryl S 100	Activity recovery was 35%	1999	[21]
Heterodimeric platelet-derived growth factor	Superdex 75	Activity recovery was more than 75%	1999	[22]
Lysozyme	Superdex 75	Activity recovery was 90% at a protein concentration of 17 mg/mL	2001	[14]
Urokinase plasminogen activator	Sephacryl S 300	Activity was more than 5 times of dilution method	2000	[23]
Lysozyme	Sephacryl S 100	Activity recovery was near 100% at a concentration of 40 mg/mL	2001	[24]
Urokinase plasminogen activator fragment	Sephacryl S 300	Activity recovery was 15.3%	2000	[25]
Lysozyme	Sephacryl S 100	Activity recovery was 80%	2002	[15]
Lysozyme	Superdex 75 HR	Activity recovery was >90%	2003	[16]
Bovine carbonic anhydrase B	Superdex 75	Activity recovery was 85%	2003	[26]
recombinant <i>Pseudomonas</i> <i>Xuorescens</i> lipase	Sephacryl S 200	Refolding yield was 14%	2005	[27]
B lymphocyte stimulator	Sephacryl S 200	Refolding yield was 30%	2005	[28]
Lysozyme	Superdex 75 HR	Activity recovery was nearly 100%	2006	[29]
Lysozyme	Sephacryl S 100	Refolding yield was 96%	2006	[19]
rhG-CSF	Superdex 75	Specific activity was 1.2×10^8 IU/mg, mass recovery was 30%	2006	[30]

was also used to refold proteins [18,19], this gave new inspiration to the development of SEC. Table 1 shows some examples of protein folding by SEC.

3.2. HIC

As pointed out previously, in 1991–1992, one of the authors [10] refolded several denatured proteins and rhIFN- γ using HIC. In 1997, HIC was applied to the refolding and purification of several HIV protease mutants by Du-Pout-Merck Co. [31,32]. So far, many proteins, such as recombinant human interferon- γ (rhIFN- γ) [10,33–35], bovine insulin [36], lysozyme [37], recombinant bovine prion protein [38] were successfully refolded using HIC, and good results were obtained. Additionally, in 2004 and 2006, the refolding with simultaneous purification of rhIFN- γ [34] and recombinant human granulocyte colony-stimulating factor (rhG-CSF) [39] were reported, respectively, downstream processes for their production in biotechnology were simplified greatly. Li et al. [40,41] refolded the originally denatured lysozyme and recombinant staphylococcus aureus elongation factor G (EF-G) separately using HIC with several urea gradients. Recently, they refolded consensus interferon (C-IFN) using HIC with a dual-gradient of decreasing guanidine hydrochloride concentration and increasing PEG concentration [42]. Compared with dilution refolding, the gradient HIC process, in the presence of PEG, gave about

2.6-folds of increase in specific activity, 30% increase in soluble protein recovery. Urea denatured recombinant human stem cell factor (rhSCF) produced by *E. coli* were renatured with simultaneous purification using a high performance hydrophobic interaction chromatographic (HPHIC) column packed with packing materials with an end group of PEG400 [48]. The refolded rhSCF had a good bioactivity and a high purity. Table 2 shows some examples of protein folding by HIC.

3.3. IEC

Protein folding by IEC was introduced by Creighton [49,50] in 1986, he used a refolding system consisting of three buffers, in which a decreasing gradient of urea concentration was used to refold protein, then an increasing gradient of salt concentration was performed to elute the refolded protein. Suttner et al. [51] used 0.01 mol/L NaOH solution to solubilize the inclusion body of papilloma virus HPV16 E7MS2 fusion protein, and successfully refolded the solubilized target protein using Mono Q strong anion exchange chromatographic column. Stempfer et al. [52] fused a polycation tag containing hexa-arginine onto α -glucosidase, and refolded this fusion protein by IEC with a polyanionic support. This method provides a novel protocol for proteins with very few charges, but the fusion and cleavage of the tag was relatively complex. Kweon et al. [53] refolded cyclodextrin glycosyltransferase fused with

Table 2
Examples of protein refolding by HIC

Refolding proteins	Refolding results	Years	References
rhIFN- γ	Activity recovery was 2–3 times of dilution method, purity was more than 85%	1991, 1992	[10,33,43]
HIV protease mutants		1997, 1998	[31,32,44,45]
Bovine insulin	Refolding yield was 66%	2002	[36]
Recombinant bovine Prion protein	Mass recovery was 87%, purity was 96%	2004	[38]
Recombinant Human proinsulin	Mass recovery was 94%, purity was 90%	2003	[46]
rhIFN- γ	Activity recovery was 2–3 times of dilution method	2001	[47]
Lysozyme	Activity recovery was 94.6%	2003	[37]
rhIFN- γ	Specific activity was 1.3×10^8 IU/mg, purity was >95%	2004	[35]
Recombinant human stem cell factor	Purity was 94%, specific activity of 1.2×10^6 IU/mg	2006	[48]
rhIFN- γ	Injection mass was about 2.0 g, injection volume was 700 mL, purity was >95%, specific activity was 5.7×10^7 IU/mg	2002	[12]
rhG-CSF	Injection mass was about 1.5 g, injection volume was 200 mL, purity was 95.4%, specific activity was 2.3×10^8 IU/mg, mass recovery was 36.9%	2006	[39]
C-IFN	Mass recovery was more than 80%	2006	[42]

10 lysine residues at the C-terminus (CGTK10ase) using strong cation exchange chromatography (SCX) with SP Sepharose gel, the refolded yield was approximately 100% and the protein concentration after elution was 2.5 mg/mL, the initial protein concentration was 7.5 mg/mL. This method is similar to that introduced by Stempfer et al. [52]. Li et al. [54] improved the refolded yield of lysozyme using urea and pH dual gradient SCX with soft gel SP Sepharose. In 2002, Cho [55] used Expanded bed adsorption chromatography (EBA) packed with a weak anion exchange resin to refold proteins, it simplified the production procedure for proteins in inclusion bodies. In 2005, Machold et al. [56] used preparative continuous annular chromatographic (P-CAC) packed with DEAE Sepharose to refold α -lactalbumin continuously. Liu et al. [57] recently proposed a relatively versatile refolding method using IEC with a silica-based weak cation exchanger, very high mass and bioactivity recoveries were obtained for denatured lysozyme. In this method, 4.0 mol/L urea was a constituent of the equilibration and elution buffers, and ammonium sulfate which is good for maintaining the stability of native proteins was selected as the elution agent. A similar method was also applied to rhG-CSF [58,59]. Lu et al. [60] refolded recombinant dual human stem cell factor using IEC similar to Creighton's method, the target protein obtained had a purity of 90%, and a refolded yield of 19.46%. Denatured/reduced bovine serum albumin (BSA) which contains 17 pairs of disulfide bonds was renatured using strong anion exchange chromatography (SAX) with a Q Sepharose column [61]. BSA was eluted after an incubation of 40 h in the SAX column, its refolded yield and mass recovery were 55 and 67%, respectively, this is one of the most complex proteins refolded by LC. Examples for protein folding by IEC are listed in Table 3.

3.4. AFC

Specific affinity interactions between ligands and target proteins are responsible for reducing aggregates between dena-

tured protein molecules and increasing the refolded yield. AFC applied to protein folding can be classified into three types according to their ligands: immobilized metal ion affinity chromatography (IMAC), immobilized liposome chromatography (ILC), and immobilized molecular chaperone chromatography (IMCC). In 1994, Phadtare et al. [69] immobilized the molecular chaperone GroEL on the surface of a stationary phase and used this column on tublin and glutamine synthetase. In 1997, Altamirano et al. [70] immobilized histidine-fused GroEL (191–345) with 17 amino acid residues at the N-terminus on Ni-NTA resin through a chelating interaction, and used the column packed with this modified resin to refold indole 3-glycerol phosphate synthase (IGPS) mutants IGPS (49–252) denatured by 8 mol/L urea, its mass recovery was 92%, and the refolded protein had a bioactivity of 100%. They also used this affinity column to refold cyclophilin A, its mass recovery was 84% and its bioactivity recovery was 98%. In 1999, they immobilized molecular chaperone/disulfide isomerase/proline PPI on agrose resin and formed a tri-component stationary phase, they used this column to refold scorpion toxin Cn5 [71], which could not be refolded at all using other methods. Its mass recovery was 87% and its bioactivity recovery was near 100%, and thus they called this method as *refolding chromatography*. They also used this method to refold another recombinant protein [72]. Gao et al. [73] refolded rhIFN- γ using an immobilized molecular chaperone fragment. Guan et al. [74] immobilized mini-molecular chaperone sht GroEL (191–345) on activated Sepharose Fast Flow gel and used a column packed with this modified gel to refold rhIFN- γ , the results showed that this method was very useful for the refolding of rhIFN- γ . When 100 μ L of rhIFN- γ solution with a protein concentration of 17.8 mg/mL was injected into the column, the mass recovery of the target protein was 74.25% and its bioactivity was 6.74×10^6 IU/mL.

IMAC is based on the affinity interaction between the ligands and the histidines tagged at the end of the target proteins. When protein molecules are adsorbed on the IMAC column,

Table 3
Examples for protein refolding by IEC

Proteins	Stationary phases	Refolding results	Years	References
Cytochrome <i>c</i>	CM-cellulose	Mass recovery was more than 80%	1986	[49,50]
Ovalbumin	DEAE-cellulose	Refolding yield was 50%	1990	
Trypsin inhibitor	CM-cellulose	Mass recovery was more than 90%		
Fused α -glucosidase	Heparin Sepharose	Bioactivity recovery was four times of dilution	1996	[52]
Papilloma virus HPV16 E7MS2 fusion protein	Mono Q		1994	[51]
Lysozyme	Silica-based WCX	Activity recovery was approximately 100% when initial protein concentration was up to 20 mg/mL	2003 2005	[62] [57]
Lysozyme	SP Sepharose	Activity recovery was approximately 95% when initial protein concentration was up to 40 mg/mL	2002	[54]
Recombinant lysozyme	SP Sepharose FF	Activity recovery was approximately 100% when initial protein concentration was up to 4 mg/mL	2002	[63]
Single-chain Fv-cellulose binding domain proteins	Cellulose	Refolding yield was 60%	1999	[64]
rhG-CSF	Q Sepharose FF	Specific activity was 2.3×10^8 IU/mg, mass recovery was 43%, purity was 97%	2005	[58]
Recombinant secretory leukocyte protease inhibitor	DEAE-cellulose	Protein concentration was enhanced 6.4-fold than dilution method, activity recovery was 46%, mass recovery was 96%	1996	[65]
α -Lactalbumin	Fractogel EMD DEAE	Refolding yield was 84%	2005	[66]
CGTK10ase	SP Sepharose	The refolding yield was approximately 100% and the protein concentration after elution was 2.5 mg/mL	2004	[53]
Recombinant LK68	Q-Sepharose Hi-Trap	Refolding yield was 68%, which is 1.7-fold of dilution method	2005	[67]
rhGH-GST	STREAMLINE DEAE	Refolding yield was 84%	2002	[55]
EGFP	Q Hyper Z	Refolding yield was 90%	2005	[68]
Recombinant dual human Stem cell factor	DEAE Sepharose FF	Refolding yield was 19.46%, purity was 90%	2005	[60]

hydrophobic interactions between denatured protein molecules are prevented. Under a high concentration of denaturants, the histidine tail of the target protein can still retard on its surface of IMAC, therefore it can accomplish refolding and purification simultaneously [75–77]. For the refolding of Ykt6p SNARE by

IMAC, its mass recovery was 88%, and its purity was 94% [78]. Aequorin was adsorbed onto Ni-NTA agarose in batch mode, after that the agarose beads were packed into column, then the adsorbed aequorin was refolded by IMAC [75], crowding of denatured proteins on the top of the column was avoided in this

Table 4
Examples for protein refolding by AFC

Proteins	Stationary phases	Refolding results	Years	References
Tublin	Immobilized GroEL		1994	[69]
(His) ₆ -LECT2	Ni-NTA	Refolding yields was 81%	2003	[84]
(His) ₆ -voltage-dependent anion-selective channel	Talon		2003	[76]
(His) ₆ -aequorin	Ni-NTA	Specific activity was 2.2×10^{10} RLU/mg	2003	[75]
(His) ₆ -interleuin-15 receptor α -chain	Ni-NTA	Mass recovery was 6-fold of dilution method	2003	[85]
Recombinant Toc75	Ni-chelated Sepharose FF		1998	[86]
(His) ₆ -exopolyphosphatase	Ni-chelated Sepharose FF	Mass recovery was 51%	2003	[77]
Recombinant bovine prion	Ni-NTA	Mass recovery was 11%	2003	[79]
Rv2430c	Ni-NTA		2004	[87]
Hsp-antigen fusion protein	Ni-agarose	Refolding yield was 34.5%	2004	[88]
rhG-CSF	Cu-chelated	Specific activity was 2.3×10^8 IU-mg ⁻¹ , mass recovery was 36.4%	2004	[80]
Lysozyme	Immobilized liposome	Activity recovery was 100%	2000	[81]
Bovine carbonic anhydrase	Immobilized liposome	Activity recovery was 83%	1998	[83]
Lysozyme	Immobilized GroEL	Activity recovery was 81%	2000	[89]
Fragment 450–650 of the spike protein of SARS-coronavirus	Ni-NTA	Mass recovery was 31.5%	2005	[90]
rhIFN- γ	sht GroEL (191–345)	Mass recovery was 74.25%	2006	[74]
IP10-scFv fusion protein	Ni-chelating	Refolding yield was 45%	2006	[91]

manner, and aggregates decreased. Tien and co-workers [79] used the octapeptide repeated sequence in recombinant bovine prion as a natural affinity tag, and refolded the protein by Ni-NTA IMAC and the target protein with correct structure was obtained, its mass recovery was 11%. Wild rhG-CSF without any affinity tag was also refolded successfully using IMAC [80].

Yoshimoto et al. [81–83] separately refolded bovine carbonate anhydrase, lysozyme and ribonuclease A using ILC. They thought that liposome could be regarded as a kind of aqueous two-phase system and can function as an artificial molecular chaperone, it has a high selectivity for different conformations of proteins denatured with different concentration of denaturants, and retention of proteins with different molecular conformations on the column has a relationship with the local hydrophobicity. During protein folding, the ILC can combine with protein folding intermediates, thus aggregates between denatured protein molecules were prevented and refolding yield was improved. Some examples for protein folding using AFC are listed in Table 4.

4. Apparatus

4.1. Unit of simultaneous renaturation and purification of proteins

For PFLC, a series of problems exists, such as aggregates would form when loading the denatured protein solution. When aggregates formed, the back pressure of the column would increase significantly, even blocking the column. Additionally, mass and bioactivity recoveries of the target protein would decrease. This problem is more important in large scale PFLC. Geng and co-workers [92,93] designed and manufactured in a series of units for simultaneous renaturation and purification of proteins (USRPP), also called chromatographic cake, its length is very short, only 1.0–5.0 cm, but its diameter is up from 2.0 to 50 cm or even larger. Its cross section is very large, so the increase in the column back pressure is not obvious when a few precipitates accumulate on the filter or on the top of column bed. This unit has very good resolution for proteins in laboratory (Fig. 2a) and large scales (Fig. 2b), it has been applied to the refolding of many proteins, and was used for the refolding with simultaneous purification of rhIFN- γ [34] and rhG-CSF [39] at an industrial scale. When the USRPP with a size of 10 mm \times 200 mm i.d. was used, rhIFN- γ with a purity of more than 95%, and a specific bioactivity of 8.9×10^7 IU/mg can be obtained by using USRPP within 2 h, bioactivity recovery can reach 24-fold that of traditional methods, the time it takes was only half that of the traditional method. When using the USRPP with a dimension of 10 mm \times 300 mm i.d., 700 mL of rhIFN- γ solution extracted by 7.0 mol/L GuHCl containing 2.0 g of total protein could be processed in one run with a flow rate of 120 mL/min [34]. And the USRPP was also used to refold rhG-CSF with simultaneous purification at large scales [39].

USRPP has a very good performance, but its price is relatively high due to packing with supports with small particles and using stainless steel for cake bodies. Based on the fact that the retention of biopolymers in LC is dominated by the contact surface

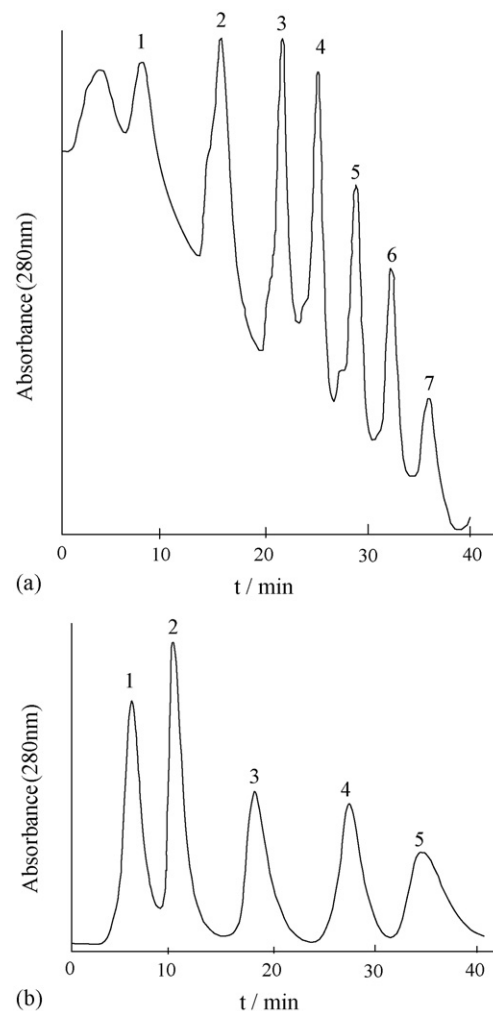


Fig. 2. Chromatogram of standard proteins separated by USRPP [7]. (a) USRPP with a dimensionless of 10 mm \times 50 mm i.d., 1, cytochrome C; 2, myoglobin; 3, ribonuclease A; 4, lysozyme; 5, α -chymotrypsin; 6, α -mylase; 7, insulin. (b) USRPP with a dimensionless of 10 mm \times 200 mm i.d., (1) cytochrome c; (2) myoglobin; (3) lysozyme; (4) α -amylase; (5) insulin.

area between biopolymers and the stationary phase employed [7,94,95,96], a short column packing with small particles (i.e., less than 10 μ m) should theoretically have an identical resolution to that of a longer column packed with the same kind packing with large diameter particles (i.e., 50–100 μ m), but the latter is much cheaper than the former. Thus, a series of simple and cheap columns were manufactured [3,39,97], in which large particulates were packed and the HPHIC column packing materials are silica-based, it is much cheaper than the USRPP. Compared to Fig. 2a, although it still has a good resolution for protein (Fig. 3), its resolution is a little worse. It can be used either for investigating chromatographic conditions in laboratory scale, or as a pre-column for other uses.

4.2. Expanded bed adsorption chromatography

Expanded bed adsorption chromatography (EBA) is a newly developed chromatographic technique in recent years and it

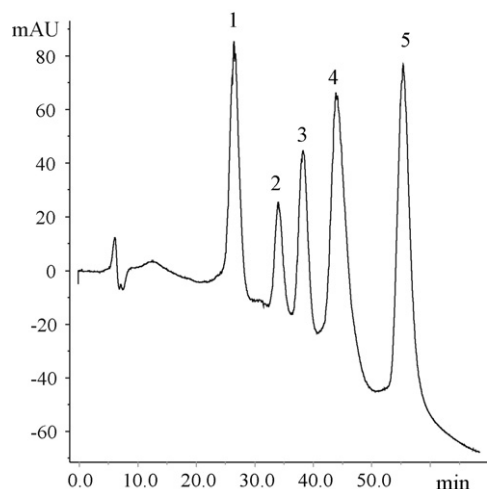


Fig. 3. Chromatogram of five standard proteins separated by Kelin[®] fast protein purification column [3] (1) cytochrome *c*; (2) myoglobin; (3) lysozyme; (4) α -amylase; (5) insulin.

is a large-scale industrial chromatographic technique. It is an alternative to traditional clarification (centrifugation, tangential, micro- and ultrafiltration) and the first chromatography step. The work of refolding proteins using EBA was initiated by Mannen et al. [98], they immobilized molecular chaperone on the chromatographic support and using EBA to refold denatured proteins. In 2002, Cho et al. [55] used EBA column packed with STREAMLINE DEAE resin to refold the *E. coli* cell homogenate of the fusion protein of recombinant human growth hormone (rhGH) and glutathione *S*-transferase (GST) fragment, its refolding yield was up to 84%. Choi et al. [67] compared the refolding efficiencies of an EBA and a packing bed for recombinant LK68, the results indicated that both were comparable. Cabanne et al. [68] refolded enhanced green fluorescent protein (EGFP) expressed in *E. coli* using EBA with an anion exchanger, good results were obtained. Jin et al. [99] used EBA packed with Streamline SP strong cation exchange chromatographic resin to refold rhIFN- γ solubilized by 8 mol/L urea with simultaneous purification, its mass recovery was 52.7%, and its specific bioactivity was 8.18×10^6 IU/mg.

Applying EBA to protein folding not only can reduce aggregates and improve refolded yield, but can also increase purity, reduce steps during refolding process, and lower production costs. EBA provides PFLC with an alternative routine to overcome the increase in column back pressure resulting from protein precipitation.

4.3. Continuous annular chromatography

Continuous annular chromatography (CAC) is also a new chromatographic technique. It can allow sampling and separation to be performed continuously, and it is a very important preparative chromatographic technique. Schlegl et al. [17] reported a continuous matrix assisted protein folding system based on SEC refolding and continuous annular chromatography (CAC). This system consisted of a preparative CAC and an ultrafiltration system, the CAC was used for protein folding and

separation, the ultrafiltration was used to recover protein aggregates formed during protein folding, and then the recovered aggregates were re-injected into the CAC for refolding again. This system can help make denatured proteins be refolded into their native states quantitatively and continuously. When using Superdex 75 PrepGrade as a gel to refold bovine α -lactoalbumin, a refolded yield of only 30% was obtained by using the normal batch SEC, but the refolded yield was increased to 41% by using the continuous system when the recycle rate was 65%. If the aggregates could be quantitatively solubilized and recycled to the sample solution, the refolded yield might have a potential to achieve nearly 100%. Lanckriet and Middelberg [100] also refolded lysozyme by using CAC. Machold et al. [56] refolded α -lactoalbumin in continuous operation mode using a preparative continuous annular chromatographic (P-CAC) column packed with DEAE Sepharose resin. A solution containing 2 mol/L GuHCl was used to dissolve protein aggregates and precipitates in the column during refolding. The re-dissolved proteins were recovered by ultrafiltration and were re-injected into the chromatographic system for refolding again; the refolded yield was enhanced to a certain extent. Continuous operation of protein refolding by IEC was achieved in this method, and it represents a very good idea for recovery of protein precipitates during PFLC. However, P-CAC is not stable, especially its flow rate is unstable, its peak wiggle, and it is not easy to operate.

4.4. Simulated moving bed chromatography

Simulated moving bed chromatography (SMB) is also a continuous chromatographic process. It has some advantages such as high reproducibility, low solvent cost, low product dilution ratio, therefore, its operational cost is relatively low, but its one-off investment is relatively high. For separation at preparative and productive scales, low operational cost is in the highest flight compared to high one-off investment, therefore, the separation cost of SMB is lower than batch chromatography [101]. This technique has been used for large scale separation in many fields. Park et al. [18,19] used four-zone SMB in SEC mode to refold denatured/reduced lysozyme continuously, both its bioactivity and mass recoveries were more than 90%. During the process, the productivity was high, cost of refolding buffer was low and support use rate was high. Compared to batch SEC, the concentration of lysozyme obtained by SMB was high, and the cost of adsorbents was low.

It can be seen from the above that PFLC at a large scale and at an industrial scale has been performed from various points of view, and relatively good results were achieved. However, work in this field is only underway just now, and much research should be carried out to further develop this method.

5. Factors effecting the PFLC

The expenses for manufacturing therapeutic proteins are very high and thus it is desirable to reduce these. Besides selecting the kind of LC for protein folding, the optimization of the renatured condition for each LC is also significant.

5.1. Denaturant concentration in the mobile phase

A suitable concentration of denaturant in sample solution and/or mobile phase can reduce a denatured proteins chances of aggregating to a minimum, or prevent precipitates appearing altogether facilitating protein elution during PFLC, thus the refolded yield would be enhanced. Urea is the mostly commonly used denaturant added to mobile phases. Muller and Rinase [22] refolded platelet-derived growth factor by using SEC, it was found that aggregates increased from 10 to 60% when the mobile phase changed from 0.5 mol/L GuHCl to 0.5 mol/L NaCl. Liu et al. [57] found that bioactivity recovery increased and then decreased with increasing urea concentrations during the refolding of lysozyme by high performance weak cation exchange chromatography (HPWCX), with the highest recovery at 4.0 mol/L urea. Little bioactivity recovery was obtained when the urea concentration was less than 1.0 mol/L. Similar results were obtained for the refolding with simultaneous purification of rhG-CSF using SAX with Q Sepharose FF gel, but the most suitable urea concentration is 3.0 mol/L [58,59]. It can be seen that urea concentration is very critical for the PFLC, especially for those proteins susceptible to aggregation, but different urea concentrations are required for different proteins.

5.2. Stationary phase

It is believed that the stationary phase adsorbs denatured protein molecules playing an important role not only in reducing aggregates during PFLC, but also in associating protein folding by means of three functions [3,12], so the stationary phase plays a very critical role during protein folding by LC. Fahey et al. [25] found that the types of stationary phase had a significant effect on the refolding of urokinase plasminogen activator by SEC. It was thought that the fraction range of the stationary phase was responsible for the refolding results. Gu et al. [26] obtained results similar to those above. Wang et al. [38] refolded a recombinant bovine normal prion protein fragment [rbPrP (104–242)] with simultaneous purification using a HPHIC with three different types of stationary phases (phenyl, PEG600 and tetrahydrogen furfural), the results indicated that the types of stationary phase had a significant effect on the mass recovery and purity. Denatured lysozyme was also refolded by HPHIC using the above three types of stationary phase [37], it was found that the highest refolded yield was obtained when PEG600, which has the weakest hydrophobicity, was used as the stationary phase. Geng et al. [34] found that types of stationary phase also had a very significant effect on the refolding of rhIFN- γ , the best results were achieved when using PEG200 as the stationary phase, which has the weakest hydrophobicity among the seven investigated types of stationary phase. Results obtained by Li et al. [40] indicated that ligands with strong hydrophobicity were susceptible to causing misfolding of EF-G, thus resulting in irreversible adsorption and lower refolded yields. Machold et al. [66] found that various refolded yields were obtained with different anion exchangers for denatured α -lactoalbumin, and the refolding time was much different with these exchangers. It also indicated that refolded yields of lysozyme depended sig-

nificantly on the types of stationary phase during its refolding using HPWCX [102]. Various proteins have various structures and characteristics, they require different stationary phases for their refolding, and therefore a suitable stationary phase should be selected from various stationary phases. Additionally, stationary phases with new structures and characteristics should be developed to broaden the types of stationary phases, thereby accelerating the maturation of the PFLC technique.

5.3. Flow rate

Flow rate can affect the contact time of denatured proteins to the stationary phase of a chromatographic column. It can also affect the rate of denatured protein molecules entering into the column and the rate of decreasing the denaturant concentration, thus resulting in different refolded yields. Harrowing and Chaudhuri [103] investigated the effect of flow rates on the refolded yields of β -lactamase by SEC, it was found that the mass recovery increased with increasing the flow rate. Fahey et al. [23] found the same phenomenon during the refolding of urokinase plasminogen activator using SEC. But Gu et al. [26] found that aggregates reduced and the bioactivity recovery increased with a decrease in flow rate. Guan et al. [74] refolded rhIFN- γ using gels immobilized with mini-molecular chaperone sht GroEL (191–345), they found that both mass recovery and specific bioactivity increased with a decrease in the flow rate. Liu et al. [29] thought that it was the key for protein folding using SEC to reduce aggregates before denatured protein entered the column and to make protein stay for enough time in the column. They used higher flow rates during the process when the protein was moving from injection valve to the top of column, then lowering the flow rate to make the protein stay long enough in the column, the results demonstrated that the aggregates were reduced significantly and that the refolding results were very good. Liu [102] refolded lysozyme using HPWCX, they found that the bioactivity recovery increased somewhat with an increase in the flow rate, approaching the highest refolded yield at a flow rate of about 2.0 mL/min, but stayed static after that. Li et al. [54] found that bioactivity recovery increased initially and then decreased with an increase in the flow rate during the refolding of lysozyme using IEC. Geng et al. [34] found that the lower flow rate was favorable for improving mass of rhIFN- γ during its refolding using HPHIC. It can be seen that the effects of flow rate on the refolded yield is not consistent, this may have something to do with refolding methods, but it is affirmative that flow rate has an important effect on PFLC.

5.4. Salts and pH

The kinds of salt in the mobile phase of HIC have not been found to affect protein folding significantly [34], but the kinds of salt in the mobile phase of IEC affect it seriously [57]. However, some differences as well as some similarities between effects of pH on the protein folding using LC and dilution method were found to contribute to protein folding, because pH can affect both retention and elution of proteins during chromatographic processes. Wang et al. [38] found that the most suitable mobile

phase pH was 7.0 during the refolding of rbPrP (104–242). Kweon et al. [53] refolded CGTK10ase using SCX, the results indicated that refolded yield and specific bioactivity decreased drastically when pH was below 7, and they approached approximately 100% between pH 7 and pH 8.5, but its mass recovery almost remained constant between pH 6 and pH 8.5. Generally, mobile phase pH is selected in the range from 7 to 9 due to the fact that weak basic circumstances facilitate protein folding.

5.5. Additives

Additions of small molecular additives help proteins to refold into their native state; this method is also used in PFLC. Introduction of a detergent to a buffer for protein folding can reduce aggregates during refolding of exopolyphosphatase using IMAC [77]. Li et al. [40] found that inclusion of 50% glycerol in the refolding buffer could markedly improve the refolded yield of lysozyme. A suitable concentration of glycerol could also benefit the refolding of rhG-CSF using IMAC or SEC [30,80]. This is probably because the addition of glycerol favors the formation of correct refolded intermediates. The combination of PFLC and refolding additives can have an additive effect, and thus hasten the protein folding; therefore more experiments should be performed in the future.

5.6. Sample loading

Similar to protein folding by dilution method, the injected total mass of unfolded protein can also affect protein folding. It was found that the refolded yields decreased by increasing the initial protein concentration during the refolding of carbonate anhydrase and urokinase plasminogen activator by using SEC [23,26]. Guan et al. [74] obtained a similar result during the refolding of rhIFN- γ by using AFC. Li et al. [54] found that the bioactivity recovery decreased by increasing the injection mass during the refolding of lysozyme using IEC, the bioactivity recovery decreased from 100 to 62% as injected mass increased from 2 to 30 mg. Liu et al. [57] found that the bioactivity recovery increased gradually by increasing the initial protein concentration when the initial protein concentration was relatively low, but decreased by further increasing the initial protein concentration, this is consistent with the results obtained by Stempfer et al. [52]. Langenhof et al. [61] found that both refolded yield and mass recovery decreased by increasing the injection mass during the refolding of BSA using IEC. This is because the higher injected mass of protein would affect the adsorption of the denatured protein molecules by the stationary phase, thus resulting in increasing aggregation of the partially refolded protein molecules. Partial refolded intermediates also form at higher local protein concentration during elution process. But protein concentrations after elution and production efficiency would improve as injected protein mass increased; however, protein purity would decrease to a certain extent. Therefore, several factors, such as refolded yield, production efficiency and protein purity must be considered together when investigating injection mass.

6. Summary and future

PFLC is a newly developed method which enables many biochemical and physicochemical processes, and performs protein folding which was originally in normal buffer by liquid chromatography. It has many advantages compared with other methods for protein folding, but it is not as simple to operate as LC. Actually, many technical difficulties and theoretical problems must be overcome. Although the presence of the stationary phase in an LC system brings many advantages for protein folding, because the mechanism of protein folding carried out in the usual buffer has not been fully understood yet, much more complicated theoretical problems stemming from PFLC are required to be solved. Thus, PFLC is only at the starting point of its development cycle. However, because it has so many advantages, such as easy operation, high automatization, easy scale up, refolding of proteins at higher concentrations and also purifies target proteins simultaneously during protein folding, scientists may be encouraged to pay more attention to develop new theories and explore new technologies to facilitate this method. Thus, it can be expected that PFLC will have a very bright future. However, a lot of difficult problems are encountered during the folding of a denatured/reduced protein. It involves both thermodynamic problem (correct forming of many disulfide bonds existed in a protein molecule) and kinetic problem (rapid forming of disulfide bonds). An approach is to synthesize new chromatographic packing materials with good properties (having good capability of refolding and separating proteins) and low costs, such as to prepare stationary phases binding to a pair oxidized/reduced agents, such as glutathione in oxidation form (GSSG) and its reduced form (GSH) to make the forming of disulfide bonds during chromatographic process. Because ligands of many kinds of LC including HIC, IEC, AFC, have hydrophobic region, the presence of hydrophobic region of an amino acid sequence can help to form a correct microdomain, resulting in each thiol, or at least most of thiols approaching a right place for subsequential thiol pairing. If a suitable catalyst can be found to accelerate the oxidation of each thiol, whole refolding of the protein can be accomplished during the process of elution. If several thiols existed in a denatured/reduced protein, some of these thiols could be rapidly oxidized to form correct disulfide bonds on a column, because a corrected domain has thermodynamically stable structure, the remaining thiols cannot freely move and can be permitted to stand enough time to continuously perform oxidation, or refolding for several hours, even overnight after being eluted out of column. An appropriate regeneration method for these types of stationary phase to make the GSSG column regeneration should be established. It is also important to find out fast and low-cost disulfide pairing methods for industrial production, to avoid use of expensive agents, such as glutathione, molecular chaperones, and enzymes for protein folding. To find out more effective ways to dissolve the deposit formed by aggregates/precipitates on the packing bed, resulting in recovering the target protein and regenerating chromatographic column. Further broadening the applications of PFLC to various standard proteins and recombinant proteins in inclusion bodies, especially those that have large molecular weight or more than four disul-

vide bonds, and to investigate factors effecting PFLC to provide more data for the optimization of protein folding, to thoroughly develop PFLC for the industrial scale, to investigate and to summarize rules for scaling-up PFLC; to design a new and cheap equipment and to facilitate PFLC, and to combine PFLC and other refolding methods such as artificial molecule chaperones to develop new PFLC methods. We do believe that PFLC has a bright future.

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