Mechanism of simultaneously refolding and purification of proteins by hydrophobic interaction chromatographic unit and applications

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Abstract The hydrophobic amino acid residues of a denatured protein molecule tend to react with the particles of the stationary phase of hydrophobic interaction chromatography (STHIC). These hydrophobic interactions prevent the denatured protein molecules from aggregating with each other. The STHIC can provide high enough energy to a denatured protein molecule to make it dehydration and to refold it into its native or various intermediate states. The outcome not only depends on the specific interactions between amino acids, the structure of STHIC, but also depends on the association between the STHIC and mobile phase. The mechanism of protein refolding and the principle of its quality control by HPHIC were also presented. By appropriate selection of the chromatographic condition, several denatured proteins can be refolded and separated simultaneously in a single chromatographic run. A specially designed unit, with diameter much larger than its length, was designed and employed for both laboratory and preparative scales. That unit for the simultaneous renaturation and purification of proteins (USRPP) had the following four functions: to completely remove denaturant, to renature proteins, to separate renatured proteins from impurities, and to easily recycle waste denaturant. The efficiencies of refolding and purification of proteins by the USRPP are almost comparable to a usual long chromatographic column in laboratory. In preparative scale, USRPP can be easily, rapidly, and economically applied requiring a low pressure gradient. As an example, recombinant human interferon-γ is employed to elucidate the application of the preparative USRPP.

Keywords: protein folding, mechanism of protein folding, quality control, hydrophobic interaction chromatographic unit, purification, biotechnology, interferon-γ.

High performance hydrophobic interaction chromatography (HPHIC) was employed to simultaneously refold and purify human recombinant interferon gamma (rhIFN-γ) in 1991[1] and several other proteins in 1992[2,3] by HPHIC and size exclusion chromatography (SEC). SEC[4], ion exchange chromatography (IEC)[5] and affinity chromatography (AFC)[6,7] were employed for protein refolding in 1994. HPHIC has also been successfully developed for renaturing and purifying of HIV-1 protease[8,9].

Although there exist different mechanisms for the retention and/or refolding of proteins based on SEC and IEC, it is believed that the primary contributions of the stationary phases are to remove the denaturant, to adsorb protein molecules in the unfolded state, and to reduce and/or

prevent the aggregation of denatured protein molecules[10,11].

Because of a specific interaction between protein and AFC ligands, such as chaperone and/or chaperone systems[6,7,12,13], Ni^{2+}-chelating[14], and liposome[15], Altamirano et al.[12,13] applied a ternary refolding matrix (minichaperone/DSBA/PPI-aggarose) to renature the denatured and reduced scorpion toxin Cn5. Scorpion toxin Cn5 was obtained with 87% mass yield and recovered 100% bioactivity by this method but otherwise could not be refolded at all. This method was termed as a new kind of "LC-Refolding Chromatography"[12]. A review paper about the development of protein refolding by liquid chromatography (LC) was recently published[16].

From 1995, a series of papers about protein refolding with calling "artificial chaperones" in solution have been published[17—23]. The basic idea of the artificial chaperones is to imitate the chaperone action but to use low molecular weight as folding assistants to instead the real chaperones themselves. A detergent, such as polyethylene glycols (PEG), or modified PEG, linking to antibody, or antigen can firstly interact with denatured protein and form a complex to prevent from the aggregation of the unfolded protein molecules. The unfolded protein molecules are then released from the complex and finally to spontaneously refold in a suitable environment. Wickner et al.[24] recently reported that both chaperones and proteases recognized hydrophobic regions exposed on unfolded polypeptides. When molecular chaperones promoted proper protein folding and prevented protein aggregation, energy-dependent proteases eliminated irreversibly damaged proteins. For that reason, both of them could be used as quality control process for protein folding[24].

Based on the stoichiometric displacement model for retention (SDM-R) of proteins in HPHIC[25], the retention mechanism of proteins in HPHIC can be elucidated as that the strong hydrophobic interactions between the protein molecules and mobile phase at high salt concentration to push the protein molecules to contact the stationary phase of hydrophobic interaction chromatography (STHIC) with hydrophobic region and, at the same time, the hydrophilic region faces to mobile phase[2,25], while with the gradient elution running and the decreasing salt concentration in mobile phase, the adsorbed protein would be desorbed at low salt concentration owing to the decreases in push forces from the mobile phase. The retention model of proteins in HPHIC was derived by using eight chemical equilibriums and one of them is the formation of the complex between protein molecules and the ligands on the STHIC. When the protein molecules are adsorbed by the STHIC, a stoichiometric number of water molecules is squeezed out at the contact region between the protein and STHIC. This is the essence of the SDM-R of proteins in HPHIC[25].

Recently, Perkins et al.[26] measured the amount of water displaced from the contact region between protein molecules and STHIC. Employing theoretical and experimental methods, they were able to measure the amount of water instantaneously lost from the hydrophobic region of a protein molecule when it was adsorbed by the STHIC. The author and Regnier[27] recently also quantitatively measured the displaced methanol by insulin and found the effect on the displacement caused by the dynamics of mass transfer at the interface between two phases[28] in reversed-phase liquid chromatography. The main questions relate to how could the HPHIC simulta-

neously refold and separate proteins, and how could the HPHIC employ the quality control process of the protein refolding?

The principle of protein refolding by HPHIC was briefly elucidated by the previous paper[2]. The fact that the denatured proteins could be caught by the STHIC to prevent from the aggregation, or precipitation of the unfolded proteins lied a foundation of protein refolding by other LC methods, except SEC[16,29].

In the present paper, based on the principle of protein refolding by HPHIC in the previous paper[2], the mechanism of protein refolding by HPHIC was intensively investigated. To make this method for the renaturation with simultaneous purification enlarge to industry scale, a unit of simultaneous renaturation and purification of proteins (USRPP) was designed and tested by recombinant human interferon-γ (rhIFN-γ).

1 Experimental

1.1 Apparatus and materials

All chromatographic operations in analytical scale were carried out by an LC-10A high-performance liquid chromatograph (Shimadzu, Kyoto, Japan). A preparative chromatographic system (Waters Delta Prep 300, Millipore, USA) consisting of a pump unit (Waters), a system controller (Waters 600E), a dual-wavelength absorbance detector (Waters 2487) and a bench auto-balance recorder (Dahua instrument and gauge factory, Shanghai, China) was employed. The shells of the USRPP (10×200 mm I.D. and 10×300 mm I.D.) were made of stainless steel and the tops and bottoms of the both have radical solvent distributors made of plastics.

All of silica base-HPHIC packings (particle size, 6.5 μm; pore diameter, 30 nm; Lanzhou Institute of Chemical Physics, Lanzhou, China) were prepared and packed in both chromatographic column and the USRPP in our institute. The polymer base-column was synthesized in an empty stainless tube (50×8 mm I.D.) and called continuous rod column.

1.2 Chemicals

Cytochrome C (Cyt-C, horse heart, type III), myoglobin (Myo, horse heart), ribonuclease A(RNase A, bovine pancreas type I-A), lysozyme (Lys, chicken egg white), α-amylase (α-Amy, bacillus anthracis type IIA), α-chymotrypsin (α-Chy, bovine pancreas), and insulin (Ins, bovine pancreas) were purchased from Sigma Co.(St. Louis USA). Deionized water was prepared with Barnstead E-pure (Barnstead Co. Ltd. U.S.A). Other chemicals employed are of analytical grade.

Mobile phase in HPHIC consisted of solution A, 3.0 mol/L ammonium sulphate-0.050 mol/L potassium dihydrogenphosphate (pH 7.0) and solution B, 0.050 mol/L potassium dihydrogenphosphate (pH 7.0).

1.3 Preparations of standard protein solutions

The concentration of each native standard protein was 5.0 mg/mL in pure water. The partially

and fully denatured standard protein solutions were prepared to dissolve each protein in guanidine hydrochloride (GuHCl) solution with various concentrations and then standing for 24 h.

1.4 Preparation of rhIFN-γ extract

The method for producing the inclusion bodies of rhIFN-γ (pBV 220/DH5α) expressed by *E. coli* was taken from the doctorial thesis by Shen[30]. After *E. coli* were disrupted with buffer consisting of 20 mmol/L PBS + 1 mmol/L EDTA + 0.2 mg/mL lysozyme (pH = 7.4), the inclusion bodies were washed three times by the buffers: (i) 20 mmol/L PBS + 1 mmol/L EDTA + 2 mol/L urea + 1 mol/L NaCl buffer A: (pH = 7.4); (ii) 20 mmol/L PBS + 1 mmol/L EDTA + 0.5% Triton X-100 + 1 mol/L NaCl (pH = 7.4); (iii) 20 mmol/L PBS + 1 mmol/L EDTA + 1 mol/L NaCl (pH = 7.4), respectively. Finally, the inclusion bodies were dissolved in 7.0 mol/L GuHCl solution. After incubation at 4℃ for 24 h with full agitation, the supernatant of rhIFN-γ was obtained by centrifuging it at 20000 r/min.

1.5 Chromatographic procedure

The packed column, or USRPP was equilibrated with a new mobile phase, at least, for 15 min at each selected flow-rates before injecting a sample solution. The selection of flow rate for concentration gradient elution depended on the size of column or USRPP, and was detected at 280 nm. The eluted out fractions of the aim proteins were collected for the measurements of the bioactivity and mass recoveries. Based on the various sample sizes injected required in various circumstances, the final concentrations of each proteins in the collected fractions were different. The start and final concentrations for protein refolding by HPHIC were listed in the notes of figure and table in the paper.

1.6 Measurement of enzyme bioactivity

The measurements of enzyme bioactivity were followed the references as: lysozyme with *Micrococcus Cell*[31], α-amylase with starch[32], ribonuclease A with yeast RNA[33], and α-chymotrypsin with acetyl-L-tryrosineethyl (ATEE)[34]. The bioactivity assay for rh-IFN-γ was done by CPE inhibitor with WISH cell and VSV virus[2,35].

2 Results and discussion

2.1 Units for simultaneous renaturation and purification of proteins (USRPP)

A series of pictures of different units for simultaneous renaturation and purification of proteins (USRPP) were designed and are shown in fig. 1(a). These units having a shell of stainless steel and packed with various packings had the same length, or thickness (10 mm), but different diameters, ranging from 5.0 to 300 mm. These units could be used either as analytical purpose or preparative scales. Experiments were conducted to evaluate their resolution and ability to recover protein bioactivity data. Five standard proteins were used to test the units. Resolution of these protein samples by a preparative scale unit with a diameter of 200 mm is shown in fig. 1(b). While

the resolution of small solutes was determined by the length of the column, the resolution of biopolymers was not determined likewise[10,36]. Lysozyme and ribonuclease A, which had denatured by 7.0 mol/L GuHCl, were refolded by a series HPHIC columns with a length ranging from 5.0 to 150 mm. Their bioactivity recoveries are shown in table 1. A conclusion here is that the resolution and recovery of proteins are basically independent of column length.

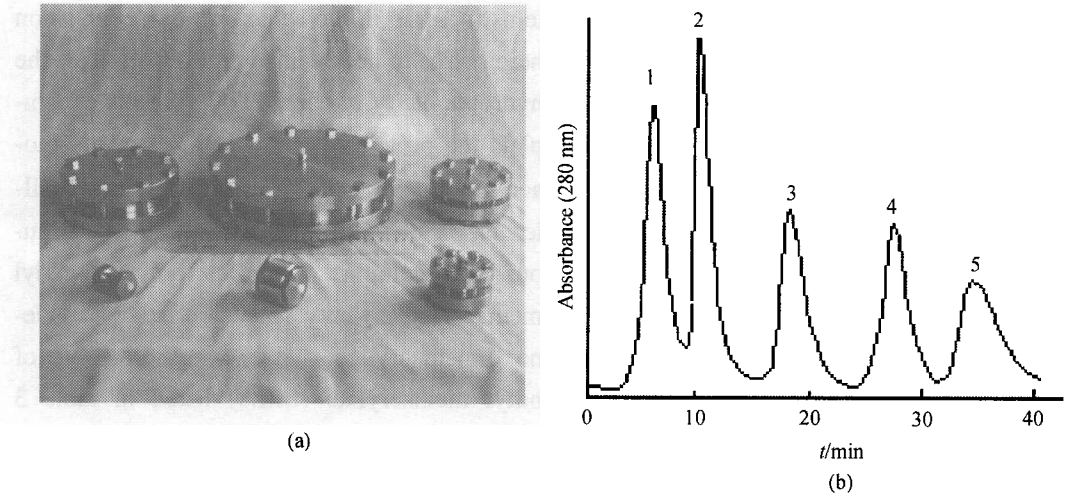

Fig. 1. Photo of the USRPP and chromatogram of five standard proteins. (a) Photo of a set of USRPP in laboratory and preparative scales; (b) chromatogram of five standard proteins separated by the USRPP with 10×200 mm I. D. sample size, 200 μL mixture solution containing total proteins 41.5 mg, consisting of cytochrome-C (5.0 mg) (1); myoglobin (7.5 mg) (2); lysozyme (1.5 mg) (3); α-amylase (7.5 mg) (4); insulin (20.0 mg) (5). The linear gradient elution was from 100% solution A [3.0 mol/L ammonium sulphate-0.05 mol/L potassium dihydrogenphosphate (pH 7.0)] to 100% solution B [0.05 mol/L potassium dihydrogenphosphate (pH 7.0)] at a flow rate of 100 mL/min for 40 min with a delay for 10 min. Chart paper speed, 2.5 mm/min; detection wavelength, 280 nm, AUFS: 0.05.

Table 1 Comparison of bioactivity recoveries of lysozyme and ribonuclease A with various column lengths[a)]

Column length/mm	5.0	10.0	25.0	50.0	75.0	100.0	125.0	150.0
Lysozyme (%)	98.0	95.9	95.6	93.7	97.5	95.3	93.2	97.3
Ribonuclease A (%)	94.9	88.6	88.6	85.8	91.5	88.6	87.3	89.2

a) 10 μL denatured lysozyme of 9.5 mg/mL and 20 μL ribonuclease A of 6.0 mg/mL by 7.0 mg/L GuHCl solution for 24 h were directly injected into the HPHIC column (linked ligands of polyethylene glycol 600) with various column lengths. Final concentrations: 0.063 mg/mL for lysozyme and 0.060 mg/mL for ribonuclease A.

One key advantage in using HPLC for protein refolding is to suppress the nonspecific interaction of partially, or completely fold, to reduce aggregation of protein molecules. In order to do so, the interactions between the protein molecules and the stationary phase have to be thermodynamically and kinetically favorable. Thermodynamically, protein adsorption to the stationary phase has to compete with protein aggregation. However, if the adsorption was too strong, it was difficult for a protein to be eluted out from the column. From our observations, kinetically, adsorption was always faster than aggregation, anyway.

2.2 Hydrophobicity of the STHIC

The effects of the hydrophobicity of four kinds of STHIC on the completeness of lysozyme and ribonuclease A refolding are indicated in table 2. The chromatograms of the lysozyme by lin-

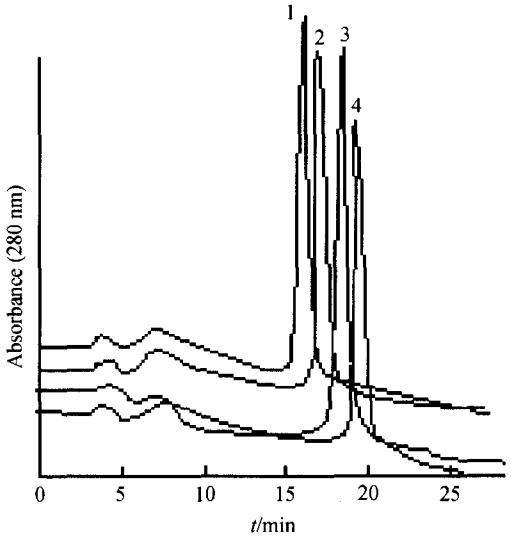

Fig. 2. Chromatograms of lysozyme from four kinds of HPHIC with various hydrophobicities columns (4.0 mm I.D. ×100 mm). 1, PEG-400; 2, PEG-600; 3, PEG-800; 4, phenyl group. Except flow rate of mobile phase being 1.0 mL/min, the other chromatographic conditions are the same as that shown in fig. 1(b). AUFS: 0.08. Sample size: 20 μL 5.0 mg/mL lysozyme and final concentration in collected fraction was 0.10 mg/mL.

ear gradient elution with the four columns are shown in fig. 2. The hydrophobicity of their end groups increases as the following order: PEG-400 < PEG-600 < PEG-800 < phenyl group. The bioactivity recoveries of both proteins increase in the same order. A conclusion here seems to be obtained that the stronger the hydrophobicity of the STHIC, the more complete the protein refolding. However, the bioactivity recoveries of α-Amy refolding by silica based polyglycol (PEG)-600 and continuous rod columns made of poly (glycidyl methacrylate-co-butyl methacrylate-co-ethylene dimethacrylate) with various contents of butyl methacrylate (BMA) listed in table 3 show a contrary result. The Arabic numbers 5, 11, and 17 represent the contents of butyl methacrylate in the packings, respectively. The more the BMA, the stronger the hydrophobicity. The order of increasing hydrophobicity was as follows: PEG-600 < HICP-5 < HICP-11 < HICP-17.

Because column HICP-5 is more hydrophobic than that of PEG-600, HICP-5 has the higher

Table 2 Bioactivity recoveries of refolded lysozyme and ribonuclease A increasing with moderate hydrophobicity of STHIC[a)]

No.	Ligands	Lysozyme(%)	Ribonuclease A(%)
1	-(CH_2-CH_2-O)400	85.8	83.4
2	-(CH_2-CH_2-O)600	88.5	85.8
3	-(CH_2-CH_2-O)800	90.5	93.6
4	-O-CH_2-O-phenyl	98.5	101.1

a) The 20 μL solutions of the denatured Lys and RNase A with 5.0 mg/mL in 7.0 mol/L GuHCl solution were directly injected into the four kinds of HIC columns, respectively. Final concentrations: 0.10 mg/mL for lysozyme and 0.050 mg/mL for ribonuclease A.

Table 3 Bioactivity recoveries of refolded α-amylase decreasing with the increasing in the hydrophobicity of STHIC[a)]

Columns	C_{GuHCl}/mol • L^{-1}			
	0.2	0.4	0.6	0.8
DXF-600	118.0	108.0	39.0	0.00
HICP-5	105.4	106.8	104.0	96.2
HICP-11	86.7	89.0	84.4	44.3
HICP-17	–	–	–	–

a) PEG-600, polyglycol-600; HICP, hydrophobic interaction chromatographic packings with polymer consisting of poly(glycidyl methacrylate-co-butyl methacrylate-co-ethylene dimethacrylate); Arabic numbers 5, 11, and 17 represent the contents of butyl methacrylate in the packings, respectively. Sample size: 20 μL of 5.0 mg/mL α-Amy in 7.0 mol/L GuHCl solution. Final concentrations: 0.050—0.034 mg/mL. –, α-Amylase was not eluted.

bioactivity recovery of α-Amy. That is, the result is the same as that shown in table 2. However, with the continuously increasing the hydrophobicity or the content of BMA of the STHIC, the bioactivities of α-Amy decrease until to zero for any concentration of GuHCl solution with HICP-17. Because the hydrophobicity of the HICP-17 column is as strong as that of a column of reversed-phase liquid chromatography, the native α-amylase is fully denatured by the HICP-17. A reasonable conclusion is that the STHIC must have a moderate hydrophobicity for protein refolding.

It should be pointed out here that the bioactivity recovery of the refolded α-Amy by the stationary phase packings DXF-600 was found to be 118%. It seems unreasonable. This is actually true, because the 100% bioactivity of α-Amy was taken from the native α-Amy that had been storied for over two years and partly lost bioactivity and it was renatured by HPHIC method in this study. Altamirano et al.[13] reported similar result that they made a sample that had lost all activity on storage in solution for five years and regained 100% activity by affinity chromatography.

2.3 Ligand structure of STHIC

The chromatograms of four standard proteins obtained from three ligands of PEG-600, tetrahydrofurfuryl alcohol (THFA), and phenyl group, as shown in fig. 3, are identical to each other.

The retention times of linear gradient for α-Chy are 23.44, 23.55, and 23.72 min, respectively. It means that the three kinds of columns have the same, or at least, a comparable hydrophobic strength. However, the bioactivity recoveries of the renatured α-Chy which had originally been denatured with 1.70 mol/L GuHCl are 94.4%, 82.1% and 65.3%, respectively. This fact can only be explained by the different kinds of ligands of the STHIC. In other words, compared with the ligand PEG-600, that of THFA is more favorable for α-Chy refolding.

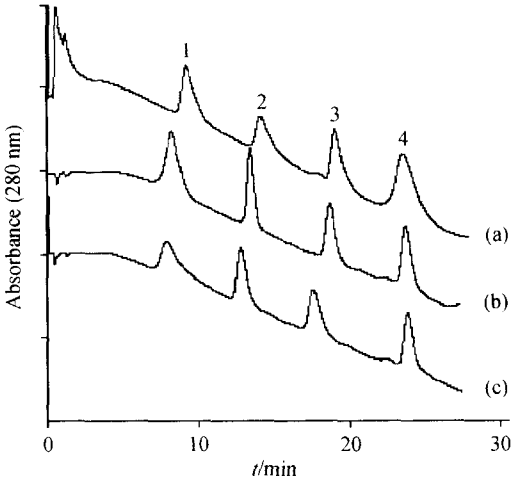

Fig. 3. Chromatograms of four proteins from three kinds of HPHIC with a comparable hydrophobicity but different ligands. (a) PEG-600; (b) THFA; (c) phenyl group. 1, Cytochrome-C; 2, myoglobin; 3, lysozyme; 4, α-chymotrypsin. AUFS: 0.08. The concentration of each protein was 5.0 mg/mL. The absolute amounts of them are: 40, 30, 15 and 30 μg for 1, 2, 3, and 4 and their final concentrations are 0.133, 0.100, 0.075 and 0.10 mg/mL, respectively. Except flow rate of mobile phase being 0.8 mL/min, the other chromatographic conditions are the same as that indicated in fig. 2.

Based on the above discussion, it is concluded that an STHIC with a suitable hydrophobic strength and ligand structure to the hydrophobic amino acid residues of the unfolded protein molecules would greatly facilitate protein refolding. We term this method as "hydrophobic interaction-refolding chromatography (HIRC)".

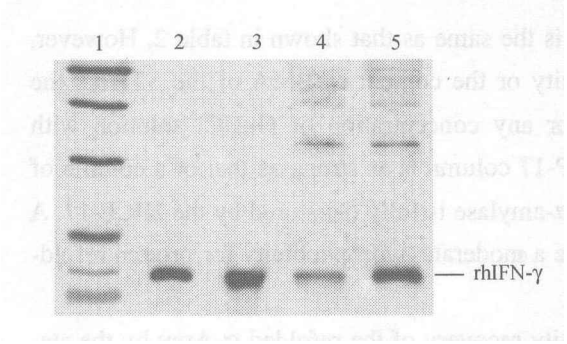

Fig. 4. SDS-PAGE (silver dying) of rhIFN-γ elutes in different stages. 1, Marker (14400, 20100, 31000, 43000, 66200, 97400 Da); 2 and 3, collected fractions of the renatured and purified rhIFN-γ in the range of 90—180 and 0—90 min after going through a chromatographic pie of 10×300 mm I.D.; 4, fraction of rhIFN-γ after break-though volume of sample; 5, extract of rhIFN-γ by 7.0 mol/L guanidine hydrochloride before chromatography.

2.4 Refolding with simultaneous purification of the recombinant human therapeutic recombinant human interferon-γ (rhIFN-γ) by the USRPP(10 mm×300 mm I.D.)

The unit of simultaneous renaturation and purification of proteins (USRPP) of 10 mm×300 mm I.D. was firstly equilibrated with a selected mobile phase consisting of 60% solution A and 40% solution B at the flow rate of 100.0 mL/min, and then 700 mL rhIFN-γ extract in 7.0 mol/L GuHCl solution containing total proteins of 2.04 g under the condition of a suitable ratio of mobile phase to sample solution was continuously pumped into this USRPP. After eluting out some impurities, at least 20 min, by isocratic elution, a linear gradient was done from 60% solution A to 100% solution B for 90 min with 90 min delay. The two collected fractions from 0—90 and 90—180 min of the renatured and purified rhIFN-γ were desalted twice for testing its purity and bioactivity. From fig. 4, the purity of rhIFN-γ tested by SDS-PAGE[19] was over 95% and the specific bioactivity was over 5.7×10^7 IU/mg, which coincides with the criteria of rhIFN-γ for Chinese Biological Products in terms of the purity and specific bioactivity. However, the renatured and purified rhIFN-γ can still not be suitable for a drug, because it has to be desalted further. Comparing the total bioactivity of the recovery of the rhIFN-γ in 7.0 mol/L GuHCl solution before and after the sample injected into the hydrophobic interaction refolding chromatogram method, the total bioactivity recovery of the rhIFN-γ is raised over 62 folds after the HPHIC. Compared with the usual elution method for the bioactivity recovery of protein renaturation being 5%—20%, the renaturation efficiency by using this hydrophobic interaction pie is a really powerful tool for protein renaturation.

Besides the above advantages, the low mass recovery has to be considered. Because the slight solubility and high hydrophobicity of the denatured interferon-γ in mobile phase, when the denatured rhIFN-γ in the exact of the 7.0 mol/L GuHCl solution contacts the mobile phase containing ammonium of sulphate with high concentration, a little precipitate of the rhIFN-γ forms either on the filter surface of the inlet, or on the top of the column bed. Although the precipitate is hard to re-dissolve in the mobile phase for its renaturation, in Chinese words, "very done meats that is still in pot". Anyhow, the precipitate still stays on the USRPP and would be re-dissolved with next chromatographic run. In case of the precipitate accumulating too much in it, it can be washed out by GuHCl, or urea solution and to do renaturation with the USRPP again. Thus, the mass loss of rhIFN-γ can be recovered in this way.

In addition, compared with the down-stream technology of recombinant human therapeutic proteins, in which it usually takes several days and several steps to obtain a qualified biological product that only has 5%—20% bioactivity recovery, the presented method in this paper is really fascinating.

The USRPP, as shown in fig. 5, simultaneously has four functions as follows: to completely remove denaturing agent, to renature several proteins, to separate the renatured proteins from impure proteins, and to easily recycle waste denaturing agent. This is really a dream of separation scientist for a long time.

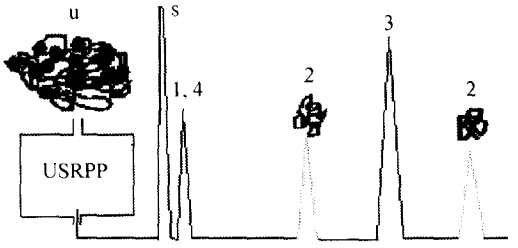

Fig. 5. Scheme of four functions of "unit of simultaneous renaturation and purification of proteins (USRPP)". 1, Denaturant solution; 2, refolded protein; 3, impure proteins; 4, recycling waste denaturant.

2.5 Mechanism of protein refolding by HPHIC

It is well known that, in a solution with a high salt concentration, any nonpolar particles tend to be squeezed out of the solution due to hydrophobic interactions (HI) between the nonpolar particles and the aqueous salt solution. Thus, the hydrophobic region of a protein molecule is pushed by HI and seeks to contact the hydrophobic region of the other particles. The same thing is true for an unfolded protein molecule adsorbed by STHIC. Because a specific STHIC only has a moderate and fixed hydrophobicity, only a small number of hydrophobic regions from different protein molecules could possibly be adsorbed by STHIC, due to their hydrophobicities and steric effect. Thus, the STHIC may select which hydrophobic amino acid regions to form microdomains of protein molecules. Different microdomains may represent native states and various intermediate states of the refolded protein molecules adsorbed by the STHIC. Fausnaugh-Pollitt et al.[37] reported that chromatographic retention in HPHIC is determined by amino acids on a single surface of the protein opposite to its catalytic slit. Thus, a correctly refolded protein should have the same retention time as that of its native states.

Protein refolding which is accomplished by the protein originally denatured by GuHCl, or urea solution to the polypeptides is injected in an HPHIC column and eluted with specific gradient elution. With rapidly and completely removing the denaturants, the polypeptides would be firstly adsorbed by the STHIC at a high concentration of salt and then desorbed at low concentration of salt to refold into native state and various intermediate states of the protein. Different refolded products would be further separated by the STHIC. If the fraction eluted from the STHIC is collected at the same retention time as that of the native protein, the purely and correctly refolded protein would be obtained, resulting in separating from its intermediates. Therefore, STHIC behaves as an important role for quality control of protein refolding. It is believed that the quality control for protein refolding by the STHIC mainly depends on the matching between the hydrophobic strength, ligand structures of STHIC, and the hydrophobic region of the polypeptides.

Some similarities in terms of the actions for protein refolding between STHIC and "artificial

chaperone" method exist. First, the STHIC having ligands consisting of the PEG modified by a hydrophobic group[25] can interact with the unfolded protein molecules and form complex of protein-ligands to prevent from the formation of aggregation, or precipitate of the protein. Second, the unfolded protein molecules can be released from the complex by adding other chemicals, or changing the environment to refold. In addition, the STHIC also acts in a similar manner to molecular chaperones for hydrophobic interaction and also for the quality control of protein refolding. Furthermore, the STHIC has more contributions to protein refolding. It is reported[38] that when a component transferred from solution to a solid surface, a chemical potential jump occurs. When unfolded polypeptides are adsorbed by STHIC, they would obtain energy from the STHIC. Energy provided by STHIC was recently reported to be as high as (838 ± 36) kJ/mol at GuHCl concentration being 2.8 mol/L for the unfolded α-amylase[39]. Comparing with the usual folded energy of a protein molecule in a buffer solution, 2—20 kJ/mol, the STHIC indeed provides much higher energy to the unfolded protein molecules. When the concentration of the GuHCl is 7.0 mol/L, the STHIC can provide even more energy to the unfolded α-Amy than what was reported[39].

From the above-mentioned, it is clear that a specific STHIC combines the advantages of both molecular chaperones and proteases[24]. The STHIC can provide high enough energy at molecular level to unfolded protein molecules and at the same time pay a role for quality control simultaneously during one chromatographic run. The reason for STHIC providing much higher energy during the adsorption process of the protein molecules can be stated as follows:

First, although it was reported that the interior of some protein molecules contains water and contributes to the molecularly conformational stability[40], the interior of the most native protein molecule has no free water. On the one hand, when a protein molecule is denatured, the protein molecule must be unfolded. The unfolded protein molecule, as shown in Plate (a), would take place hydration in an aqueous salt solution. Water molecules (blue color) surrounding the unfolded protein molecule make it more stable. So, thermodynamically, energy is released in the hydration process of an unfolded protein molecule to lower its potential energy corresponding to the decreases in entropy of water in the state of hydration form. On the other hand, dehydration of the hydrolyzed protein molecule corresponding to the increases of water in the original existence as hydrolyzed form occurred spontaneously the increases in entropy of water as the denatured environment is removed, even though this process usually is processed at a very slow rate. When an external energy is added to a soluble protein molecule, the dehydration process would be accelerated. When an STHIC adsorbs a protein molecule on its surface, it facilitates dehydration of the protein molecule at a relatively rapid speed. Therefore, STHIC must provide energy to the protein molecule during this process. The schematic model of the dehydration process of a hydrated protein molecule on an STHIC surface is also shown in Plate (a). From Plate (a), it is seen how are the water molecules in the hydrated state squeezed out from the contact region between the unfolded protein molecule and the STHIC surface[25,26].

Second, it was well known that a hydrophobic packet of a native protein molecule is buried inside the molecule. It is reasonable to believe that protein refolding should start by forming this inner hydrophobic packet. Thus, the formation of a structurally correct hydrophobic inner region that consisted mostly of the hydrophobic side chain of the amino acid is very important. In HPHIC, unfolded protein molecules are adsorbed on the surface of an STHIC, with hydrophobic amino acids binding to the STHIC. These high density hydrophobic amino acid residues could potentially function as the inner hydrophobic packet needed for the protein molecule to build on to form its correct 3-D structure. It is also possible that incorrect 3-D structure of the protein molecule can also be formed. This process of the formations of the microdomains and intermediates of the protein is schematically illustrated in Plate (b). The hydrophobic amino acid residue (red color) binds to the STHIC surface and the hydrophilic amino acid residues (yellow color) face to the mobile phase. The unfolded protein molecules bind very tightly on the surface of the STHIC to form a stable complex so that even though there are a few hydrophobic regions left in other regions of the protein molecules, the protein molecules tightly binding to the STHIC could not interact with each other. As a result, protein molecules aggregation do not occur at this stage. Because the peptide backbone is rigid, as shown in Plate (b), energy provided by STHIC can propagate along the peptide chain to the other amino acid residues of the unfolded protein molecules. Thus, amino acids residues which originally do not contact the STHIC due to the interactions among those amino acid residues are able to be dehydrated. It is believed that the process of forming microstructures is accompanied by the dehydration process[41].

When unfolded protein molecules in hydrated state (Uh) are injected into the HPHIC column, as shown in Plate (c), most of the protein molecules are adsorbed and dehydrated (green color, 1a) simultaneously. Because the partition coefficient of any protein is not infinite, only a very small amount of the protein is still in the mobile phase and it would slowly become dehydrated (black color, 1b) also as normally happens in a solution. For those adsorbed and unfolded protein molecules in the dehydration state, some would form intermediates which may contain correct hydrophobic configuration (blue and green colors, 2a), or some of them may contain the wrong hydrophobic configuration (black, 2a). Because those intermediates would stick tightly to the STHIC surface, the protein molecules with correct hydrophobic configuration cannot continue to fold to its native state, whereas protein molecules with wrong hydrophobic configuration cannot be corrected by the STHIC alone.

Third, continuously changing constituents of the mobile phase in HPHIC helps restore the unfolded protein molecules to their native states. Different evolution steps of protein refolding are also illustrated in Plate (c). Under a gradient elution from a high salt concentration to a low salt concentration with pH = 7.0, both correct and incorrect intermediates in the adsorbed state would be desorbed by the mobile phase at different retention times. The correct, thermodynamically stable intermediates may continue to fold into their native states under suitable mobile phase conditions. However, the incorrect intermediates, thermodynamically unstable, must disappear very

quickly in the mobile phase and become random polypeptide coil (2b). The random coil, such as the protein refolding usually in a solution, can further form other correct, or incorrect, intermediates (3b) in the mobile phase.

All the native state protein molecules and other intermediates having correct and incorrect hydrophobic regions would be re-adsorbed by the STHIC. As shown in 3a of Plate (c), the foregoing process is repeated many times. As a result, more native state protein molecules (blue color) would be generated with time, and fewer molecules with incorrect hydrophobic configuration would remain (black color, 3b). In the end, most protein molecules would refold properly as shown in 4a and 4b of Plate (c). Different protein molecules would need a very specific, or a narrow range of salt concentration to refold. A gradient elution is able to successfully refold several kinds of protein molecules by means of the foregoing manner at one run.

The conclusions of the associations between the STHIC and the mobile phase employed are that (i) the adsorbed unfolded protein desorbs as the decreases in salt concentration, or increases in water concentration, (ii) some incorrect microdomains of the protein are corrected together with the mobile phase employed by adsorption and desorption for many times, (iii) the separation of completely refolding of the protein and in case of some intermediates with incompletely refolding of the protein can be accomplished.

In addition, as long as the unfolded protein molecules are fully refolded, or even partially refolded, the specific elution salt concentration range in the mobile phase employed for them must be the same as their corresponding protein molecules in native state. This characteristic indicates that one chromatographic run may involve the simultaneously refolding and separation of different protein molecules.

In conclusion, the mechanism of protein refolding and the principle of the quality control for protein refolding by HPHIC can be elucidated as follows: The unfolded protein molecules are pushed by hydrophobic interaction force from mobile phase at high salt concentration to move forward to an STHIC and tightly contact the STHIC with a nonpolar 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 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 hydrated state from both the hydrated unfold 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 decreases in salt concentration, or the increase in water concentration in the mobile phase. The protein molecules with incorrect microdomains would be corrected by their spontaneously disappearing in the mobile phase due to their unstable thermodynamics. With the adsorption and desorption of the protein for many times during gradient elution, the incorrect microdomains would be getting less and less, while the protein molecules with correct microdomains would be getting more and more,

resulting in the protein to accomplish completely refolding. The completely refolding of the protein can be separated from that in case of some stable intermediates, or the incompletely refolding of the protein. Several unfolded proteins can be simultaneously refolded and, at the same time, to accomplish the separation of those protein each other.

It can be also pointed out that there are cases observed in which the random coil of the protein molecules in the mobile phase may be aggregated before being re-adsorbed by the STHIC. In this case, the protein molecules could not be refolded at all; or at least, only partially refolded by the HPHIC. In addition, if the STHIC did not provide high enough energy to the unfolded protein molecules, or the stereo structure of the ligands of the STHIC is not very comparable with the target-protein, the protein molecules cannot totally (or only partially) refold. Furthermore, if the STHIC provides too high energy to the unfolded protein molecules, some other stable, intermediates which may not exist in nature may form.

There are some more issues that need to be addressed here. The formation of aggregates may occur during sample injection and/or before protein molecules contacting the stationary phase when the sample solution is mixed with the mobile phase. If aggregation and/or precipitation forms before the protein molecules contact the stationary phase, it may occur either in sample loop, or on the top of the HPLC column. As a result, the recoveries of both mass and bioactivity of the target protein would be compromised. In addition, the column could also be blocked. This is especially important in the renaturation and purification of some recombinant therapeutic proteins which are very hydrophobic. Because of the initially very high salt concentration employed in HPHIC (2.5—3.0 mol/L ammonium sulphate solution), some precipitates would occur, especially in the preparative scale HPHIC. In order to reduce the time of sample staying in a sample loop, and the time sample being transferred from the sample loop to the column, a lower dead column volume and a higher flow rate were desired. As the above-pointed, by using the USRPP with much bigger diameter and shorter length we made, this problem can be solved, because the precipitate of proteins only blocks a very small area, i.e., 1/100 of total cross section area either on the filter outside the column, or on the top of the stationary phase inside the HPLC column. As a result, the increases in the back pressure of the USRPP would not be usually observed. The formed precipitate can be dissolved by suitable solution including denaturant solution. The unfolded protein from the precipitate dissolution can be refolded by the HPHIC again.

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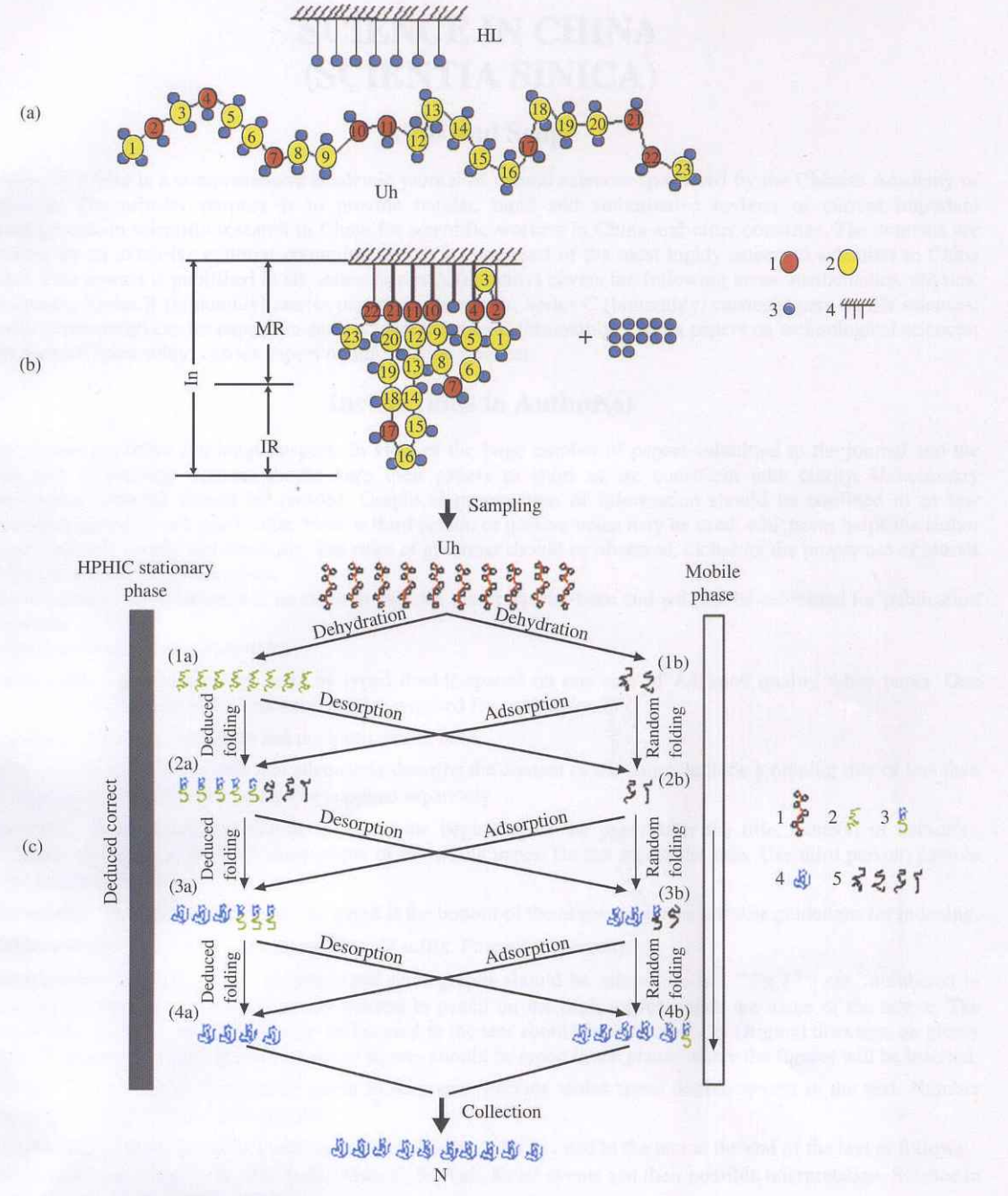

(a) and (b) Schematic model of dehydration and formation of protein intermediates in HPHIC. (a) Hydrated protein and HPHIC stationary phase, (b) formation of intermediate containing microdominate and deduced region. 1, Hydrophobic amino acid residues; 2, hydrophilic amino acid residues; 3, water molecules; 4, ligands of HPHIC stationary phase brush. HL, Hydrated ligands; Uh, unfolded hydrated-amino acid sequence; MR, microdomain region; IR, induced region; In, intermediate. (c) Schematic model of steps for the association between HPHIC stationary and mobile phases for proteins refolding. a, Refolding on the STHIC; b, random refolding in mobile phase. 1a and 1b, Dehydrated unfold protein molecules in adsorbed and desorbed states, respectively; 2a, correct (blue and green colors) and incorrect (black color) intermediates formed on STHIC; 2b, intermediates formed in solution with random folding; 3a, native protein (blue color) and correct intermediates (blue and green colors) on the STHIC; 3b, native protein refolding in mobile phase (blue color) and correct intermediates (blue and black colors), and wrong intermediates (black color); 4a and 4b, protein completely refolding on the STHIC and in the mobile phase, respectively. 1, Hydrated-unfold protein; 2, dehydrated-unfold protein; 3, intermediates containing correct microdominates and deduced structures; 4, correctly refolded protein; 5, wrong folded intermediates.