

Expression and Purification of SAK-fused Human Interferon Alpha in *Escherichia coli*

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Abstract

A method for improved refolding and purification of *E. coli* derived human Interferon - α (rhIFN α 2b) from inclusion bodies as a Staphylokinase (SAK) fusion protein is described. Such a fusion protein did not require the supplementation of rare codons for expression and was found to be stable at 37°C. The optimal conditions of refolding involved the use of a mild denaturing agent without the need for any other agents to prevent aggregation. The SAK-rhIFN α 2b fusion protein was successfully purified using two steps of purification and was cleaved using enterokinase into two fragments namely SAK and IFN. Both the proteins were found to be biologically active showing proper folding of both the fusion partners. The cleaved IFN showed similar retention time on RP-HPLC as the bacterial derived untagged purified IFN as well as similar molecular weight on Agilent 2100 Bioanalyzer indicating the right processing of the IFN after enterokinase cleavage. The expression levels of SAK-IFN were found to be two folds higher than that observed with untagged IFN under similar experimental conditions.

Keywords: IFN α 2b; Anti-viral assay; Fusion proteins; Enterokinase cleavage; Stability

Introduction

Interferons (IFNs) are natural cell-signaling proteins produced by the cells of the immune system of most vertebrates in response to challenges such as viruses, parasites and tumor cells. They belong to the large class of glycoproteins that are produced by a wide variety of cells in response to the presence of double-stranded RNA, a key indicator of viral infection (Pfeffer, 1997; Pestka et al., 1987).

After, the medical potential of IFN was recognized, FDA approved the drugs namely rhIFN α 2a (Roferon A) and IFN α 2b (Intron A) for treatment of malignant tumors and viral diseases (Gutterman, 1994; Lauer and Walker, 2001; Mahan et al., 2002; Motzer, 2002). Interferon therapy is used (in combination with chemotherapy and radiation) as a treatment for many cancers, AIDS related Kaposi's sarcoma, and chronic hepatitis B and C (Remington, 1995). More than half of hepatitis C patients treated with interferon respond with viral elimination (sustained virological response), better blood tests and better liver histology.

The expression of IFN α cDNA was achieved directly in *E. coli* soon after it was first cloned (Goeddel et al., 1980; Nagata et al., 1980; Pestka, 1983; Mizoguchi et al., 1985; Barron and Narula, 1990). The *E. coli* recombinant protein expression system has been the system of choice for the production of IFN α

since IFN α genes do not have introns, and the non-glycosylated IFN is known to be bioactive. Since, *E. coli* could be grown to high cell densities, and strains used for recombinant protein production are generally regarded as safe, it is a preferred expression host for large-scale fermentations (Rabhi-Essafi et al., 2007). A recent paper on methodologies to select a host cell for production of therapeutics is available (Yin et al., 2007) and various merits of bacterial expression system has been outlined here.

The human IFN gene is known to contain rare codons for certain amino acids due to which it is expressed only in cell lines that are supplemented with rare codons (Garcia et al., 1996). Although IFN protein expressed in large amount in *E. coli* often precipitates into insoluble aggregates called inclusion bodies (Swaminathan and Khanna, 1999; Beldarraín et al., 2001; Srivastava et al., 2005), the basic problem with purification of interferon has been the observation of dimerization during refolding, downstream operations and also after storage at 4°C, hence requiring slow refolding strategies that are laborious and not cost-effective.

The stability of interferon in aqueous solution and the development of stable dosage forms of alpha interferons remains a great challenge for pharmaceutical scientists because of the poor stability of these biomolecules. This poor stability frequently increases the probability of protein degradation during some pharmaceutical processes such as purification, separation, storage and also due to proteolysis, oxidation, deamidation including aggregation, precipitation, and adsorption (Ruiz et al., 2006; Sharma and Kalonia, 2004).

IFN fusion proteins have been created to render them soluble in *E. coli*. Some of the well studied interferon fusions include GST-IFN (Rabhi-Essafi et al., 2007), HSA-IFN (Zhao et al., 2007) and the latest report on the fusion of IFN with the antimicrobial peptide as IFN-CM4 fusion by Li et al., (2009). An inherent problem with all these fusion protein systems is the difficulty in removing the fusion tag due to non-specific and incom-

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plete proteolytic cleavage. Moreover, the use of proteases such as factor Xa or thrombin, are non-specific and do not result in authentic N terminus of the protein of interest after the proteolytic cleavage.

Although the Human Genome Science Inc. recently have presented data on Albuferon, a long-lasting recombinant human serum albumin-interferon-alpha2b fusion protein, its structure and biological activities studied have shown instability of such fusions and such a protein was later stabilized by having IFN at the N terminus of the HSA fusion (Zhao et al., 2007). Such a fusion protein was purified using a series of chromatography steps like dye affinity chromatography, hydrophobic interaction chromatography, ion exchange chromatography and size-exclusion chromatography (Huang et al., 2007).

In this paper, we report a strategy that combines both approaches to make the IFN protein without the need of codon requirement and also making the most effective and easy purification protocols followed by a simple cleavage with enterokinase to offer IFN preparation with expected authentic N terminus. We also show that the cleaved IFN from such fusions is biologically active and this indicates promising scalability of the same process in manufacturing scale since such clones express higher yield of IFN α as a molar ratio is equal to that produced as IFN alone in *E. coli*.

Material and Methods

Reagents and chemicals

E. coli hosts such as BL21A1 and enterokinase was from Invitrogen, USA, while the other hosts such as BL21(DE3) and BL21(DE3) codon plus cells were from Stratagene, USA. DH5 α competent cells and restriction endonucleases like BamHI and EcoRI were procured from Bangalore Genei Pvt. Ltd, India while NdeI was from New England Biolabs, USA. PCR clean up kit, urea, glass beads (425-600 μ m) and other fine chemicals were from Sigma, USA while pET21a vector was from Novagen, USA. Synthetic gene coding for human IFN alpha 2b and Staphylokinase were procured from GenScript, USA. All chromatographic resins were purchased from GEHealthcare, Sweden.

Cloning of IFN α 2b in pET21a vector

IFN α 2b gene was PCR amplified using the forward primer 5' CCG CCG GGA TCC GAT GAT GAT GAT AAA TGT GAC CTA CCA CAA ACC CAC 3' that introduces a BamHI site at the 5' end of the gene and a reverse primer 5' CCG CCG GAA TTC AAG CTT TCA TTA CTC TTT AGA TCT TAA 3' containing the EcoRI site at the 3' end of the gene. PCR was performed in 50 μ l reaction volumes by initial denaturation at 95°C for 5 min followed by 5 cycles of 95°C for 30 sec, 45°C for 30 sec, 72°C for 30 sec and 25 cycles of 95°C for 30 sec, 63°C for 30 sec, and 72°C for 30 sec. After final extension of at 72°C for 7 min, the PCR product was gel purified and after digestion with BamHI and EcoRI was ligated to pET21a vector for 16 h. The ligation mix was then introduced into DH5 α competent cells and the recombinant clones were screened by colony using IFN gene specific primers. The PCR positive clones were later selected by restriction digestion and the selected clone was designated as pET21a-IFN.

Cloning of staphylokinase (SAK) into pET21a-IFN construct

SAK gene was PCR amplified from the synthetic gene using 5' CCG CCG GAA TTC CAT ATG TCA TTC GAC AAA GGA 3' and 5' CCG CCG GAA TTC TTA TTT ATC ATC ATC ATC GGA TCC TTT CTT TTC TAT AAC AAC 3' as the forward and reverse primer respectively. These primers were designed such that SAK will have enterokinase (DDDDK) recognition site. The amplification reaction was performed in 50 μ l volume as above using 57°C as the annealing temperature. The SAK PCR product was gel purified, digested with NdeI and BamHI and into pET21a-IFN construct at similar sites to create an N terminal fusion of IFN as SAK-IFN. The ligation mix was used for transformation of DH5 α cells and colonies were screened with colony PCR for SAK gene using gene specific primers. The clones were further confirmed by restriction analysis NdeI/EcoRI digestion. The construct was designated as pET21a-SAK-IFN.

The nucleotide sequence of the selected clones was checked by automated DNA sequencing analysis at BioServe Technologies, Hyderabad, India.

Expression of IFN and SAK IFN fusion proteins from pET21a-IFN and pET21a-SAK-IFN clones

BL21A1 cells and BL21(DE3) codon plus cells were independently transformed using pET21a-IFN and pET21a-SAKIFN plasmids. The cultures were then inoculated into 30 ml Luria Bertani (LB) with ampicillin at 100 μ g/ml and incubated at 37°C at 200 rpm till A_{600nm} was nearly 0.5 to 0.6. The cells were induced with 13 mM arabinose and 1 % lactose in case of BL21A1 cells while 1 mM IPTG was used in case of BL21(DE3) codon plus cells. After 4 hours of induction at 37°C at 200 rpm, the induced cells were pelleted, lysed with glass beads and the soluble and insoluble cell fractions were separated. Suitable aliquots of both the soluble and insoluble fraction were checked for expression on 12% SDS-PAGE gel followed by silver stain.

For large scale purification, the induction of pET21a-SAK IFN was carried out in 500 ml in LB amp under similar conditions.

Purification of IFN2b and SAK-IFN fusion protein

The IFN expressed from pET21a-IFN clone was purified as per the protocol described by Srivastava et al., (2005) and is not presented in this paper. The bacterial inclusion bodies containing SAK-IFN protein was solubilized in 6M Urea, pH 12.0 for 1 h at RT in the ratio of 40 ml of denaturation buffer/g of inclusion body. The denatured protein was then refolded in 10 mM Tris-Cl, pH 8.0 with continuous stirring at 4°C keeping the dilution ratio of 1:10. The refolded fusion protein after centrifugation at 13,000 rpm for 30 minutes was dialyzed against 100 volumes of cold 10 mM Tris-Cl, pH 8.0 buffer for 16 hours at 4°C and loaded onto an anion exchange column (Q-Sepharose, GE Healthcare), washed with the equilibration buffer till the absorbance (A_{280nm}) became nil and the bound proteins were eluted using increasing concentrations of sodium chloride (0- 1M) in the same buffer. The fractions containing the proteins of interest, were pooled and dialyzed against 10 mM Tris-Cl, pH 8.0 overnight in cold and loaded onto a hydrophobic interaction chromatography (Butyl Sepharose) with 1.0 M ammonium sulphate in 10 mM Tris.Cl, pH 8.0 (HIC equilibration buffer). After washing the column with the HIC equilibration buffer, the bound proteins were eluted

using decreasing concentrations of ammonium sulphate and all the samples were analyzed on SDS-PAGE.

In-vitro cleavage of the SAK-IFN fusion protein using enterokinase (EK)

The enterokinase cleavage reaction was performed as per manufacturers' instruction. Briefly, the reaction comprised of 4 units of EK (Invitrogen, USA) in a total volume of 50 μ l with 1.25 μ g of the purified fusion protein along with the enterokinase buffer (1X). The reaction was allowed to be carried out at 37°C for 16 hours after which they were analyzed on SDS-PAGE followed by silver stain for extent of cleavage and further analysis.

RP-HPLC and agilent 2100 bioanalyzer analysis of EK digest of purified SAK-IFN

Shimadzu LC-2010 CHT system was used for the RP-HPLC runs. Quantitation was carried out using the LC solution software. The analysis used ACE C18 0.25m \times 4.6 mm, 5 μ m column at 1ml/min flow rate. The gradient chromatography was carried out with 0.2% TFA in 30% acetonitrile as buffer A and 0.2% TFA in 80% acetonitrile as buffer B. Samples for analysis were injected 50 μ l each and detected at 210 nm.

The Agilent 2100 bioanalyzer separates biomolecules based on the gel electrophoresis principle replicated into a chip format (www.agilent.com). The Protein 80 kit was used for the analysis of proteins, ranging between 5 and 80 kDa. The samples were prepared and analyzed following the manufacturer's protocol. The data were represented in an electropherogram by plotting fluorescence intensity units (FU) versus retention time in seconds.

SAK activity and IFN α 2b anti-viral activity

The SAK activity was performed using a chromogenic substrate assay as reported by Mandi et al., (2009) while the anti-viral activity was done using WISH cells and Encephalomyocarditis virus challenge method by cytopathic effect method and using standard NIBSC interferon preparation at National Institute of Virology, Pune, India as per the published protocol (Yousefi et al., 1985).

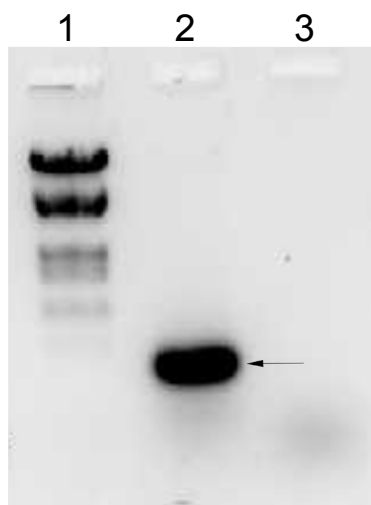


Figure 1: PCR amplification of human IFN α 2b from the synthetic gene. Lane 1: lambda EcoRI/HindIII marker; lane 2: PCR product of IFN gene; lane 3: negative control.

Results

Cloning of pET21a-IFN and pET21a-SAK-IFN

Figure 1 shows the PCR product of IFN α 2b from the synthetic DNA. While the results shown in Figure 2a and 2b show the plasmid map of pET21a-IFN clone and small scale expression of the IFN from pET21a-IFN in BL21A1 (without rare codon supplementation) and in BL21(DE3) codon plus cells (with rare codon supplementation) respectively. pET21a-SAK-IFN clone (Figure 3a) showed SAK-IFN expression even in BL21A1 cells (Figure 3b).

Quantitation of rhIFN from clones of pET21a-IFN and pET21a-SAK-IFN by densitometry

The clones of pET21a-SAK-IFN showed nearly 2.5 fold high expression as compared to untagged IFN in BL21(DE3) codon plus cells as judged by densitometry (Figure 4). Since the molecular weight of the SAK-IFN fusion is \sim 34 kDa and untagged IFN is 19 kDa, the molar ratio of the amount of IFN in the SAK-IFN fusion would be \sim 1.8 folds higher than untagged IFN.

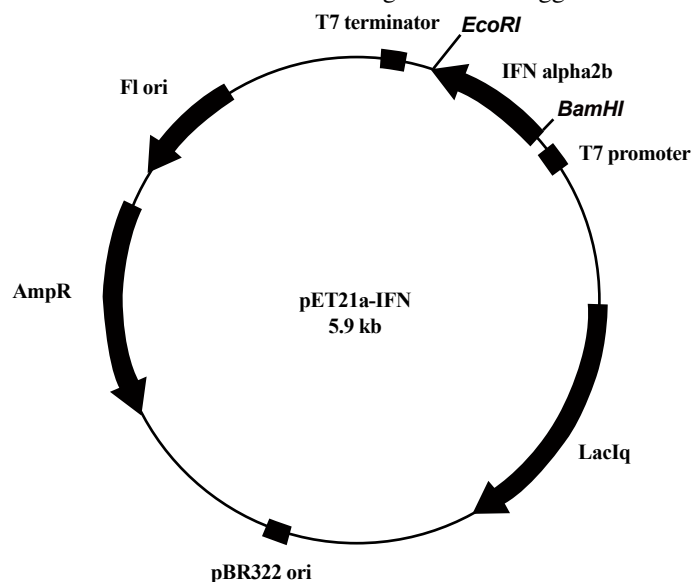


Figure 2a: Plasmid map of pET21a-IFN.

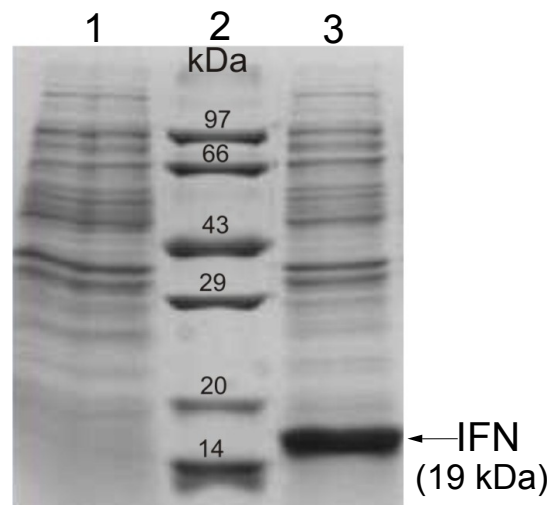


Figure 2b: SDS-PAGE analysis of IFN expressed from pET21a-IFN clone in BL21A1 (lane 1); lane 2: medium range molecular weight marker (14 to 97 kDa); lane 3: IFN from pET21a-IFN clone in BL21 (DE3) codon plus cells.

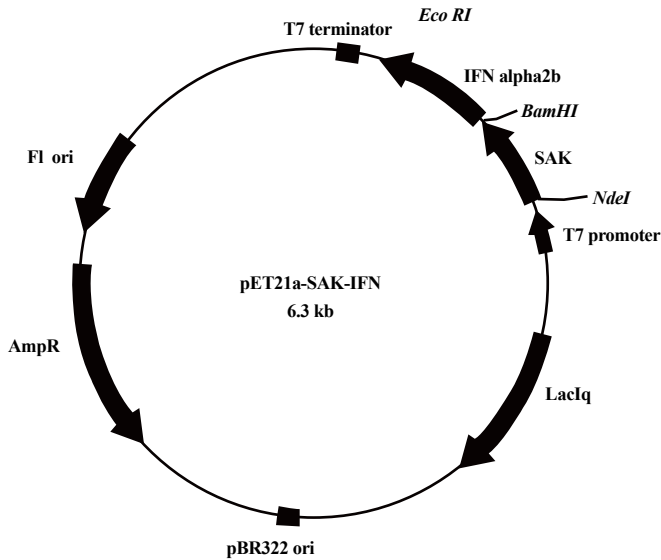


Figure 3a: Plasmid map of pET21a-SAK-IFN.

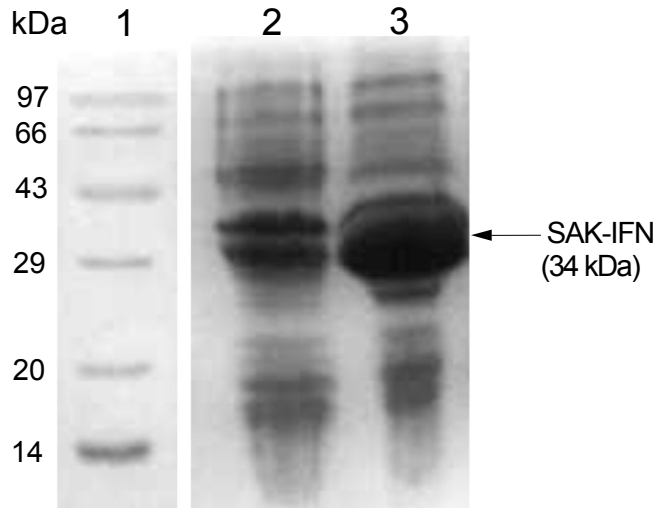


Figure 3b: SDS-PAGE analysis of IFN expressed from pET21a-SAK-IFN clone in BL21A1 (lane 2) and BL21 (DE3) codon plus cells (lane 3). Lane 1: medium range molecular weight marker (14 to 97 kDa).

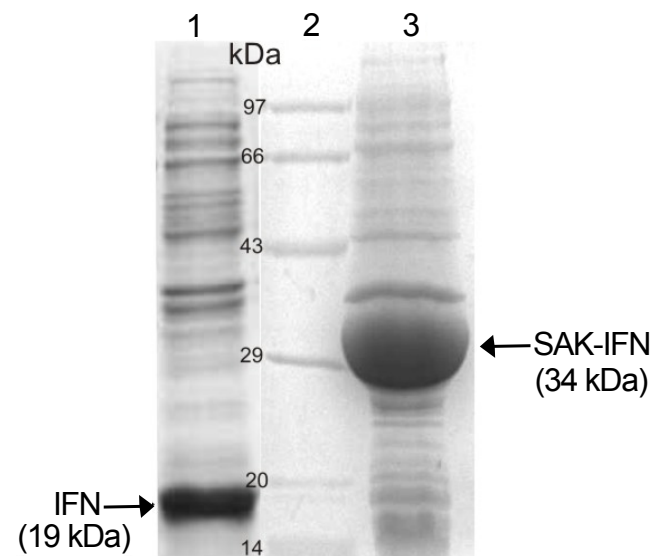


Figure 4: Comparison of expression of IFN from pET21a-IFN clone (lane 1) and pET21a-SAK-IFN clone (lane 3) in BL21(DE3) codon plus cells under the conditions described in materials and methods section. Lane 2: medium range molecular weight marker (14 to 97 kDa).

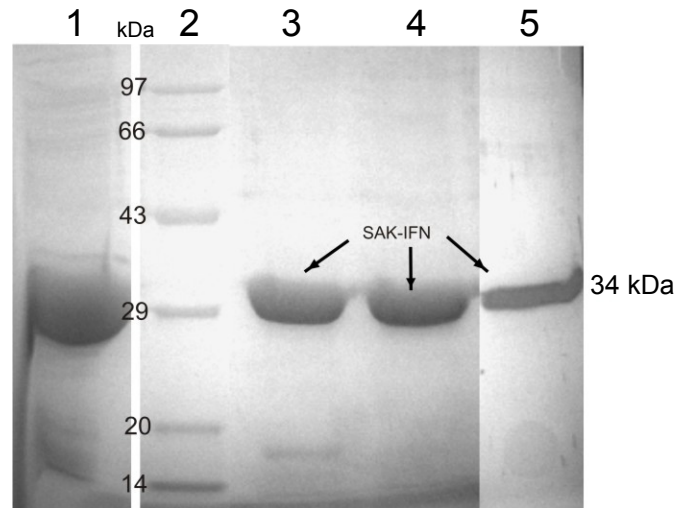


Figure 5: SDS-PAGE showing purification profile of SAK-IFN fusion. Lane 1: refolded SAK-IFN; lane 2: is medium range molecular weight marker (14 to 97 kDa); lane 3 and 4: Q Sepharose eluates; lane 5: Butyl Sepharose eluate. Note the purity of the SAK-IFN fusion protein (>95%).

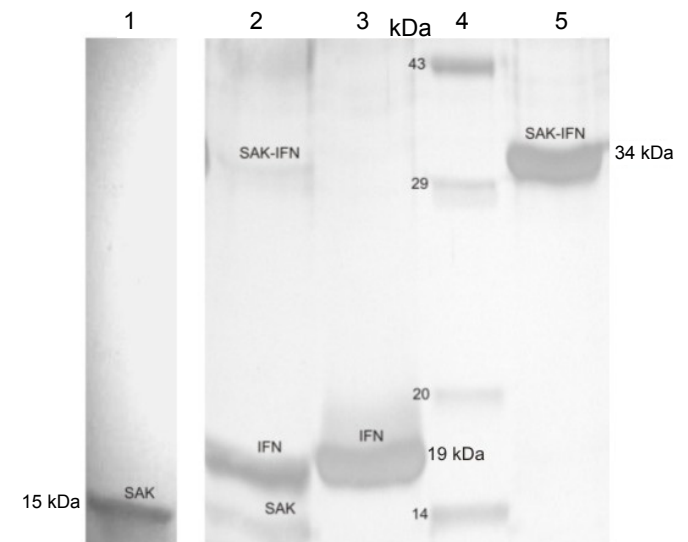


Figure 6: Enterokinase cleavage of SAK-IFN purified fusion protein. Lane 1: Purified SAK protein, lane 2: SAK-IFN fusion after enterokinase digestion; lane 3: bacterial derived rhIFN α 2b; lane 4: medium range molecular weight marker (14 to 97 kDa); lane 5 is untreated SAK-IFN fusion protein.

Purification of SAK-IFN fusion protein and EK cleavage

SAK-IFN purified protein was purified to nearly 80% after a single anion exchange step as seen in Figure 5, lane 3 while after the second step of HIC chromatography, the protein was nearly homogenous (>95% pure) as detected by silver stain SDS-PAGE (Figure 5, lane 5). Such a purified IFN fusion protein was also found to cleave easily using Invitrogen’s enterokinase into SAK and IFN respectively with no non-specific cleavage as shown in Figure 6, lane 2. It is interesting to see the exact matching of both the SAK and the IFN cleaved fragments with the purified SAK and IFN preparations generated in-house showing the specificity of the enterokinase protein on the SAK-IFN fusion cleavage. The purified SAK-IFN protein showed higher stability at 37°C for 17 hours (Figure 6, lane 5) while the untagged IFN showed slight aggregation (Figure 6, lane 3).

The fusion protein was shown to have both the SAK activity

(209200 units/ml) and IFN activity of 2.03×10^9 IU/mg fusion protein indicating that both IFN and SAK are folded rightly when present in the fusion protein.

RP-HPLC and Agilent 2100 bioanalyzer analysis of EK digest of purified SAK-IFN

The results on the RP-HPLC pattern of purified bacterial IFN and enterokinase digested purified SAK-IFN indicated similar retention times (Figure 7a. peak 1 and 2) and identical molecular weights as seen on Agilent 2100 bioanalyzer (Figure 7b, peak 1 and 2).

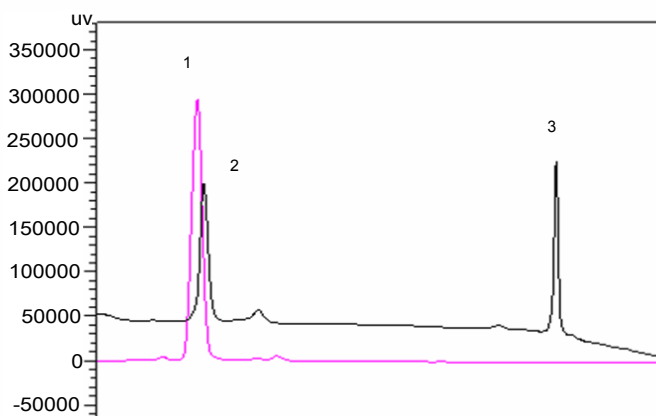


Figure 7a: RP-HPLC profile of purified bacterial IFN and enterokinase digested SAK-IFN. Peak 1: Purified IFN; Peak 2: IFN from EK digested SAK-IFN; Peak 3: Undigested SAK-IFN.

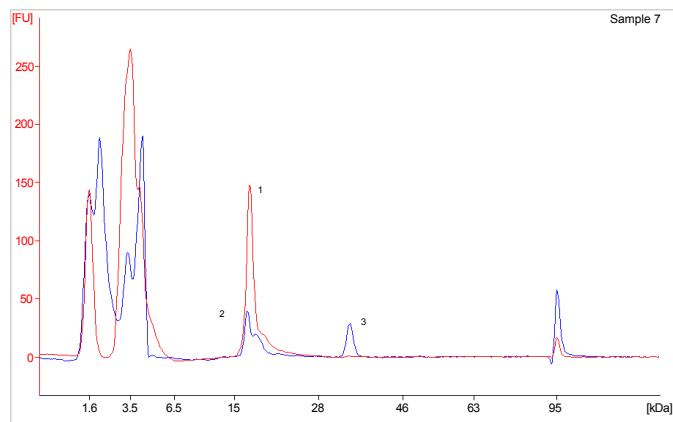


Figure 7b: Profile of bacterial IFN and enterokinase digested SAK-IFN on Agilent 2100 Bioanalyzer. Peak 1: Purified IFN; Peak 2: IFN from EK digested SAK-IFN; Peak 3: Undigested SAK-IFN.

Discussion

In this article, we report a novel method to hyper express hIFN as a staphylokinase fusion. Since one can easily assay SAK activity using the simple chromogenic assay, one could adopt the SAK assay as a measure of successful refolding of the SAK-IFN fusion and this appears to have a promising application for in-process testing of IFN in manufacturing scale. This is more so since the regular anti-proliferative and the anti-viral assays for interferon requires special infrastructure and training and is also time-consuming.

The SAK-IFN α 2b fusion protein was refolded and purified by a two step process from the SAK-IFN fusion protein without the use of harsh chemicals and other lengthy refolding proto-

cols. There are also no special additives like reducing or oxidizing agents required in any of the purification steps disclosed here making the refolding and the purification process easy and cost-effective. Various published purification protocols for IFN in literature (Honda et al., 1987) indicate the use of redox conditions for effective refolding and hence our protocol appears relatively simple and cost-effective. Moreover, since IFN α 2b is known to aggregate and dimerize in aqueous solutions, and our present study shows negligible levels of such forms with the SAK-IFN fusion protein, one might find this as an alternate and a stable protein for long term storage prior to enterokinase cleavage for final step of processing.

Albuferon, a long-acting interferon resulted from the direct genetic fusion of human albumin and interferon- α 2b (HSA-IFN- α 2b) is known to migrate as doublets on non-reducing SDS-PAGE and prone to form covalent aggregates in aqueous solution. To alleviate the structural perturbation of IFN- α 2b by HSA, IFN- α 2b-HSA fusion protein, in which IFN- α 2b was located at the N-terminus, was shown to be homogeneous and stable at 37 °C for at least 10 days. The results with SAK-IFN, described here, reflect the improved homogeneity and stability of C terminal fusions of IFN- α 2b when SAK is used as a fusion partner at the N terminus making our work novel.

The observations of similar retention times of purified bacterial IFN and IFN cleaved from SAK-IFN fusion protein on RP-HPLC indicate similar hydrophobicity of both the protein types. Our data on usefulness of the Agilent Bioanalyzer in differentiating the rightly processed protein preparations vs incorrectly processed fusion proteins (Somani et al., 2009; Deshpande et al., 2009) could also be applied for the observations of similar molecular mass obtained for both the IFN preparations reported here.

Efforts are on to separate both the fusion partners using conventional ion exchange columns and also to optimize the purification protocol to make a cost-effective protocol for large scale manufacturing.

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