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High-performance hydrophobic interaction chromatography as a tool for protein refolding

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ABSTRACT

A method for the refolding of previously unfolded proteins with a concentrated solution of denaturing agent is presented, involving the use of high-performance hydrophobic interaction chromatography (HPHIC) to separate the denaturing agent completely from the unfolded protein and to provide a suitable environment for its refolding. The retention, peak shape and peak height in HPHIC and size-exclusion chromatography, UV spectra, circular dichroic spectra and bioactivity were used to test the possibility and the completeness of the protein refolding. The proposed method permits the extracted solution from *Escherichia coli* cells to be injected directly into the HPHIC column and, at the same time, the refolding and purification of the proteins to be effected. The renaturation and purification of recombinant human interferon – γ from *E. coli* cells is one example of the application of the method in biotechnology.

INTRODUCTION

About 20 years ago, Anfinsen [1] reported the significant discovery of the spontaneous refolding of ribonuclease as it moves into aqueous solution from 8.0 mol/l urea solution containing β-mercaptoethanol in which ribonuclease had been reduced and denatured to its unfolded state. Since then many workers have studied protein refolding in two steps: first to make the protein unfold and then to make it refold [2]. Therefore, means of making the protein refolding and obtaining information about its refolding and renaturation are of significant interest [3].

Many kinds of therapeutic proteins can be produced from recombinant DNA technology either in the plant or in the laboratory. A new and convenient technique for extraction these therapeutic products from the bacterium *Escherichia coli* is to use a denaturing agent in a suitable concentration [4]. In some instances 7.0 mol/l guanidine hydrochloride (GuaHCl) or 8.0 mol/l urea solution is needed to extract very strong hydrophobic proteins in the unfolded state. Therefore, to achieve protein refolding completely from a concentrated solution of denaturing agent is vital not only for theoretical studies in molecular biology, but also for lowering the cost of these therapeutic proteins in industry. However, the refolding is usually not complete and this seriously influences the output and benefit of therapeutic proteins in industry.

According to Anfinsen [1], the three-dimensional structure of a protein molecule is based on its primary structure, *i.e.*, the sequence of amino acid residues. If the denaturing agent is removed from its mother solution containing the denatured protein, the hydrophobic environment of water or a dilute aqueous solution of salt of suitable viscosity, ionic strength, etc., will make the denatured protein refold spontaneously to its thermodynamically stable state so as to be identical with its native form, even if this refolding process takes place very slowly. High-performance hydrophobic interaction chromatography (HPHIC) is a powerful tool for the separation and purification of biopolymers [5] and may satisfy these conditions during gradient elution with a mobile phase consisting of both a concentrated salt solution and water. In previous work [6] we used HPHIC for the renaturation and separation of recombinant human interferon-γ (rIFN-γ)

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and obtained good results in terms of the completeness of its refolding and purification, with the simple operation.

In this work, we investigated the refolding of protein only unfolded with a denaturing agent and studied the possibility of protein refolding using HPHIC; the mechanism was not considered. Measurements of both the conformational changes of protein molecules, including UV spectrophotometry, circulair dichroism (CD) and size-exclusion chromatography (SEC), and its bioactivity were used to test the possibility and the completeness of the refolding of a denatured protein. An example of the renaturation and purification of a therapeutic protein *E. coli* cell in biotechnology is presented.

EXPERIMENTAL

Equipment

An LC-9A liquid chromatograph (Shimadzu, Kyoto, Japan) was used, consiting of two pumps (LC-6A), a variable-wavelength UV–visible detector (SPD-6AV), a column oven (CTO-4A) and a recorder (R-112). Stainless-steel columns (100–150 × 4.0 mm I.D. and 250 × 7.9 mm I.D.) were used. The size-exclusion column (diol 150) was bought from Shimadzu. The HPHIC packings (XDF-GM, silica linked to ligands consiting of a polyethylene glycol chain and with a hydrophobic end-group) used were made and packed in our laboratory. Equipment for optical measurement consisted of a Jasco J-20c automatic recording spectropolarimeter (Japan Spectroscopic), a UV–VIS spectrophotometer (Perkin-Elmer) and a dual- wavelength thin-layer chromatographic scanner (CS-930, Shimadzu). Equipment for studying the cytopathic effect (CPE) inhibition for rIFN-γ included a carbondioxide incubator (Sheldon Manufacturing), an inverted microscope (Chongqing Optical Instrument Factory) and a super clean bench.

Chemicals

Egg white lysozyme (LYS), ribonuclease (RNase) and bovine serum albumin (BSA) were purchased from the Institute of Biochemicals (Shanghai, China) and α-amylase (α-AMY, *Bacillus anthracis*, type II A) from Sigma (St. Louis, MO, USA). Other chemicals were of analytical-reagent grade. Water was re-distilled (quartz).

Mobile phase

HPHIC eluent consisted of solution A, 3.0 mol/l ammonium sulphate–0.020 mol/l potassium dihydrogenphosphate (pH 7.0), and solution B, 0.020 mol/l potassium dihydrogenphosphate (pH 7.0). The SEC eluent was 0.020 mol/l Tris–0.10 mol/l sodium chloride (pH 8.0).

The reagents used for the CPE inhibition assay of rIFN-γ were of analytical-reagent grade. The cells and virus used for the CPE inhibition assay of rIFN-γ were WISH cell and VSV virus. The medium used for assay was Eagle's–BSA (90:10) containing 250 U/ml of penicillin and 250 U/ml of streptomycin (pH 7.2–7.4, adjusted with sodium hydrogencarbonate).

Protein refolding with HPHIC

A denatured protein solution in 7.0 mol/l GuaHCl or 8.0 mol/l urea was injected directly into the HPHIC column (150 × 4.0 mm I.D.), which had already been equilibrated with eluent A. A 20-min linear gradient with a flow-rate of 1.0 ml/min from 100% eluent A to 100% eluent B and followed by a 10-min delay was applied. The collected fractions were then tested for the completeness of refolding of protein.

CPE inhibition assay of rIFN-γ [7]

The procedure is as follows.

(1) Seed cells: add 100 μl of medium containing suspended cells (600 000 cells/ml) to each well in a 96-well culture plate. Incubate the cells at 37°C for 24 h to obtain a monolayer.

(2) Incubate the cells with dilutions of rIFN-γ. Remove the growth medium and add rIFN-γ dilution (in the medium) to monolayers at 100 μl per well. Incubate the cells at 37°C for 24 h.

(3) Infect the cells with virus. Remove the rIFN-γ dilutions, then add VSV virus (100–400$TCID_{50}$, one $TCID_{50}$ equals 50% of tissue culture infective dose) at 100 μl per well. The viruses are diluted in medium. Incubate at 37°C for 24 h.

(4) Measure the viral effect. When CPE appears more than 75% destroyed in IFN-free wells, score an estimate of CPE by microscopic observation of each infected culture.

(5) Calculate the rIFN-γ titres by the Reed–Muehch method.

RESULTS AND DISCUSSION

Although it was about 60 years ago when Wu and Yang [8], Anson and Mirsky [9] and Northrop [10] defined the "native structure" for a protein not only by the activity, but also by some physical characteristics and chemical properties, this may still be valid today. The bioactivity of a protein and its molecular conformational changes are simply connected with each other. Four proteins, RNase, LYS, α-AMY and BSA, were selected as the representatives of three types of proteins. The first two have a low molecular mass (less than 20 000), the third has an irreversible process for thermal denaturation and the fourth one has a higher molecular mass (67 000). The renaturation and purification of rIFN-γ from *E. coli* cells was selected as a representative application in biotechnology. The results were very successful.

Retention and refolding with HPHIC

According to the stoichiometric displacement model for retention (SDM-R) of solute [11], Kunitani *et al.* [12] reported that the changes in the molecular conformation of protein in reversed-phase liquid chromatography (RPLC) necessarily cause changes in the contact surface area between the stationary phase and protein molecules, and so do both its Z value (the number of displacing agent molecules required to displace a protein from ligand) and retention. Karger and co-workers also confirmed this point in both RPLC [13] and HPHIC [14,15]. We also tested their conclusion again using both HIC and RPLC [16]. Benedek *et al.* [13] also reported that the changes in the peak height of native and unfolded protein indicate the changes in its molecular conformation in RPLC if the unfolded protein has only one kind of conformation. Hence the changes in either the retention or the peak height of a protein may be considered as important parameters for the characterization of conformational changes of protein molecules.

Fig. 1 shows four chromatograms of BSA and LYS in their native forms and refolded from their unfolded state. Fig. 1a and c represent the refolded and the native BSA, respectively, and Fig.b and d represent the refolded and the native LYS, respectively. The chromatographic profiles of the two native proteins and the corresponding profiles of the refolded proteins are the same in terms of retention, peak shape and peak height. This is very unusual, because LYS is a relatively small enzyme and has a molecular mass of 14 600, but BSA is a three times larger protein than LYS. From the traditional point of view, the BSA refolding should be much more difficult than the LYS refolding. The results in Fig. 1 show that HPHIC may have a special function for protein refolding.

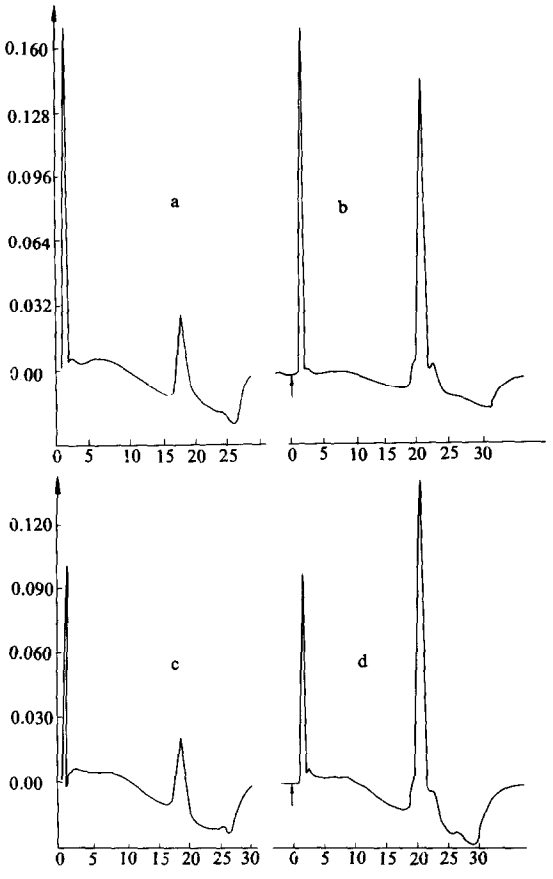

Fig. 1. Comparison of results of HPHIC between native and refolding proteins. Linear gradient elution from 100% solution A [3.0 mol/l ammonium sulphate–0.020 mol/l potassium dihydrogenphosphate (pH 7.0)] to 100% solution B [0.020 mol/l potassium dihydrogenphosphate (pH 7.0) at a flow-rate of 1.0 ml/min for 20 min with a delay for 10 min. Chart paper speed, 2.5 mm/min; detection wavelength, 280 nm, 0.64 a.u.f.s. Peaks: a and c = native and refolded BSA, respectively; b and d = native and refolded LYS, respectively. y-axis: Absorbance at 280 nm; x-axis: time in min.

If the three parameters for a chromatogram can

be used to characterize the native, unfolded or refolded state of a protein, we may conclude that the two kinds of proteins unfolded with 7.0 mol/l GuaHCl can really be refolded using HPHIC. The reasons why this occurs need to be considered.

Factors affecting protein refolding with HPHIC

It is very difficult to answer the above question exactly, but it would be useful to consider a few possible answers. We first used SEC to examine the possibility and the completeness of the refolding of an unfolded protein in the same way as that with HPHIC, in order to find out the reason why HPHIC does it so well. Because an ideal SEC column should never display any interactions between the stationary phase and a protein molecule, even if the protein molecule has polar groups and electrostatic charge, the retention of a protein on an SEC column depends only on the size and shape of the protein molecules [17]. For the same protein, changes in its retention would indicate conformational changes if the same column and the same mobile phase are used. GuaHCl or urea should be removed during the SEC process and therefore, if the protein refolds in this instance, it is due only to the removal of the denaturing agent. Hence SEC should be a means of investigating the reasons for the change in molecular conformation of proteins.

Fig. 2 shows the comparative size-exclusion chromatograms of the three types of protein, LYS, RNase and BSA, where the sample solutions were injected in both (a) the native and (b) unfolded forms. Peaks 1, 2 and 3 represent BSA, RNase and LYS, respectively. BSA, RNase and LYS have different chromatographic profiles, retentions, peak heights and peak shapes in the two instances. The difference between the two retention times for LYS in Fig. 2a and b is more than 2 min. Based on the facts that the retentions of BSA and RNase in Fig. 2a and b are the same, but their peak shapes are different, we cannot conclude that refolding of either BSA or RNase with SEC would be possible or complete. Comparing Figs. 1 and 2, we know that the protein refolding cannot be accomplished only by removing the denaturing agent by SEC, or at least not in a short time. Although the compositions of the mobile phases for HPHIC and SEC are different, the latter may be more in favour of protein refolding. Hence we may conclude that the reason

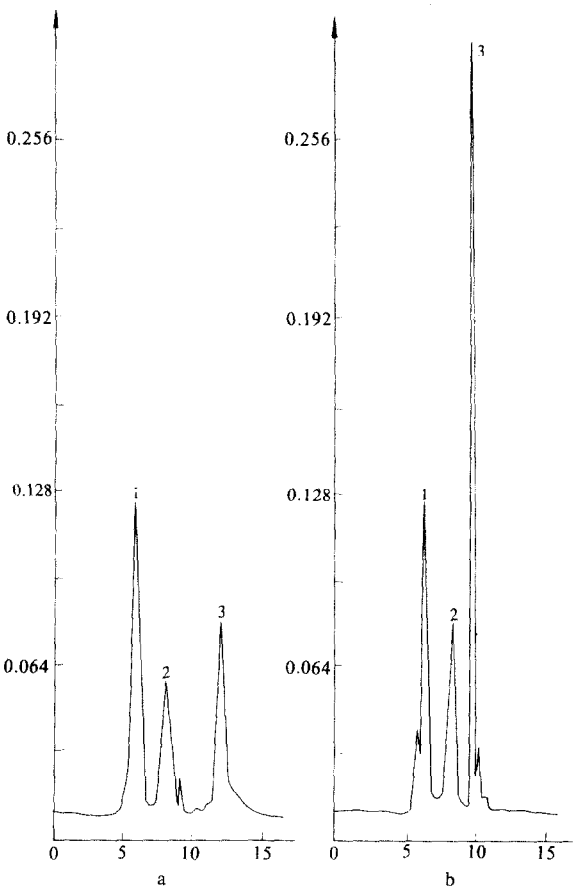

Fig. 2. Comparison of results of SEC between native and unfolding proteins. Column, Shimadzu diol 150 (250 × 7.9 mm I.D.); mobile phase, 0.020 mol/l Tris–0.10 mol/l NaCl (pH 7.0); Flow-rate 1.0 ml/min; chart paper speed, 2.5 mm/min; detection wavelength, 280 nm; 0.32 a.u.f.s. Proteins: 1, BSA; 2, RNase; 3, LYS. (a) Native proteins; (b) unfolded proteins. Axes as in Fig. 1.

for refolding with HPHIC is not or not only due to the removal of denaturing agent during the chromatographic process.

We need a full understanding of the mechanism of retention of proteins in HPHIC. In other words, we must know how protein molecules bind to and desorb from an HPHIC column and also what happens during this whole adsorption–desorption process. Based on the SDM-R of proteins HPHIC [18], we also need to know the contributions of the contact surface and the continuous conformational change of a protein during its refolding. The hydro-

phobicity of a salt solution of high concentration is high enough to push the protein molecules into contact with the surface of the HPHIC stationary phase, and to be adsorbed by it. Because HPHIC offers a higher hydrophobicity on this surface than that in the mobile phase, more hydrophobic amino acid residues in the protein molecule would face the surface of the stationary phase whereas more hydrophilic amino acid residues would face to the mobile phase. In this case, in the specific contact region, the hydrophobic forces make part of the unfolded protein molecules form a local configuration. The local configuration of the unfolded protein molecule may become a potential "seed" to continue its folding when it leaves the surface and passes into the mobile phase which has less hydrophobicity than that previously, as the hydrophobicity in the mobile phase changes continuously during gradient elution. Which part of a given unfolded protein molecule will contact the surface of the stationary phase? It really depends on both its primary structure, *i.e.*, its amino acid sequence, and the nature of HPHIC. Fausnaugh-Pollitt *et al.* [19] concluded that chromatographic retention in HPHIC is determined by amino acids on a single surface of the protein opposite to its catalytic left. A reasonable assumption is that an unfolded protein molecule makes contact in HPHIC also with the same region and it becomes a "seed" to continue refolding until complete refolding of this protein molecule has occurred. However, for any single unfolded protein molecule, it may contact the hydrophobic surface of the stationary phase with an incorrect hydrophobic part of the molecule because of the effect of Brownian motion. If this happens, the "wrong seed" or "ill-seed" may also grow. However, it is short-lived, because it is thermodynamically unstable and it tends to adopt a stable state, or any stable kind of intermediate configuration. Therefore, the above elucidation of the exact refolding of an unfolded protein molecule is only in terms of statistics. In addition, the continuous changes in the hydrophobicity and water concentration, and also the viscosity of the eluent during gradient elution in HPHIC, may provide a suitable environment for refolding of a given protein. Hence the hydrophobic surface of an HPHIC stationary phase and a mobile phase with a variable composition may not only provide energy to effect refolding but may also provide a infinite chance for unfolded protein molecules to find their right "seed" and their thermodynamically stable state. Consequently, it accelerates the refolding process.

Application and limitations of protein refolding with HPHIC

If the above explanation of the reasons for protein refolding with HPHIC is reasonable, it seems that HPHIC can effect the refolding of any kind of

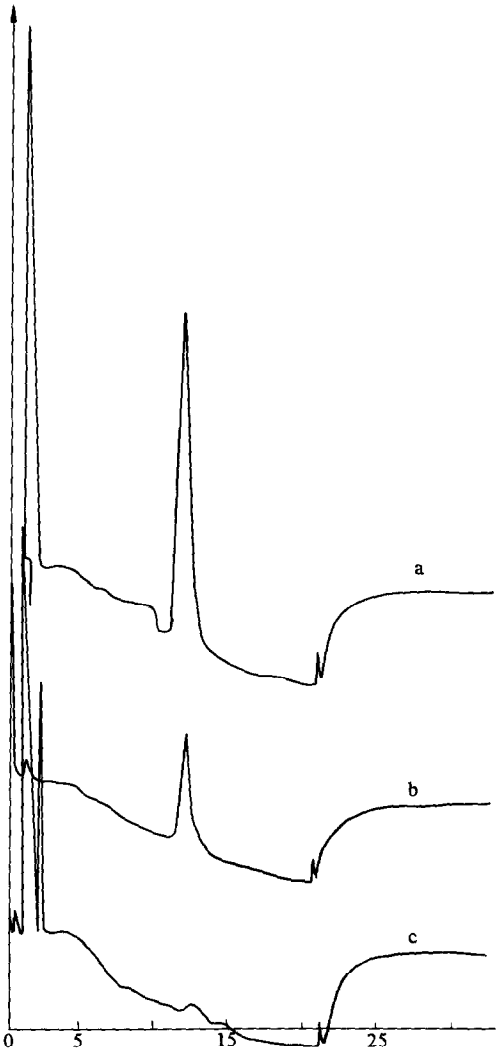

Fig. 3. Comparison of results of HPHIC of α-amylase: (a) native (b) unfolded with GuaHCl; (c) unfolded by boiling for 15 min. Chromatographic conditions as in Fig. 1. Time scale in min.

unfolded protein, such as thermal, acid or base, and even biological denaturation. However, Fig. 3 shows a comparison of α-amylase refolding chromatograms for three cases. Fig. 3a, b and c represent the native α-amylase, the unfolded α-amylase with 7.0 mol/l GuaHCl and the unfolded α-amylase after boiling for 15 min, respectively. The chromatographic conditions were as in Fig. 1. Although the retentions and shapes are identical for each peak, the peak heights are different. After boiling for 15 min, α-amylase aggregated and became a suspension, therefore, the height of peak c is the smallest of the three, but we do not know the reason why peak c has the characteristics of native α-amylase or where it comes from. It may come from either incomplete unfolding of α-amylase during boiling or from incomplete refolding with HPHIC. Anyhow, peak b shows incomplete refolding of α-amylase unfolded with 7.0 mol/l GuaHCl and we do not understand the exact reason. Fig. 3. demonstrates that HPHIC is not a universal method for refolding of every kind of protein from their unfolded states by any kind of denaturing method, even with GuaHCl. Nevertheless, we cannot draw the conclusion from Fig. 3 that HPHIC may effect refolding only of some proteins and not of other unfolded proteins either with a denaturing agent or in other ways. However, we used only one kind of mobile phase and chromatographic conditions to do the refolding of four kinds of proteins after having them unfolded in two ways. The composition hydrophobicity, pH, ionic strength, viscosity and temperature should be optimized for each protein to make complete refolding of the proteins possible, but this is beyond the scope of this paper.

UV spectra

UV spectrophotometry is one of the most important tools for investigating the changes in the molecular conformation and refolding of proteins [2].

Without injecting a sample, we ran the same gradient elution and collected the eluate in the same time interval as if the unfolded standard RNase had been injected. The collected eluate was divided into two parts: the first was used as the solvent for standard RNase and the second was used as the blank solution for UV spectral measurement of the refolded RNase. Also, a suitable standard RNase was dissolved in 7.0 mol/l GuaHCl and its UV spectrum was compared with that of the refolded RNase.

Fig. 4 shows a comparison of the UV spectra of RNase of (a), the native form, (c) the unfolded form, denatured with 7.0 mol/l GuaHCl, and (b) the refolded form after HPHIC of the unfolded RNase. The maximum absorption wavelength (202.5 nm) in (a) and (b) are the same, but are different from that in (c). The intensity in (b) is slightly lower than that in (a) probably because its mass recovery is not 100%. Anyhow, the refolding of unfolded RNase with 7.0 mol/l GuaHCl appears to be almost complete. The measurement of UV spectra is as usually reliable as the wavelength is longer than 200 nm [20]. Therefore, the shift of the maximum absorption wavelength towards the UV re-

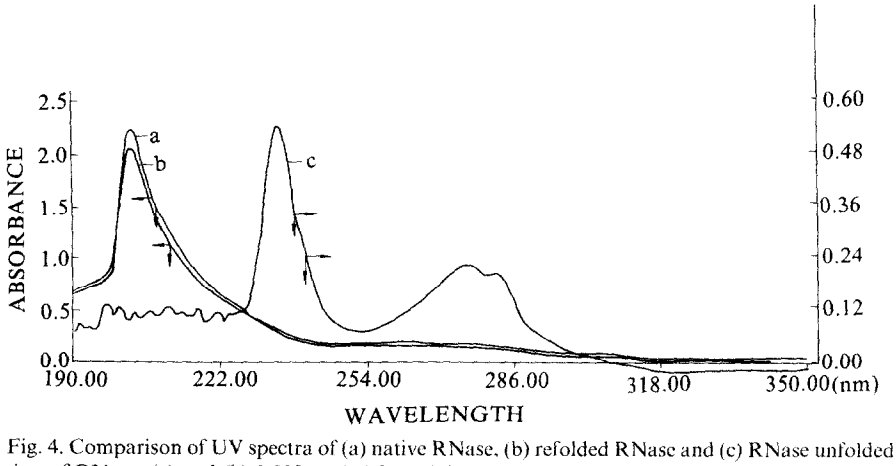

Fig. 4. Comparison of UV spectra of (a) native RNase, (b) refolded RNase and (c) RNase unfolded with 7.0 mol/l GuaHCl. Concentration of RNase: (a) and (b) 0.083 mg/ml for left-hand scale; (c) 0.50 mg/ml for right-hand scale.

gion can be attributed completely to changes in the molecular conformation of the RNase, as the sample and blank solutions had the same composition except for the proteins in the sample in (a) and (b). We obtained similar results in corresponding experiments for LYS and BSA which had been unfolded with both 7.0 mol/l GuaHCl and 8.0 mol/l urea.

Circular dichroic spectra

When a protein unfolds, the UV spectrum shows changes in the aromatic amino acids (tyrosine, tryptophan, phenylalanine) on exposure to the solvent, while CD spectra can denote changes in secondary structures (α-helices, β-pleated sheet and antiparallel β-sheet) of the unfolded protein, *i.e.*, CD spectra give some information concerning the global transition of a protein. Hence CD is also a very important method for investigating the variation of the molecular conformation of a protein.

Fig. 5 shows a comparison of the CD profile of the native (solid line) and the refolded (dotted line) RNase which has been unfolded with 7.0 mol/l GuaHCl and then refolded with HPHIC. The two CD spectra are identical in terms of their maximum absorption wavelength, absorption intensity and spectroscopic profile, although we did not calculate the changes in ellipticity. The composition of the solution containing native RNase is identical with that of the refolded RNase, hence Fig. 5 again indicates that HPHIC may effect refolding of unfolded RNase.

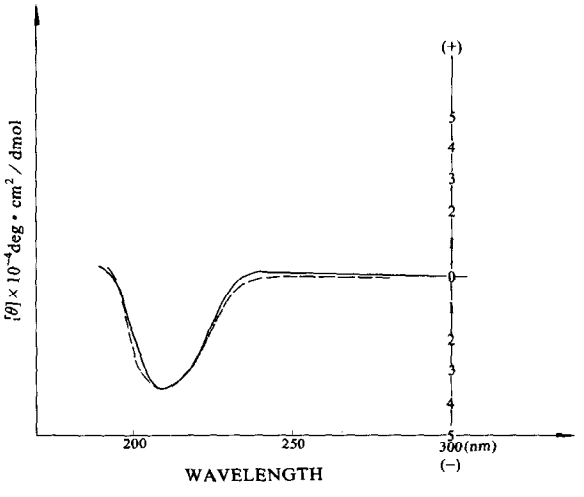

Fig. 5. CD spectra of (solid-line) native and (dashed line) refolded RNase.

Applications of protein refolding with HPHIC in biotechnology

An important factor complicating the recovery of rIFN-γ from *E. coli* is its interacellular location. An alternative to the commonly used method of releasing it by mechanical disruption is to permeate chemically the cells with a concentrated denaturing agent. These methods have several undesirable properties, *e.g.*, the extensive fragmentation of the cells makes the subsequent centrifugation difficult and nearly all of the soluble cellular proteins are released, resulting in difficulties in downstream production in industry.

However, GuaHCl can make the cell permeable to proteins without causing extensive breakage of the cell and can dissolve protein from *E. coli* membrane fragments [4]. The cell membrane acts as a molecular sieve which retards RNA and most of the DNA inside the cells. Hence it is possible to simplify the technology for the purification of therapeutic proteins in biotechnology. However, it is then necessary to separate GuaHCl from rIFN-γ. Several means can be used to effect renaturation of therapeutic protein, *e.g.*, common dialysis, dilution with an approprite aqueous solution to adjust hydrophobicity, viscosity and ionic strength or the removal of sodium dodecyl sulphate (SDS) with mild neutral detergents [21], but only low recoveries of bioactivity are normally obtained.

Fig. 6 shows the chromatogram of the solution of rIFN-γ extracted with 7.0 mol/l GuaHCl from *E. coli* cells. This extracted solution was injected directly into the HPHIC column (250 × 7.9 mm I.D.). The shaded areas show the recovery of the bioactivity of rIFN-γ. The major peak in Fig. 6 is rIFN-γ containing some impurities. Its retention time is about 33 min. Because rIFN-γ easily forms inclusion bodies, it is very difficult to dissolve it with the usual media, and hence to measure its real native bioactivity. Therefore, we have to make a comparision with the bioactivity of rIFN-γ in the sample before injection into the HPHIC column. The average bioactivity recovery of the renaturated rIFN-γ with HPHIC is about 280%. This is due to the comparison of the recovery of the bioactivity of rIFN-γ between the dilution method mentioned

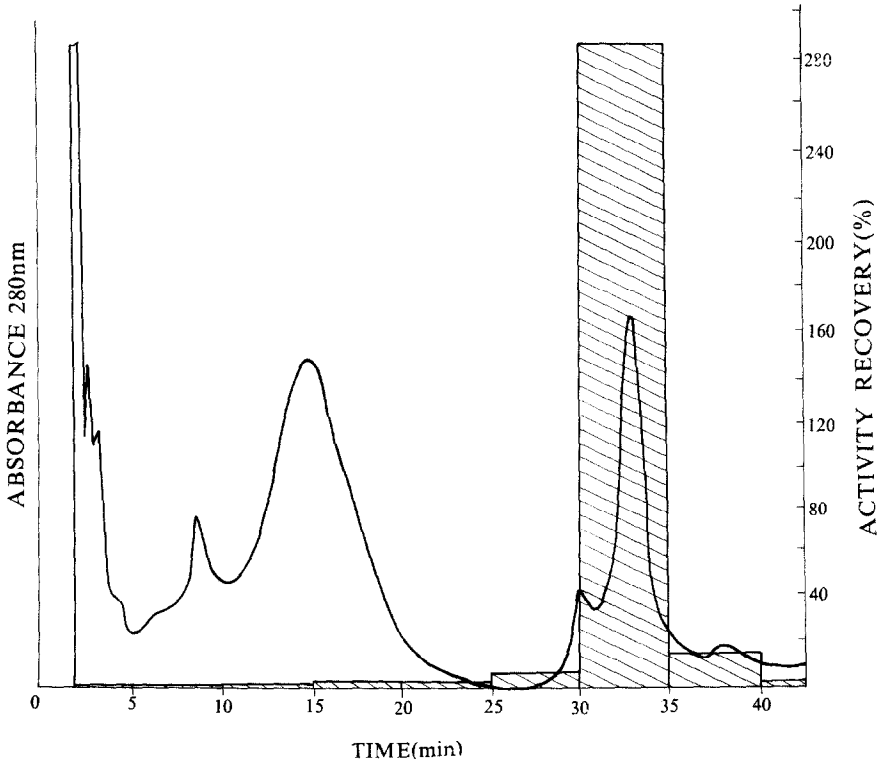

Fig. 6. HPHIC of rIFN-γ solution extracted from *E. coli* cells. Chromatographic conditions as in Fig. 1. Sample size, 1.0 ml; flow-rate, 2.0 ml/min. The shaded areas represent the bioactivity recovery in collections in every 5 min (right-hand scale).

above and the HPHIC refolding method in this work, because the CPE inhibition assay, in fact, makes many folds during dilution of the original extracted solution. For this sample, it was eluted up to 2000–4000 fold. The concentration of GuaHCl in this instance is so low that its influence on bioactivity may be ignored. However, it is still necessary to check further the bioactivity recovery with other methods [22].

Fig. 7 shows the results of SDS–polyacrylamide gel electrophoresis (PAGE). The right lane is the original extracted solution form *E. coli* cells and the left lane shows the major peak containing rIFN-γ in Fig. 6 using standard protein. The molecular mass of protein in the main spot is calculated to be about 17 000 (data not shown), as expected for rIFN-γ. The purity of refolded rIFN-γ is about 85% as determined by dual-wavelength thin-layer chromatographic scanner (not shown). We also did refolding and purification of an extracted solution of interleukin-2 and recombinant human interferon-β from *E. coli* cells using the same chromatographic conditions as in Fig. 6, and obtained a positive result, *i.e.*, their unfolded forms can be refolded and purified. However, the results are not comparable to those for rIFN-γ.

The bioactivity recovery of rIFN-γ refolded with HPHIC is so high that it is difficult to explain it only by removing GuaHCl from the purification system. As was pointed out above, the HPHIC system provides desirable conditions which can be further optimized for rIFN-γ refolding. rIFN-γ is a small protein with a molecular mass of less than 20 000, it is a very strong hydrophobic protein without any disulphide bond. The refolding of rIFN-γ should be much easier than that of other proteins, even small proteins, such as LYS and RNase.

Both Figs. 6 and 7 show that HPHIC is a powerful tool not only for protein refolding but also for the separation and purification of proteins.

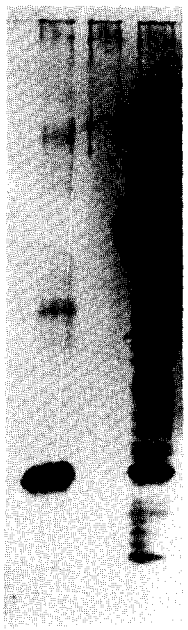

Fig. 7. SDS-PAGE of rIFN-γ. The large spot in the left-hand lane denotes the SDS-PAGE of the purified rIFN-γ from the peak at 33 min in Fig. 6. The right-hand lane represents the SDS-PAGE of the original solution extracted from *E. coli* cells with 7.0 mol/l GuaHCl.

CONCLUSIONS

HPHIC can be used to accomplish the refolding of a protein unfolded by a concentrated solution of denaturing agent, such as 7.0 mol/l GuaHCl and 8.0 mol/l urea solution. For some protein the refolding may be complete.

The reason for the protein refolding with HPHIC may be partially due to the completely removal of denaturing agent and to the establishment of a suitable environment in which its hydrophobicity, viscosity, ionic strength, etc., can be ajusted by gradient elution. The contact surface region between the surface of the HPHIC matrix with a suitable hydrophobicity and the hydrophobic part of the whole unfolded protein molecule may play the most important role in starting the protein refolding, apart from a contribution of the mobile phase to the spontaneous refolding of unfolded protein.

This method of protein refolding with HPHIC can be used for the renaturation of some therapeutic proteins extracted from *E. coli* in the biotechnological industry, whereby these proteins can be separated and purified.

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