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Refolding and purification of interferon-gamma in industry by hydrophobic interaction chromatography

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Abstract

A new technology for renaturation with simultaneous purification of the recombinant human interferon-γ (rhIFN-γ) in downstream of biotechnology is presented. The strategies to develop the new technology in industry scale were suggested. Based on chemical equilibrium and molecular interactions, the principle of rhIFN-γ refolding by HPHIC was described. The kind of stationary and mobile phases were evaluated and found the former to contribute to the rhIFN-γ refolding more than the latter. The extract containing the rhIFN-γ in gram scale in 7.0 mol L^{-1} guanidine hydrochloride solution of 700 mL was directly pumped into a unit of simultaneous renaturation and purification of proteins (USRPP, 10 × 300 mm i.d.) packed by small particle packings of hydrophobic interaction chromatography and a satisfactory recovery of bioactivity and mass of the rhIFN-γ was obtained. With flow rate 100 mL min^{-1} and a gradient elution by only one step in 4 h, the purity and specific bioactivity approach to 95% and 8.7×10^7 IU^{-1} mg, respectively. To evaluate the goodness of the presented new technology in this study, a usual method with the renaturation by dilution method firstly and then purification with a series of LC in literature was employed to compare with each other. The obtained result in terms of purity, recoveries of mass and bioactivity, cost time as well as expenses, the former is much better than the latter. Comparing the total bioactivity of rhIFN-γ in the extract before to that after the renaturation by the USRPP, the total bioactivity of rhIFN-γ increased 62-fold.

Keywords: Biotechnology; Human interferon-γ; Protein refolding; Purification technology; High-performance hydrophobic interaction chromatography; Unit of simultaneous renaturation and purification of proteins (USRPP)

1. Introduction

The general strategy used to recover active protein from inclusion bodies involves three steps: inclusion body isolation and cleaning; solubilization of the aggregated protein; and refolding of the solubilized protein.

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The efficiency of the first two steps can be relatively high. However, the renaturation yields may be limited by the accumulation of inactive misrefolded species as well as aggregates. The challenge is how to convert the inactive and misfolded inclusion body proteins into soluble bioactive products, or protein refolding, specially in industry scale (Lilie et al., 1998; Eliana et al., 1998, 2001; Misawa and Kumagai, 1999). The usually employed methods for the renaturation of inclusion body proteins include dilution method and buffer-exchange method, such as dialysis, diafiltration. Because of its simplicity, dilution of the solubilized protein is directly put into a renaturation buffer and is the most commonly used method. The main disadvantages of dilution refolding for commercial applications require larger refolding vessels and additional concentration steps after renaturation. Buffer exchange to remove high concentration denaturant can be accomplished by diafiltration (Varnerin et al., 1998) and dialysis (West et al., 1998) using ultrafiltration membranes.

Ten years ago, one of the authors firstly reported high-performance hydrophobic interaction chromatography (HPHIC) and size exclusion chromatography (SEC) to be a powerful tool for protein refolding (Geng et al., 1991, 2001; Geng and Chang, 1992; Guo and Geng, 2000). Several standard proteins and recombinant human interferon-γ (rhIFN-γ) were used as examples to test it. Two years later, gel filtration chromatography (Werner et al., 1994), ion-exchange chromatography (IEC) (Suttnar et al., 1994), affinity chromatography (AFC) (Phadtare et al., 1994; Taguchi et al., 1994) were also reported. Many new investigations in this field (Ala et al., 1998a,b; Creighton, 1985; Gu et al., 2001) and two review papers for the recent developments of renaturation with simultaneous purification of proteins by LC were reported (Guo and Geng, 2000; Geng et al., 2001). However, no report has been found about protein refolding in industry scale by LC.

Although there exist different mechanisms for the retention and/or refolding of proteins based on SEC, HPHIC, AFC, and IEC, it is commonly 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 from the aggregation of denatured protein molecules (Taguchi et al., 1994; Ala et al., 1998a,b; Creighton, 1985; Gu et al., 2001; Stempfer et al., 1996).

For the protein refolding by LC, a big problem is that the formation of aggregates may occur during sample injection. If it does, the back pressure of column will increase significantly, even block the column. In addition, the recoveries of both mass and bioactivity of the target protein would decrease. If we still want to work in the circumstance, it would be favorable to employ a very short column.

A question may be raised that how is about the resolution and the bioactivity recovery of proteins by using a very short column? When biopolymers are separated by HPLC, many researchers have found column length almost having no influence on the resolution (Liu and Geng, 1999; Moore and Walters, 1984) and qualitatively explained this fact with stoichiometric displacement theory for retention (SDT-R) (Geng and Regnier, 1984; Liu, 1999). Based on the SDT-R, Tennikov et al. (1998) elucidated this phenomenon as an ‘on-off’ mechanism for the good resolution of protein by an ion-exchange disc or membrane of 2 mm in thickness. Also based on the SDT-R, Belenkii et al. (1993) further proposed an ‘all or nothing principle’ for protein retention on a very short column.

One of the authors have designed a unit of simultaneous renaturation and purification of proteins (USRPP) in both laboratory and preparative scales of only 10 mm in thickness but with diameter ranging 10–500 mm (Liu, 1999; Liu and Geng, 1997). The obtained recoveries of bioactivity and mass of proteins by using the USRPP can be comparable to usual chromatographic column. With gradually increasing the diameter and keeping its thickness as 10 mm, the shape of this kind column does not like a usual chromatographic column, but like a ‘cake’, and thus it is also called chromatographic cake.

If the extract of the recombinant human therapeutic proteins by guanidine hydrochloride (GuHCl) solution contacts with the mobile phase to form precipitates and to block the HPHIC column, the formed precipitates only block a very small fraction of the frits with much greater cross-section area on the top of the USRPP, not resulting in an increase in the back pressure of the USRPP significantly.

In this study, we try to simplify the production technology of the rhIFN-γ produced by *Escherichia coli* in downstream of biotechnology by using HPHIC cake in industrial scale and to explain the principle for protein refolding by HPHIC. With an optimizational

experimental condition, the results obtained by the new technology presented in this study and that by traditional one were also compared. The new technology with the USRPP by one step only in 2–4 h was found to be much better than that by usual four steps and taking 44 h.

2. Principle of protein refolding with simultaneous purification by LC

In terms of the operation, the protein refolding and usual chromatographic separation of proteins by LC is basically the same. The extract containing the aim protein in 7.0 mol L^{-1} GuHCl or 8.0 mol L^{-1} urea solution is directly injected into a suitable chromatographic column, or chromatographic cake and then the fraction containing the renatured aim protein is collected. However, the principle of the former is significantly different from the latter. From the standpoint of view of the molecular interactions, two of the authors recently reported the mechanism of simultaneously refolding and purification of proteins by HPHIC (Geng and Bai, 2002). In terms of chemical equilibrium, usual chromatographic separation only depends on the partition coefficient of protein in two phases, while protein refolding in buffer is dominated by the competition between monomer of protein in its unfolded state and a series of polymers and/or precipitates of the proteins. In other words, protein refolding in buffer involves a series of chemical equilibria. Protein refolding by LC, however, is the combination of the both. The contributions of stationary and mobile phases, as well as their association, make the series chemical equilibrium in chromatographic system move to forward its monomer in its unfolded state, which can be adsorbed by stationary phase. And then, as shown in Fig. 1, the unfolded protein molecules can be refolded to their native state by HPHIC by means of the following six steps: (1) the unfolded protein molecules (green) in mobile phase (MP) are pushed by hydrophobic interaction force from mobile phase at high salt concentration to move forward to stationary phase of hydrophobic interaction chromatography (STHIC). (2) Tight contact to STHIC with a non-polar region of amino acid residues to form stable complex and the hydrophilic parts of the unfolded protein molecules face the mobile phase. The unfolded protein molecules in this circumstance cannot aggregate. The unfolded protein molecules take high enough energy at molecular level from the STHIC and simultaneously carry out three functions (Geng et al., 2000; Giddings, 1991): (a) the STHIC can recognize a specific hydrophobic region of a polypeptide (Fausnaugh-Pollit et al., 1998) which would be favorable to refold the protein correctly, or play a role of quality control for protein refolding; (b) squeezing out water molecules (blue) in dehydrated (DH) state from both the hydrated (HY) unfolded protein and the STHIC (Geng et al., 1990; Perkins et al., 1997) and thus accelerate the dehydration process; (c) the microdomains of the protein molecules on the STHIC are formed because of the interactions among non-polar amino acid residues and non-polar groups of the STHIC (Geng and Bai, 2002). The formed microdomains may be correct (yellow) or incorrect (black). The correct microdomains would further fold into their native state and would be the inner hydrophobic packet of the native protein molecules. (3) The correct and incorrect microdomains of the protein desorb from the STHIC to convert into their corresponding intermediates (correct or incorrect) as the decreases in salt concentration, or the increases in water concentration in the mobile phase. The correct intermediates spontaneously fold into their native state (red), because it has thermodynamically stable state, while that with incorrect microdomains would either spontaneously disappear and convert into their unfolded state (green) in the mobile phase due to their

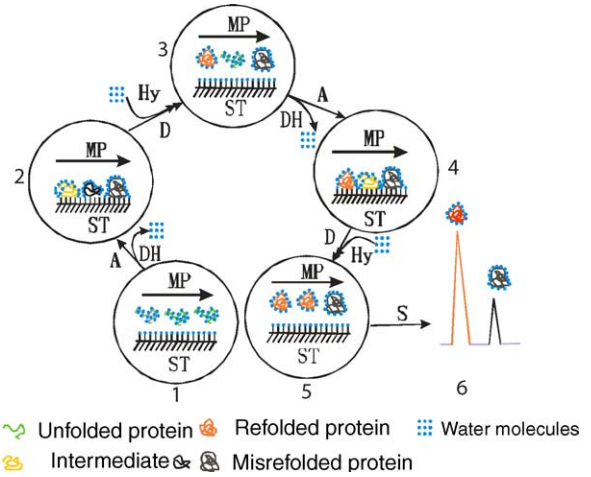

Fig. 1. Scheme of the refolding mechanism of denatured protein with HPHIC.

unstable thermodynamics, or form some very stably incorrect intermediates of the protein and thus may never disappear (black). (4) Each species of the protein will be adsorbed by the stationary phase again. Step 4 is different from step 2 is that the latter only has many kinds of microdomains, while the former has completely refolded protein (red), correct microdomains (yellow), and a few unfolded states (black) of the proteins. (5) Likewise, with the adsorption and desorption of the protein for many times during gradient elution, the unstably incorrect microdomains and/or intermediates would be getting less and less, while the protein molecules with correct microdomains and/or intermediates would be getting more and more, resulting in the protein to accomplish refolding completely. (6) The completely refolding of the protein is just the protein in its native state and both have the same retention (red peak) and it can be separated from that in case of some stably wrong intermediates (black peak), hence also playing a role of quality control for protein refolding in this step. In addition, several unfolded proteins can select their suitable environment condition (composition of mobile phase) by themselves with gradient elution and thus be refolded with simultaneous separation from each other. The mechanism of protein refolding by HPHIC was reported in detail by Geng and Bai (2002).

In short words, along the pathway of protein refolding shown in Fig. 1, the adsorption of protein on the LC stationary phase makes the chemical equilibria in solution move from its precipitates, and/or polymers to its monomer in unfolded state, while the protein completely refolding because of the three functions indicated shown in step 3 of this section, makes chemical equilibrium from the monomer in unfolded state move or refold to its native state.

3. Strategies

All considerations for developing the strategy for the new production technology of the rhIFN-γ in industrial scale should satisfy with the foregoing seven points: (1) an ideal USRPP simultaneously having four functions (Geng and Bai, 2002) should be used. They are to completely remove denaturing agent, to renature the aim protein, to separate the renatured proteins from impure proteins, and to recycle waste denaturing agent easily; (2) an ideal technology for the renaturation and purification of therapeutic protein produced by *E. coli* should include the USRPP to simplify the production technology as shorter and simpler as possible; (3) the optimization production technology enlarges to industry scale without big problems; (4) the kind of LC including stationary and mobile phases should be selected according to the character of the aim protein. In the study, the molecules of the rhIFN-γ have very strong hydrophobicity and do not contain disulfide bonding. On one hand, the refolding of rhIFN-γ should be mainly dominated by hydrophobic interaction forces and has no other problem of misbinding of disulfide bond. On the other hand, the strong hydrophobicity of rhIFN-γ makes it have strong retention, resulting in a favorable separation from impure proteins with weak or middle hydrophobicity. Thus, HPHIC would be firstly chosen; (5) to prevent from that in case of the formed precipitates either squeezing into inside of soft media or staying in the large space among packing particles, which not only leads to very high pressure, but is also very hard to dissolve the formed precipitates with large particles. Thus, rigid based packings should be packed in the USRPP. Commercial non-rigid based media of HIC packing is usually hard to satisfy this request; (6) a solution containing denaturing agent in the presence of dithiothreitol (DTT) should be used to periodically dissolve the precipitates deposited on the stationary phase to prolong the USRPP life. In addition, with re-injecting the washed out solution containing the rhIFN-γ (of course, it is in unfolded state) into the USRPP to be renatured again, the losses of the mass and bioactivity of the rhIFN-γ would decrease. (7) Compared to purification, protein renaturation should be more important. If it is possible, both renaturation and purification of protein by using the USRPP should still be afforded to do the best for each. Thus, the particle diameter of the packings of HPHIC must be very small, i.e. 5 μm, or less.

4. Experimental

4.1. Apparatus

An LC-10AT vp high-performance liquid chromatograph (Shimazu, Japan) consists of SPD-10A detector (UV and visible wavelength), LC-10Atvp pumps, SCL-10Avp system controller, DGU-12A

degas unit and workstation. A preparative chromatography 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.

Three sizes of USRPP-HPHIC (10 mm × 100 mm i.d., 10 mm × 200 mm i.d. and 10 mm × 300 mm i.d.) made of stainless steel and HPHIC columns were bought from Shaanxi Xida Kelin Gene-Pharmacy Co. Ltd. (Xi'an, China, www.kelin2y.com).

4.2. 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 II-A) and insulin (Ins, bovine pancreas) were purchased from Sigma Co. (St. Louis, MA, USA) and prepared into aqueous solutions of 5.0 mg mL^{-1}. Other chemicals employed were of analytic grade and purchased from Xi'an Chemical Co. (Xi'an, China) and Third Jiaozuo Chemical Factory (Jiaozuo, Henan Province, China), respectively. Deionized water was prepared with Barnstead E-pure unit (Barnstead Co. Ltd., USA).

Mobile phases consisted of the following: solution A, 3.0 mol L^{-1} ammonium sulphate–0.050 mol L^{-1} potassium dihydrogen phosphate (pH 7.0); solution B, 0.050 mol L^{-1} potassium dihydrogen phosphate (pH 7.0).

Buffer A: 20 mmol L^{-1} PBS + 1 mmol L^{-1} EDTA + 0.2 mg mL^{-1} lysozyme (pH 7.4).
Buffer B: 20 mmol L^{-1} PBS + 1 mmol L^{-1} EDTA + 2 mol L^{-1} urea + 1 mol L^{-1} NaCl (pH 7.4).
Buffer C: 20 mmol L^{-1} PBS + 1 mmol L^{-1} EDTA + 0.5% Triton X-100 + 1 mol L^{-1} NaCl (pH 7.4).
Buffer D: 20 mmol L^{-1} PBS + 1 mmol L^{-1} EDTA + 1 mol L^{-1} NaCl (pH 7.4).

4.3. Preparation of rhIFN-γ extract

The method for producing the inclusion body of rhIFN-γ (pBV 220/DH5α) expressed by *E. coli* was taken from the Doctorial thesis by Shen (2001). The bacteria was produced with a 50 L fermenter (B. Brauwn Co, Germany). The yield of the rhIFN-γ 14.0 g dry cell L^{-1} with fed-batch fermentation. The bacteria was put into buffer A and crashed by ultrasonic processor in an ice-water bath and then centrifuged at 18,000 rpm for 15 min. The isolated inclusion body was washed once for each of buffer B, buffer C and buffer D, respectively. After that, the clean inclusion body was dissolved in 7.0 mol L^{-1} GuHCl solution. After incubation at 4 °C for 24 h with full agitation, the extract of the rhIFN-γ was obtained by centrifuging at 20,000 rpm.

4.4. Bioactivity assay

The bioactivity assay for the rh-IFN-γ was done by CPE inhibitor with WISH cell and VSV virus (Lengyel, 1981).

4.5. Chromatographic procedure

The USRPP and chromatographic columns of HPHIC were initially equilibrated with solution A, at least for 15 min at each selected flow-rate before injecting a sample solution and then a linear gradient elution of 0–100% solution B at different times was performed at a selected flow rate and detected at 280 nm. The selection of flow rate depends on the size of column, or the USRPP and detected at 280 nm. The eluted fractions of the aim proteins were collected for the measurements of the recoveries of bioactivity and mass of the rhIFN-γ.

5. Results and discussion

5.1. Resolution of USRPP in industrial scale

The resolution of USRPP in analytical scale was reported to be almost comparable to the usual chromatographic column (Liu et al., 1999). With the diameter of the USRPP increasing, we need to know whether the resolution of the USRPP may become worse significantly or not and thus it should be firstly tested. Because of the very expensive rhIFN-γ, the resolution was tested by standard proteins. Fig. 2 shows an almost baseline separation of six standard proteins on the USRPP-HPHIC of 10 × 100 mm i.d. at flow rate of 20 mL min^{-1}. Fig. 3 also indicates an almost baseline

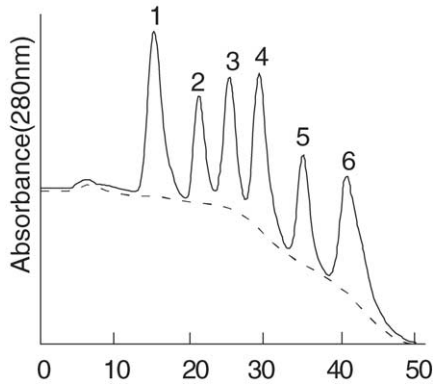

Fig. 2. Chromatogram for the separation of six proteins by a USRPP-HPHIC of 10 × 100 mm i.d. Stationary phase: hydrophobic interaction chromatographic column (end group PEG-600); linear gradient elution: 100% solution A, 3 mol L^{-1} ammonium sulphate–0.050 mol L^{-1} potassium dihydrogenphosphate (pH 7.0) to 100%; solution B, 0.050 mol L^{-1} potassium dihydrogenphosphate (pH 7.0) in 40 min with 10-min delay. Flow rate: 20 mL min^{-1}; sensitivity: 0.1AUFS. (1) Cyt-*c*; (2) Myo; (3) RNase A; (4) Lys; (5) α-Amy; (6) Ins.

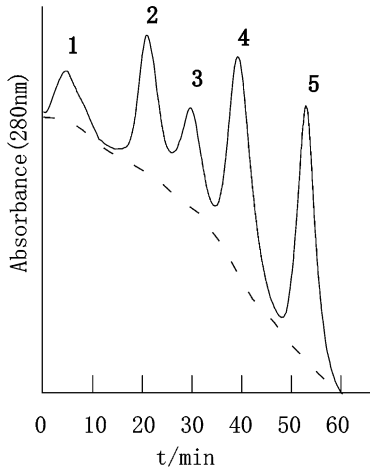

Fig. 3. Chromatogram for the separation of five proteins by the USRPP-HPHIC of 10 × 300 mm i.d. Except the flow rate, 120 mL min^{-1}, linear gradient, 0–100%B, in 60 min, the other chromatographic conditions are the same as those shown in Fig. 1. (1) Cyt-*c*; (2) Myo; (3) RNase A; (4) Lys; (5) α-Amy.

separation of five standard proteins with the USRPP of 10 × 300 mm i.d. at flow rate of 120 mL min^{-1}. Compared to the resolution obtained from Figs. 2 and 3, although the resolution is better for the former than the latter, both are quite satisfactory.

5.2. Stationary phases

As pointed out above, the contribution of stationary phase to protein refolding including quality control is not only to retard or diminish the formations of the polymerization and precipitate of the unfolded proteins, but also has other three functions by steps 5 in Section 2, or steps 2, 4, and 6 shown in Fig. 1. Thus, the effect of the kind of stationary phase on the renaturation of the rhIFN-γ should be investigated. As shown in Table 1, seven kinds of silica-based STHIC with various hydrophobicities and end groups were selected to test the recoveries of mass and bioactivity, respectively. To save expensive sample of the rhIFN-γ, the seven packings were packed in usual chromatographic column (150 × 4.6 mm i.d.).

Table 1
The quality results for rhIFN-γ fractions from seven columns packed with different packing materials for various ligands

Packing material with different ligands	Total mass (mg)	Total bioactivity ($\times 10^7$ IU)	Specific bioactivity ($\times 10^7$ IU mg^{-1})	Bioactivity recovery (%)	Purity (%)	Mass recovery (%)	Mass losses (%) (column)	Mass losses (%) (no retention)
rhIFN-γ extract	1.7	0.14	0.06	100	56.7	–	–	–
PEG-200	1.6	7.1	4.3	5043	>95.0	>93.7	0	<6.3
PEG-400	1.4	3.4	2.5	2450	>95.0	>78.2	9.5	<12.3
PEG-600	1.2	3.8	3.1	2714	>95.0	>69.3	21.7	<9.0
PEG-1000	1.5	3.7	2.5	2642	>95.0	>85.6	5.3	<9.1
Furfural	1.1	2.8	2.5	2000	86.0	58.6	33.4	8.0
Pyridine	1.9	2.5	1.3	1764	82.6	94.3	4.8	0.9
Phenyl	0.03	0.5	17	364	>95.0	>1.7	92.8	5.5

Column size, 150 × 4.6 mm i.d.; sample size,700 μL rhIFN-γ extract in 7.0 mol L^{-1} GuHCl containing 1.664 mg total rhIFN-γ.The nonlinear gradient elution was from 100% solution A [3.0 mol L^{-1} ammonium sulphate–0.05 mol L^{-1} potassium dihydrogenphosphate (pH 7.0)] to solution B [0.05 mol L^{-1} potassium dihydrogenphosphate (pH 7.0)] at a flow rate of 1.5 mL min^{-1} for 35 min with a delay for 15 min.

Four parameters, bioactivity recovery, mass recovery, purity, and specific bioactivity, shown in Table 1 can be employed to evaluate the goodness of the packings of HPHIC. The bioactivity assay of the rhIFN-γ is evaluated by comparing the bioactivity with that of the standard rhIFN-γ (specific bioactivity 1×10^7 IU mg^{-1}) and its standard solution of concentration 1×10^3 IU mL^{-1} and real sample in 7.0 mol L^{-1} GuHCl solution were prepared. While carrying out the bioactivity assay, the standard solution of the rhIFN-γ was diluted 10-fold, while the real sample solution was diluted 4×10^3-fold, i.e. the final concentration of GuHCl in sample solution was 1.75×10^{-3} mol L^{-1}. The renaturation of the rhIFN-γ by usual dilution method requires to dilute the real sample in 7.0 mol L^{-1} GuHCl solution up to 100-fold, i.e. the final concentration of GuHCl is only 7×10^{-3} mol L^{-1}, which is four-fold of that for bioactivity assay of the rhIFN-γ. However, as it is well known, the renaturation of the rhIFN-γ with dilution method by means of this manner is complete, so does the real sample for the bioactivity assay. Because of this, the bioactivity assay method in this study can be considered as the renaturation of the rhIFN-γ by the usual elution method. If the total bioactivity and mass of the rhIFN-γ in the exact in 7.0 mol L^{-1} GuHCl, or before renaturation of the rhIFN-γ by the USRPP-HPHIC are separately referred to as 100%, the bioactivity recoveries of the rhIFN-γ obtained from the seven stationary phases are raise up to 2.6- to 49-fold after this renaturation. However, for the rhIFN-γ renaturation by dilution method, the obtained mass recovery was only 11.2%. The result in Table 1 indicates that the renaturation efficiency for the rhIFN-γ by different stationary phases is quite various and some of them are excellent.

Each mass recovery of the rhIFN-γ shown in Table 1 is totally less than 100%, specially for the stationary phase with end group of phenyl which is only 1.7%. This fact indicates some of the rhIFN-γ loss during the chromatographic process. It may cause from either no retarding of the species of the denatured rhIFN-γ as sample injection, or the irreversible adsorption of the rhIFN-γ because of the formations of some polymers and/or precipitates of the rhIFN-γ on the stationary phase.

In order to find out where the mass loss of the rhIFN-γ is going, a specially strong washing agent containing denaturing agent in the presence of dithiothreitol (DTT) was used to clean up the HPHIC column after usual gradient elution. The collected fractions were passed through a Superdes 75 grade GPL column and then it was determined for the recoveries of mass and bioactivity of the rhIFN-γ again. Table 1 separately shows the mass losses of the rhIFN-γ because of the irreversible adsorption and/or no retention on stationary phase. Except PEG-600, the end groups furfural and phenyl, the irreversible adsorption of other packings of the HPHIC is totally less than 10%. Except PEG-400, the mass losses of the rhIFN-γ because of the no retention on other six packings of the HPHIC are less than 10%. These results indicate that except phenyl group, the losses of mass and bioactivity are not very significant.

From Table 1, except the end groups of pyridine and phenyl, the purity of the purified rhIFN-γ by using other five stationary phases are totally greater than 95%. Except phenyl, each of the specific bioactivity of the renatured rhIFN-γ by the other six stationary phases is comparable with each other. Based on both the purity and specific bioactivity of the rhIFN-γ, the renatured and purified rhIFN-γ with four kinds of end groups, PEG-200, PEG-400, PEG-600, and PEG-1000 coincides with the criteria of the rhIFN-γ for Chinese biological products. For a comprehensive evaluation of the seven stationary phases, the end group PEG-200 is the best.

The specific bioactivity of the rhIFN-γ obtained from the end group phenyl shown in Table 1 is 10-fold of that from the other six packings. A question is now raised that why the specific bioactivity of the rhIFN-γ obtained from this stationary phase with end group of phenyl is so high?

It may be attributed to the formation of the dimer of the rhIFN-γ, which has much higher specific bioactivity than its monomer. Because comparing to other six kinds of packings, the end group of phenyl has the strongest hydrophobicity, the unfolded rhIFN-γ in monomer state adsorbed on the stationary phase is so strong that 92.8% of the mass of the rhIFN-γ cannot be eluted from the surface of the HPHIC stationary phase by the solution B, but it does by the very strong washing agent mentioned above. The dimer of the rhIFN-γ has hydrophobicity weaker than that of its monomer and thus can be eluted by the solution B. However, compared to the amount of the monomer of the rhIFN-γ in the HPHIC system, that of the dimer is only a little bit in it.

Table 2
Effects of mobile phase on the renaturation of the rhIFN-γ by LC[a]

Salts (pH = 7.0)	Total rhIFN-γ mass (mg)	Total bioactivity ($\times 10^7$ IU)	Specific bioactivity ($\times 10^7$ IU mg^{-1})	Bioactivity recovery (%)	Purity (%)	Mass recovery (%)
Extract (7 mol L^{-1} GuHCl)	0.713	0.075	0.06	100	56.7	–
KH_2PO_4	0.704	3.2	4.3	4243	>95.0	>93.8
NaCl	0.692	0.574	0.83	766	>95.0	>92.1
NaAc	0.670	1.08	1.61	1438	>95.0	>89.3
Tris	0.639	1.03	1.61	1372	>95.0	>85.1
NH_4Cl	0.567	1.18	2.08	1572	>95.0	>75.5
NaH_2PO_4	0.531	1.29	2.43	1720	>95.0	>70.7
NH_4Ac	0.347	1.39	4.02	1860	>95.0	>46.3

[a] Except salts, all chromatographic conditions are the same as that shown in Table 1.

5.3. Mobile phase

Mobile phase also plays an important role for protein refolding by LC (Table 2). Without the high enough energy provided by the changes in the continuous composition of mobile phase with gradient elution, which makes the adsorbed unfolded protein molecules or their intermediates desorb from the stationary phase, both the phases would tightly stay on the stationary phase forever, hence never accomplishing a complete chromatographic process. Three functions of mobile phase in the circumstance of a HPHIC system are: (1) instantaneously and completely removing the denatured agent from chromatographic system and thus a favorable environment for protein refolding may be obtained; (2) mobile phase with high salt concentration has strong hydrophobicity, pushing the unfolded proteins to the surface of the STHIC, leading to the dehydration of protein molecules and the formation of microdomains of proteins; and (3) proving a suitable composition and associating with the stationary phase to correct some of that in case of the formed wrong intermediates of the proteins.

As described above already, a gradient elution in HPHIC usually consists of solution A of very strong hydrophobicity and solution B with strong hydrophilicity. The former should be selected to adsorb each intermediate of the protein and thus should have as strong hydrophobicity as possible. For the latter, it should associate with the solution A to make a suitable environment favorable for the protein refolding.

Ammonium sulphate is theoretically and practically proved to be the best salt for making the solution A and thus without many other choices (Geng et al., 1990). However, based on the hydrophobicity of proteins, the start concentration of ammonium sulphate for the gradient elution may be selected in the range of 1.0–3.0 mol L^{-1}. The stronger the hydrophobicity of the protein, the lower concentration of solution A should be selected. Some impure proteins with weak or middle hydrophobicity cannot be adsorbed as sample injecting in this circumstance, resulting in increasing sample size, or the USRPP loading.

Many buffers may be employed for the solution B. Seven kinds of salts, potassium dihydrogen phosphate, sodium chloride, sodium acetate, tris(hydroxymethyl) aminomethane (Tris), ammonium chloride, sodium dihydrogen phosphate, and ammonium acetate were employed to prepare 0.05 mol L^{-1} buffer with end group PEG-200 which was used as the stationary phase packed in a column (150 × 4.6 mm i.d.). The four parameters, the recoveries of bioactivity, mass, specific bioactivity, and purity of the rhIFN-γ shown in Table 2 were used to evaluate the goodness of the seven mobile phases. Except the solution B made of sodium dihydrogen phosphate, ammonium acetate and ammonium phosphate having the least mass recovery and that of sodium chloride having the least bioactivity recovery, the obtained results are almost comparable. Compared to stationary phase, mobile phase contributes to protein refolding by HPHIC not very significantly. For the solution B made of potassium dihydrogen phosphate, the mass recovery was found to be greater than 93%, and the specific bioactivity of the rhIFN-γ 4.3 × 10^7 IU mg^{-1}. Compared to other six buffers, it is the best one. Thus, 3.0 mol L^{-1} ammonium sulphate, together with 0.05 mol L^{-1} potassium dihydrogen phosphate with

pH 7.0, and 0.05 mol L^{-1}potassium dihydrogen phosphate with pH 7.0 were separately selected as the solutions A and B of the mobile phase employed in this study.

5.4. Flow rate

When flow rate of mobile phase is limited in a suitable range, it is very simple to find out a suitable flow rate of small solutes in usual HPLC. However, when flow rate of mobile phase is too high, because of slow mass transfer in the two phases, the mass recovery of solutes, specially for proteins with high molecular mass, will decrease significantly. For protein refolding, besides mass transfer, the dissociation of various polymizations and the dissolution of the precipitates of proteins also involve dynamic problem and thus are affected by the flow rate of mobile phase. Because it is very complicated problem, it is hard to expect theoretically. The optimization flow rate of mobile phase has to be found by experiment.

Fig. 4 shows the chromatograms of the renatured and purified rhIFN-γ under 10 mL min^{-1}, 20 mL min^{-1}, and 30 mL min^{-1} by a USRPP-HPHIC (10 × 200 mm i.d.). From Fig. 4, when the flow rate of mobile phase is 10 mL min^{-1}, the resolution of the rhIFN-γ from other impure proteins is the best.

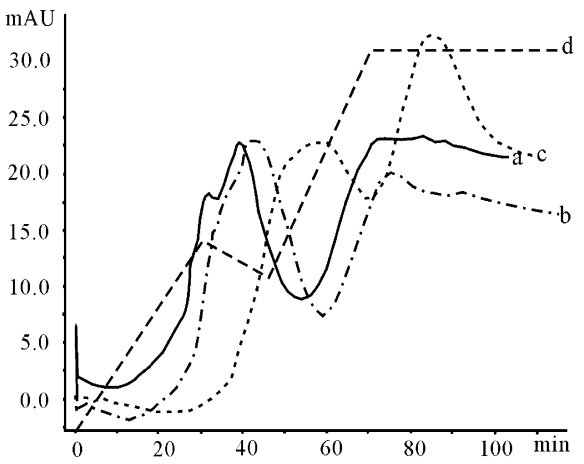

Fig. 4. Chromatogram of the renatured and purified rhIFN-γ under different flow rates by USRPP-HPHIC (10 × 200 mm i.d.). (a) 30 mL min^{-1}, (b) 20 mL min^{-1}, (c) 10 mL min, (d) non-linear gradient elution profile, (*) rhIFN-γ. Sample size: 12 mL 7 mol/L GuHCl extract containing total protein 48 mg.

5.5. Comparison between traditional and new productive technologies

In a traditional method the renaturation and purification of the rhIFN-γ are separately carried out. For example, a procedure reported in reference (Zhang et al., 1992) was employed to compare to the new technology presented in this study. The amount of about 180 mg total proteins from the extracted rhIFN-γ solution by 7 mol L^{-1} GuHCl was used for testing both the methods. For the traditional method, the rhIFN-γ firstly renatured by dilution method with standing for 24 h and then applied onto different LC columns for its purification. The recoveries of the bioactivity and purity for each step are listed in Table 3.

From the results shown in Table 3, the traditional technology for renaturation and purification of the rhIFN-γ consists of four steps. One of them is for the renaturation by dilution method; other three steps for purification are of chromatographic purification, ion exchange chromatography, immobilized metal ion affinity chromatography, and size exclusion chromatography. It took totally about 44 h to accomplish the whole process including renaturation but excluding desalting and removing pyrogen. With the four steps, the purity of the rhIFN-γ was 95% and bioactivity recovery was found only 2.6-fold of that of the original extract. The specific bioactivity was obtained as 3.51×10^7 IU mg^{-1}.

An USRPP-HPHIC (PEG-600) of 10 × 200 mm i.d. (bed volume, 314 mL) was employed to renature and purify the rhIFN-γ by using the same sample as that by

Table 3
The obtained results by traditional technology of renaturation and purification for rhIFN-γ (Zhang et al., 1992)

Steps	(1) Dilution renaturation	(2) IEC	(3) IMAC	(4) SEC
Time (h)	24	9	3	8
Bioactivity recovery (%)	3702	793	397	264
Purity (%)	35.9	58.5	77.0	95
Specific bioactivity (IU $mg^{-1} \times 10^7$)	2.48	5.85	4.4	3.5

Total rhIFN-γ in 7 mol L^{-1} GuHCl, 180.5 mg; IEC: *S*-Sepharase FF (50 mm × 50 mm i.d.; bed volume 98 mL). IMAC: Ni(II)-chelating Sepharose FF (60 mm × 26 mm i.d. bed volume 32 mL); SEC: Superdex 75 Prep grade (60 mm × 26 mm i.d.; bed volume 32 mL). Flowrate: 5.0 mL min^{-1}.

the tradition method pointed above. After equilibration with solution A for 20 min at flow rate of 10 mL min^{-1}, 45 mL of the extract of rhIFN-γ in 7 mol L^{-1} GuHCl were pumped into the USRPP-HPHIC and a nonlinear gradient of 100% A to 100% B within 70 min at 10 mL min^{-1} was run (shown in Fig. 4). The fraction of the rhIFN-γ was collected and then was desalted by Superdex 75 prep grade GPC column. It only took 2 h excluding desalting and removing pyrogen, the recoveries of bioactivity, purity, and specific bioactivity were measured to be 62%, greater than 95%, and 8.9 × 10^7 IU mg^{-1}, respectively.

5.6. Simultaneous purification and renaturation of rhIFN-γ by the USRPP (10 × 300 mm i.d.)

An USRPP-HPHIC of 10 × 300 mm i.d. was firstly equilibrated with a selected mobile phase at the flow rate of 100 mL min^{-1}, and then the rhIFN-γ extract of 700 mL in GuHCl solution containing total proteins of 2.0 g was continuously pumped through this USRPP-HPHIC. After eluting out some impurities by isocratic elution, taking at least 20 min, a linear gradient elution, 50%A–100%B from 120 to 210 min, holding 100%B to 300 min was employed. Fig. 5 shows the chromatogram of the renatured and purified rhIFN-γ. The three collected fractions from 0 to 130 min, 130 to 220 min, and 220 to 320 min were desalted twice for testing the purity and bioactivity of the rhIFN-γ. From the SDS-PAGE (shown in Fig. 6), the first collected fraction was not found any rhIFN-γ. This fact indicates that the mass and volume loadings of the USRPP are enough for mass loading total 2 g of proteins and volume loading of 700 mL GuHCl, respectively. In the last collected fraction, a very little rhIFN-γ was found, indicating that the rhIFN-γ in the optimization chromatographic condition can be almost completely washed out and separated from impure proteins completely. From Figs. 5 and 6, the second collected fraction (130–220 min) contains the renatured and purified rhIFN-γ. The purity and the specific bioactivity are over 95% and 8.7 × 10^7 IU mg^{-1}, respectively. These also coincide with the criteria of rhIFN-γ for Chinese biological products in terms of the purity and specific bioactivity; for the USRPP with this size, it only takes, as shown in Fig. 5, 4 h to renature with simultaneously purifying the rhIFN-γ.

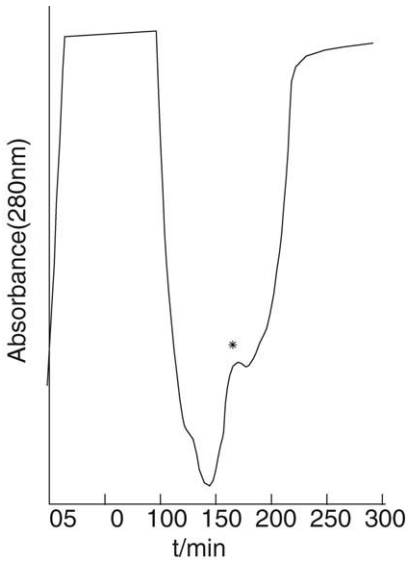

Fig. 5. Chromatogram for the renaturation and simultaneous purification of rhIFN-γ by USRPP-HPHIC (10 × 300 mm i.d.). Flow rate, 100 mL min^{-1}; injection and equilibration in 0 120 min; gradient elution, 50%A–100%B from 120 to 210 min; holding 100%B to 300 min; (*) indicate target peak.

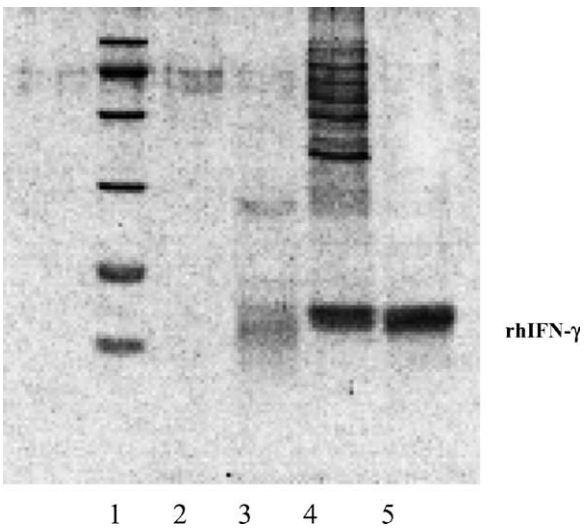

Fig. 6. The SDS-PAGE (Silver staining technique) of rhIFN-γ eluted in different stages. (1) Marker (14,400 Da, 20,100 Da, 31,000 Da, 43,000 Da, 66,200 Da, 97,400 Da); (2) and (3) fractions of rhIFN-γ in 220–310 min and before 130 min by a USRPP of 10 × 300 mm i.d.; (4) extract of rhIFN-γ by 7 mol L^{-1} GuHCl before chromatography; (5) collected rhIFN-γ fraction in 130–220 min.

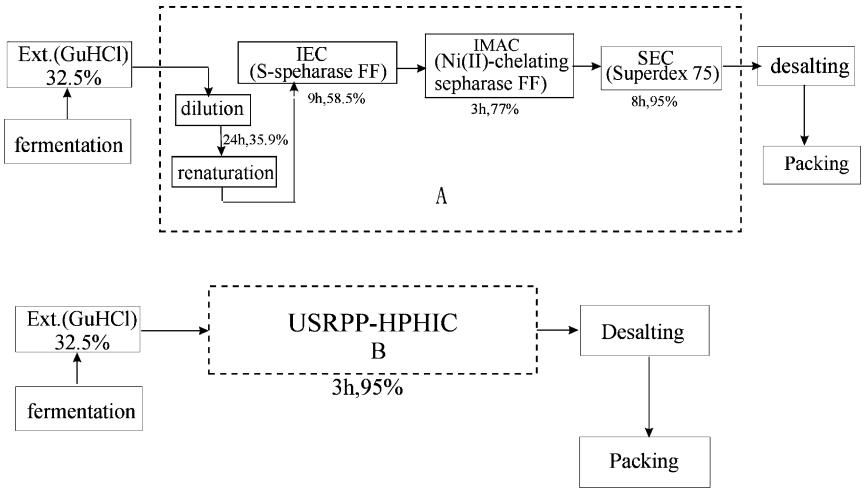

Fig. 7. Scheme for the comparison of the new and usual production technologies of the rhIFN-γ produced by *E .coli* with USRPP. (A) Usual production technology (Zhang et al., 1992); four steps, 44 h, purity, >95%; increases in bioactivity recovery, 1.6-fold; (B) new production technology; one step, 3 h; purity, >95%; increases in bioactivity recovery, 61-fold.

Comparing the total bioactivity recovery of the rhIFN-γ in 7 mol L^{-1} GuHCl solution before and after the sample injected into the unit, the total bioactivity recovery of the rhIFN-γ is raised over 62-fold after the USRPP-HPHIC. Compared to the usual elution method in terms of the bioactivity recovery of protein renaturation usually being 5–20%, the renaturation efficiency by using this USRPP-HPHIC is a really powerful tool for protein renaturation.

Fig. 7 shows the comparison between the usual and the new production technology of the rhIFN-γ in industrial scale. Fig. 7A shows the usual production technology consisting of four steps denoted inside of a dash line rectangular and it takes total 44 h to obtain the purity of only 95%. As pointed above, the bioactivity recovery is 2.6-fold of the original total bioactivity in the extract of GuHCl solution. Fig. 7B shows the new production technology presented only by one step in this study denoted also by a dash line rectangular and takes 2–4 h to obtain the purity being more than 95%. Furthermore, the bioactivity recovery is 62-fold of the original total bioactivity in the extract of GuHCl solution.

In this study the renaturation with simultaneous purification of the rhIFN-γ only by the USRPP-HPHIC is reported. Actually, other scientists with HPHIC column also accomplished the renaturation with simultaneous purification successfully. However, it was only in laboratory scale (Geng et al., 1991, 2001; Geng and Chang, 1992; Guo and Geng, 2000; Ala et al., 1997, 1998a,b; Jadhav et al., 1997) but not in industry scale.

6. Conclusion

(1) Based on chemical equilibrium and molecular interactions, the principle of rhIFN-γ refolding by HPHIC is presented. It would be expected to be suitable for other proteins, but a specifically experimental condition for a given protein should be carefully found out.

(2) The unit of simultaneous renaturation and purification of proteins of high-performance hydrophobic interaction chromatography (USRPP-HPHIC) with 10 mm in thickness and 300 mm in diameter can be employed for the renaturation with simultaneous purification of rhIFN-γ in industry scale. With the USRPP-HPHIC having different diameters but only 10 mm in thickness, a satisfactory separation efficiency of biopolymers can be obtained.

(3) The rhIFN-γ originally in the extract of 7 mol L^{-1} GuHCl solution can be directed into the USRPP-HPHIC, and only with one step and 2–4 h, the obtained purity and the specific bioactivity can approach to 95% and the request. Comparing to the

total bioactivity in the extract, or before that it was injected into the USRPP-HPHIC, the bioactivity recovery raises upto about 62-fold.

(4) The contributions of stationary phase was not only to retard the formation of precipitates of protein, but to providing energy, squeeze out water, and form microdomains. Both stationary and mobile phases were found to contribute to the protein refolding, but the effect of the former was more significant than the latter.

(5) Because of some dynamic problems of mass transfer of protein molecules between two phases, the flow rate of mobile phase was also found to affect on the resolution and renaturation of the rhIFN-γ.

(6) With the comparison to usual renaturation method with, or without LC, the new production technology is much better.

(7) It is believed that with the increases in the thickness being 50 mm and the diameter being 500–1000 mm, the USRPP loading may increase significantly and can still keep a satisfactory resolution and renaturation of therapeutic proteins produced by *E. coli* in biotechnology.

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