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Poster Presentation

Over-expression and single-step purification of human $IFN_{\alpha 8}$ and human $IFN_{\alpha 2b}$ reveals the highest antiviral activity of human $IFN_{\alpha 8}$

Julio César Sánchez García*[1], Alejandro Miranda Ariza[1], Alexis Musacchio Lassa[2], Luis Javier González[2] and Vladimir Besada Perez[2]

Address: [1]Process Control Department, Center for Genetic Engineering and Biotechnology, P.O. Box 6162, C.P. 10600, Havana C., Cuba and [2]Division of Physical-Chemistry, Center for Genetic Engineering and Biotechnology, P.O. Box 6162, C.P. 10600, Havana C., Cuba

* Corresponding author

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Background

Human alpha interferons ($_{Hu}IFN_{\alpha}$) comprise a multigene family, originally identified as proteins responsible for the induction of cellular resistance to viral infections, subdivided into 13 different subtypes ($IFN_{\alpha 1, \alpha 2, \alpha 4, \alpha 5, \alpha 6, \alpha 7, \alpha 8, \alpha 10, \alpha 13, \alpha 14, \alpha 16, \alpha 17}$ and $_{\alpha 21}$). The genes that encode these proteins are clustered on chromosome 9 in human [1]. There have been reports of obvious differences in the relative biologicals activities among IFN_{α} subtypes [2,3] and it was found that the activity varied greatly depending on the target cells, the IFN_{α} subtypes and even if the proteins were compared on base of both units of biological activity or mass [4]. The $_{Hu}IFN_{\alpha 2b}$ and $_{Hu}IFN_{\alpha 8}$ are among the IFN subtypes with highest antiviral activity. The first one was licensed by the Food and Drug Administration in 1986 (USA) for the treatment of hairy cell leukemia [5]. On the other hand, the $_{Hu}IFN_{\alpha 8}$ has shown the highest biological activity in several *in vitro* assays [6-8].

Several progresses in the fundamental understanding of transcription, translation, and protein folding in *Escherichia coli*, together with the availability of improved genetic tools (including new mutants) are making this bacterium more valuable than ever for the expression of complex eukaryotic proteins. Several strategies have been required to enhance rare codons gene expression [9,10].

Homogenous $_{Hu}IFN_{\alpha}$ preparations were originally obtained in 1978 for further chemical and physical characterization [11]. Thereafter have been introduced new high performance chromatography techniques useful to obtain enough quantity of these proteins for their chemical, biological and immunological studies [12]. Although high quantities of these proteins could be obtained, these procedures involve costly and laborious purification protocols.

In our previous report we attempted to over express the $_{Hu}IFN_{\alpha 8}$ in *E. coli* without success [13]. In the present work, the heterologous expression of both $_{Hu}IFN_{\alpha 8}$ and $_{Hu}IFN_{\alpha 2b}$ was significantly improved. These proteins were purified by one step and low cost SDS-PAGE procedure and used for detailed chemical and physic characterization. Both $_{Hu}IFN_{\alpha}$ subtypes obtained were used to compare its antiviral activity by using an *in vitro* model involving Hep-2 cells challenged with Mengo virus [14].

Results

Comparing the rare codon compositions of both $_{Hu}IFN_{\alpha}$ subtypes, two clusters of 30 amino acids, containing five minor triplets, were found (see Figure 1). For the $_{Hu}IFN_{\alpha 2b}$ gene, the minor codons, represented only by AGG/AGA (that encodes for Arg) are present in both clusters. However, besides the minor triplets that encodes for Arg, in the $_{Hu}IFN_{\alpha 8}$ gene one minor codon AUA (ATA triplet, that encodes for Ile) is present in the first cluster, while in the second one, the CUA (CTA triplet, that encodes for Leu) exists.

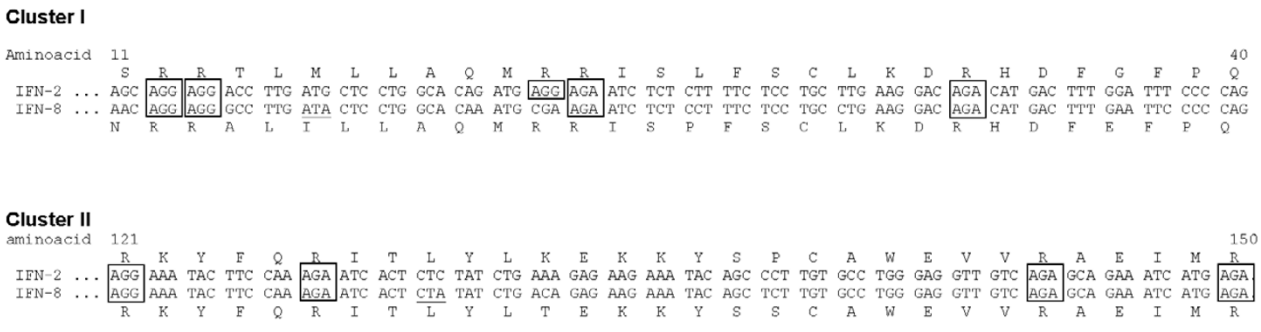

Figure 1
Clusters of 30 aminoacid presents in both $_{Hu}$IFN$_{\alpha}$ subtypes. In boxes, the AGA/AGG triplets coding for Arginine. The ATA and CTA triplets coding for Isoleucine and Leucine, respectively, are underlined.

Taking into account the minor codon composition in both $_{Hu}$IFN$_{\alpha}$ genes, *E. coli* BL21-codonplus-RIL cells (Stratagene, USA) were used to increase the heterologous polypeptides expression levels. *E. coli* BL21-codonplus-RIL cells were transformed with the plasmids pALF8-4 and pAGUA-4 [13] coding for $_{Hu}$IFN$_{\alpha 8}$ and $_{Hu}$IFN$_{\alpha 2b}$, respectively. This strain carries out additional copies of the *dna*Y, *ile*Y and *leu*W $_{t}$RNAs in an additional Cm^{r} pACYC derivative plasmid. The last two tRNA decode the AUA and CUA minor codons, respectively. The obtained transformants were grown in M9 minimum medium. For $_{Hu}$IFN$_{\alpha 8}$ transformants, the protein expression level increased from 1% (with *E. coli* BL-21/pALF8-4) up to 25% (with *E. coli* BL-21 CP RIL/pALF8-4) of total bacterial proteins based on densitometric analysis of scanned gels (see Figure 2). Similar results were obtained with the $_{Hu}$IFN$_{\alpha 2b}$ Amp^{r}-Cm^{r} transformants, the expression levels increased from 5% (with *E. coli* BL-21/pAGUA-4) up to around 20% (with *E. coli* BL-21CPRIL/pAGUA-4) of total host proteins.

The purity of purified $_{Hu}$IFN$_{\alpha}$ subtypes was estimated over 98%, based on the homogeneity observed in the silver

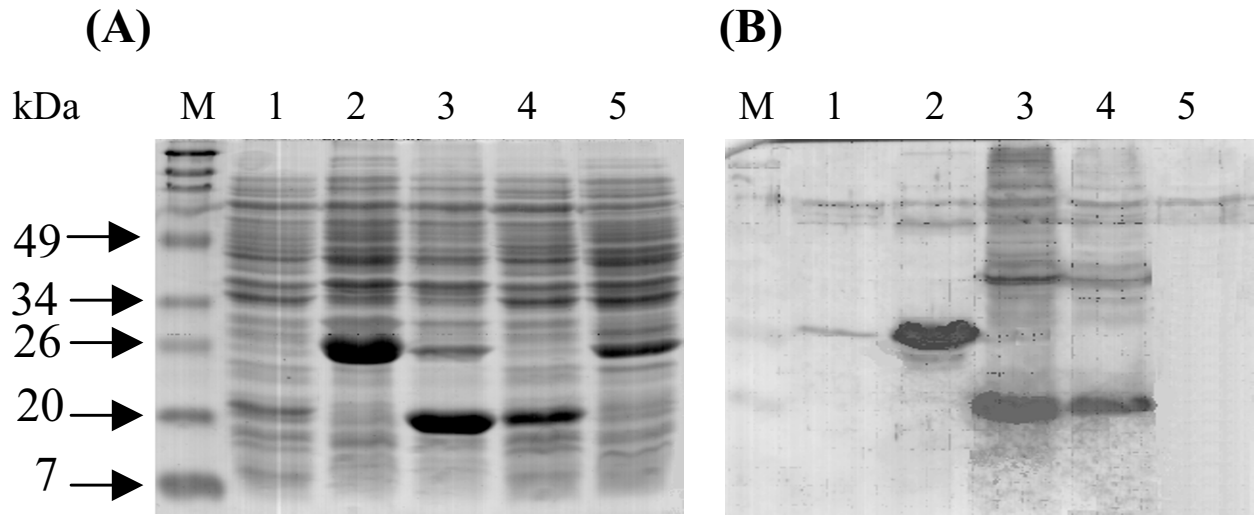

Figure 2
Expression of both $_{Hu}$IFN$_{\alpha}$ subtypes in *E. coli* cells grown in minimum media. **(A)** 12.5% SDS-PAGE stained with Coomassie blue, and **(B)** Immunoblotting analysis using a mixture of rabbit polyclonal antibodies specific for both $_{HU}$IFN$_{\alpha 2}$ and $_{HU}$IFN$_{\alpha 8}$. Samples: (1) *E. coli* BL-21/ pALF8-4, (2) *E. coli* BL-21CP RIL/ pALF8-4, (3), *E. coli* BL-21CP RIL/ pAGUA-4, (4) *E. coli* BL-21/ pAGUA-4, (5) *E. coli* BL-21CP RIL. The molecular weight markers (M) are indicated (M).

Table 1: Antiviral potency of both purified $_{Hu}$IFN$_{\alpha}$ subtypes.

Sample	Titer 1	Titer 2	Titer 3	Titer 4	Titer 5	Titer average
$_{Hu}$IFN$_{a8}$	5.1	4.5	4.8	3.9	4.1	4.48 (3.86-5.09)[a]
$_{Hu}$IFN$_{a2b}$	2.3	2.8	2.5	3	2.1	2.54 (2.08-2.99)[a]
E. coli BL-21 CP RIL	nd	nd	nd	nd	nd	nd

The titer values are expressed in 10^6 international units. nd, not antiviral activity detectable.
[a] Values in parenthesis correspond to confidence limits ($p < 0.05$).

stained SDS-PAGE and by RP-HPLC analysis (see Figure 3).

The antiviral potency of both $_{Hu}$IFN$_{\alpha}$ subtypes was compared in an *in vitro* assay with one microgram of each protein challenged with mengo virus [14]. Results of five independent experiments are shown in the table 1. The $_{Hu}$IFN$_{\alpha 8}$ had 1,46 fold more antiviral activity than $_{Hu}$IFN$_{\alpha 2b}$ ($p < 0,05$) in this **biological system**.

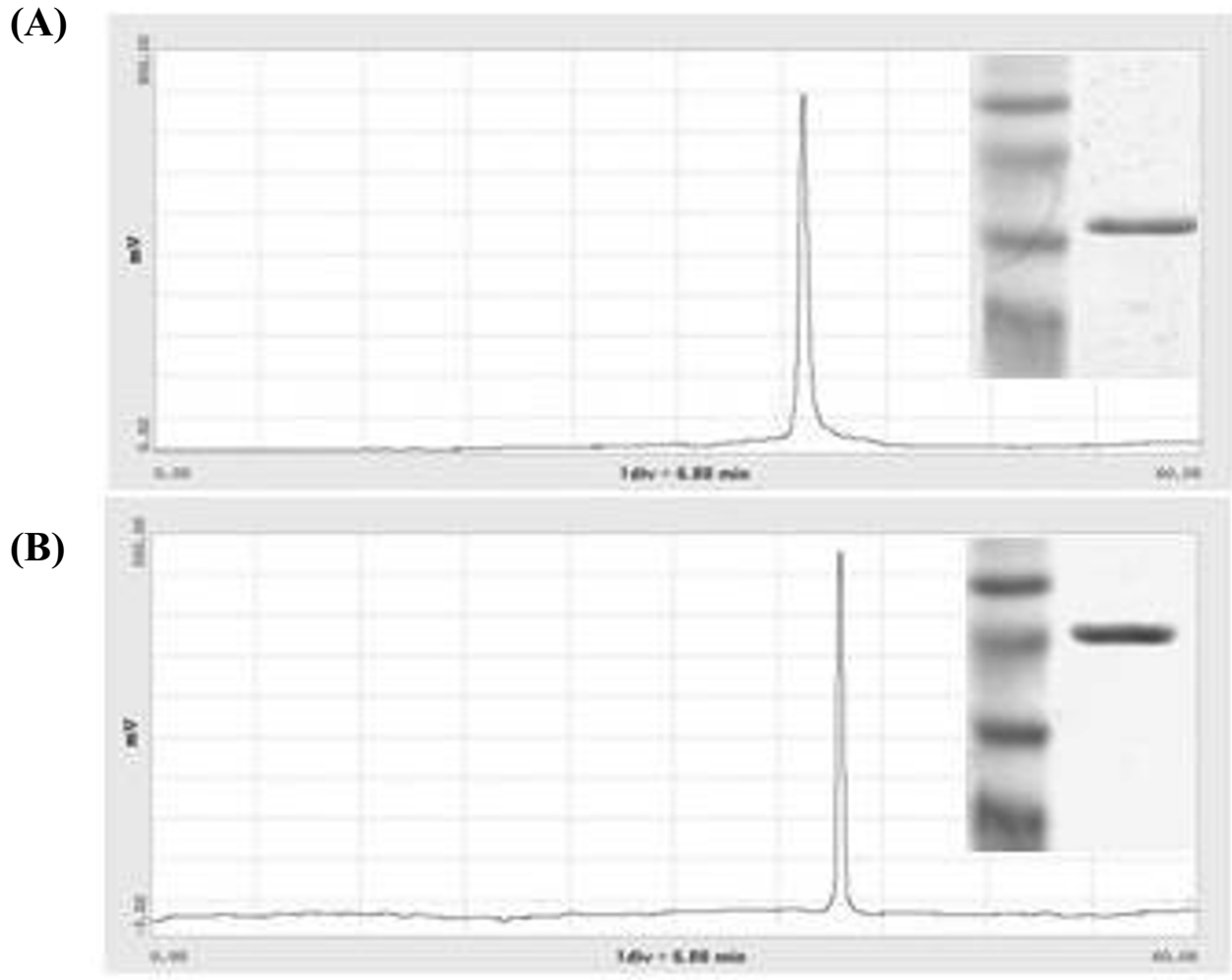

Figure 3
(**A**). RP-HPLC analysis of purified $_{HU}$IFN$_{\alpha 2}$ (A) and $_{HU}$IFN$_{\alpha 8}$ (B). The molecular weight of markers shown in SDS-PAGE are: 34 kDa, 26 kDa, 20 kDa and 7 kDa, respectively. (**B**). The LC-MS analysis of the tandem digestion tryptic/V8 revealed that cysteine residues of $_{Hu}$IFN$_{\alpha 8}$ and $_{Hu}$IFN$_{\alpha 2b}$ are correctly linked by disulfide bridges ((Data not show).

Conclusion

In summary, this work highlights several issues to obtain human proteins with high molecular homogeneity avoiding costly and tedious purification procedures. Therefore, we recommend this experimental strategy to obtain humans proteins with high therapeutic potentials. The highest antiviral activity of $_{Hu}IFN_{\alpha 8}$ shown in vitro add new evidences to take into account this proteins as strong therapeutic candidate.

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