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Purification and Characterization of Recombinant Human Interferon Alpha 2a Produced from *Saccharomyces cerevisiae*

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Abstract: The recombinant human interferon alpha 2a (rhIFN-α2a), expressed in *Saccharomyces cerevisiae*, was purified from insoluble aggregates. The inclusion body of rhIFN-α was solubilized by guanidine salt in the presence of disulfide reducing agent. The refolding of denatured rhIFN-α2a was achieved by simple dilution. The authentic interferon alpha, which has two correctly matched disulfide bonds, was seperated from incompletely oxidized IFN-α and dimeric IFN-α by use of a CM-Sepharose column, followed by size exclusion columns at two different pH conditions. The purified protein has been subjected to detailed physicochemical characterization including sequence determination. Unlike other rhIFN-α2a from *E. coli* reported, the rhIFN-α2a from *S. cerevisiae* has no methionine residue at its N-terminus originating from the start codon, ATG. The pI of the protein was determined to be 6.05 with a single band in the pI gel, which demonstrated that the purified rhIFN-α was homogeneous. The structural study using circular dichroism showed that the protein retains its three dimensional structure in the wide range of pH conditions between pH 3 and 9, and only minor strucural deformation was observed at pH 1.0.

Key words: antiviral activity, N-terminal Met, interferon alpha, purification, refolding

Interferons are a group of cellular proteins which act as potent biological response modifiers *in vivo*. It has been demonstrated that they can elicit antiviral activity, inhibit cell proliferation and modulate immune responses (Stewart, 1979). These biological properties have brought interferons into clinical use as therapeutic agents. Especially, interferon alpha is widely used for the treatment of chronic hepatitis, Kaposis sarcoma, and many other carcinomas.

Interferon alpha is produced by almost all cell types when stimulated by various viruses, bacteria, mycoplasma and protozoa (Sen *et al.*, 1992). It consists of either 165 or 166 amino acids depending of subtypes, of which more than 20 different types are known (Pestka, 1986). The production of IFN-α by recombinant technology for therapeutic use has been focused on IFN-α2a and IFN-α2b, which differ by a single amino acid at position 23. IFN-α2a has Lysine at residue 23 while IFN-α2b has Arginine at the same position. IFN-α has two disulfide bonds; one between Cys1 and Cys 98 and the other between Cys29 and Cys 138. Of the two disulfide bridges, the Cys29-Cys138 bond is known to be critical for interferon's biological activity (Morehaed *et al.*, 1984).

Many attempts have been made to clone and express rhIFN-α in microorganisms. The fisrt successful production of IFN-α2a was demonstrated by Goeddel *et al.* (1980) in recombinant *E. coli*. Because IFN-α was expressed as insoluble aggregates in either recombinant *E. coli* or recombinant yeast, many efforts were made to refold and purify the protein with a native-like structure. During the refolding process, it was known that several kind of variants were obtained, namely slow monomer, fast monomer, and oligomers, as judged under nonreducing conditions by SDS-polyacrylamide gel electrophoresis (Tarnowski *et al.*, 1986). The fast monomer indicates the protein with two disulfide bonds while the slow monomer represents the molecule with one disulfide bond (i.e. partially oxidized IFN-α). The specific activity of purified rhIFN-α2a in bovine MDBK cells was $2\sim2.2\times10^8$ IU/mg of protein (Pestka and Tarnowski, 1985; Thatcher *et al.*, 1986) and partially oxidized rhIFN-α2a showed about 80% of the activity of the fully oxidized form (Morehead *et al.*, 1984).

While characteristics of rhIFN-α2a from *E. coli* have been well reported, rhIFN-α2a from *Saccharomyces cerevisiae* have not. In this paper, we report the optimal conditions of refolding for the insoluble rhIFN-α2a

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from yeast cells, and furthermore the purification method to obtain a homogeneous IFN-α2a suitable for pharmaceutical purposes. The detailed physicochemical properties of the purified IFN-α2a are also described.

Materials and Methods

Materials

Saccharomyces cerevisiae strain transformed with a vector coding rhIFN-α2a was provided by LG chemical Ltd. (Cho *et al.*, 1992). Phenylisothiocyanate and phenylthiocarbamyl amino acids were purchased from Pierce (Rockfold, USA) and acetonitrile from Burdick & Jackon (USA). C_{18} μ-bondapak column and PicoTag column were purchased from Waters (Milford, USA). Chemicals used for the experiments were all reagents grade.

Protein concentrations were determined by the BCA (Pierce) method with bovine serum albumin as standard. International standard rhIFN-α2a (Gxa 01-901-535) was obtained from NIAID (National Institute of Allergy and Infectious Diseases) and its specific activity was 2.0×10^8 IU/mg when assessed by CPE/dye-uptake assay.

Refolding of rhIFN-α

The cultured yeast cells were disrupted with glass beads in the buffer (20 mM Tris-HCl, 1 M Urea, 1 mM EDTA, 1 mM PMSF, pH 8.0) and the precipitate was collected by centrifugation at 15,000 g. The insoluble aggregates were washed consecutively with washing buffer (20 mM Tris-HCl, 1 M Urea, 1 mM EDTA, 0.1% Triton X-100, pH 8.0) and 2 M guanidine-HCl. After centrifugation, the aggreagtes were solubilized in three volumes (v/w) of solubilizing buffer (20 mM Tris-HCl, 6 M guanidine-HCl, 100 mM 2-mercaptoethanol, pH 8.0). After removing the pellet by centrifugation the supernatant was diluted to six times the original volume with 20 mM Tris-HCl buffer at pH 8.0 and clarified by centrifugation. The supernatant was dialyzed against DEAE buffer (20 mM Tris-HCl, 0.12 M NaCl, pH 8.0).

Purification of rhIFN-α

The clear solution was concentrated and then loaded onto a DEAE cellulose column (Whatman Inc.) equilibrated with DEAE buffer. The flow-through fraction was collected and dialyzed against CM buffer (50 mM sodium acetate at pH 4.5). The protein solution was loaded onto a CM-Sepharose column (Pharmacia Co.) equilibrated with the same buffer. After washings with CM buffer containing 0.15 M NaCl, the proteins were eluted from the column with the buffer containing 0.175 M NaCl. The fractions containing the fully oxidized form as judged by nonreducing SDS-PAGE were collected and then concentrated with YM-10 membrane (Amicon Co.). The concentrated solution was applied to a S300 column (Pharmacia Co.) equilibrated with 20 mM ammonium acetate, 0.1 M NaCl at pH 5.0. The pH of the collected monomer fraction was raised to pH 7.2 with 1 M NaOH, and then loaded to a S100 column (Pharmacia Co.) equilibrated with phosphate buffered saline solution at pH 7.2. The eluents were analyzed by SDS-PAGE under both reducing and nonreducing conditions.

C-terminal sequencing

Enzymatic method was used for the C-terminal determination. rhIFN-α2a was made to react with Carboxypeptidase P (Boehringer mannheim Co.) at 37℃. After 1 min, 2 min, 5 min, and 10 min, solutions were taken out and the reaction was stopped by addition of acetic acid to a final 80% concentration. The amino acid composition of the samples was determined by the ABI 420AH analyzer.

Size exclusion HPLC

Protein PAK 125 column (Waters Co.) was used for size exclusion HPLC. Samples were introduced into the column equilibrated with 10 mM sodium phosphate, 0.14 M NaCl at pH 7.2. The elution flow rate was 0.5 ml/min. and the eluent was monitored at 280 nm.

CD measurement

CD spectra of the protein were determined by a Jasco J-600 spectrophotometer. The cell path length used was 10 mm. The results were recorded as mean residue molar ellipticity (θ) with units of $deg\cdot cm^2\cdot dmol^{-1}$ and calculated from the following equation;

$$(\theta)=mdeg\times MRW/10\times l\times c$$

where mdeg was the measured ellipticity in millidegrees, MRW was the mean residue molecular weight, l was the optical path length of the cell in cm, and c was the protein concentration in mg/ml.

pI determination

pI was determined with ready-made gel (pH 3~10) and electrode solutions prepared by Novex (San Diego, USA). Focusing was performed sequentially at 100 V for 1 h, 200 V for 1 h and finally 500 V for 30 min. The gel was fixed with the fixing solution (3.5% sulphosalicylic acid, 11.5% trichloroacetic acid) and stained with Coomassie Brilliant Blue R-250.

Antiviral activity

In order to determine the specific activity of rhIFN-α2a, antiviral activity was measured by use of Vesicular stomatitis virus (VSV) as a challenge virus and MDBK cell as a host cell. The antiviral activity of rhIFN-α2a was determined by cytopathic effect/dye uptake method as described previously (Kim and Park, 1992).

Results

Refolding

When IFN-α2a was expressed in recombinant yeast cells, the protein was made to relatively solid insoluble aggregates. They were solubilized by high concentration of guanidine salts, but other mild chaotropic agents such as urea was not sufficient to solubilize the inclusion body. In the experiments, 6 M of guanidine salt in the presence of disulfide bond reducing agent yielded complete solubilization of the precipitated protein. Prior to the solubilization of inclusion body, a washing step was implemented in order to remove contaminating materials effectively. Refolding of the solubilized protein was achieved by six fold dilution of the solution, followed by extensive dialysis with 20 mM Tris-HCl buffer at pH 8.0.

Purification

Because interferon alpha contains 4 Cysteins which make two disulfide bonds, many variants with different combinations of disulfide bond formation could be produced during the refolding process. Three forms of interferon alpha variants were observed, during the refolding procedure, as judged by non reducing SDS-PAGE (Fig. 1). The high molecular weight IFN-α variant migrated on the gel as a dimeric molecule (lane 3 in Fig. 1). The slow monomer (lane 2 in Fig. 1) represents a partially oxidized IFN-α with only one disulfide bond formed and the fast monomer (lane 1 in Fig. 1) indicates an authentic IFN-α with two disulfide bonds. The results shown in Fig. 1 were also observed in the refolding experiments with IFN-α expressed in recombinant *E. coli* (Tarnowski *et al.*, 1986).

The seperation of IFN-α variants was accomplished by use of CM-Sepharose ion exchange column (Fig. 2). The partially oxidized IFN-α was eluted from the column at a lower salt condition, followed by the fully oxidized native form. The dimeric IFN-α was eluted at a much higher salt concentration. The stepwise elution of the proteins in CM-Sepharose described in Materials and Methods provided several advantages over gradient elution. Operation time was greatly saved without sacrificing resolution power, thus enabling increasing the scale of chromatography column.

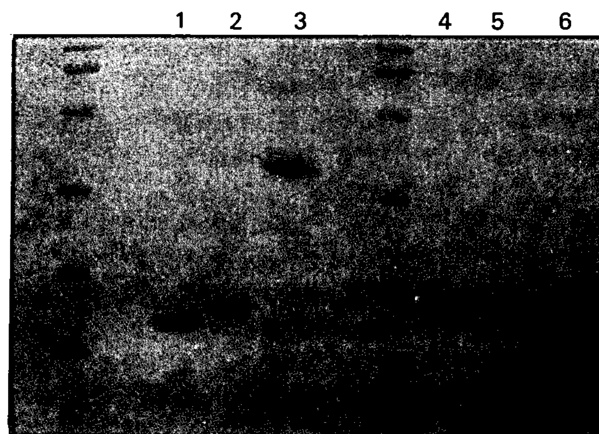

Fig. 1. Reducing and nonreducing SDS-polyacrylamide gel electrophoresis of the rhIFN-α2a variants. Lanes 1~3 were nonreducing PAGE and Lanes 4~6 were reducing PAGE. Lane 1 and 4: fast monomer, Lane 2 and 5: slow monomer, Lane 3 and 6: dimer. The molecular weight markers were Mr 10000~100000 standards (Bio-Rad; lysozyme, soybean trypsin inhibitor, carbonic anhydrase, ovalbumin, bovine serum albumin, phophorylase B). A blank lane was required between molecular weight marker and nonreducing fast monomer to prevent artifacts arising form diffusion of 2-mercaptoethanol.

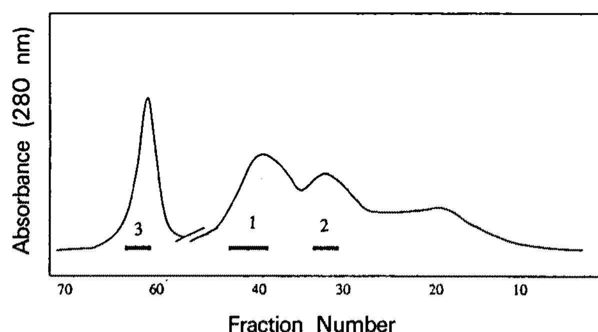

Fig. 2. Chromatogram of CM-Sehparose. 1: fast monomer, 2: slow monomer, 3: dimer. Experimental conditions were as described in Materials and Methods.

The dimeric form of IFN-α and other high molecular weight contaminating proteins were further removed by S300 gel filtration column at low pH.

Concentration-dependent subunit interaction at high pH

It has been well known that rhIFN-α shows pH dependent oligomerization (Shire *et al.*, 1983). Therefore when gel filtration chromatography was performed at pH greater than 5.0 with *E. coli*-derived rhIFN-α, resolution disappeared (Tarnowski *et al.*, 1986). In our experiment at pH 7.2, it was found that polymerization of IFN-α2a was also concentration dependent (Fig. 3). In this figure, when 0.27 mg/ml of rhIFN-α was loaded

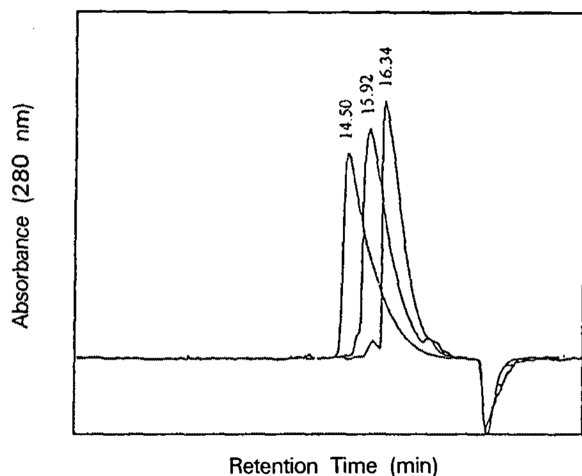

Fig. 3. Chromatogram of size exclusion HPLC at pH 7.2 at different interferon concentration. The numbers on the chromatogram are the elution times. The retention times of 14.5 min, 15.92 min and 16.34 min represent the results obtained from respectively 2.7 mg/ml, 0.68 mg/ml and 0.27 mg/ml of rhIFN-α2a.

to a sizing HPLC column, the protein appeared at the molecular weight of 19 K dalton, which coincided with the size of monomeric rhIFN-α2a. But at the concentration of 0.68 mg/ml and 2.7 mg/ml of the proteins, they appeared as 23.5 K dalton and 44.6 K dalton, respectively.

We used this phenomenon for the purification of rhIFN-α2a. The protein solution obtained from a S300 gel filtration column contained the contaminating proteins whose sizes were similar to rhIFN-α2a. Thus, when we applied rhIFN-α2a solution on to a S100 gel filtration column at higher protein concentration (over 10 mg/ml) at pH 7.2, we could remove the lower molecular weight contaminants. The purity assessed by the C_{18} reverse phase HPLC method demonstrated that there are no detectable protein contaminants in the purified rhIFN-α2a (data not shown). The antiviral activity of the purified rhIFN-α2a was more than 2.8×10^8 IU/mg of protein as assessed by the CPE/dye uptake method.

Primary structure determination

In order to confirm that the cDNA sequence of rhIFN-α2a was correctly translated in yeast cells, the amino acid analysis, N-terminal sequencing, and C-terminal sequencing were performed with the purified rhIFN-α2a.

The amino acid composition of each amino acid agreed well with the expected values based on cDNA sequence (data not shown). The amino acid sequence of 27 N-terminal amino acids were as follows;

X-Asp-Leu-Pro-Gln-Thr-His-Ser-Leu-Gly-Ser-Arg-Arg-Thr-Leu-Met-Leu-Leu-Ala-Gln-Met-Arg-Lys-Ile-Ser-Leu-

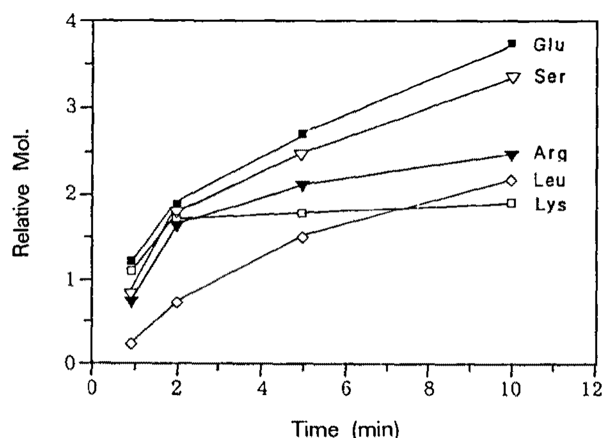

Fig. 4. C-terminal sequencing of rhIFN-α2a by Carboxypeptidase P method. The experimental conditions were as described in Materials and Methods.

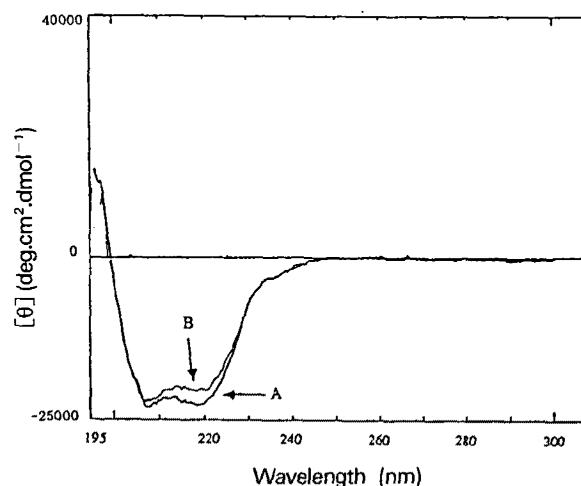

Fig. 5. CD spectra of the authentic (A) and partially oxidized (B) rhIFN-α2a at pH 7.2. The concentrations of the sample were 84 μg/ml (A) and 58.6 μg/ml (B), respectively.

Phe

where X indicates blank in the first cycle of sequencing. Because Cys bridged to another Cys bond can not be detected in the Edman reaction, we assumed that the first residue of the protein was disulfide bonded Cys. The amino acid sequencing after reduction and carbarmylation of rhIFN-α2a proved that X was disulfide-bonded Cys. This result clearly demonstrated that N-terminal Met translated from the start codon of IFN-α2a was processed completely in *S. cerevisiae*.

By use of the enzyme method described in Materials and Methods for the C-terminal amino acids, we could identify 5 amino acids (Fig. 4). The sequence was -Leu-Arg-Ser-Lys-Glu, which was expected from the cDNA sequence. The result showed that C-terminal amino acids were not digested by the carboxypeptidases in yeast during expression.

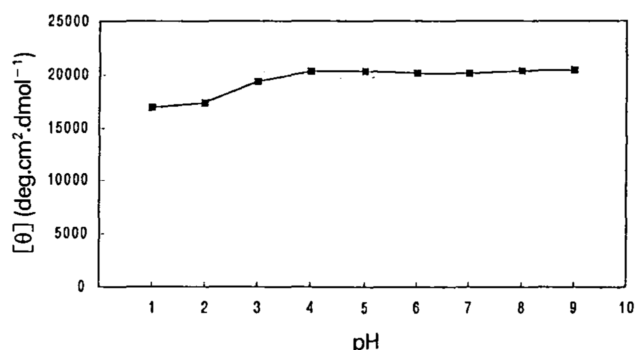

Fig. 6. CD spectra (at 222 nm) of the rhIFN-α2a in the range of pH 1.0~9.0. The concentration of the sample was 62.6 μg/ml in 6 mM sodium citrate-borate-phosphate, 50 mM NaCl. The pH was adjusted with 1 M NaOH and 1 M HCl.

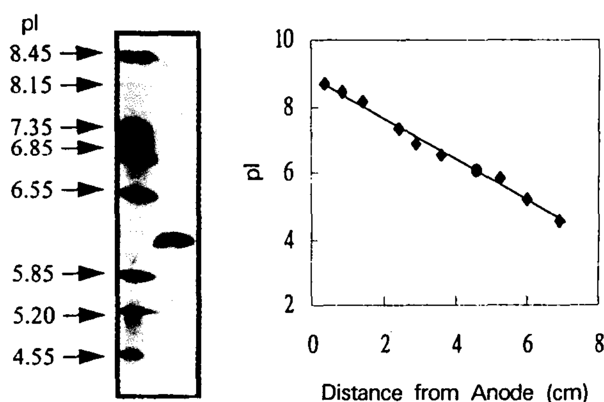

Fig. 7. Isoelectric focusing result of the rhIFN-α2a purified from *Saccharomyces cerevisiae*. ◆: standard proteins, ●: rhIFN-α2a.

CD determination

The secondary structure of the purified rhIFN-α2a was determined by circular dichroism spectroscopy. The CD spectrum in Fig. 5 indicates that rhIFN-α2a consists mainly of an α-helix (about 60%). The partially oxidized rIFN-α2a obtained during purification as a by- product was also an α-helical protein whose helix content was only slightly lower than that of the authentic rIFN-α.

The pH dependent protein stability was studied by measuring α-helix content (Fig. 6). The result showed that rhIFN-α was stable (or no change of helix content) between pH 3.0 and pH 9.0. Even at an extreme acidic condition (pH 1.0), the helix content was reduced by no more than 16% compared to that at pH 7.0.

pI determination

Homogeneity of the purified rhIFN-α2a was assessed by pI determination. A single band at pI 6.05 was observed (Fig. 7). The pI of yeast-derived rhIFN-α2a was almost identical to the result obtained from *E. coli*-derived rhIFN-α2a (Wetzel *et al*., 1981). However, the published results showed that the minor bands at acidic positions were also observed with *E. coli*-derived rhIFN-α. The single band obtained with yeast-derived IFN-α2a demonstrated that the purified protein is extremely homogeneous and pure.

Discussion

The recombinant interferon alpha is effective in treating viral diseases and carcinomas. IFN-α is the only drug which has been approved by FDA for the treatment of chronic hepatitis B and chronic hepatitis C (Dorr, 1993). Aside from the enormously important therapeutic applications of the protein per se, rhIFN-α has been a good candidate for the studies of protein refolding and structure-function relations.

When recombinant yeast cells were subjected to non-reducing SDS-PAGE after fermentation, it was found that most of the rhIFN-α was present in the covalently attached polymerized form. For the refolding of rhIFN-α2a expressed from recombinant *Saccharomyces cerevisiae*, we adopted a spontaneous oxidation method where the inclusion body was solubilized by a chaotropic agent and subsequantely diluted with an appropriate buffer. This early refolding implemented for rIFN-α was necessary because extensive purification procedures were required in order to remove variants with improper disulfide connections. In the washing step, washing of insoluble aggregates by a buffer containing 0.1% Triton X-100 effectively removed yeast-originated lipids and further washing with a 2 M guanidine HCl eliminated much of the yeast proteins. In detergent washing, the use of tween was not as effective as triton to serve the purpose. The elimination of contaminating materials in washing steps brought forth the improved refolding yields.

The addition of mercaptoethanol was also neccesary for the complete solubilization of insoluble aggregates, thus resulting in the better recovery of refolded protein. Another important factor for proper refolding was the choice of dilution buffer. Because IFN-α is a relatively hydrophobic protein, salt concentration in the buffer affected the refolding process. The dilution buffer described in Materials and Methods was chosen from many different buffer conditions for the maximum recovery of refolding yield.

The theoretical variants expected from a protein containing 4 cysteines are three fully oxidized forms. In rhIFN-α, only one of the fully oxidized forms was observed after refolding, that is, an authentic form of IFN-α with two disulfide bonds at Cys1-Cys98 and Cys29-Cys138. On the other hand, partially oxidized rIFN-α2a and the dimeric form of rIFN-α2a were also observed during refolding (Fig. 1). Amino acid sequencing showed that this partially oxidized form of rIFN-α has a disulfide

bond between Cys29 and Cys138, rendering Cys1 and Cys98 free from oxidation (data not shown). This result agrees with the previous reports that in *E. coli*-derived rIFN-α2a, the disulfide bond interaction between Cys29 and Cys138 was more favorable than that of Cys1 and Cys98 (Morehead *et al.*, 1984). It was observed in our experiments that the partially oxidized form of rIFN-α2a was quite stable and the majority of the molecule slowly converted to the dimeric form of rIFN-α upon storage. It, therefore, becomes clear that complete elimination of partailly oxidized rIFN-α is necessary during purification because dimeric rIFN-α elicits unwanted immune response when rhIFN-α2a is used for therapeutic purposes.

Interferon alpha has been known to exist in a monomeric form in the human body. The recombinant IFN-α, however, interacts to make a non-covalent oligomer *in vitro* under physiological conditions. At physiological pH, the oligomrization of IFN-α2a was concentration dependent (Fig. 3). The oligomerization of rhIFN-α2a was also observed in native electrophoresis (data not shown). In Fig. 3, the molecular weight of rIFN-α at a concentration of 0.68 mg/ml was the same as the size of two molecules of rIFN-α. Thus, in the experimental conditions described, the transition concentration of monomer-dimer subunit interaction of rIFN-α was between 0.27 mg/ml (or 14.2 μM) and 2.7 mg/ml (142 μM). The finding that rIFN-α undergoes concentration dependent oligomerization at physiological pH can be implemented for the purification. The contaminating proteins with similar molecular weights to rIFN-α2a were effectively removed by size-exclusion chromatography at pH 7.2. The purity of rhIFN-α2a obtained through the process described in Materials and Method was more than 99.5% as assessed by double staining SDS-PAGE (i.e. Coomassie staining, followed by silver staining).

In the results, what was noteworthy was that *Saccharomyces cerevisiae*-derived rhIFN-α did not have a N-terminal Met residue originating from the start codon. It was reported that in *E. coli*-derived rhIFN-α, the N-terminal Met was incompletetly removed (about 50%) in the cell (Wetzel *et al.*, 1981), so that it existed as a mixture. This result implies that yeast-derived rhIFN-α may be a better choice for therapeutic purposes than *E. coli*-derived rhIFN-α because the recombinant drugs with N-terminal Met frequently induce antibodies in human beings after repeated injections. It has been well documented that the patients treated with recombinant Met -human growth hormone showed higher antibody formations than the patients treated with methionyl free recombinant human growth hormone (Hibi *et al.*, 1987).

The secondary structure of rhIFN-α2a shown in Fig. 5 clearly demonstrates that the protein structure after refolding was almost the same as that of natural IFN-α. Even if the three dimensional structure of IFN-α has not been revealed yet, it can be expected from many other structural studies that IFN-α consists of an α-helix (Bewley *et al.*, 1982). Fig. 5 indicates that the rhIFN-α2a is an α-helical protein without β-stranded secondary structure. Fig. 6 also demonstrates that rhIFN-α2a is extremely pH stable. Even under acidic conditions where most proteins are easily denatured, rhIFN-α2a still possesses the majority of its three dimensional structure.

Finally, the refolding procedure and the purification methods reported in this manuscript were suitable for the production of rhIFN-α2a for pharmaceutical usage. The efficacy of the refolding and the purification method were indirectly confirmed by the specific activity of the purified rhIFN-α2a. The antiviral activity was 2.8 $\sim 3.0 \times 10^8$ IU/mg of the protein as determined by CPE/dye-uptake inhibition assay, while that of the international standard (recombinant hIFN-α2a) from *E. coli* obtained from NIAID was 2.0×10^8 IU/mg. The refolding process is relatively simple, yet very effective. The purification method was also convenient for increasing the scale of production and more advantageous than other cumbersome techniques such as the immunoaffinity chromatography used for the production of *E. coli*-derived rhIFN-α2a (Secher *et al.*, 1980).

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