Analysis of the physical homogeneity and biological activity of recombinant human interferon-gamma

V. A. Bumelis, Ž. Bumelienė, G. Gedminienė, V. Smirnovas, J. Sereikaitė, I. Medelytė

Vilnius Gediminas Technical University, Saulėtekio 11, LT-2040 Vilnius, Lithuania

We describe here analysis of recombinant human interferon-gamma (HuIFN-γ) homogeneity and biological activity under storage conditions at a temperature of –20°, 37°, 50 °C in absence and presence of protease inhibitors. Appearance of aggregate forms has been found to decreased significantly the biological activity of HuIFN-γ. The highest aggregation level and the lowest biological activity were established in protein solutions treated with PMSF and in the cases of incubation at 50 °C.

Key words: human recombinant interferon-gamma, biological activity, protease inhibitors

INTRODUCTION

The stability of recombinant proteins has become an increasingly important consideration as more protein therapeutics are developed. The complicated structure of recombinant proteins maked these substances higly susceptible to degradation. Recombinant proteins often undergo a denaturation-renaturation cycle during extraction or purification. Therefore, determination of the physical homogeneity, the state of aggregation in a final preparation is essential to prove the quality [1]. Various identity tests, such as RP-HPLC, tryptic mapping and polarized fluorometry [2] are applied for identification of interferons and establishment of their purity. In most cases SDS-PAGE is used for determing the physical homogeneity, and the potency of recombinant protein is evaluated by testing its antiviral activity. We have previously shown that stability of recombinant human interferon-gamma (HuIFN-γ) depends on storage temperatures [3]. HuIFN-γ was stable at –20 °C, but showed some degrading and aggregative forms under incubation temperatures 37 °C and 50 °C.

In the current paper we discuss the correlation between the physical homogeneity and biological activity (potency) of recombinant interferon-gamma under storage conditions.

MATERIALS AND METHODS

Acrylamide/Bis powder, Ammonium persulfate, Bromphenol blue, Gel drying solution, 2-mercaptoethanol, Premixed buffer 10xtris/gly/SDS, SDS solution 10%, TEMED, Low Mol Mass standard, Acetic acid glacial, EDTA-$Na_2 \times H_2O$, Formaldehyde 37%, Glycerol, Ethanol, Silver nitrate, Sodium carbonate, Sodium Thyosulphate, PMSF, Cell lines L-41, Mice encephalomyocarditis virus (MECV), Dulbecco Minimum Essential Medium (DMEM), Fetal bovine serum (FBS), Trypsin, Dimethylsulphoxide, Reference standard for recombinant HuIFN-γ 87/586, NIBSC. All chemicals used in the study were commercial products from Bio-Rad, Merck, Sigma compannies and were of guaranteed grade.

The recombinant HuIFN-γ was produced in *E. coli* and purified to homogeneity as described previously [4]. SDS-PAGE was performed essentially by Laemmly [5]. Detection of monomeric and other forms of HuIFN-γ was described previously [3]. The antiviral activity of HuIFN-γ was determined in a standard assay, employing L-41 cells and mice encephalomyocarditis virus [6]. HuIFN-γ activity units were expressed according to the reference standard 87/586, NIBSC.

RESULTS AND DISCUSSION

Investigation of the homogeneity of Hu IFN-γ has been analysed using a gel electrophoretic approach. The results presented earlier [3] demonstrated that the protein aggregated at a temperature of 37 °C and 50 °C. It is known that the primary stucture of HuIFN-γ reveals the potential sites of proteolytic degradation [7]. Although during the initial purification steps the majority of protease contaminants are removed, some trace amounts of proteolytic en-

zymes can co-purify with the protein of interest. Many proteases are known to have a molecular weight in the range of 20–30 kDa. In order to avoid the possible influence of the proteolytic enzyme, we used some inhibitors such as EDTA, PMSF and copper sulfate at a final concentration of 2 mM. In this study we compared the biological activity of HuIFN-γ solutions after incubation for appropriate time at 37 °C and 50 °C in presence and absence of the mentioned inhibitors. The data on biological activity (potency) and electrophoresis analysis are presented in Table and Figure.

The initial solution of recombinant HuIFN-γ stored at –20 °C served as control and demonstrated high homogeneity (Figure, lanes 1, 7, 13, 19) and biological potency, which was equated as 100%. The results of this study showed, that the potency of interferon after incubation at 37 °C for one day remained at the level of control. SDS-PAGE displayed a homogeneity of recombinant HuIFN-γ, (lanes 2, 8, 20). An exeption was the case with PMSF. The lost of antiviral activity reached about 30% (after 1 day of incubation) and a minor component of oligomeric form was observed (lane 14). After prolonged incubation (7–28 days, lanes 15–18) the relative intensity of the band of higher molecular mass of HuIFN-γ increased as the biological potency decreased from 35% to 6,7% (incubation for 7–28 days). The loss of activity was noted in all cases when aggregate forms appeared. The appearance of aggregate forms decreased the antiviral activity (lanes 4–6, incubation for 14–28 days), lost of activity

Table 1. **Evaluation of the biological activity of recombinant Hu IFN-γ**

Time of incubation days	Antiviral activity, % Temperature of incubation							
	37 °C				50 °C			
	without	EDTA	PMSF	$CuSO_4$	without	EDTA	PMSF	$CuSO_4$
1	106.41	102.24	68.43	109.77	11.97	8.06	2.68	10.42
7	82.85	76.12	35.26	84.13	1.27	0.32	0.27	0.44
14	22.79	24.84	12.26	40.06	0.21	0.08	0.09	0.56
21	20.83	19.63	8.81	19.23	0.01	0.01	0.01	0.01
28	22.44	19.23	6.68	24.04	0	0	0	0
84	0.17	0.23	0.01	0.96	0	0	0	0

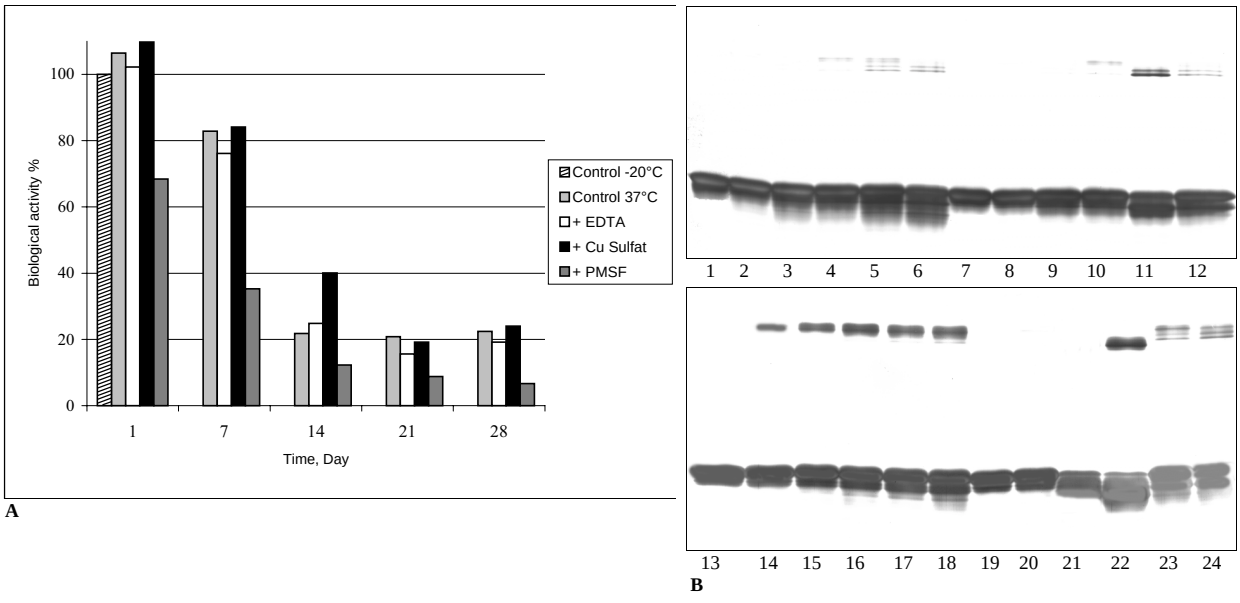

Figure. **Dependence of biological activity (A) and physical homogeneity (B) of Hu IFN-gamma on storage time and temperature (+37 °C).** Samples were incubated appropriate time (days) in absence and presence EDTA, PMSF, copper sulfate for 1, 7, 14, 21, 28 days. **A**. Biological activity of Hu IFN-γ, %. **B**. SDS-PAGE: Intact Hu IFN-gamma appears as a predominant band, 17.2 kDa, corresponding to its monomeric state. Incubation of protein leads to the appearance of bands of higer molecular mass, 31–35 kDa. Lanes***: 1, 7, 13, 19*** – IFN-γ Control (–20 °C);); *2, 3, 4, 5, 6* – Hu IFN-γ without supplements 1, 7, 14, 21, 28 days; *8, 9, 10, 11, 12* – with EDTA for 1, 7, 14, 21, 28 days, *14, 15,16, 17, 18* – with PMSF for 1, 7, 14, 21, 28 days; 20, 21, 22, 23, 24 – with copper sulfate for 1, 7, 14, 21, 28 days

reached up to 22–24% in other cases. Estimation of biological potency and physical state of HuIFN-γ incubated at 50 °C revealed a decrease of potency by about 90–93% (after one day of incubation) and showed high heterogeneity on SDS-PAGE [3]. The biological activity was lost practically totally after seven days of incubation at 50 °C.

Thus, it may be concluded that the biological potency of recombinant HuIFN-γ was inactivated via formation of aggregate forms. The formation of aggregates was much faster at 50 °C than at 37 °C. Presence of PMSF in protein solution accelerated formation of oligomeric forms and decreased noticeably the biological activity of HuIFN-γ.

ACKNOWLEDGEMENTS

Authors are sincerely thankful to Dr. B. Starkuvienė for her interest and useful discussions during this work.

References

1. Bordens R, Grossberg SE, Trotta PP, Nagabhushan TL. Seminars in Oncology 1997; 24: 41–51.
2. Brochon JC, Tauc P, Merola F, Schoot BM. Anal. Chem. 1993; 65: 1028–34.
3. Bumelis VA, Bumelienė Ž, Gedminienė G et al. Biologija 2002; 2: 37–41.
4. Dudich IV, Zavyalov VP, Bumelis AV et al. Mol Biol 1992: 26: 441–50.
5. Laemmli UK. Nature 1970; 227: 680–5.
6. Ito M, J. Interferon Res. 1984; 603–8.
7. Haelewyn J, Michiels L, Verhaert P et al. Biochem J 1997; 324: 591–5.

V. A. Bumelis, Ž. Bumelienė, G. Gedminienė, V. Smirnovas, J. Sereikaitė, I. Medelytė

ŽMOGAUS REKOMBINANTINIO INTERFERONO-GAMA HOMOGENIŠKUMO IR BIOLOGINIO AKTYVUMO ANALIZĖ

S a n t r a u k a

Tirtas žmogaus rekombinantinio interferono-gama (Hu IFN-γ) homogeniškumas ir biologinis aktyvumas saugant preparatą –20°, 37° ir 50°C temperatūroje su proteazių inhibitoriais ir be jų. Nustatyta, kad susidarę agregatai mažina Hu IFN-γ antivirusinį aktyvumą. Labiausiai aktyvumas prarandamas esant PMSF tirpaluose. PMSF ir 50°C temperatūra skatina rekombinantinio baltymo agregaciją ir mažina jo aktyvumą.