Investigation of thermal stability of recombinant human interferon-gamma

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Our studies in the structure and biological function of recombinant human interferon gamma (IFN-γ) showed that IFN-γ was stable at –20 °C if to analyse the quantity of its monomeric form (MW 17240 Da). The protein aggregated and degraded at a temperature of +37 and +50 °C. The degradants became visible in SDS- PAGE after 5 days and oligomeric forms after 10 days (+37 °C). Their molecular mass measured by electrophoresis method were 16000–11000 Da for degradants and 33000, 31000 (+37 °C), 35000–32000 (+50 °C), for oligomeric forms. The protease inhibitor PMSF stimulated the appearance of dimeric forms and EDTA exhibited a slight suppression of the degradants.

Key words: human recombinant interferon-gamma, thermal stability, proteolytic degradation, protease inhibitors

INTRODUCTION

Human interferon-gamma (HuIFN-γ), one of the three major classes of human interferons, has been shown to have a broad range of biological effects. It is a glycoprotein naturally secreted by activated T lympfocytes and monocytes [1]. HuIFN-γ is suitable as an antiviral, antiproliferative, immunomodulatory human therapeutic agent, particularly for the treatment of kidney tumours and chronic granulomatosis and glioblastoma (brain cancer) [2]. Recombinant HuIFN-γ produced in bacteria *Escherichia coli* is not glycosylized [3].

As our previous investigations [4] have shown, recombinant human interferon-gamma is dimeric in solution at pH 7–4 as revealed by analytical gel-filtration. It was proven by circular dichroism that decreasing pH to 5.0 has not affected the secondary and tertiary structures of the HuIFN-γ macromolecule. It was established that heat denaturation process of gamma interferon obeys the two-state transition model and can be described as the first-order reversible reaction. Temperature dependence of the denaturation–renaturation rate constants was shown to be consistent with the Arrhenius law. The equilibrium value of the denaturation temperature was found. Effective enthalpy of denaturation was determined both by thermodynamic and kinetic approaches. The data obtained showed that in the pH range 7–4 the dimeric HuIFN-γ structure could be considered as a single cooperative thermodynamic domain. Thus, it may be concluded that gamma-interferon is necessary for the existence of the corresponding tertiary structure of a macromolecule.

The development of stable and effective pharmaceutical protein requires not only careful handling at every stage of recombinant HuIFN-γ purification, but also its storage under proper conditions. The complicated structure of recombinant proteins makes these substances highly degradable. Therapeutic peptides and proteins can degrade by several physical and chemical pathways. In most cases, more than one pathway of physical and / or chemical instability is responsible for the degradation [5]. Of all the interferons, the stability, especially thermal, of HuIFN-γ is the lowest [6]. This low stability of HuIFN-γ renders it difficult to use as a human therapeutic agent. It is generally known that proteins must be stored in an appropriate temperature to retain activity and prevent degradation or aggregation. During storage the degraded forms and oligomers can be observed. Such solutions of HuIFN-γ may have a lower potency and cause thrombosis if injected [7].

The aim of our investigation was to analyse the thermal stability of recombinant HuIFN-γ and to cle-

ar up the reasons for degradation. To assess the stability of recombinant HuIFN-γ solution, we have incubated samples at different temperatures such as –20 °C, +37 °C and +50 °C. The degrades and aggregates were evaluated by the SDS-PAGE method.

MATERIALS AND METHODS

Acrylamide/Bis powder (Bio-Rad), ammonium persulfate (Bio-Rad), bromphenol blue (Bio-Rad), gel drying solution (Bio-Rad), 2-mercaptoethanol (Bio-Rad), premixed buffer 10xtris/gly/SDS (Bio-Rad), SDS solution 10% (Bio-Rad), TEMED (Bio-Rad), low mol mass standard (AmershamPharmacia-Biotech), acetic acid glacial (Merck), EDTA-Na_2xH_2O (Merck), formaldehyde 37% (Merck), glycerol (Merck), ethanol (Stumbras), silver nitrate (Merck), sodium carbonate (Merck), sodium Thyosulphate (Merck), PMSF (Merck).

The lot of solution of recombinant HuIFN-γ was obtained from "Biotechna" in freezed form. The solution was formulated in succinate, mannitol, TWEEN-20, pH 5.0 buffer at a concentration of 0.3 mg/ml. Before the experiment recombinant HuIFN-γ solution was warmed up under ambient temperature. The samples were dispensed in 1 ml portion in glass vials that were then kept at –20 °C, +37 °C, +50 °C for appropriate incubation time with or without proteolytic enzyme inhibitors. The treatment with protease inhibitors EDTA and PMSF was performed at a concentration of 2 mM. At the end of the exposition period the samples were analysed by the SDS-PAGE method [8]. Aliquots containing 5 μg of the sample were prepared for SDS-PAGE in reducing and non-reducing conditions.

Detection of degraded, aggregated and monomeric forms of recombinant HuIFN-γ was performed after staining gels with silver. Evaluation of the molecular mass of various forms and their percentage was performed with the ScanPack 3 program.

RESULTS AND DISCUSSION

Recombinant human interferon-gamma was purified from *E. coli* by a series of chromatographic procedures and prepared as described earlier [4]. Recombinant proteins often undergo a denaturation–renaturation cycle during extraction and purification.

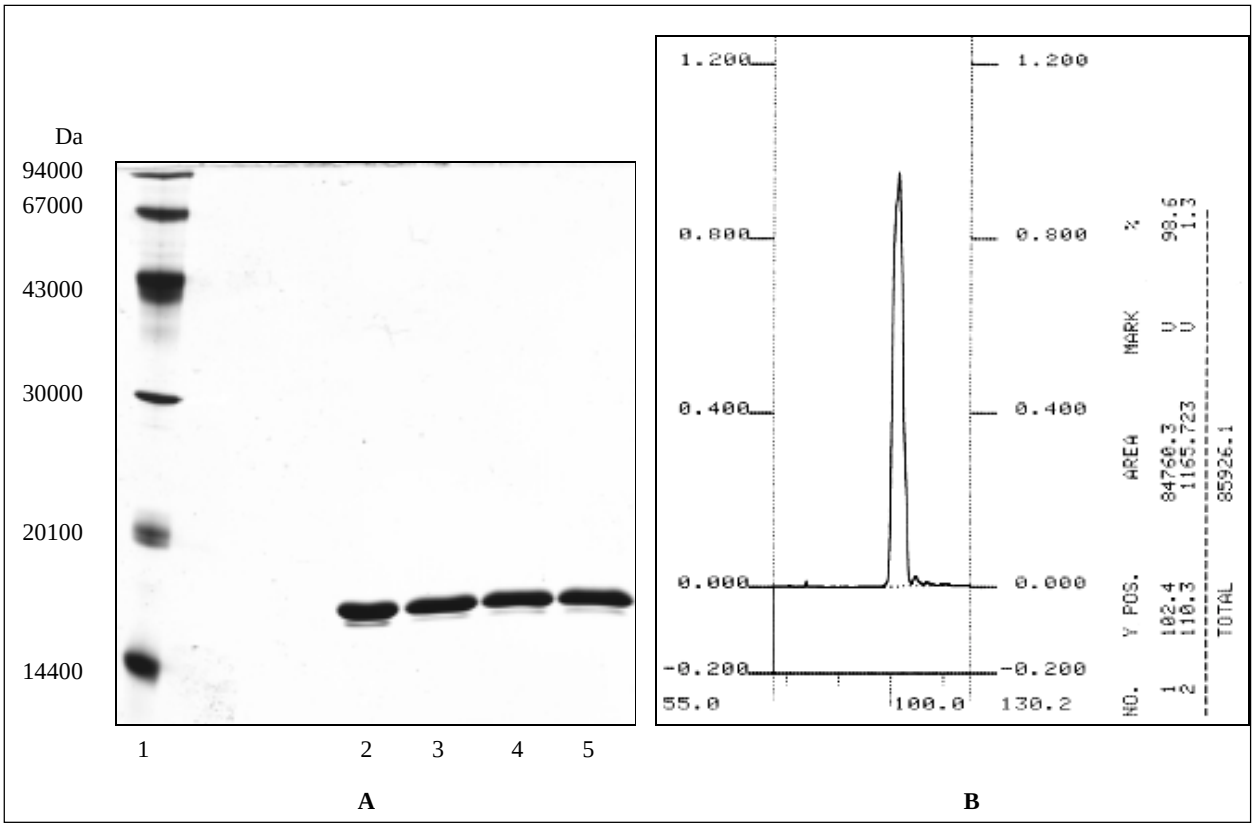

Fig. 1. **A**. Investigation into the homogeneity of recombinant HuIFN-γ by SDS-PAGE method. Lanes: 1 – molecular weight markers; 2–4 – different batches of purified recombinant HuIFN-γ; 5 – standard of recombinant HuIFN-γ. All the samples of recombinant HuIFN-γ were stored at –20 °C. Purified HuIFN-γ samples (5 μg) were resolved on 15% SDS-PAGE and silver-stained. **B**. Results of densitometric scanning

Therefore, determination of the physical homogeneity and the state of aggregation in the final preparation is essential to prove the quality.

Investigation into the homogeneity of recombinant HuIFN-γ has been analysed using gel electrophoretic approaches in combination with densitometry scanning. Initial solution of HuIFN-γ was formulated at pH 5.0. Recombinant HuIFN-γ is known to aggregate at a neutral to slightly alkaline pH and the resulting solutions may form visible precipitates. Such solutions may have a lowered potency. Formulation of a liquid dosage form buffered around pH 5.0 was found to prevent such aggregation. The pH-dependence on the aggregation of HuIFN-γ has been exhaustively analysed by Mulkimer and Wetzel [9].

The batches of recombinant HuIFN-γ showed a high quality of homogeneity revealed by the SDS-PAGE method in reducing and non-reducing conditions. Recombinant HuIFN-γ whose homogeneity electrophoresis view and results of densitometric scaning are presented in the example was used in thermo-stability analysis (Fig. 1 A; Lane 2, Fig. 1 B). The densitometry scanning has shown 98.6% of protein in monomeric form and an additional little fragment which contained about 1.4% of total protein. Deterioration of recombinant HuIFN-γ during storage at various temperatures (–20 °C, +37 °C, +50 °C) becomes apparent when aged samples are analysed by SDS-PAGE electrophoresis. The method has demonstrated the formation of hydrolysis products as well as minor amounts of higher molecular weight products (Fig. 2).

The visual evaluation and densitometric scanning of gels showed that during storage of recombinant HuIFN-γ at +37 °C for five days and more there were visible lower fragments than monomeric form (Fig. 2A, Lanes 4–8); after ten days we could see aggregate forms (Fig. 2A, Lanes 5–8). The aggrega-

Table 1. **Quantitative changes of the monomeric form of recombinant human interferon-gamma**

Day	Storage at +37 °C		Storage at +50 °C	
	Monomeric form, %	Other forms, %	Monomeric form, %	Other forms, %
0	98.6	1.4	98.6	1.4
1	98.5	1.5	96.9	3.1
5	97.5	2.5	85.1	14.9
10	93.5	6.5	78.3	21.7
15	92.4	7.6	75.3	24.7
20	91.1	8.9	70.5	29.5
25	90.7	9.3	62.2	37.8

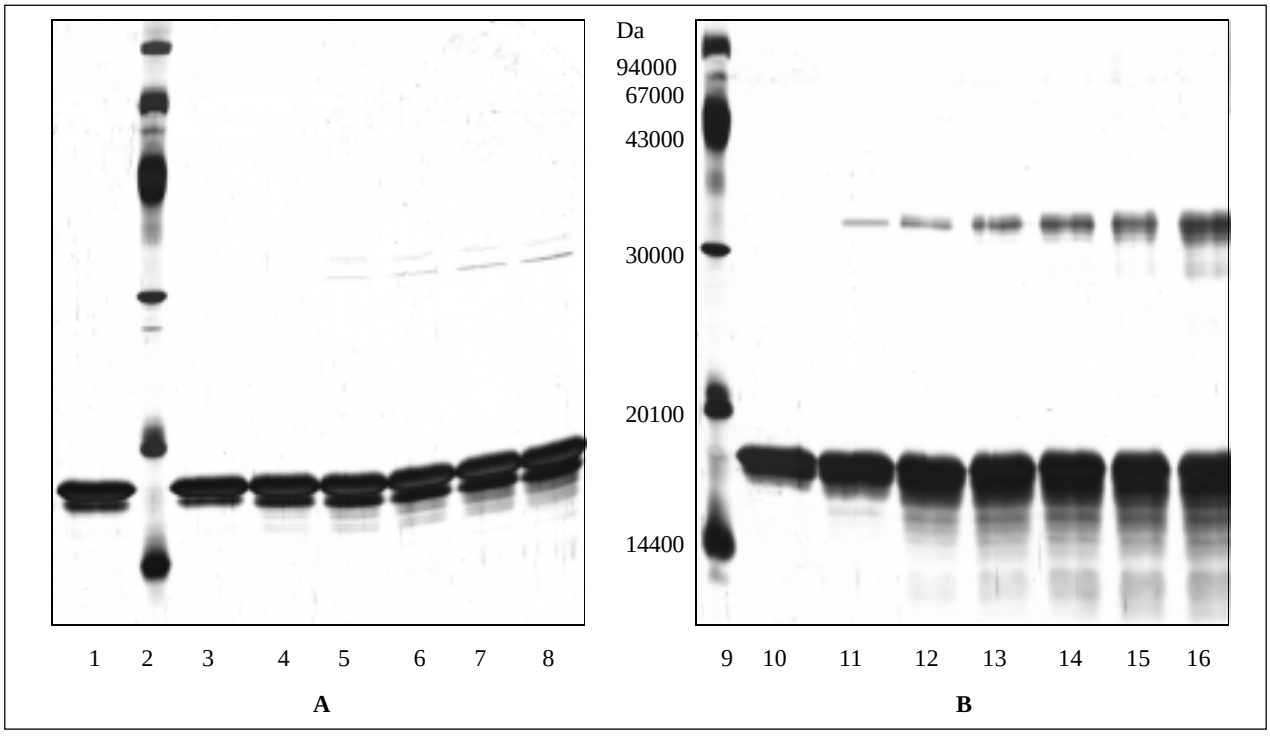

Fig. 2. Investigation into the stability of recombinant HuIFN-γ after incubation at +37 °C (**A**) and +50 °C (**B**) for 1, 5, 10, 15, 20 and 25 days. Recombinant HuIFN-γ stored at –20 °C served as control (Lanes 1,10). Lanes: 2, 9 – molecular weight markers, 1, 10 – recombinant HuIFN-γ (–20 °C); 3–8 – samples after incubation at +37 °C for 1, 5, 10, 15, 20, 25 days respectively; 11–16 – samples after incubation at +50 °C for 1, 5, 10, 15, 20, 25 days respectively

te forms of the protein analysed appeared very soon – after one day under storage at +50 °C (Fig. 2B, Lane 11). Quantitative changes of the monomeric form of pharmaceutical protein HuIFN-γ is shown in Table 1

Evaluation of the molecular mass of the fragments was carried out. The SDS-PAGE method with standard mass molecular protein and ScanPack 3.0 program was used. Figures 3 and 4 show the typical densitograms of recombinant HuIFN-γ whose aliquots were stored at –20 °C (Fig. 3) and +37 °C (Fig. 4)

The molecular mass of aggregate or other forms of recombinant HuIFN-γ was evaluated by SDS-PAGE method and the data are presented in Table 2.

In order to make certain that the variation of HuIFN-γ-gamma forms is related to the impact of protease, an experiment was conducted with two protease inhibitors, EDTA and PMSF. In comparison,

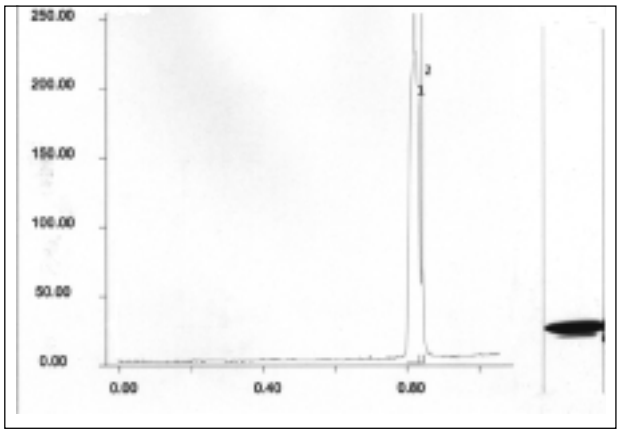

Fig. 3. Densitogram of recombinant HuIFN-γ stored at –20 °C. On the right side of the picture the electrophoresis view of scanned protein is shown

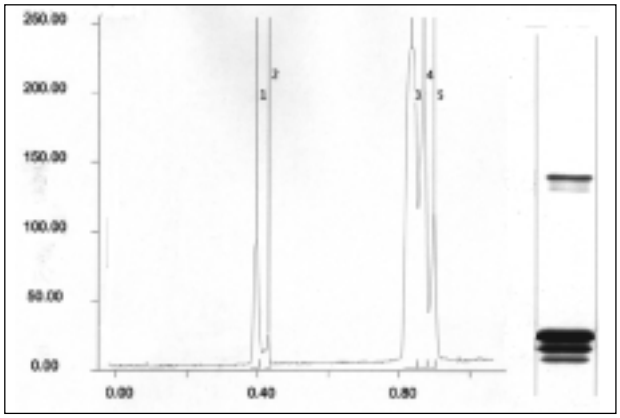

Fig. 4. Densitogram of recombinant HuIFN-γ stored at +37 °C for ten days. On the right side of the picture the electrophoresis view of scanned protein is shown.

Table 2. **Evaluation of molecular mass of recombinant human interferon-gamma**

Storage temperature		Oligomeric form, Da	Monomeric form, Da	Other forms, Da
–20 °C		Not observed	17240	16700
+37 °C	1 day	Not observed	17300	16050
	5 days	Not observed	17030	16160 15850
	10 days	32610 30870	16860	16030 15340
	15 days	33520 31700	17470	15420 14610 13760
	20 days	33520 31870	17400	15250 14610 14020 14970
	25 days	33205 31343	17500	14800 14070 13530 12630
+50 °C	1 day	34400	17690	15980
	5 days	34850	17510	15690 14670
	10 days	3233	17100	16440 15760 14890
	15 days	35220	17640	14800 13670 12350 11350
	20 days	33050	17330	14720 13630 12290 11130
	25 days	33730	17670	14610 13500 12130 10800

similar experiments were also carried out with or without protease inhibitors. An estimation of the theoretical values and the values presented in Table 2 and their comparison suggested that the protein degradation could be influenced by proteolytic enzymes. It is highly probable that the appearance of fragments lower than the monomeric form of 17000 Da in HuIFN-γ preparation could be connected with proteolytic degradation. It is known that the primary structure of HuIFN-γ reveals the potential sites of proteolytic degradation. The most sensitive places for some proteolytic enzymes are known

as Lys–Arg (128–129), Arg–Lys (129–130), Lys–Arg (130–131), Ser–Glu (132–133), Met–Leu (134–135). What is known about the carboxyl is that terminal heterogeneity of human and murine IFN-γ can be due to the proteolytic degradation during and after secretion [7]. During the initial purification phases the majority of protease contaminants are usually removed. However, trace amounts of protease can still cause a considerable proteolysis and some proteases can co-purify with the protein of interest, because many proteases have a molecular weight in the range of 20000–30000 Da.

On the other hand, recombinant HuIFN-γ produced in mammalian cells is glycoproteins. The glycosylation sites appear to afford some proteolytic protection. In contrast to the other cytokines and interferons, interferon-gamma does not contain any cystein. If compared to human interferon α, β and ω which can be heated to 100 °C [10] without any loss of biological activity, the thermal stability of recombinant HuIFN-γ is low.

The use of proteolytic enzyme inhibitors such as EDTA and PMSF could elucidate this presumption. This is shown in Fig. 5.

To sum it up, the obtained results were unexpected. Contrary to our expectations, the degradation of HuIFN-γ was not stopped either with EDTA or PMSF. The serine protease inhibitor PMSF accelerated the aggregation significantly. It is likely that the hydrolysis of the peptide chain of HuIFN-γ is not related to proteolysis and could be a nonspecific chemical process, where Arg plays a very important role. The question of investigation into the stability of recombinant HuIFN-γ is still in progress.

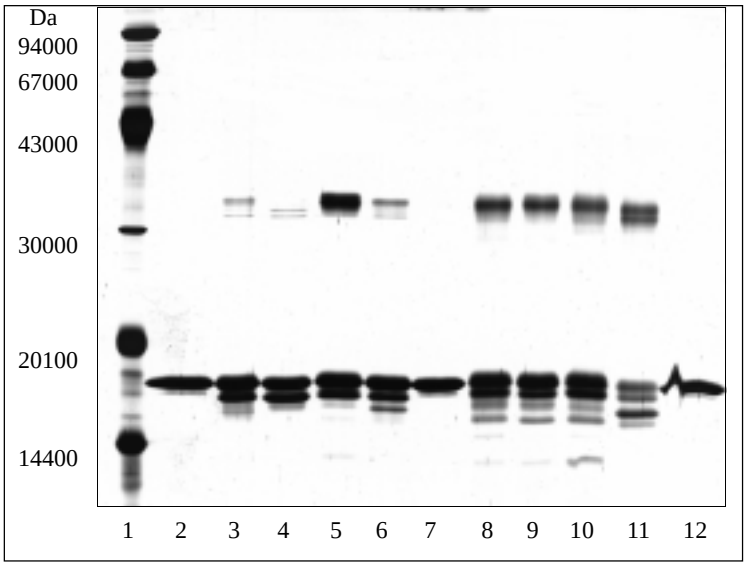

Fig. 5. Investigation into the recombinant HuIFN-γ stability in presence of protease inhibitors. Lanes: 1 – molecular weight markers; 2, 7, 12 – recombinant HuIFN-γ control (storage at –20 °C); 3, 8 – recombinant HuIFN-γ in absence of protease inhibitors at +37 °C and +50 °C; 4, 9 – with EDTA at +37 °C and +50 °C. 5, 10 – with PMSF at +37 °C and +50 °C.

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REKOMBINANTINIO ŽMOGAUS GAMA-INTERFERONO TERMINIO STABILUMO TYRIMAI

S a n t r a u k a

Baltymų PAAG-SDS elektroforezės metodu nustatyta, kad gamainterferono (IFN-g) tirpalas, saugomas –20°C temperatūroje, nepakito pagal monomerinės (molekulinė masė 17240 Da) formos kiekį. Baltymas kinta saugant jo tirpalą aukštesnėje temperatūroje (+37°C ir +50°C): atsiranda dimerų ir degradantų. Oligomerinės formos bei mažesnės molekulinės masės fragmentai susidaro po paros +50°C temperatūroje. Saugant baltymo tirpalą +37°C temperatūroje, degradantai stebimi po 5 parų, o agreguotos formos susidaro po 10 parų. Susidariusių oligomerinių formų bei degradantų molekulinė masė buvo 33000 Da ir 31000 Da (+37°C) bei 35000–32000 Da ribose (+50°C). Už monomerinę formą mažesnių degradantų molekulinė masė buvo 16000–11000 Da ribose. Panaudojus proteazių inhibitorius paaiškėjo, kad IFN-γ sąveikaudamas su PMSF sudaro dimerines formas, o EDTA šiek tiek pristabdo degradantų susidarymą.