

STUDY OF OPPORTUNISTIC INFECTIONS IN HIV INFECTED PATIENTS

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UNIVERSITY OF MUMBAI

FOR THE DEGREE OF
MASTER OF SCIENCE
IN
MICROBIOLOGY

SUBMITTED BY
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UNDER THE GUIDANCE OF
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MUMBAI

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**INDIGENOUS PRODUCTION AND APPLICATION OF
RECOMBINANT INTERFERON ALPHA USING RECOMBINANT
DNA TECHNOLOGY**

**A THESIS SUBMITTED
TO THE UNIVERSITY OF MUMBAI
FOR THE Ph.D. (SCIENCE) DEGREE
IN MICROBIOLOGY**

**SUBMITTED BY
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Indigenous production and application of recombinant interferon alpha using
recombinant DNA technology

A thesis submitted
To the university of Mumbai
For the Ph.D. (science) degree
In Microbiology

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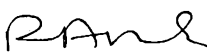
Statement by the candidate

As required by the University Ordinance 770, I wish to state that the work embodied in this thesis titled, **Indigenous production and application of recombinant Interferon alpha using Recombinant DNA technology** forms my own contribution to the research work carried under the guidance of Dr. (Mrs.) R.A.Deshmukh at the Haffkine Institute for Training, Research And Testing, A.D.Marg. Parel, Mumbai-400012. This work has not been submitted for any other degree of this or any other University. Whenever reference have been made for previous works of others, it has been clearly indicated as such and included in the bibliography.

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To
GOD

I

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INTRODUCTION

Indigenous production and application of recombinant Interferon alpha using Recombinant DNA technology

1. INTRODUCTION

1.a Interferon:

Interferon (IFN) belongs to the cytokine family of proteins-the chemical messengers of the immune system, produced by virus-infected cells and capable of helping other cells to resist the virus. Isaacs and Lindenmann first discovered the molecule in 1957 [1,5,11,20,21]. This was followed by similar findings by Nagano and Kojima in 1958, which initiated subsequent studies of the IFN system in details [1,5,11,20,21]. The IFN response represents an early host defense, one that occurs prior to the onset of the immune response. IFN binds to the specific receptor on the cell surface, thus inducing distinct proteins inside the cell [2,6,9,11,20,21,25]. Interferon also inhibits replication of tumor cells by influencing cell communication besides breaking down unstable cellular messenger RNA. Therefore, the molecule is used against malignant cells of the skin and immune system such as hairy cell leukemia, chronic myeloid leukemia, non-Hodgkin's lymphomas, malignant melanoma, renal cell carcinoma, and HIV-related Kaposi's sarcoma [1,2,5,6,9-21]. It initiates the synthesis of the enzyme oligoadenylate synthetase after binding with specific cellular receptors and further catalyzes the synthesis of small oligonucleotides, which plays an active role in breaking down the single-stranded viral RNA, thus preventing viral multiplication. As soon as IFN attaches to a cell receptor, various other cells of the immune system are stimulated and attach themselves to viral

fragments on the surface of infected cells, inducing an immune response. In this way infecting viruses are destroyed and uninfected cells are protected from the attack [1-3,5,6,9,11,12,20,21,25,26,32-36,37-46].

1.b Classification and Properties:

IFNs are a multigene family of inducible cytokines [1,3,5,20,21,50]. They are commonly grouped into two types. Type I IFNs are also known as viral IFNs and include IFN- α (leukocyte), IFN- β , (fibroblast), and IFN- ω . Type II IFN is also known as immune IFN (IFN- γ). The viral IFNs are induced by virus infection, whereas type II IFN is induced by mitogenic or antigenic stimuli. Most types of virally infected cells are capable of synthesizing IFN- α/β in cell culture [1,3,5,20,21,50]. IFN- γ is synthesized only by certain cells of the immune system including natural killer (NK) cells, CD4 Th1 cells, and CD8 cytotoxic suppressor cells. The natural IFN- α -producing cells appear to be precursor dendritic cells. Purified CD4CD11c⁻ type 2 dendritic cell precursors (pDC2s) from human blood produce up to 10^3 times more IFN in cell culture than do other blood cells following microbial or viral challenge [1,3,5,20,21,50-55].

IFN- α genes can be divided into two groups: an immediate-early response gene (IFN- $\alpha 4$), which is induced rapidly and without the need for ongoing protein synthesis, and, a set of IFN- α genes, consisting of IFN- $\alpha 2$, IFN- $\alpha 5$, IFN- $\alpha 6$, and IFN- $\alpha 8$, that display delayed induction and are synthesized more slowly and require protein synthesis [50]. The large number of viral IFN genes in the human includes 13 IFN- α genes, 1 IFN- β gene, and 1 IFN- ω gene [1,3,5,20,21,50]. They all lack introns and are clustered on the short arm of chromosome 9 in the human and chromosome 4 in the mouse [50]. The single IFN- γ gene

possesses three introns and maps to the long arm of chromosome 12 in the human, and chromosome 10 in the mouse [50]. Although some IFNs are modified post translationally by N- and O-glycosylation, the major human IFN- α subspecies are not glycosylated. The IFN- α gene products appear to function as monomers, whereas IFN- β and IFN- γ appear to function as homodimers [1,3,5,20,21,50-55].

Table 1. IFN classification and properties

IFN Type	IFN categories	Receptor type	Prototypic cell of origin	Direct Anti-proliferative effects	Stimulates MHC class I expression	Stimulates MHC class II expression	Stimulates NK cell activation
Type I	Alpha (α)	I	Leukocyte	Yes	Yes	No	Yes
	Beta (β)	I	Fibroblast	Yes	Yes	Slightly	Yes
	Omega (ω)	I	Leukocyte	Yes	Yes	No	Yes
	Tao (τ)	I	Ovine				
			Trophoblast				
Type II	Gamma (γ)	II	T-cells	Yes	Yes	Yes	Less than type I
			NK cells				IFNs, delayed

1.c Structure:

Human IFN- α has the apparent molecular weights ranging from 16,000 to 27,000 Da [1,3,5,20,21,50]. Most of these species have a signal peptide sequence of 23 amino acid residues and a mature amino acid sequence of 166 amino acid residues. The region between 139 to 151 is strongly conserved among the IFN- α species and IFN- β [1,3,5,20,21,50]. Most of the IFN- α species have four-conserved cysteine at positions 1,

29, 98/99 and 138/139. The first residue of human IFN- α species is probably a cysteine as determined by reduction and alkylation studies [1,3,5,20,21,50]. The secondary structure of several IFN- α species molecules has a large α -helix content and essentially no β -sheet. It is postulated that the sugar moiety of IFN- α species is covalently linked to the protein by o-glycosylation. At least 23 different variants of IFN-alpha are known [1,3,5,20,21,50]. The individual proteins have molecular masses between 19-26 kDa and consist of proteins with lengths of 156-166 and 172 amino acids [1,3,5,20,21,50]. Many IFN-alpha subtypes differ in their sequences at only one or two positions. The disulfide bond 29/138 is essential for biological activity while the 1/98 bond can be reduced without affecting biological activity. In contrast to IFN-gamma, IFN-alpha proteins are stable at pH 2. The various species of human IFN- α are closely related in amino acid sequences with homologies in the range of 80 to 100 % [1,3,5,20,21,50]. The molecular weight of the recombinant human IFN- α species is about 19 kDa consisting of 166 (165 for IFN- α 2) amino acid residues lacking any N-glycosylation (α 14 has N-glycosylation). Target analysis of human IFN- α suggests that the functional unit is a monomer [1,3,5,20,21,50].

The Mol. Wt. of human IFN- β is 20,027 Da and is made up of a mature protein of 166 amino acid residues and a signal peptide of 21 amino acids [1,3,5,20,21,50]. Human IFN- β has three cysteines at positions 17,31, and 141. The α -helix content appears to be less than that of the IFN- α species (47%). IFN- β has been clearly demonstrated to be a glycoprotein [1,3,5,20,21,50]. Glycosylation is not required for biological activity in

vitro. The protein contains a disulfide bond Cys31/141 required for biological activity. IFN-beta, like IFN- alpha is stable at pH 2 [1,3,5,20,21,50].

In case of Human IFN- γ , the complete primary amino acid sequences are in two forms 25KD and 20KD [1,3,5,20,21,50]. IFN- γ is made up of a signal peptide of 20 amino acid residues and a mature protein of 146 amino acid residues. The first residue of the N-terminus of IFN- γ is glutamic acid in the form of pyroglutamate. IFN-gamma is a dimeric protein with subunits of 146 amino acids. The pI is 8.3-8.5. Glycosylation is not required for biological activity. IFN-gamma contains two cysteine residues, which are not involved in disulfide bonding. [1,3,5,20,21,50]. There are at least six different variants of naturally occurring IFN-gamma. They differ from each other by variable lengths of the carboxyterminal ends. In contrast to IFN-alpha and IFN-beta, IFN-gamma is labile at pH 2. IFN-gamma does not display significant homology with the other two interferons , IFN-alpha and IFN-beta. Murine and human IFN-gamma show approximately 40 percent sequence homology at the protein level. [1,3,5,20,21,50].

1.d Antiviral Activity:

The interferons exhibit a huge number of biological effects. The antiviral activity led to the name interferon and serves to define the unit of interferon activity. On purification of the natural human leukocyte interferons (IFN- α), it was found that all fractions that exhibited antiviral activity also exhibited anti-growth activity [1,5,6,8,9,11-21,48,50-55]. This observation was confirmed with purified recombinant interferons and extended to other activities like: stimulation of cytotoxic activities of lymphocytes and macrophages,

natural killer cell activity as well as increase in expression of some tumor-associated antigens [52]. The antiproliferative and antitumor activities of interferon have led to the application as an antitumor agent. The interferons also modulate cellular differentiation. A major effect of interferons is their modulation of antigens of the major histocompatibility complex (MHC). All interferons induce an increase in surface expression of class I MHC antigens [1,5,6,8,9,11-21,48,50-55].

Expression of the *Fc* receptors is also stimulated by interferon. Alterations in surface antigens may be an important mechanism by which interferon can modulate cellular interactions. The interaction of the interferons with their receptors determines the biochemical events and their modulation of cellular functions. This is a complex process just in the beginning to be dissected [1,5,6,8,9,11-21,48,50-55]. The role of IFN- α as a disease marker and marker for immunotherapeutic approaches has been demonstrated for a number of different indications and pathological situations:

- During the acute phase of a viral infection IFN- α levels are significantly elevated in the majority of patients. IFN- α levels fall significantly during the period of convalescence at the time viral infection is indicated by seroconversion tests [1,5,6,8,9,11-21,48,50-55].
- Increased levels of IFN- α were found in the majority of patients suffering from inflammatory arthropathies like juvenile polyarthritis, rheumatoid arthritis, ankylosing spondylitis, polychondritis, psoriatic arthritis, polymyalgia rheumatica and scleroderma. Elevated IFN- α levels have also been shown for other

autoimmune disorders like systemic lupus erythematosus and systemic vasculitis [1,5,6,8,9,11-21,48,50-55].

- Serum IFN- α levels can help to distinguish between children with nonspecific abdominal pain or mesenteric adenitis, and those with acute appendicitis [50-55].

Mode of Antiviral Action:

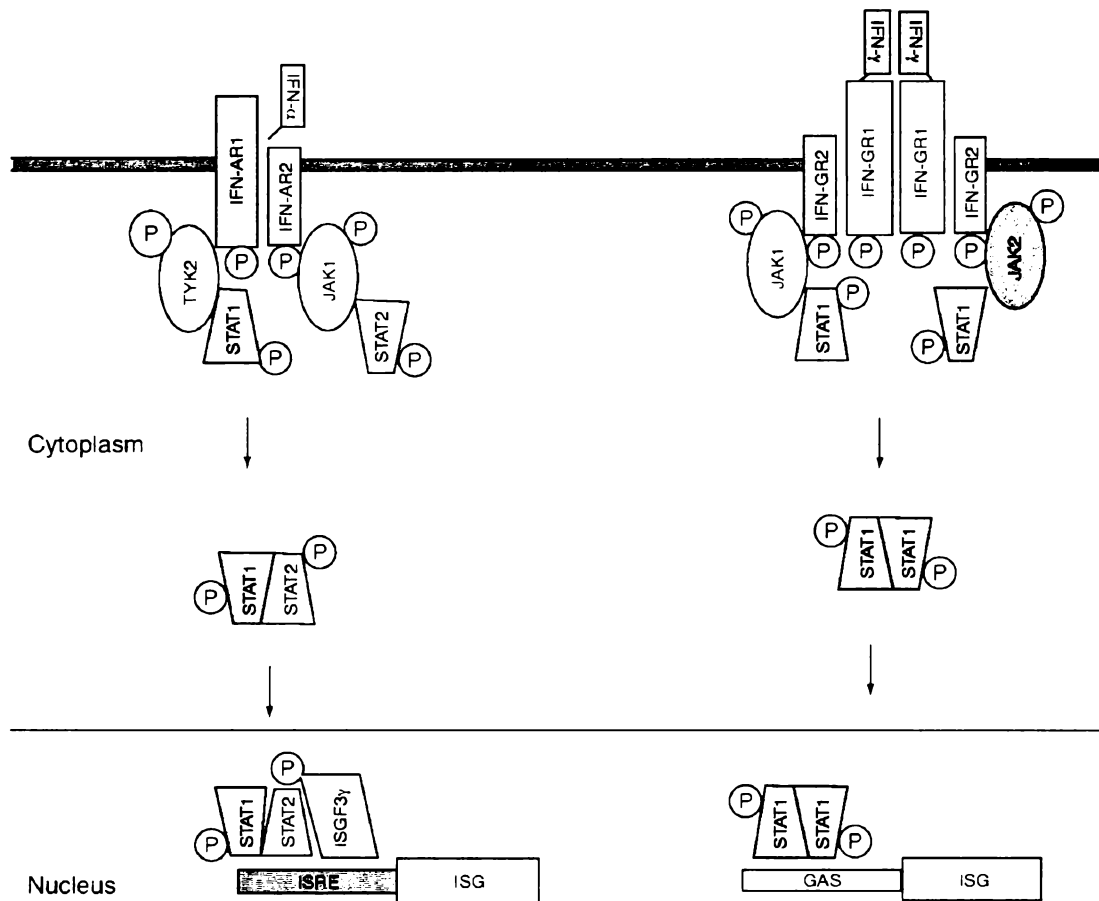
(A) Signal Transduction System (JAK-STAT):

IFN α mediates its signal transduction by binding to its receptor, which rapidly become tyrosine phosphorylated. Phosphorylation of the receptor is regulated by two Janus kinases, Tyk2 and Jak1, which become activated upon stimulation of IFN α [2,4-6,7,9,20,21-25,32-34,39,42-45,50]. The activated Tyk2 and Jak1 then phosphorylate three STAT proteins. Activated STAT1 and 2 associates with a 48-kDa protein (p48) to form the interferon-stimulated gene factor-3 (ISGF-3) complex, which binds specifically to the IFN α -stimulated response element (ISRE), resulting in gene transcription [2,4-6,7,9,20,21-25,32-34,39,42-45,50]. In addition, IFN α treatment induces STAT3 phosphorylation, and activated STAT3 can bind to the interferon responsive element (IRE) sequence, forming a protein-DNA complex distinct from the ISGF3-ISRE complex [6,7,9,20,21-25,32-34,39,42-45,50].

Steps in the Signal Transduction System (JAK-STAT):

1. Binding of interferon α / β causes dimerization of the receptor subunits, IFNAR1 and IFNAR2.
2. Tyk2 gets phosphorylated by JAK1 (Janus Kinase), another JAK kinase, which is bound to IFNAR2.
3. Activated Tyk2 then in turn phosphorylates JAK1.
4. The activated JAK kinases, Tyk2 and JAK1, subsequently phosphorylate IFNAR1 and IFNAR2 at specific tyrosine residues.
5. STAT2 then binds to specific phosphorylated residues on IFNAR1.
6. Upon docking, STAT2 gets phosphorylated at a conserved tyrosine residue (Y701) by the JAK kinases.
7. STAT1 gets subsequently phosphorylated at Y690.
8. The phosphorylated STATs dissociates from the receptor heterodimer and binds to p48 (IRF9), a member of the interferon regulatory factor (IRF) family, forming the major interferon transcription factor, known as ISGF-3.
9. ISGF-3 translocate to the nucleus and binds to specific regulatory DNA sequences (ISRE-Interferon stimulated response elements) and initiates transcription of several interferon-inducible genes.
10. STAT1: 2 heterodimers as well as STAT1: 1 homodimers also form and are capable of driving the expression of a minority of interferon-stimulated genes (ISGs), independently of p48. In this case, they binds to different DNA regulatory sequences than ISRE called GAS (gamma activated sequence elements), which are usually found in the promoters of interferon gamma stimulated genes.

Figure 1. Steps in the Signal Transduction System (JAK-STAT)



(B) Protein Kinase R (PKR) system:

IFN also initiates its antiviral action by the Protein Kinase R (PKR) system [1,2,5-7,9,12,20-25,27,28,31,32-35,37-39,41-46,50]. PKR is a 551 amino acid serine-threonine kinase mainly found in an inactive form in the cytoplasm of most cells and in the nucleus. It gets active due to IFN stimulation. This action of interferon is mediated through an ISRE and GAS in the promoter region of the PKR gene. It plays a major role in viral genome translation step and has also been involved in regulating cell proliferation, playing a role as tumour suppressor and in signal transduction. The other systems which

are involved in the IFN mode of action are 2-5A oligosynthetase / RNase L, The Mx system, Guanylate Binding Protein (GBP), 9-27 and nitric oxide synthase (iNOS) [1,2,5-7,9,12,20-25,27,28,31,32-35,37-39,41-46,50].

(C) Oligosynthetase/RNase L system:

The oligosynthetase/RNase L system is multienzyme system operating only in the cytoplasm is composed of three separate components: 2-5A synthetase, endoribonuclease RNase L and 2-5A phosphodiesterase, induced by IFN. The system is activated by dsRNAs, probably of viral origin. 2-5A synthetase activates RNase L, which degrades all single-stranded RNA inhibiting viral replication. 2-5A phosphodiesterase regulates the entire process [1,2,5-7,9,12,20-25,27,28,31,32-35,37-39,41-46,50].

(D) Mx proteins:

Mx proteins are interferon-inducible, high-abundance 70-80kD GTPases of the dynamin superfamily found both in cytoplasm and nucleus. They interfere with replication at the transcriptional and other levels in influenza and other negative-stranded viruses replication [1,2,5-7,9,12,20-25,27,28,31,32-35,37-39,41-46,50].

(E) Guanylate Binding Protein (GBP), 9-27 and nitric oxide synthase (iNOS):

The anti-viral systems Guanylate Binding Protein (GBP), 9-27 and nitric oxide synthase (iNOS) are interferon-stimulated proteins, which inhibit VSV replication, protect macrophages from infection by several viruses etc. [1,2,5-7,9,12,20-25,27,28,31,32-35,37-39,41-46,50].

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(F) Cell death/ Apoptosis:

Interferons in conjunction with dsRNA, TNF or LPS are a potent inducer of cell death. Over expression of PKR induces apoptosis. Interferons have the ability to arrest cell growth especially tumour cells. STAT1 is believed to be involved in this step since it is often deficient in tumors [1,2,5-7,9,12,20-25,27,28,31,32-35,37-39,41-46,50].

(G) Immunomodulatory activity:

Interferon gamma is the predominant immunomodulatory interferon. Type I interferons are mainly antiviral cytokines but also utilize the immune system to eradicate viral infection. NK cells are a heterogeneous subpopulation of lymphocytes capable of lysing target cells without prior sensitisation. Alpha interferons augment NK activity in vitro, except for subtype alpha-7. It has been suggested that interferon alpha enhances humoral immunity by increasing proliferation of B cells, which secrete antibodies against invading antigens. Interferon alpha also enhances the CD8⁺ cytotoxic T cell (CTL) response. Type I interferons appear to upregulate IL-12R β , a component of IL-12 receptor on Th cells, thereby upregulating the production of CTLs and enhancing the Th1 responses [1,2,5-7,9,12,20-25,27,28,31,32-35,37-39,41-46,50].

Figure 2. Role of IFN after virus infection of cells

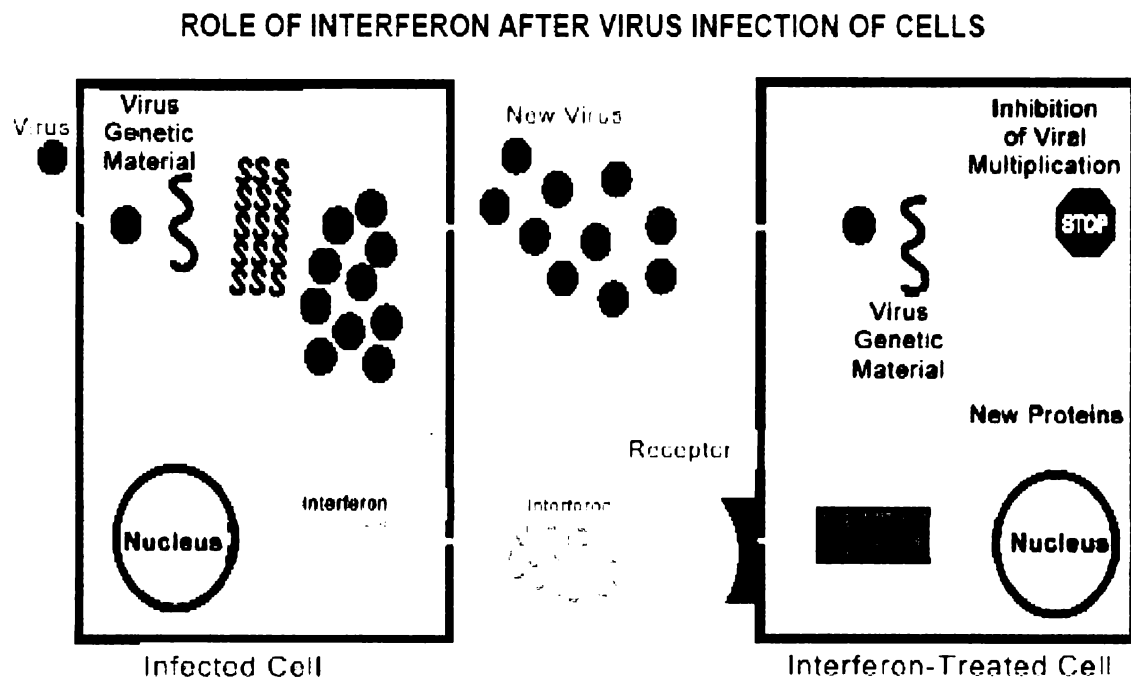
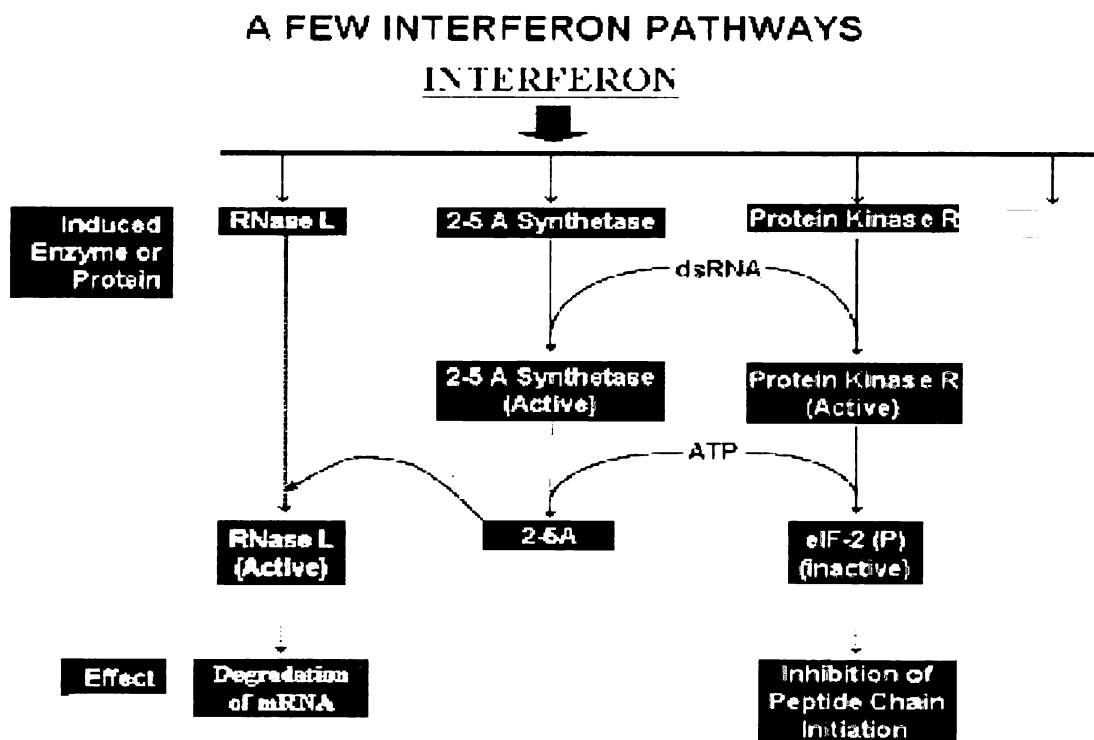


Figure 3. A few IFN pathways



1.e Inducers of IFN:

Specific inducers also produce interferons in the cells. Interferon inducers are the agents having a wide range of antiviral, etiotropic and immunomodulating activity [1,5,6,10,14,20,21,30,31]. They stimulate the synthesis of the body's own IFN, which has no antigenicity unlike the most widely used recombinant IFN.

IFN inducers cause IFN circulation at the therapeutical levels; at the same time to achieve such an effect requires multiple administration of large-dose of exogenous agents. Some IFN inducers includes IFN synthesis in particular cell populations and organs, which has certain advantages over the polyclonal stimulation of IFN immunocytes.

The induction of interferon usually follows exposure to viruses, double-stranded RNA, polypeptides, cytokines, T-cell-specific antigens, staphylococcal enterotoxin A, and the combination of phytohemagglutinin and phorbol ester. Other inducers include certain microorganisms (e.g., viruses, Rickettsia, Mycoplasma, coliform bacteria), microbial extracts, certain dyes (e.g., methylene blue, acridince orange), and synthetic polymers [1,5,6,10,14,20,21,30,31].

Usually, interaction of a virus/inducer with a vertebrate cell results in secretion of IFN by the cells within a few hours after induction. The production may cease 28 to 48 h later [1,5,6,10,14,20,21,30,31]. Both DNA and RNA viruses are capable of inducing IFNs when tested under appropriate conditions. RNA viruses, especially togaviruses,

paramyxoviruses and reoviruses are considered to be generally good inducers. A great number of synthetic inducers like Anionic polymers (polycarboxylates, sulfates and phosphates), polynucleotides, low molecular weight inducers (tilorone, cationic dyes, propanediamine, carboxymethylacridanone etc.) are known [1,5,6,10,14,20,21,30,31]. This ever-expanding list has made it difficult to explain the basic [common] mechanisms, if any, behind their IFN inducing properties. Perhaps most extensively studied synthetic IFN inducer is polyriboinosinic acid: polyribocytidylic acid (poly I: C) [1,5,6,10,14,20,21,30,31].

Priming is one of the non-antiviral actions of IFNs. Treatment of cells with low doses of IFN for several hours prior to induction results in quicker production as well as enhanced yields. Repeated stimulation of a cell with an inducer does not result in the production of several crops of IFN [1,5,6,10,14,20,21,30,31]. This phenomenon is known as 'hyporesponsiveness'. Although the mechanism of this phenomenon is not understood, has shown that it can be overcome, at least in vivo, by either judicious use of different inducers or by administration of prostaglandins.

1.f Routes of IFN administration:

Oral delivery of the IFNs is not practical due to proteolytic degradation [1,2,5,8,9,11-21,48,52-55]. Subcutaneous and i.m. absorption of IFN- α and IFN- γ is >80%, and 30%-70%, respectively. These routes of administration result in a protracted distribution phase, with maximal serum or plasma concentrations occurring after 1 to 8 h, followed by measurable concentrations for 4 to 24 h after injection for both IFN- α and - γ

[1,2,5,8,9,11-21,48,52-55]. After i.m. administration, IFN- β levels peak between 3 and 15 h, and then decline at a rate consistent with a 10-h elimination half-life. At therapeutic dosages of 0.25 mg s.c. serum concentrations of IFN- β are low or undetectable. Intravenous administration of IFN- α or - β results in a biexponential decrease in serum concentration, and IFN- γ levels decline monoexponentially [1,2,5,8,9,11-21,48,52-55]. Terminal elimination half-lives range from 4-16 h for IFN- α , 1-2 h for IFN- β and 25 to 35 min for IFN- γ . Serum concentrations are generally measurable for between 8 and 24 h after i.v. injection of IFN- α and up to 4 h after i.v. injection of IFN- β and - γ . The relationship between dose and biological response varies among disease types

1.g Clinical Applications of interferon:

Evidence supports a benefit to IFN- α therapy in patients with early chronic-phase CML (Chronic Myeloid Leukemia) [1,2,5,8,9,11-21,48,52-55]. Combination of IFN- α 2b, hydroxyurea, and ara-C is also effective in overall survival and cytogenetic response. IFN- γ has also been used as therapy in chronic-phase CML. IFN- α was first used in the treatment of patients with follicular lymphoma in the early 1980s. The role of IFN- α as maintenance therapy for patients with low-grade non-Hodgkin's lymphoma (NHL) who achieved tumor bulk reduction after cytoreductive chemotherapy has also been evaluated [1,2,5,8,9,11-21,48,52-55]. Later it was recommended that IFN- α therapy be used in combination with chemotherapy in clinically aggressive follicular NHL, and as maintenance therapy in patients with high tumor burden after cytoreductive therapy.

IFN has been extensively used in the treatment of Renal Cell Carcinoma (RCC) [1,2,5,8,9,11-21,48,52-55]. It has been found that IFN- α 2b is more effective against

metastatic RCC than medroxyprogesterone acetate (MPA) and vinblastine (VBL), which are generally used against RCC. IFN monotherapy in patients with metastatic RCC provides a modest but significant prolongation of survival with manageable side effects. IFN monotherapy in patients with metastatic melanoma induces responses in approximately 15% and CR rates on the order of 5%. Recently, IFN- α has been used in the context of combination therapy (e.g. with IL-2 and Tamoxifen) for the treatment of metastatic melanoma [1,2,5,8,9,11-21,48,52-55]. The FDA approved the uses of IFN- α in stage III and stage IIB melanoma.

IFN has been used in the treatment of HIV-associated KS since 1981. Initial treatment regimens employed doses in the 20×10^6 U/d range, which were associated with significant response rates, but also high levels of toxicity. The combination of lower dosages of IFN- α and zidovudine (AZT) was found to be effective in treating HIV-associated KS, including those with lower CD₄ counts, but with dose-limiting hematological side effects. GM-CSF as an adjunct to combination IFN- α /AZT therapy resulted in an improved end-of-study absolute neutrophil count. A phase I/II study assessed effectiveness of IFN- γ therapy in the treatment of HIV-associated KS. In summary IFN- α , either as monotherapy or in combination with AZT, shows activity in HIV-positive patients provided they possess relatively elevated CD4 (+) lymphocyte counts (i.e., greater than $150/\text{mm}^3$). For more advanced disease, combination chemotherapy is currently the accepted means of treatment for KS [1,2,5,8,9,11-21,48,52-55]. IFN has a major protective role against influenza virus, hepatitis virus, coxsackievirus B1, etc. Much depends on the dynamics of the disease. Clinically, effective prophylaxis was demonstrated against rhinovirus infection of human volunteers,

also reduced CMV reactivation in seropositive patients undergoing kidney transplants has also been observed. IFN had also reported to be successful in treating genital warts and juvenile laryngeal papillomatosis [1,2,5,8,9,11-21,48,52-55].

More recently, synthetic alpha-interferon was licensed for use in the treatment of hepatitis B carriers and it is also being used for the treatment of hepatitis C carriers with chronic active hepatitis. Both IFN-alpha and IFN-beta are also used against Herpes zoster, Papilloma virus and Rhino Virus (prophylactic only).

IFN-gamma is also used against Lepromatous leprosy, Leshmaniasis, Toxoplasmosis, Chronic granulomatous disease (CGD), and Warts. Interferon has unspecific effect and can therefore stop all kinds of virus infections [1,2,5,8,9,11-21,48,52-55].

Scientists have now tried to stimulate the production of Interferon in cells in different ways, in order to be able to stop a virus infection quickly for example of measles or of influenza. Thereby trials have been made to apply nucleic acid in the environment of the cell, hoping that the cell would suspect that this nucleic acid was a virus and therefore start an Interferon production. The use of IFN against chronic hepatitis B and HIV has lately drawn immense attention.

Interferon-alpha 2b (Intron A) was first shown to be effective in chronic hepatitis B virus patients in 1988 [1,2,5,8,9,11-21,48,52-55]. The FDA subsequently licensed it for the treatment of chronic hepatitis B viral infection. In addition to its direct anti-viral effects, interferon works against hepatitis B virus by stimulating the body's immune system to clear the virus. For the treatment of chronic hepatitis B, a four to six month course of

interferon-alpha is given. Successful treatment, therefore, changes the infection from a reproducing (active) to a non-reproducing (inactive) state. Interferon alfa-2a (Roferon-A; Hoffmann-La Roche), interferon alpha-2b (Intron-A; Schering-Plough) and interferon alfacon-1 (Infergen; Intermune) are all approved in the United States for the treatment of adults with chronic hepatitis C as single agents. The recommended dose of interferons alfa-2b and alpha-2a for the treatment of chronic hepatitis C is 3,000,000 units three times a week, administered by subcutaneous or intramuscular injection. Treatment is administered for six months to two years. More recently peginterferon alpha, sometimes called pegylated interferon, has been available for the treatment of chronic hepatitis C [1,2,5,8,9,11-21,48,52-55]. The addition of ribavirin to interferon alpha is superior to interferon alpha alone in the treatment of chronic hepatitis C. Interferon-alpha is effective for severe, Zidovudine-resistant, HIV-1-related Thrombocytopenia [1,2,5,8,9,11-21,48,52-55]. Results of three important trials of Hepatitis C (HCV)/HIV co-infection were presented by Bill Valenti, at the 11th Conference on Retroviruses and Opportunistic Infections (CROI) on February 9, 2004 in San Francisco. The trial was based on the use of Pegylated Interferon and Ribavirin Treatment for HCV-HIV Co-Infection. The data supports the safety and efficacy of pegylated interferon/ribavirin for the treatment of patients with HIV/HCV co-infection and represent a major step forward in the treatment of coinfecting patients. While Sustained Virological Response was lower in coinfecting patients compared to HCV monoinfected patients, this result was not unexpected. However, the combination of pegylated interferon plus ribavirin appears to improve the chances of Sustained Virological Response in coinfecting patients. Pegylated interferon's safety profile and tolerance, as well as its weekly administration, make it the interferon-

of-choice in HIV/HCV coinfecting patients. Pegylated interferon alfa-2a (PEG-INF, Pegasys) shows better characteristics than the unmodified interferon (IFN) used to date [1,2,5,8,9,11-21,48,52-55].

Pegylated interferon alfa-2a is a branched polyethylene glycol (PEG) molecule with an average molecular weight of around 43 KD is attached to the IFN molecule. The modified drug has a biological half-life around ten times greater than the 8.5-hour half-life of the unmodified drug and therefore needs to be injected only once weekly as compared with three times weekly in case of hepatitis C infection. PEGs are nontoxic and amphophilic, i.e. soluble both in water and in most organic solvents. A PEG mass of 40 to 50 KD is sufficient to increase the size of a small molecule to such an extent that it is less readily excreted through the kidneys and therefore persists in the body for longer. In addition, as they are more or less surrounded by the attached PEGs, pegylated proteins are less rapidly broken down by the body's enzymes than are unmodified proteins [1,2,5,8,9,11-21,50,51,55-58].

IFN therapy against certain bacterial and protozoal infections:

It is established that interferons (IFN) play a defensive role during viral infections. There is also growing evidence that the IFN system can be a defense mechanism during certain bacterial and protozoal infections [1,12,20,21]. The availability of recombinant murine gamma IFN (IFN- γ) in large amounts made it possible for several investigators to show that recombinant murine IFN- γ could protect mice against bacteria such as *Listeria monocytogenes*, *Mycobacterium bovis* (BCG), and *Mycobacterium intracellulare* in vivo [1,12,20,21]. Therapeutic efficacy of interferon alfa-2a in the treatment of Behçet disease

has also been reported recently. It was concluded that Interferon alfa-2a is an effective alternative treatment for Behçet disease, particularly for the management of the mucocutaneous lesions of the disease. Potential therapeutic applications of interferon (IFN) in pulmonary diseases and the Churg-Strauss syndrome (CSS) have also been reported [1,11,12,20,21].

1.h Side Effects of the Treatment:

IFN treatment has four major toxic effects; constitutional, neuropsychiatric, hematologic, and hepatic [1,2,5,8,9,11-21,48,52-55]. Toxicity can be divided into acute and chronic manifestations. In the acute case, the patients experience fevers, chills and rigors, headaches, myalgias, malaise, Transaminitis and neutropenia. The chronic symptoms include fatigue, anorexia, and neuropsychiatric symptoms. Nervous system toxicities include somnolence, confusion, lethargy, dizziness, impaired mental status, numbness and tingling. Patients with cancer have a higher risk of developing clinical depression and dysfunction in the endocrine system [1,2,5,8,9,11-21,48,52-55]. Neuroendocrine disturbances, includes perturbation of the hypothalamic-thyroid-adrenal axis, alteration in dopamine and serotonin production, alterations in the secretion of IL-1, IFN- β and IFN- γ effecting the levels of ACTH, prolactin, growth hormone and cortisol levels in patients. There have been reports of alterations in the levels of sex hormones during IFN therapy, and male sexual dysfunction does occur. A few patients with hepatitis C have developed symptoms of Grave's disease while on IFN- α . Antithyroid antibodies occur in up to 16% of women and 1.5% to 3% of men in the general population, and individuals expressing baseline antithyroid antibodies have a 60% risk of developing clinical thyroid disease during the course of therapy. Treatment of MS patients with IFN- β was shown to induce

antithyroid autoantibodies and symptoms of overt hypothyroidism in a small series of patients [1,2,5,8,9,11-21,48,52-55].

Table2. Currently available interferon perpetrations and clinical application

IFN type	Trade name	Manufacturing technique	FDA-approved indications
IFN- α 2a	Roferon A	rDNA	NHL
			Hairy cell leukemia
			CML
			AIDS-related KS
			Chronic hepatitis C
IFN- α 2b	Intron A	rDNA	Follicular lymphoma
			Hairy cell leukemia
			AIDS-related KS
			Malignant melanoma
			Condyloma accuminata
			Chronic hepatitis B
			Chronic hepatitis C
IFN alfacon-1	Infergen	rDNA	Chronic hepatitis C
IFN- α n3	Alferon N	Purified from human leukocytes	Condyloma accuminata
IFN- β 1a	Avonex	rDNA (Chinese hamster ovary cells)	Relapsing-remitting MS
IFN- β 1b	Betaseron	rDNA (<i>E. coli</i>)	Relapsing-remitting MS
IFN- γ	Actimmune	rDNA	Chronic granulomatous Disease

1.i interferon perpetrations and clinical application:

The Biotechnology Industry has invested heavily in these agents and the worldwide market for interferons continues to increase. Alpha Interferon (IFN- α) is a synthetic version of a protein [53], which occurs naturally in the body and plays an important role in the functioning of the immune system. In the test tube, it inhibits HIV by interrupting the last stage of viral reproduction. IFN- α treatments have previously been shown effective against a rare leukemia and genital warts. It has also been proved that IFN- α is an effective anti-Kaposi's Sarcoma agent in-patient with less advanced stages of immunologic decline [1,2,5,8,9,11-21,48,50,52-55]. Currently, IFNs for therapeutic use are produced from two major sources, natural IFNs from human leukocytes or leukocyte cell lines and recombinant IFNs produced in bacterial cells. Natural IFNs are considered to be superior as they consist of the entire complement of IFNs and have the proper structure, but they are expensive and time-consuming to produce [1,2,5,8,9,11-21,48,50,52-55]. Bacterially produced recombinant IFN is cheaper and more efficient to make. Thus, a method for enhancing the quality and the production of recombinant IFN would be advantageous.

At the end of 2004 the world market for Interferon was valued at \$5.9 billion, a growth of 13% over 2003. The market has been growing at an average annual growth rate of 17% over the previous 5 years. The interferon market is application driven and clearly demarcated along the lines of the type of the interferon molecule. At 60% share, interferon Beta accounts for the main bulk of the market followed by interferon Alfa at 37% market share. Besides, its side effects and the high cost of basic research in this

field, IFN could be a key molecule in the drug market in the near future. An innovative and an industry-oriented research could certainly lead to a better IFN biomolecule.



1.1 BACKGROUND OF THE WORK

Modern medical intervention in many non-infectious diseases involves therapy-using proteins, often to replace the normal production of specific proteins, which have been diminished in the patient. Classic examples include insulin, growth hormone, and blood clotting factors such as factor IX. These proteins have traditionally been purified from animal sources, such as insulin from pig and cattle pancreas, growth hormone from pituitary glands from human cadavers and growth hormones and blood clotting factors (factor IX) from human blood donors.

These processes have given rise to serious ethical and practical problems. Insulin from animal sources is not identical to human insulin and some patients reject animal insulin. In addition, usage of insulin was exceeding the ability to supply insulin from animal sources, and the risk of infection by non-human pathogens has been highlighted by BSE (*Bovine spongiform encephalitis*), which is a major consideration in the proposed use of animal organs in human medicine. With treatment using proteins derived from human sources, such as growth hormone and factors IX there are no problems of mismatch between the treatment protein and the required human protein but the risk of infection from emerging pathogens has proved extremely high with HIV, hepatitis C and potentially BSE causing additional tragedy in these cases, and the problems of supply are even greater than in the case using animal sources. In addition to these traditional protein therapies there are new treatments for a wide range of disease- processes, including cancer. Proteins like the interferons, which may play a role in defense against infections

by viruses and aberrant cancer cells, and selective proteins like ricin, is proposed for use in specific target, directed therapy [65].

Genetic engineering has provided the tools to enable the production of proteins for therapy, which overcomes many of the problems with traditional production methods. The gene coding for the specific protein, whether the human insulin gene or the gene for one of the protein chains ricin from *Ricin communis*, can be cloned and expressed in a suitable host strain. *E coli* has been a popular choice as it is easy to grow and is well understood [56,57,60-63,65,67,72-76,78-81,88,89,92]. Proteins expressed in *E.coli* fermentation at high cell density and high yield should overcome problems of supply so that as much protein as required for treatment can be produced, although still at a cost. Appropriate good manufacturing practice and stability control will obviate the risk of infection, by known or unknown agents, from production of the protein. There are virtually no ethical problems. However, there are still some residual problems.

Especially in the case of human proteins, the protein synthetic machinery is not identical in prokaryotes and eukaryotes and post-translational modification of proteins, which frequently takes place in higher organisms, does not in *E coli*. Although *E coli* remains a valuable organism for the high level production of recombinant protein, not every gene can be expressed efficiently in this organism because of significant structural features of the gene sequence, the stability and translational efficiency of mRNA, the ease of protein folding, degradation of the protein by host cell proteases, major differences in codon usage between the foreign gene and native *E coli* proteins, and potential toxicity of the

protein to the host [56,57,60-63,65,67,72-76,78-81,88,89,92-96,103,105-109]. The major drawbacks of *E. coli* as an expression system includes the inability to perform many of the posttranslational modifications found in eukaryotic proteins, the lack of a secretion mechanism for the efficient release of protein into the culture medium, and the limited ability to facilitate extensive disulphide bond formation [56,57,60-63,65,67,72-76,78-81,88,89,92-96,103,105-115]. Therefore, even though the protein sequence is identical the protein product is not. In these cases other, usually more complex hosts have to be used e.g. *Pichia* (yeast) or mammalian cell cultures. The possibility of pos-translational modification using cell free systems after protein production also exists. For those proteins, which do not require post-translational modification, production of native protein still requires correct folding and complete translation. Misfolding and protein truncation are minimal during normal growth and protein synthesis in *E. coli*, however high yield production of heterologous protein can lead to both these problems affecting protein quality. High-level protein production of recombinant protein is important to enable cost-effective production and protein purification.

Proteins contain within their complete amino acid sequence all of the information necessary for attaining their functional three-dimensional structure. But all newly synthesized proteins face challenges in reaching their native state within the crowded environment of the cell. While some domains of a nascent chain might be capable of folding spontaneously, the folded structure cannot be obtained until the entire domain is synthesized [74,90,91,111]. This time lag increases the chance that hydrophobic sequences normally buried in the interior of the protein will become exposed, resulting in protein aggregation. About 40 amino acids of the nascent chain are protected from the

cytosol by the ribosome exit tunnel. When the chain leaves the tunnel, molecular chaperones bind preventing aggregation. Molecular chaperones are ubiquitous and highly conserved proteins that help other polypeptides to reach their native conformation without becoming part of the final structure [56-59,67-72,74,75,89,92,96,97,102-105,109,115]. They are not true folding catalysts, since they do not accelerate folding rates. Instead, they prevent off-pathway aggregation reactions by transiently binding hydrophobic domains in partially folded or unfolded polypeptides collectively designated as non-native proteins.

For the vast majority of polypeptides, folding is a spontaneous process directed by the amino acid sequence and the solvent conditions. Yet, even though the native state is thermodynamically favored, the time-scale for folding can vary from milliseconds to days. While protein folding in the absence of kinetic barriers is extremely fast, such barrier, which includes disulfide bond formation, cis/trans isomerization of the polypeptide chain around proline peptide bonds, preprotein processing, and the ligation of prosthetic groups can significantly delay correct folding of proteins [56-59,67-72,74,75,89,92,96,97,102-105,109,115]. The presence of kinetic barriers results in the accumulation of partially folded species, or folded intermediates, that contain exposed hydrophobic 'sticky' surfaces, which promote self-association. The self-association of folding intermediates is the basis for protein aggregation in vitro and for the formation of inclusion bodies [67,74,78,89].

1.1a. Recombinant protein production leads to the formation of inclusion bodies:

Rapid production of recombinant proteins can lead to the formation of insoluble aggregates designated as inclusion bodies [67,74,78,89]. These are large, spherical particles, which are clearly separated from the cytoplasm, and result from the failure of the quality control system to repair or remove misfolded or unfolded protein. The formation of inclusion bodies does not correlate with (1) the size of the synthesized polypeptide, (2) the use of the fusion construct, (3) the subunit structure and (4) the relative hydrophobicity of the recombinant protein. Overproduction by itself (the increase in the concentration of the nascent polypeptide chains) can be sufficient to induce the formation of inclusion bodies. These aggregates do not consist of pure recombinant polypeptide chains, but contain several impurities such as host proteins (RNA polymerase, outer membrane proteins), ribosomal components and circular and nicked forms of plasmid DNA. In addition, they might contain the small Heat Shock proteins IbpA and IbpB [56-59,67-72,74,75,89,92,96,97,102-105,109,115].

1.1b. Strategies to prevent the formation of inclusion bodies:

Strategies to prevent the formation of inclusion bodies are aimed to slow down the production of recombinant proteins and include (1) low-copy number vectors, (2) weak promoters, (3) low or high temperature, (4) co-expression of molecular chaperones, (5) use of a solubilizing partner, and (6) fermentation at extreme pH values.

A lower level of protein synthesis from a weaker promoter or from a strong promoter under conditions of partial induction is found to result in a higher amount of soluble protein and greater specific activity. Growth at lower temperatures is a well-known

technique for facilitating correct folding. The reason why a lower temperature favors the native state is related to a number of factors, including a decrease in the driving force for protein self-association, a slower rate of protein synthesis, changes in the folding kinetics of the polypeptide chain, etc [57,60,65,67,68,74,78,92,98,99,108,112,117,129].

The aggregation of proteins secreted into the periplasmic space can be suppressed by growing cells in the presence of relatively high concentrations of polyols or sucrose, a non-metabolizable sugar for *E. coli*. In the optimal concentration range, these additives do not affect cell growth, protein synthesis or export and, therefore, they influence directly the physiochemical processes that result in protein-protein association. Polyols and sucrose do not permeate through the cell membrane and consequently cannot exert a direct effect on the folding of cytoplasmic proteins. An increase in the osmotic pressure, however, leads to the accumulation of osmoprotectants, such as glycine betaine, which have an effect similar to sucrose in stabilizing the native protein structures. It has been shown that cells grown in the presence of sorbitol at 25 °C produce 400-fold higher levels of recombinant protein than control cultures [56,60,61,67,68,78,93,94,98,106-112,117,129].

Vector plasmids are tentatively divided into four classes based on their copy number (the copy number is defined as the number of plasmid copies per chromosome): very high-copy-number vectors are present in more than 100 copies per chromosome (pUC vectors), high-copy-number vectors (15-60 copies; pBR322), medium-copy-number vectors (about 10 copies; pACYC177, pACYC184 and pSC101) and low-copy-number vectors (1-2 copies; mini-F). Here, medium-copy-number vectors might reduce the

amount of recombinant protein sufficiently to prevent their aggregation. Alternatively, high-copy-number vectors can be used in combination with a weak promoter such as the wild-type *lac* promoter.

Reducing the growth temperature down to 25 or 20 °C also lowers the productivity of the cells. Co-expression of folder chaperones such as the DnaK or the GroE system might help in some cases to keep the recombinant proteins soluble. Solubilizing partners are other proteins, which are fused to the recombinant proteins and keep the hybrid proteins soluble. When three different proteins known to increase the solubility (maltose-binding protein [MBP], glutathione-S-transferase [GST] and thioredoxin [TRX] were fused to six different recombinant proteins, MBP turned out to be superior [56,57,59-61,67-72,74-79,81,88,89,92-100,102,104,105,108-115,129].

Sometimes, it might be desirable to produce recombinant proteins as inclusion bodies. How can active proteins become recovered from aggregates? This involves a four-step procedure. During the first step, the inclusion bodies are harvested by cell lysis and centrifugation of the cell lysate at 5,000 to 12,000x g. Under these conditions, the protein aggregates will be present in the pellet. The second step involves solubilization of the inclusion bodies by resuspension of the pellet in a buffer with a denaturant agent such as 6 M guanidinium chloride or 6-8 M urea. During the next step, the solubilized polypeptide chains are purified by ion exchange chromatography in the presence of nonionic denaturants such as urea. The fourth and last step results in vitro protein folding. Folding can be aided by the addition of low-molecular weight folding enhancers such as 1.0-1.3 M guanidiniumchloride, 2 M urea or polyethyleneglycol. If the recombinant protein

contains one or more disulfide bonds, generation of native bonds can be sustained by addition of reduced and oxidized glutathione.

1.1c. Role of Molecular Chaperones:

Cells have evolved an elaborate protein quality control system, which consists of molecular chaperones, and ATP-dependent proteases acting together to prevent aggregation, assist refolding and degrade misfolded polypeptides.

Molecular chaperones are divided into two distinct classes, folder and holder chaperones. Both classes of chaperones interact with non-native polypeptide chains through exposed hydrophobic surfaces, and while folder chaperones mediate their refolding in an ATP-dependent process, holder chaperones bind non-native proteins and prevent their aggregation [56-59,67-72,74,75,89,92,96,97,102-105,109,115,129].

In the cytoplasm of *Escherichia coli* cells (and other bacterial species), there are two multi-component chaperone complexes with broad specificity. The first comprises the 60-kDa-Heat Shock protein GroEL (60 kDa) and the smaller accessory protein GroES (10 kDa). GroEL forms a characteristic doublet of heptameric rings, which, during the catalytic cycle, associate one or two heptameric rings of GroES. The GroEL chaperone has a very broad specificity and is essential for viability. The second complex comprises DnaK and the two co-chaperones DnaJ and GrpE (the KJE complex). Nascent polypeptide chains are most probably recognized and bound by DnaK.

There are many examples that over-expression of molecular chaperones in *Escherichia coli* can facilitate the assembly of heterologous proteins. GroESL co-expression was

found to increase protein expression at 30 °C, but not at 37 or 42 °C; the KJE complex conferred a more substantial increase in the expression of soluble proteins at all temperatures tested. Addition of 3% ethanol was shown to have a synergistic effect with chaperone co-expression and led to the production of protein that was nearly all soluble. For any given recombinant protein, only the chaperone that interacts productively with an aggregation-prone folding intermediate will have a beneficial effect on the production of native protein. Unfortunately, the current substrate-chaperone match has to be found by trial and error.

Two important holder chaperones are the trigger factor and the small Heat Shock proteins IbpA and IbpB (inclusion body binding proteins A and B). The trigger factor occurs at about 20,000 copies per exponentially growing cell, and is found at the exit tunnel of the ribosomes where it binds to virtually all nascent polypeptide chains to prevent their premature folding. In addition to its holder chaperone activity, it acts as a peptidyl-prolyl cis/trans isomerase (PPIase). These enzymes catalyse the interconversion between cis and trans forms of the peptide bond preceding proline residues [56-59,67-72,74-76,89,92,96,97,102-105,109,115,129].

ATP-dependent proteases recognize non-native proteins in the cytoplasm and degrade them to peptides of a length of about ten amino acid residues. The current model for proteolytic degradation involves three steps: (1) Recognition: The protease selects a protein for degradation, either because it has an accessible tag located at the N- or C-terminus or because an internal degradation signal has become exposed. (2) Translocation: ATP-hydrolysis promotes both unfolding and translocation into the

proteolytic chamber (dual role of ATP). (3) Proteolysis: Proteins are hydrolysed to small peptides, which are released from the chamber into the cytoplasm. Five different ATP-dependent proteases have been identified in *Escherichia coli* (Lon, ClpAP, ClpXP, ClpYQ and FtsH where only FtsH is essential) which all form ring-like structures [56-59,63,67-72,74,75,89,92,96,97,102-105,109,115,129].

1.1d. Regulated gene expression requires an inducible or repressible system:

Tight expression of transcription of recombinant genes is often desirable or necessary since leaky expression can be detrimental or even lethal to cell growth. Regulated gene expression requires an inducible or repressible system, and therefore, all expression systems are based on controllable promoters. Promoters allowing constitutive expression turned out not to be adequate for the production of recombinant proteins due to two main reasons: First, they do not allow the production of toxic proteins and second, even non-toxic proteins produced at physiological concentrations can be deleterious to the cells when produced at higher levels. One prominent example are integral membrane proteins which, when overproduced, cause jamming of the inner membrane leading to cell death. Four regulatable promoter systems are widely used, where three are based on the repressors already mentioned (*LacI*, *TrpR* and phage λ cI) and the fourth on a phage RNA polymerase.

The *lac* system consists of the promoter/operator region preceding the *lac* operon and the *LacI* repressor encoded by the *lacI* gene. In the absence of an inducer, the *Lac* repressor binds to its operator situated immediately downstream from the promoter as a homotetramer. One of the many promoter mutations isolated has been termed *lacUV5*.

The promoter strength of *lacUV5* has increased 2-5 fold, and mutations increasing the promoter strength are called promoter-up mutations in general. Based on the *lacUV5* and the *trp* promoters, an artificial promoter was constructed termed P_{tac} (from *trp* and *lac*) .

In the case of the P_{lac} , the P_{lacUV5} and the P_{tac} promoters, the repressor is inactivated by addition of isopropyl- β -D-thiogalactopyranoside (IPTG). This compound binds to the active *LacI* repressor and causes dissociation from its operator. IPTG has two advantages over lactose: First, its uptake is not dependent on the *Lac* permease (it diffuses through the inner membrane) and second; it cannot be cleaved by β -galactosidase preventing turn-off of transcription. The *lacI* gene is either part of the expression plasmid or it is present within the chromosome. Since the wild-type level of the *LacI* repressor is not sufficient to repress expression of the recombinant gene in the absence of IPTG, two derivatives have been isolated resulting in an increase in the amount of repressor based on promoter-up mutations called *lacI^q* and *lacI^{q1}*. Expression systems based on the *trp* system make use of synthetic media with a defined tryptophan concentration. The concentration is chosen in such a way that the system becomes self-inducible when the tryptophan concentration within the cells falls below a threshold level. Additionally, 3- β -indole-acrylic acid can be added which inactivates the *TrpR* repressor and inhibits charging of tRNA^{trp} by tryptophanyl-tRNA synthetase [57,60,61,64,73,78,87,93,95,98,106,107,109,112,116,126,128,129,131].

The third system makes use of the bacteriophage λ repressor cI. This repressor is synthesized from the λ prophage and prevents expression of all the lytic genes by interacting with two operators termed O_L and O_R . These two operators overlap with two

strong promoters, P_L and P_R , respectively, and as long as the cI repressor is bound to its two operators, binding of RNA polymerase is prevented. Expression vectors carry the cI repressor gene and either $P_L O_L$ or $P_R O_R$. The wild-type cI repressor protein can be inactivated by UV-irradiation or treatment of the cells by mitomycin C. A more convenient way is the application of a temperature-sensitive version of the cI repressor called cI857. Therefore, *E. coli* cells carrying a λ -based expression system are grown to mid-exponential phase at low temperature and then transferred to high temperature to induce expression of the recombinant gene.

The most widely applied expression system makes use of the phage T7 RNA polymerase that recognizes only promoters found on the T7 DNA, and not promoters present on the *E. coli* chromosome. Therefore, the expression vectors contain one of the T7 promoters (normally the promoter present in front of gene 10) to which the recombinant gene will be fused. The gene coding for the T7 RNA polymerase is either present on the expression vector itself or on a second compatible plasmid or integrated into the *E. coli* chromosome. In all three cases, the gene is fused to an inducible promoter allowing its transcription and translation during the expression phase. The T7 RNA polymerase offers three advantages over the *E. coli* enzyme: First, it consists of only one subunit, second it exerts a higher processivity, and third it is insensitive towards rifampicin. The latter characteristic can be used especially to enhance the amount of recombinant protein by adding this antibiotic about 10 min after induction of the gene coding for the T7 RNA polymerase. During that time, enough polymerase has been synthesized to allow high-level expression of the recombinant gene, and inhibition of the *E. coli* enzyme prevents further expression of all the other genes present on both the plasmid and the

chromosome. Since all promoter systems are leaky, low-level expression of the gene coding for T7 RNA polymerase may be deleterious to the cell in those cases where the recombinant gene codes for a toxic protein. Expressing the T7-encoded gene for lysozyme can inhibit these polymerase molecules present during the growth phase. This enzyme is a bifunctional protein that cuts a bond in the cell wall of *E. coli* and selectively inhibits the T7 RNA polymerase by binding to it, a feedback mechanism that ensures a controlled burst of transcription during T7 infection [57,60,61,64,73,78,87,93,95,98,106,107,109,112,116,126,128,129,131].

Another expression system not widely used so far is induced upon a cold shock. When a mid-exponential phase culture of *E. coli* is rapidly transferred from 37 °C to the 10-15 °C temperature range, the synthesis of most cellular proteins significantly decreases, while that of about 15 cold-shock proteins is transiently upregulated [57,60,61,64,73,78,87,93,95,98,106-108,112,116,126-129,131]. CspA, the major cold-shock protein, is virtually undetectable at 37 °C, but more than 10% of the total protein synthesis is devoted to its production 1 h following the temperature downshift. The *cspA* mRNA is transcribed with a 150 nucleotide-long 5' untranslated region that confers high instability to the transcript at 37 °C ($t_{1/2} \sim 10$ s) but the transcript stability increases by two orders of magnitude upon transfer of the cells to 15-10 °C. A vector has been constructed based on the *cspA* promoter followed by its untranslated region to express recombinant proteins at low temperatures. Very recently, it could be shown that while the growth rate of an *E. coli* strain dropped rapidly as incubation temperatures decreased to 20 °C, addition of the *groESL* operon of *Oleispira antarctica*, isolated from Antarctic seawater, allowed 3-fold faster growth at 15 °C and an even 36-fold faster at 10 °C. Such an

engineered *E. coli* strain could produce high amounts of correctly folded recombinant protein at low temperatures.

1.1e. Factors responsible for the reduced expression of recombinant gene:

If expression of the recombinant gene is low, several factors may be responsible for the reduced expression: (1) stability of the mRNA, (2) occurrence of secondary structure(s) near the 5' end of the mRNA, (3) rare codons and (4) weak Shine Dalgarno sequence. mRNA molecules are relatively short-lived with a half-life of around 2 min. The following factors are involved in and influence the degradation of transcripts: exonucleases, endonucleases, secondary structures and ribosome-binding sites. In *E. coli*, two exonucleases have been identified, RNase II (*rnb*) and polynucleotid phosphorylase (*pnp*); both attack mRNA molecules at their 3' end. No exonuclease has been identified attacking from the 5' end. 3' → 5' degradation of transcripts by one of the two exonucleases (which are functionally redundant) can be delayed by secondary structure(s) present at or near the 3' ends. Some of these stem-loop structures may act as stabilizers when fused to heterologous mRNAs. This has been shown for the element present within the transcription terminator of the crystal protein gene of *Bacillus thuringiensis*, which had increased the half-life of the human interleukin-2 and of a penicillinase and thereby the final protein yields. Major endonucleases involved in cleavage of transcripts are RNase E, RNase II and RNase P. All three recognize elements, mainly stem-loop structures within the transcripts, and cleave at or near these secondary structures with two different consequences: in most cases, the endonucleolytic cut will lead to the inactivation of the transcript, while in rare cases this cut is part of a processing reaction involving polycistronic mRNAs. RNase E seems to be the most powerful endonuclease,

which, together with other proteins (exonuclease, RNA helicase, and enolase), constitutes the RNA degradosome. A stabilizing element for the 5' end of transcripts is the 5' untranslated region of the *E. coli* ompA mRNA which prolongs the half-life of a number of heterologous mRNAs in *E. coli* [57,60,61,64,73,78,87,93,95,98,106-108,112,116,126-129,131].

Secondary structures at the 5' end sequestering the Shine-Dalgarno and/or the start codon within a double-stranded stem significantly reduce translation of that transcript since it will be barely recognized by the 30S ribosomal subunit. Appropriate computer programs can detect mRNA secondary structures. There are two experimental solutions to this problem, exchange of nucleotides to prevent formation of inhibitory secondary structures or using a construct allowing translational coupling. Translational coupling requires at least a one-nucleotide overlap between the stop and the start codon, e.g. UGAUG, of the upstream and the downstream gene. If translating ribosomes arrive at the stop codon they slide back a few nucleotides on the transcript till they reach the Shine-Dalgarno sequence of the downstream gene. Translation of the downstream gene is normally prevented by a secondary structure near the end of the upstream gene sequestering the Shine-Dalgarno sequence of the downstream gene. This mechanism can be explored to ensure efficient translation of recombinant genes avoiding impairment of translation by secondary structures reducing binding of the 30S subunit. Vectors have been developed ensuring translational coupling of recombinant genes.

More than one codon encodes most amino acids and the relative abundance of cognate tRNAs determines codon usage. The codon usage by the different species can be quite

different. While the codons AGA and AGG are rare codons in *E. coli*, they represent frequently used codons in *Saccharomyces cerevisiae* and *Homo sapiens*. Over expression of genes with high contents of rare arginine codons may result in defective synthesis of the corresponding protein. Besides the amount, the location of rare codons within the coding region can significantly influence the translation level. It has been demonstrated that the closer AGG codons were to the initiation codon, the stronger the effect on protein synthesis [57,60,61,64,73,78,87,93,95,98,106-108,112,116,126-129,131]. They showed that single and, particularly, tandems of two to five AGG have stronger effects when placed closer to the translation start. Rare codons close to the initiator may stall the ribosome and prevent the entry of new incoming ribosomes. There are two experimental solutions to this problem: increase in the amount of the appropriate cognate tRNA or alteration of these codons to frequently used ones by sequence-specific mutagenesis.

Thus, the design of an optimal expression system for *E. coli* should be composed of DNA elements directing efficient transcription, stabilizing the transcript, powerful translation, resulting in authentic recombinant protein without any contamination by truncated or extended versions, and it should stay soluble and accumulate to about 20% of the total cellular protein. Such an expression system contains the consensus promoter recognized by the housekeeping promoter σ^{70} and can be further enhanced by addition of an UP element. Two strong factor-independent transcriptional terminators arranged in tandem prevent Readthrough transcription into neighbouring genes. The transcript itself is stabilized by inverted repeats present at both ends able to form stem-loop structures impairing endonuclease attack at the 5' end and exonucleolytic degradation from the 3' end but not translation.

Last but not least, efficient translation is assured by a strong Shine-Dalgarno sequence, an AUG start codon located about 8 bp downstream and the extended UAAU stop codon. Folding of the nascent polypeptide chains is aided by co-expression of folder chaperones. But it has to be mentioned at the end that there is no optimal expression system working with all recombinant proteins [57,60,61,64,73,78,87,93,95,98,106-108,112,116,126-129,131]. Each protein poses a new problem, and a high level of synthesis has to be optimized in each single case by empirical variation of the different parameters.

1.1f. Recombinant protein production requires an accurate understanding of the biological process:

The production of recombinant proteins rapidly and efficiently is of great importance in the biotechnology industry as a whole. The development of fermentation processes for recombinant protein production can involve several time consuming and costly stages. One of the first, and potentially the most crucial, decision in the development can be selection of a suitable combination of host strain and expression vector. Following shake flask studies the process is “scaled up” stepwise from small-scale fermenters to pilot plant production and can culminate in the definition of a large scale manufacturing process. The time to progress from small to large-scale production can be a limiting step in the introduction of a new product to the commercial market. The reduction of this time can thus be seen as a major objective.

Thus, optimization of protein production under fermentation conditions can be very time consuming and would be greatly facilitated by the use of accurate mathematical and computer models, which can predict the process of protein expression, by the organisms.

Development of such models requires an accurate understanding of the biological process involved in the expression of foreign proteins by organisms such as *E coli*. High-level protein production of recombinant proteins depends on the choice of an expression system as mentioned earlier. Cell growth characteristics, expression levels, intracellular and extracellular expression, posttranslational modifications, and biological activity of the protein of interest, as well regulatory issues in the production of therapeutic proteins plays an important role in choosing an effective expression system [56,58,60,61,64,65,68,73-75,78,86,88,92,93,95,98-100,106-108,112,116,117,128,129, 131].

High cell density culture systems can effectively increase the recombinant protein production in *E coli*. This includes, batch, fed batch, and continuous culture systems. These methods can achieve cell concentration in excess of 100g/litre and can provide cost-effective production of recombinant proteins. The composition of cell growth medium should be carefully formulated and monitored as it may have significant metabolic effects on both cells and protein production. For example, changes in temperature and changes in the culture medium can affect the translation of different mRNAs. The nutrient composition and fermentation variables such as temperature, pH and other parameters can affect proteolytic activity, secretions and production levels of recombinant proteins. It has also been found that distinct manipulation of the culture medium can have enhanced protein release into the medium. Thus supplementation of the growth medium with glycine enhances the release of periplasmic proteins into the medium without causing significant cell lysis. There are certain drawbacks in high cell

density culture systems as they suffer from limited availability of dissolved oxygen at high cell density, high carbon dioxide levels which can decrease growth rates and stimulate acetate formation, reduction in the mixing efficiency of the fermenters, and heat generation.

Another important strategy to attain elevated expression of native or heterologous proteins in *E coli* is to increase the dosage of the gene encoding that protein. As discussed above, plasmids with different copy numbers can be utilized for this purpose. Increased copy number facilitates greater cloned-gene expression, but also effects the overall metabolism of the cell as manifested in decreased growth rates and also less product formation per cloned gene copy. Information regarding the response of the cells in the presence of the plasmid helps in understanding the function of the cells containing a foreign genetic element, which imposes a parasitic burden on metabolism. This allows much more sophisticated genetic manipulations and cell environment controls to maximize cloned protein synthesis. Thus determination of plasmid-induced changes in the cellular content of individual host-cell enzymes and regulatory proteins is crucial for more systematic design and implementation of metabolic engineering. However attempts to produce large amounts of a single protein in bacteria by inserting the gene for the desired protein under the control of an effective promoter and incorporating the construct into a multicopy plasmid are often initially unsuccessful. The growth rate of cells containing plasmids suffers a setback in comparison to the growth rate of those without plasmids because of the additional strain placed on the hosts by plasmid replication, rDNA transcription, and plasmid-encoded mRNA translation. The difference can trigger

segregational instability, which leads to uneven partitioning of plasmids to the daughter cells and can yield an unproductive bioreactor.

Achievements in the field of genetic engineering have provided important steps, which may reduce the effect of growth rate differential and plasmid segregation. Controllable promoters (e.g. *λ Pl*, *LacUV5*, and *tac*), when inserted upstream of the product gene, facilitate the regulation of product expression. Insertions near the replication origin of R1 like plasmids have provided the first steps towards copy number control. Thus it is possible to construct a plasmid where both copy number and cloned gene product expression are tightly controlled and thus, so is the growth rate differential between plasmid-free and plasmid bearing cells.

The availability of strong promoters (native and synthetic) has led to high levels of recombinant protein accumulation in *E coli* [56,58,60,61,64,65,68,73-75,78,86,88,92,93,95,98-100,106-108,112,116,117,128,129,131]. But rapid accumulation of protein in either aggregated or soluble form is not uniformly observed. Recombinant protein having a different amino acid composition from that of the average *E coli* proteins was found to have low yields. Thus induction of a recombinant protein with an unusual amino acid composition can cause a temporary shortage of intracellular amino acid, particularly those in abundance in the recombinant protein. In this way, induction of recombinant proteins could cause a stringent response in *E coli*, which leads to imbalance in amino acid uptake into the elongating polypeptide relative to the endogenous amino acid synthesis rate. The depletion of the intracellular amino acid pool would result in a

change in the aminoacylated tRNA to uncharged tRNA ratio, and induction of the classical stringent response. It was also found that several Heat-Shock proteins are observed to increase during a stringent response.

An inevitable result of high-level protein expression in *E.coli* is that the cells amino acid pools, energy supply and normal growth processes are stressed. This can trigger stress-response processes, which are designed to switch the organism into a starvation resistant state and switch off growth and protein synthesis. It can also trigger repair mechanisms, which are designed to repair stress damage, which can lead to the induction of proteases which would normally degrade misfolded or denatured proteins but which may degrade recombinant proteins. Studies have shown that removing the stringent response (by using a ppGpp- deficient strain of *E.coli*) can increase the production of recombinant protein five fold [62].

However, many of the stress responses of *E.coli* could also be beneficial to recombinant protein synthesis. Recombinant protein production is strongly coupled to cell growth, but in order to achieve high cell densities it is necessary to apply fed-batch growth strategies, which lead to decreasing cell growth rates and substrate limitations. Over expression of recombinant protein can lead to depleted amino acid pools, energy depletion and accumulation of misfolded and truncated proteins, which trigger a drastic stress response. Co-ordinated expression, in which recombinant expression matched growth and metabolic potential would be the optimum and down regulation of protein synthesis would achieve this if applied progressively.

Similarly, part of the stress response exhibited by *E.coli* leads to increased production of protein chaperones, which increase the fidelity of protein folding. And the fidelity of transcription and translation is increased. So the stress response of *E.coli* has overlapping beneficial as well as deleterious effects on recombinant protein production. In recombinant protein systems with high productivity expression systems the stress response generated by over-expression is rapid and complete so that at times any beneficial affects are masked.

The Heat Shock response is triggered by response to denatured proteins and induces, primarily a response to maintain protein fidelity with increased chaperones and transcription factors but also a response to degrade protein with increased production of broad specificity proteases [56,58,60,61,64,65,68,73-75,78,86,88,92,93,95,98-100,106-108,112,116,117,128,129,131]. The effect on down regulation of protein synthesis is much less than in the stringent response.

The mechanism of an organism's adaptation to high temperatures has been investigated intensively in recent years. It was suggested that the macromolecules of thermophilic microorganisms (especially proteins) have structural features that enhance their thermo stability [132,133]. Most cell types from all known organisms respond to thermal stress by synthesizing a small number of polypeptides that have come to be known as Heat Shock proteins [134-136]. Heat Shock proteins belong to a larger class of proteins called stress proteins that are induced by a wide variety of physiological insults. At least two of

the stress proteins (HSP60 and HSP70) function as molecular chaperones that facilitate correct folding of newly synthesized polypeptides during normal cell growth [62,64,137].

What ever could be the argument, *E coli* is a versatile organism for the production of recombinant proteins. Often, however, as it is discussed earlier in this section, the recombinant protein does not reach its native, biologically active conformation within the bacterial cell but deposits as inclusion bodies [56-59,62,64,67-72,74,75,89,92,96,97,102-105,109,115,137,138]. Coproduction of individual chaperones with the target protein can also reduce deposition of the recombinant protein into inclusion bodies [134,136]. Thus in the following paragraphs few details about the Heat-Shock chaperones and its role in protein folding has been studied.

1.1g. Role of Heat Shock proteins in protein folding:

The Heat-Shock chaperones, a group of polypeptides omnipresent in all kingdoms of life, form a network to assist proper folding of cellular proteins by binding to exposed hydrophobic residues, prevent their deposition and can even dissolve deposits of misfolded proteins formed during environmental stress conditions such as excessive heat.

The best-characterized molecular chaperones in the cytoplasm of *E. coli* are the ATP-dependent DnaK-DnaJ-GrpE and GroEL-GroES systems. DnaK binds to hydrophobic regions exposed to the solvent by nascent or stress-unfolded polypeptides, thereby preventing off-pathway reactions leading to aggregation. The promiscuity of DnaK binding is well explained by the fact that it recognizes heptameric stretches of amino acids consisting of a 4–5 residues-long hydrophobic core flanked by basic residues. This

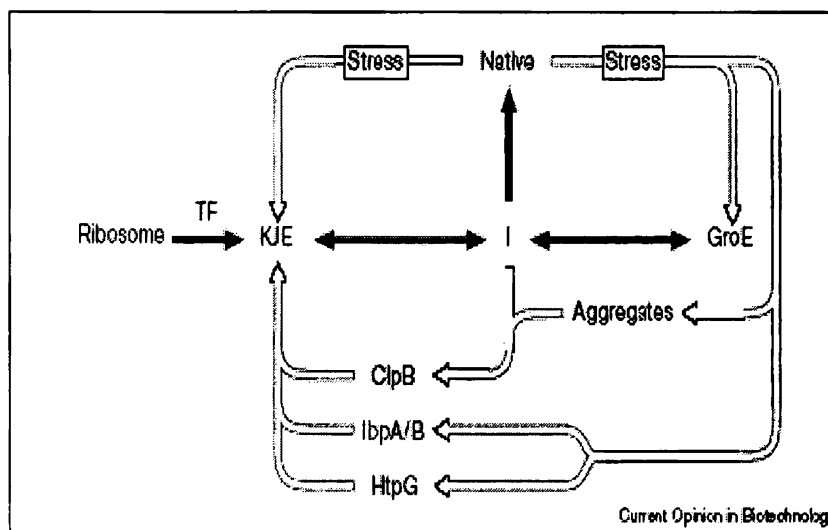
motif occurs every 36 residues on the average protein [56-59,61,63,66,67-72,74-77,79-81,88,89,92,96,97,102,104-106,109-110,115,127,129,132-138]. DnaJ, which independently binds folding intermediates, activates DnaK for tight substrate binding and might direct it to high-affinity sites. The nucleotide exchange factor GrpE mediates complex resolution: released proteins may either fold into a proper conformation, be recaptured by DnaK-DnaJ for additional cycles of interaction or be reversibly transferred to the 'downstream' GroEL-GroES chaperonins. GroEL is an ~800 kDa hollow toroid consisting of two stacked homoheptameric rings. It binds both substrate proteins and GroES (a 70 kDa domeshaped homoheptamer) via a ring of hydrophobic residues located in its apical domain. Although no clear consensus sequence has been identified, GroEL, like DnaK, appears to favor hydrophobic and basic residues in its substrates. Upon GroES binding, partially structured folding intermediates are released into the inner cavity of GroEL where they can fold in a capped and hydrophilic environment. There is extensive evidence that co-overproduction of the DnaK-DnaJ or GroEL-GroES chaperones can greatly increase the soluble yields of aggregation-prone proteins and a number of plasmids compatible with pMB1-derived cloning vectors are available for this purpose [71-73]. The process does not involve dissolution of preformed recombinant inclusion bodies but is related to improve folding of newly synthesized protein chains. It is important to point out, however, that the beneficial effect associated with an increase in the intracellular concentration of DnaK-DnaJ and GroEL-GroES is highly dependent on the nature of the overproduced protein, and that success is by no means guaranteed (and highly unlikely if the protein is inherently incapable of folding). Based on *in vitro* studies and homology considerations, a number of additional cytoplasmic proteins have been

proposed to function as molecular chaperones. They include ClpB, HtpG and IbpA/B, which like DnaK-DnaJ-GrpE and GroEL-GroES, are Heat-Shock proteins (Hsps) belonging to the σ^{32} stress regulons. Although inactivation of these Hsps has a modest effect on the ability of *E. coli* to handle thermal stress they appear to have a supporting role in cellular protein folding by acting as minor chaperones that bind folding intermediates or misfolded proteins and transfer them to the DnaK-DnaJ-GrpE team for subsequent reactivation (Figure 4). Although overproduction of IbpA/B, HtpG or ClpB did not suppress the misfolding of an aggregation-prone fusion protein in the *E. coli* cytoplasm, increased intracellular levels of these Hsps might improve the solubility of other substrates, particularly if coordinated with DnaK-DnaJ over expression. The *trans* conformation of X-Pro bonds is energetically favored in nascent protein chains; however, ~5% of all prolyl peptide bonds are found in a *cis* conformation in native proteins. The *trans* to *cis* isomerization of X-Pro bonds is rate limiting in the folding of many polypeptides and is catalyzed *in vivo* by peptidyl prolyl *cis/trans* isomerases (PPIases). Three cytoplasmic PPIases, SlyD, SlpA and trigger factor (TF), have been identified to date [75,76]. The most potent is TF, a 48 kDa protein associated with 50S ribosomal subunits that has been postulated to cooperate with chaperones to guarantee proper folding of newly synthesized proteins. Whether TF overproduction will improve the folding of recombinant proteins synthesized in the *E. coli* cytoplasm remains to be determined. It should be noted, however, that this might not be without physiological consequences as TF and SlyD overproduction lead to cell filamentation. Interestingly, co-overproduction of a leader-less version of PpiA (thus confining to the cytoplasm a PPIase that normally resides in the periplasm) has been shown to increase the yields of a

cytoplasmic fusion protein [77]. Structural disulfide bonds do not form in the cytoplasm of wild-type *E. coli* strains, as this environment is reducing and at least five proteins (thioredoxins 1 and 2, and glutaredoxins 1, 2 and 3, the products of the *trxA*, *trxC*, *grxA*, *grxB* and *grxC* genes, respectively) are involved in the reduction of disulfide bridges that transiently arise in cytoplasmic enzymes. Nevertheless, disulfide-bonded recombinant proteins can accumulate in the cytoplasm of surprisingly healthy *trxB* mutants that lack thioredoxin reductase, a protein responsible for the reduction of oxidized thioredoxins. Oxidation occurs post-translationally and is favored at low temperatures. While mutants lacking both *trxB* and genes involved in the reduction of glutaredoxins (e.g. *gshA* and *gor*) are even more efficient at accumulating oxidized recombinant proteins in dithiothreitol-free medium, they exhibit severe growth deficiencies in the absence of the reducing agent [80].

Fig. 4 A model of Chaperone mediated protein folding in the cytoplasm of the *E.coli*.

A model for chaperone-mediated protein folding in the cytoplasm of *E. coli*. The peptidyl-prolyl *cis/trans* isomerase trigger factor (TF) might be the first folding factor to interact with proteins as they emerge from the ribosome. Under non-stressful conditions (filled arrows), nascent or newly synthesized proteins enter the chaperone cycle through interactions with DnaK-DnaJ-GrpE (KJE). Folding intermediates (I) are released into a native conformation following cycles of binding and release by KJE and/or GroEL-GroES (GroE). Upon heat shock or other stressful conditions (e.g. recombinant protein expression), a number of alternate pathways become prevalent (open arrows). Misfolded proteins re-enter the KJE-GroE pathway directly or are bound by minor chaperones. HtpG and IbpA/B capture proteins as they denature. ClpB is likely to have a more general function including dissolution of preformed aggregates and/or disaggregation



of improperly associated intermediates. The minor chaperones interact with KJE to allow protein substrates to re-enter the major chaperone protein folding pathways. This

model is based on *in vitro* studies from a number of laboratories and *in vivo* data (JG Thomas, F Baneyx, unpublished data).

1.1h. Role of Heat Shock proteins in triggering immune responses:

Heat Shock proteins trigger immune response through activities that occur both inside the cell (intracellular) and outside the cell (extra cellular). Because of the normal functions of Heat Shock proteins inside the cell (such as helping proteins fold, preparing proteins for disposal, etc.), HSPs end up binding virtually every protein made within the cell. This means that at any given time, HSPs can be found inside the cell bound to a wide array of peptides that represent a 'library' of all the proteins inside the cell. This library contains normal peptides that are found in all cells as well as abnormal peptides that are only found in sick cells [59,69-72,74,75,84,88,92,96,97,102-105,109,115,127,133,134,138].

Heat shock proteins are extremely versatile and potent molecules, the importance of which to biological processes is highlighted by the high degree to which their structure and function are phylogenetically conserved. Our knowledge of the physiological role of Heat Shock proteins is currently limited; however, a better understanding of their function and thereby the acquisition of the capacity to harness their power might lead to their use as therapeutic agents and revolutionize clinical practice in a number of areas.

In spite of their high degree of conservation, Heat Shock proteins (HSP) have been shown to behave as immunodominant antigens in a large number of pathologies including infectious diseases. The HSP70 proteins from some pathogenic organisms are involved in the development of autoimmune responses; however, they have also been implicated in immunoprotection [29,59,82-85,88,102,133].

The binding properties of HSP70 protein confer to the proteins outstanding immunological properties, e.g. they have been implicated in MHC class II and in a transporter associated with antigen processing (TAP)-independent way MHC class I antigen presentation pathways. Thus, this property would allow peptides bound to HSP70 to be presented more efficiently and would thus promote stronger stimulation of antigen-specific T cells [29,59,82-85,88,102,133]. The immunostimulatory effect of HSP70 may be a consequence of stimulation of previously generated memory T cells. However, it has been reported that the strong humoral response to *M. tuberculosis* HSP70 protein itself appears to be due to a strong α β T cell response with no evidence of natural priming. An immunostimulatory activity of stripped HSP70 was also recently described: the protein was able to stimulate the antigen-specific cytotoxic activity of a CD8⁺ T cell clone in the

presence of peritoneal macrophages as antigen-presenting cells through the active secretion of IFN- γ and tumor necrosis factor (TNF)- α . This activity was not MHC dependent and was also functional *in vivo*, suggesting the existence of a new mode of T cell stimulation for these types of protein. Nevertheless, the mechanism of HSP70 action, as well as the nature of the primary target cells, remains uncertain. However, mouse HSP70 has the capacity to bind to the surface of dendritic cells and macrophages, and to be internalized by receptor-mediated endocytosis. In these cells, HSP70 are co-localized with MHC class I molecules in late endosomes, suggesting a direct interaction between HSP70 and MHC molecules. These studies indicated that antigen-presenting cells are capable of interacting with HSP70 and can be stimulated to adapt the immune response. In addition, endothelial cells also respond to HSP70, inducing the expression of adhesion molecules and cytokines that attract lymphocytes to the HSP70-expressing focus.

Increased synthesis of Heat Shock proteins (hsp) occurs in prokaryotic and eukaryotic cells when they are exposed to stress. Due to their wide distribution and their homology among different species, hsp represent target antigens of the immune response. Frequent confrontation of the immune system with conserved regions of hsp, which are shared by various microbial pathogens, can potentiate antimicrobial immunity. However, long-term confrontation of the immune system with hsp antigens, which are similar in the host and invaders, may convert the immune response against these host antigens and promote autoimmune disease.

The molecules involved in antigen recognition, i.e., immunoglobulins (Ig), T-cell receptors (TCR), and gene products of the major histocompatibility complex (MHC), are all multimeric complexes, and their assembly is promoted by distinct chaperones.

Members of the hsp family has important role in intracellular antigen-processing pathways.

Both host cells and microbes are confronted with dramatic alterations in their living conditions during infection. With these changing conditions, induction of hsp synthesis is vital for pathogen survival. Subsequently, increased pathogen hsp levels in cells lead to rapid degradation of hsp by the host processing machinery. Pathogen-derived determinants may then be efficiently presented by host cells and promote recognition of infected cells by the immune system.

Although the exact role of hsp in immunity to microbial infection is incompletely understood, hsp apparently serve as important antigens in defense against infectious agents. In fact, immune responses to hsp have been observed in infectious diseases caused by bacteria, protozoa, fungi, and nematodes, as well as in various experimental infection models [29,59,82-85,88,102,133].

Evidently, due to their high conservation among various microbial pathogens, hsp are major antigens. They are known to induce very strong humoral and cellular immune responses in numerous infections. Different hsp cognate proteins, e.g., hsp60 or hsp70, have a high degree of sequence homology among various pathogenic or nonpathogenic bacteria. For example, the hsp60 in mycobacteria is homologous to the common antigen of *Pseudomonas aeruginosa* and to hsp60 of other gram-negative bacteria, including GroEL of *E. coli* [29,59,82-85,88,102,133].

It has been proposed that hsp are particularly suited to be carriers because of their high affinity of binding to certain peptides and their involvement in various steps in antigen processing.

Cross-linking of peptides to mycobacterial hsp60 or hsp70 induced peptide-specific immune responses against malaria that were independent of adjuvants in mice and monkeys. Immunization of mice with an immunodominant viral peptide noncovalently associated with hsp70 was capable of eliciting strong peptide-specific T-cell responses. Similarly, immunization of mice with a soluble fusion protein, consisting of an ovalbumin fragment covalently linked to mycobacterial hsp70, induced a strong MHC class I-restricted CD8 T-cell response against a dominant ovalbumin T-cell epitope. Moreover, complexes of recombinant human hsp70 with a peptide representing a CD8 T-cell epitope of lymphocytic choriomenigitis virus were found to induce protective immunity in mice against lymphocytic choriomenigitis virus challenge. In a further extension of this concept, defined peptides of foreign or self-hsp60 have been used successfully as carriers for poorly immunogenic T-cell-independent carbohydrate antigens [29,59,82-85,88,102,133].

Importantly, as documented in other studies, immunization of mice with mycobacterial hsp60 conjugated to peptide or carbohydrate antigens induced hsp-specific antibodies that cross-reacted only with hsp homolog from other prokaryotes but not with the mammalian hsp60 cognate.

The capacity of hsp to serve as carrier molecules has been studied intensively in murine tumor models. The observation that tumorigenicity of a murine macrophage tumor cell

line was decreased after transfection with mycobacterial hsp60 led to the suggestion that hsp60 promoted the delivery of immunodominant tumor antigens to the cell surface and consequently facilitated the recognition and eradication of tumors by specific T cells.

In other studies, gp96, which is involved in the loading of MHC class I molecules in the ER, has been conjugated with a viral T-cell epitope. These studies showed that exogenous antigens could be chaperoned by gp96 into the endogenous processing pathway, leading to MHC class I-restricted recognition of peptides by CD₈ T lymphocytes [29,59,82-85,88,102,133]. By applying this concept, purified gp96 was also able to cross prime a CD₈ T-cell response to a minor histocompatibility antigen. The identity of a viral peptide bound to gp96 with a naturally MHC class I-presented peptide in virus-infected cells provides evidence that gp96 is capable of chaperoning immunodominant epitopes [29,59,82-85,88,102,133]. Based on this finding, gp96 may be useful in generating CD₈ T-cell responses against all kinds of intracellular antigens. In an extension of this concept, effective antitumor activity has been induced by treatment of mice with gp96 derived from autologous tumor cells without identifying the tumor-specific antigenic epitopes [29,59,82-85,88,102,133]. Use of either foreign or self-hsp as carrier molecules for antigenic determinants provides a basis for applying hsp in conjugate vaccines.

Because hsp represent dominant antigens in numerous microbial infections, a potential use of pathogen-derived hsp for vaccination has been suggested. In fact, in various infectious disease models different vaccination strategies using hsp have induced significant protection. For example, immunization of mice, with recombinant GroES and GroEL from *Helicobacter pylori* protected the animals against subsequent infection and

development of gastroduodenal disease [29,59,82-85,88,102,133]. Moreover, vaccination of mice with recombinant hsp60 from *Histoplasma capsulatum* induced protection against pulmonary histoplasmosis. Another example of a protective anti-hsp immune response has been shown in murine infection with *Y. enterocolitica*. Immunization of mice with yersinia-hsp60 induced a strong yersinia-hsp60-reactive T-cell response, which conferred protection against a challenge with yersiniae. Similarly, studies by Lowrie and coworkers suggest a protective role of mycobacterial hsp60 in murine infection with *M. tuberculosis* [29,59,82-85,88,102,133]. Transfer experiments with immune spleen cells revealed that protection was T-cell dependent. Analysis of lymphocyte subsets revealed that effective protection against *M. tuberculosis* correlated with cytolytic responses of CD₄ α β T cells, CD₈ α β T cells, and γ δ T cells.

In further experiments, naked-DNA vaccination was used. Mice, which received plasmid DNA encoding mycobacterial hsp60, were partially protected against subsequent challenge with *M. tuberculosis* [29,59,82-85,88,102,133]. Similar protection was achieved with plasmid DNA encoding mycobacterial hsp70.

Thus, it could be stated that, hsp rather than initiating anti-infectious or auto aggressive immune responses, chaperone the immune response induced by other antigens both influencing its strength and sustain it. In this way, the term “chaperone,” originally used to describe the biological function of hsp, also fits well as a description of its role as an antigen in infection and auto aggression.

Production of Heat Shock proteins together with the recombinant proteins has already been found to increase the folding and thus the solubilization of the recombinant proteins [10,12-16]. **Hence, the present study was designed to standardize an indigenous approach towards the rhuIFN α 2a production and to observe the potentially beneficial effects of Heat Shock on the rhuIFN α 2a protein production by the *E.coli* strain and also to observe the change in the biological property or the antiviral activity of the rhuIFN α 2a protein molecule when expressed under the Heat Shock condition.**

1.2 AIMS AND OBJECTIVES

AIM:

The main aim of the project was to find out that whether Heat Shock given in the exponential growth phase of the genetically modified *E.coli* strain harboring the rhuIFN α 2a gene leads to a better production of the protein molecule in terms of quantity and quality. But under this broad spectrum a number of issues has been addressed which could be of interest for the biotechnology industries engaged in rhuIFN α 2a manufacturing.

OBJECTIVES:

1. Analysis of the effect of Heat Shock on the production pattern of the rhuIFN α 2a protein molecule by Growth Curve Analysis, SDS-PAGE & IFN-ELISA.
2. Purification of the rhuIFN α 2a protein molecule by Electroelution technique.
3. Endotoxin level estimation of the Electroeluted rhuIFN α 2a protein molecule by LAL method.
4. rhuIFN α 2a bioassay against the Encephalomyocarditis Virus (EMC) on WISH Human Amnion Cell lines.
5. Antiviral Assay of rhuIFN α 2a against HIV I by Reverse Transcriptase Inhibition Assay and by P-24 antigen Inhibition Assay.
6. Primary Structure Analysis of the Electroeluted rhuIFN α 2a protein molecule.

MATERIALS AND

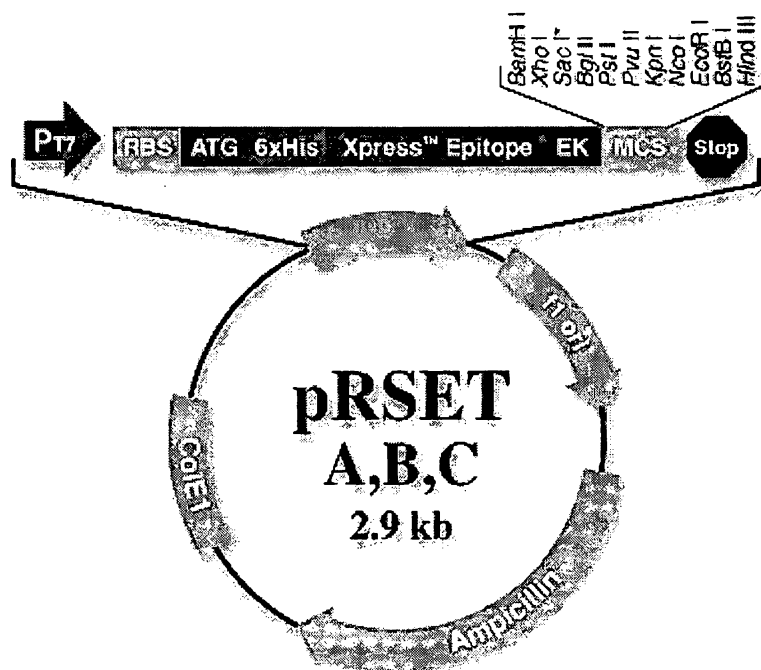
METHODOLOGY

2. MATERIALS AND METHODS

2.1 Strain used in the study:

In the recombinant *E.coli* strain used in this study, the rhuIFN α 2a [rhuIFN α 2a gene cloned into the plasmid expression vector pRSET T7 (Invitrogen) Figure 5 was procured from Centre for Biotechnology, JNU, New Delhi] coding sequence was transformed into the *E.coli* BL21 (DE3) pLysS cells (Promega). The protein expression system was under the control of an IPTG inducible *T7* promoter.

Figure 5. Plasmid expression vector pRSET T7



The pRSET vector is designed for high-level prokaryotic expression controlled by the strong bacteriophage T7 promoter. Expression is induced by the production of T7 RNA polymerase in BL21 (DE3) *E. coli*. These cells also produce T7 lysozyme to reduce basal

expression of target genes. The pRSET vector offers: Bacteriophage T7 promoter for high-level expression, T7 gene 10 sequence to provide protein stability, N-terminal polyhistidine (6xHis) tag for rapid purification with nickel-chelating resin and detection with an Anti-HisG Antibody, N-terminal Xpress™ epitope for detection with the Anti-Xpress™ Antibody and Enterokinase cleavage site for removal of fusion tag.

BL21 (DE3) pLysS allow high-efficiency protein expression of any gene under the control of a T7 promoter and having a ribosome-binding site. BL21 (DE3) pLysS is lysogenic for λ -DE3 (1), which contains the T7 bacteriophage gene I, encoding T7 RNA polymerase (2) under the control of the *lac* UV5 promoter. BL21 (DE3) pLysS also contains a plasmid, pLysS, which carries the gene encoding T7 lysozyme. T7 lysozyme lowers the background expression level of target genes under the control of the T7 promoter but does not interfere with the level of expression achieved following induction by IPTG. Genotype: F⁻, *ompT*, *hsdS_B* (*r_B-m_B-*), *dcm*, *gal*, (DE3), pLysS, Cm^r.

2.2 Preparation of competent *E.coli* BL21 (DE3) cells and transformation of
***rhuIFN α 2a* clone into the competent *E.coli* BL21 (DE3) cells:**

Media:

Luria Agar: (Hi-Media Cat. No. M575)

Luria Broth: (Hi-Media Cat. No. M557)

Supplement:

Ampicillin 100 μ g/mL

0.1M CaCl₂

A single colony was picked from a freshly grown *E.coli* BL21 (DE3) plate (16-20 hours at 37 ° C) and transferred into 100 mL of Luria Broth (20gm/1000mL D/W) in a 1-liter flask. The culture was incubated for ~3 hours at 37° C, 200rpm shaking condition. The cells were aseptically transferred to sterile, disposable, 50-ml polypropylene tube and stored in ice for 10 minutes. The cells were pelleted by centrifugation at 5000 rpm for 10 minutes at 4° C. The media from the cell pellet were decanted and the tube was kept in an inverted position for 1 minute to allow the last traces of media to drain away. The pellet was then resuspended in 10mL of ice-cold 0.1 M CaCl₂ (14.7g of CaCl₂·H₂O dissolved in 1 L of distilled water and autoclaved at 15lb/inch², and stored in aliquots.) and stored on ice. The cells were recovered by centrifugation at 4500 rpm for 10 minutes at 4° C. The supernatant from the cell pellet was then decanted out, and the tube was again kept in an

inverted position for 1 minute to allow the last traces of the medium to drain away. The pellet was then re-suspended in 2 mL of ice-cold 0.1 M CaCl_2 for each 50 mL of original culture.

Using a chilled, sterile pipette tip, 200 μL of the competent cells suspension were transferred to a sterile microfuge tube. 50ng of rhuIFN α 2a plasmid DNA in 10 μL volume was added to the tube. The contents of the tube were mixed by gentle swirling and tube was stored on ice for 30 minutes. After that, the tube was transferred to a rack placed in a circulating water bath that has been preheated to 42° C. The tube was then left in the rack for exactly 90 seconds. Immediately after that, the tube was rapidly transferred to an ice bath. The cells were allowed to chill for 1-2 minutes. The tube was then removed from the ice and 800 μL of Luria broth at 37°C was added. The cells were then gently agitated (150rpm in a rotary shaker) at 37° C for 1 hour. 100 μL of the transformed competent cells were then streaked on the ampicillin Luria Agar plates (35gm/1000mL D/W). The plates were then inverted and incubated at 37° C /12-16 hours [87].

2.3 Growth of the *E.coli* strain at Shake flask scale and sample collection:

Inoculum:

For each growth a loopful of the strain grown overnight on ampicillin Luria Agar plate was used to inoculate 10mL of Luria Broth medium supplemented with ampicillin in each 50mL flask. The cultures were grown at 37°C for approximately 15-16 hrs at 150 rpm in an Orbital Incubator Shaker. After incubation, $\text{OD}_{600\text{nm}}$ was measured and a volume calculated to give the equivalent of 100 μL of culture at $\text{OD}_{600\text{nm}}$ 1.0 and this was used for inoculation of the growth experiments.

Growth:

The inoculated 250mL culture flasks containing 50mL culture broth were incubated at 37°C/150 rpm and samples were taken every 1 hr. for 11-13 hrs. A sample of the Inoculum was taken at inoculation and a sample was taken after growth overnight. OD_{600nm} was measured for each sample.

Heat Shock:

The cultures were given the heat shock of 50° C for 5 minutes at 4 hours growth (when the OD_{600nm} reaches approximately 0.3 – 0.4). This step was performed to generate heat stress response within the strains. In another experiment the culture volume was reduced to 40mL to induce a greater heat stress to the cells.

IPTG induction:

rhIFN α 2a gene was induced [87] by 100 μ L IPTG at the 4 hours growth (when the culture biomass level reaches approximately 0.5 OD_{600nm} after the heat shock was applied to the culture.

2.4 rhIFN α 2a protein production level was estimated by SDS-PAGE and IFN α **ELISA:****Sodium-Dodecyl-Polyacrylamide Gel Electrophoresis (SDS-PAGE):****Resolving gel**

40% Acryl amide stock:	5 mL
Water (distilled)	2.3 mL

1 M Tris-HCl pH 8.8	2.5 mL
10% SDS	0.1 mL
Ammonium per sulfate	0.1 mL
TEMED (added last)	0.004 mL

Stacking gel

40% Acryl amide stock	0.67 mL
Water	2.7 mL
1 M Tris-HCl pH 6.8	0.5 mL
10% SDS	0.04 mL
Ammonium per sulfate	0.04 mL
TEMED (stir quickly)	0.004 mL

Electrophoresis buffer

SDS	1 gm
Tris	3 gm
Glycine	14.4 gm
Distilled water	1000 mL

Samples

Sample was prepared by taking 10 μ L of diluted cell pellet and 90 μ L gel loading buffer; kept in boiling water bath for 10 min.

Coomassie blue stain.

0.25% Coomassie blue	0.75 gm
10% Methanol	30 mL
10% Glacial acetic acid	30 mL
Distilled water	240 mL

Destain I

50% Methanol	150 mL
10% Glacial acetic acid	30 mL
Distilled water	120 mL

Destain II

10% Methanol	30 mL
10% Glacial acetic acid	30 mL
2% Glycerol	6 mL
Distilled water	234 mL

The most common application for protein electrophoresis is the separation of complex protein mixtures by molecular weight. This is a basic characteristic tool, as well as high-resolution method for assessing protein purity. Proteins are denatured in solutions containing SDS. The detergent coats the proteins and imparts a uniform negative charge to them thus allows proteins to be compared by molecular weight.

Hand cast gels were used to assess the protein production [87]. The gels were used in Vertical Gel Electrophoresis systems Mini Model (Bangalore Genei Pvt. Ltd.) and run at 150 V for 2 hours. The glass plates were assembled according to the manufacturer's instructions. The volume of the gel mold was determined (this information is usually provided by the manufacturer). In a conical flask, the appropriate volume of solution containing the desired concentration of acryl amide for the resolving gel was prepared. The components were mixed in the order shown. Polymerization begins as soon as the TEMED has been added. Without delay, the mixture was swirled rapidly. The acryl amide solution was poured into the gap between the glass plates. A sufficient space was left for the stacking gel (the length of the teeth of the comb plus 1 cm). Using a micropipette, the acryl amide solution was carefully over layered with 0.1% SDS (for gels containing $\geq 10\%$ acryl amide). The gel was placed in a vertical position at room temperature. After polymerization was complete (30minutes), the overlay was poured off and the top of the gel was washed several times with distilled water to remove any unpolymerized acryl amide. All the fluid was drained from the top of the gel, and then any remaining water was removed with the edge of a paper towel. The stacking gel was prepared in a conical flask; the appropriate volume of solution containing the desired concentration of acryl amide was prepared using the values given above. The components were mixed in the order shown. Polymerization begins as soon as TEMED has been added. Without delay, the mixture was swirled rapidly. A clean Teflon comb was inserted before the stacking gel solution was poured onto the surface of the polymerized resolving gel. Care was taken that no air bubbles form during pouring of stacking gel. The gel was placed in a vertical position at room temperature. After polymerization was complete (30

minutes), the Teflon comb was removed carefully. The wells were washed immediately with distilled water to remove any unpolymerized acryl amide. The gel was mounted in the electrophoresis apparatus. Tris-glycine electrophoresis buffer was added to the top and bottom reservoirs. The air bubbles that become trapped at the bottom of the gel were removed between the glass plates. 25 μ L β -Mercaptoethanol was added to 475 μ L of sample buffer prior to use. The sample was resuspended in 40 μ L of distilled water; 10 μ L was used for SDS gel electrophoresis. The sample was diluted to 90 μ L with sample buffer and heated at 95 ° C for 10 minutes to lyse the cells and dissolve the sample proteins, before loading 40 μ L on to the gels. Protein standard markers were loaded alongside the protein samples to allow determination of the apparent molecular weight of the proteins of interest. Gels were run according to the manufactures guidelines, until the dye front reached the bottom. Gels were washed three times for 5 minutes in 200 mL – 300 mL distilled water. All the water from the staining container was removed and 300 mL Bio-Safe Coomassie Stain (Biorad) was added to the gels. The gel were kept shaking for overnight on a regular Gel Rocker. It was then rinsed with 200 mL – 300 mL of distilled water. A digital image of the gels were taken using the UV-Visible Transilluminator for Gel Documentation and analyzed by the Universal Software (Zeineh Programs) from Advanced American Biotechnology (AAB).

Analysis of the Gels:

For the analysis of the gel Universal Software (Zeineh Programs) from Advanced American Biotechnology (AAB) was used to acquire images from UV-Visible Transilluminator for Gel Documentation. The images were first optimized and displayed

and were adjusted for faint or saturated images to aid visualization and printing. Once the image had been captured the gel lanes were found and adjusted manually using the ‘tools’ in the software. The band sensitivity was adjusted to omit noise. From the analysis menu in the software, Image Analysis option was selected to quantitate the bands. The Image Analysis functions to quantify the rectangle drawn around the $\text{rhuIFN}\alpha 2\text{a}$ band and help to read the intensity of the band (Table 9).

IFN α ELISA:

Materials:

- 96 well IFN α monoclonal antibody coated microtiter plate
- Biotinylated monoclonal antibody lyophilized
- Diluent
- Streptavidin HRP-Conjugate
- IFN α standard lyophilized
- Wash Buffer Concentrate 20x (Wash Buffer Concentrate 20 x was added in to 950 mL distilled water. Stored at 2° to 25°C.)
- Substrate Solution
- Stop Solution (2N Sulphuric Acid)

Interferon α enzyme immunoassay has been used (using the IFN α ELISA kit of Immunotech France; Cat No. IM3193 as per their insert) to assess the protein production (Fig. 10 & 12). This ELISA was a two immunological step sandwich type assay. In the first step the IFN α was captured by a monoclonal antibody bound to the wells of a

microtiter plate. In the second step a biotinylated monoclonal antibody was added together with streptavidin – peroxidase conjugate. The biotinylated antibody binds to the solid phase antibody-antigen complex and in turn, binds the conjugate. After incubation, the wells were washed and the antigen complex bound to the well detected by addition of chromogenic substrate. The intensity of coloration was preoporational to the IFN α concentration in the sample or the standard. The sample results were calculated by interpolation from a standard curve that was performed in the same assay as that of the sample. The curve was drawn, plotting on the horizontal axis the IFN α concentration of the standards and on the vertical axis the corresponding absorbance.

The components of the kit were allowed to equilibrate for 30 minutes at room temperature before use. The lyophilized Biotinylated monoclonal antibody and the IFN α standard (3000 IU/mL) were reconstituted with the volume of D/W stated on the vial label as per the instructions in the kit insert. From the 3000 IU/mL standard solution a fresh dilution series (0 IU/mL, 17 IU/mL, 33 IU/mL, 100 IU/mL and 300 IU/mL) were prepared using the provided diluent as per the kit instructions. 50 μ L of standard or sample and 50 μ L of diluent was added to all the wells. The plate was incubated for 2 hours at room temperature while shaking. The wells were then washed three times as per the kit instructions. After the washing step 50 μ L of biotinylated antibody and 100 μ L of Streptavidin HRP-Conjugate was added to each wells. The plate was then again incubated for 30 minutes while shaking at room temperature. The wells were washed three times as per the kit insert instruction. 100 μ L of Substrate was then added to all the wells. The plate was then incubated for 30 minutes while shaking at room temperature.

50 μ L of Stop Solution was added to all the wells. Absorbance was read at 450_{nm}. The absorbance for each sample was located on the vertical axis and the corresponding IFN α concentration was read off on the horizontal axis.

2.5 Electroelution of the rhIFN α 2a protein from SDS-PAGE gels:

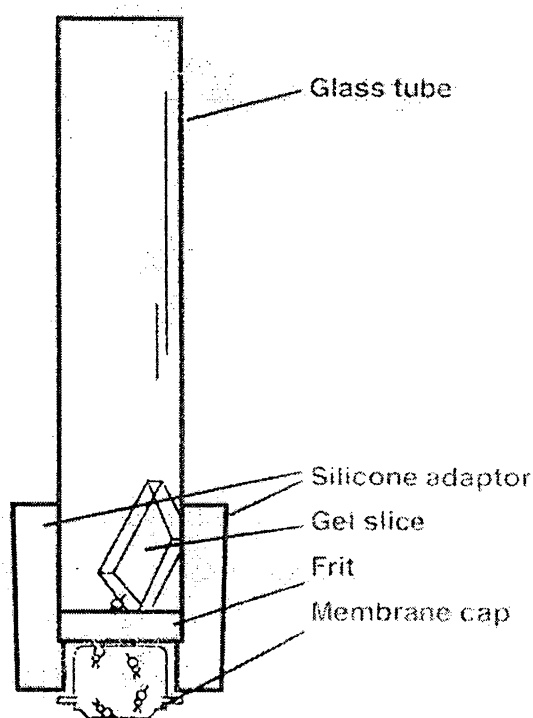
Materials:

Running buffer:

SDS	1 gm
Trizma	3 gm
Glycine	14.4 gm
Distilled water	1000 mL

The rhIFN α 2a bands obtained from the SDS-PAGE gels were Electroeluted using the Bio- Rad's Electro-Eluter (Model No.422) as per the company's protocol. Appropriate numbers of Glass Tubes were taken and one Frit was placed in the bottom of each tube. The Frits were pushed from inside the Glass Tube. Next, the Glass Tube and the Frit was pushed into the electro-eluter module. One pre-wetted membrane cap was placed in the bottom of each Silicone Adaptor. The adaptor was filled with elution buffer (Same as the Running Buffer used in the SDS-PAGE electrophoresis) [87]. The buffer was pipetted up and down to remove any air bubbles around the dialysis membrane of the membrane cap. Each tube was filled with elution buffer and the gel slices were placed in the tube. To increase sample recovery, several bands (Bands of the T5, T8, T9 and the Over night growth hour samples of the *E.coli* strain were excised and put into Electroeluter tubes)

Figure 6. Side of the Model 422 Electro-Eluter assembly



The vertical glass tube is filled with elution buffer. The negative electrode is at the top. The positive electrode is below the membrane cap. Proteins are carried by the electrical current out of the gel-slice through the frit and into the membrane cap. The molecules are retained by a dialysis membrane, which is molded into the cap.

were excised from the gel and minced and placed into the tubes. The entire module was placed into the buffer chamber. The lower buffer chamber was filled with ~600 mL of elution buffer. The upper buffer chamber was filled with ~100 mL of elution buffer. Elution is done at 8-10 mA/glass tube for 2 hours. After the elution was completed, the electro-eluter module was removed from the buffer tank. The stopper from the upper buffer chamber was removed and the buffer was allowed to drain out. The buffer left in the tube was pipetted out to the level of the Frit and the liquid was discarded. The Silicone Adaptor together with the membrane cap was removed from the bottom of the glass tube. Using a pipette, the remaining liquid in the membrane cap was transferred into

a tube. The volume was approximately 400 μ L. With another 200 μ L of fresh elution buffer, the membrane cap was rinsed. The rinsed solution was also added to the tube. This solution contains the eluted protein. The steps were repeated for each Glass Tube. The eluted (blue) protein was collected and stored at -20° C.

2.6 Refolding of the Electroeluted rhuIFN α 2a molecule by Dialysis:

Materials:

Renaturation/Refolding buffer without Urea:

20mM Tris Cl [pH 7.5-8]	2.42gm/1000mL
10-5% Glycerol	100 ml/1000mL
0.1 M NaCl	5.844gm/1000mL
5mM β -mercaptoethanol	0.4 mL/1000 mL

The Electroeluted IFN molecules were dialyzed against the refolding buffer [87]. The activity of the eluted protein was recovered by unfolding the protein with denaturants urea and then refolded by removing the denaturants by dialysis or dilution allowing the formation of native structure of protein. The dialysis bag (Dialysis Membrane – 50, Hi-Media Cat No.: LA 387) was dipped in distilled water for sometime and tied from one end. Approximately 500 μ L of eluted protein was suspended into the bag. The bag was tied from the other end. The bag was then dipped in about 25 mL of renaturation buffer containing urea (6 M) and kept overnight at 4-5 ° C. The renaturation buffer was replaced by a fresh 25mL of renaturation buffer without β -Mercaptoethanol containing (3 M) urea and kept for 8 hours at 4-5 ° C. After 8 hours, the renaturation buffer was further replaced

with a fresh renaturation buffer without β -Mercaptoethanol containing (1.5 M) urea and kept overnight at 4-5 ° C. The renaturation buffer was then again replaced with a fresh 25 mL of renaturation buffer without urea and β -Mercaptoethanol kept for 8 h at 4-5 ° C. After 8 hours, the renaturation buffer was discarded and the renatured rhuIFN α 2a protein molecule from the dialysis bag was collected in an eppendorf tube and stored at -20 ° C. The whole process was performed with intermittent stirring of the renaturation buffer in the beaker containing the dialysis bag.

2.7 Total protein estimation of the electroeluted rhuIFN α 2a and of the *E.coli* specific growth hour cell pellet by Miller's method:

Materials:

Standard: BSA (0-100 μ g/mL conc.)

Folin's solution (1 mL of 1% CuSO₄ + 1 mL of 2% sodium potassium tartarate +18 mL of 10% Na₂CO₃ in 0.5 N NaOH)

Folin's Ciocalteu reagent (1 mL Folin's Ciocalteu reagent + 10 mL D/W)

The principle behind the Miller's method of determining protein concentrations lies in the reactivity of the peptide nitrogen[s] with the copper [II] ions under alkaline conditions and the subsequent reduction of the Folin-Ciocalteu phosphomolybdic-phosphotungstic acid to hetero-polymolybdenum blue by the copper-catalyzed oxidation of aromatic acids. The color development may vary greatly with different proteins since it is almost entirely due to their content of tyrosine and tryptophan. However the reduction of Cu [II]

to Cu [I] by formation of Complex with the peptide bonds increases sensitivity and reduces unequal chromogenicities of different proteins. Insoluble proteins are dissolved in NaOH and then treated with Na_2CO_3 CuSO_4 tartarate reagent.

Heat Shock and Non Heat shock Electroeluted rhuIFN α 2a protein molecule (1: 100) and the lysed growth hour cell pellets (1:50) were treated as per the below mentioned protocol. The OD $_{660\text{nm}}$ was measured with an UV/Vis Spectrophotometer, and their concentrations were estimated by comparing the OD values to a Standard Graph plotted by using BSA as a standard. The Method is as follows:

1 mL of Folin's solution was added to the Standard and Samples, mixed well and kept at room temperature for 10 minutes. 1mL of Folin's Ciocalteu reagent was added to each test tube. The tubes were kept in water bath at 50 °C for 10 min. Optical density of the test tube were taken at 660 nm [90,218].

2.8 Endotoxin test of the Electroeluted rhuIFN α 2a by the LAL Method:

Reagents

- Limulus Amebocyte Lysate (LAL), Lyophilized
- LAL Reagent Water
- Endotoxin Standard.

The Limulus Amebocyte Lysate (LAL) test is a qualitative test for Gram-negative bacterial endotoxin [280,281]. Limulus Amebocyte Lysate as supplied (Pyrogen – Cambrex kit) was reconstituted with LAL Reagent Water and then mixed with in equal

parts with the solution being tested. After incubation, and in the presence of endotoxin, gelation occurs; in the absence of endotoxin, gelation does not occur.

Endotoxin

Proenzyme -----> Coagulase

Coagulase

Coagulogen -----> Coagulin

Gram-negative bacterial endotoxins catalyze the activation of a proenzyme in the Limulus Amebocyte Lysate [280,281]. The initial rate of activation is determined by the concentration of endotoxins present. The activated enzyme (Coagulase) hydrolyzes specific bonds within a clotting protein (Coagulogen) also present in Limulus Amebocyte Lysate. Once hydrolyzed, the resultant Coagulin self-associates and forms a gelatinous clot [280,281].

Lyophilized lysate was reconstituted by adding 1.8 mL LAL Reagent Water to the test vial. It was swirled gently for 30 seconds.

Endotoxin control was reconstituted per label directions with LAL Reagent Water.

The vial of endotoxin was vortexed for at least 15 minutes. The endotoxin was diluted with LAL Reagent Water to a concentration of 1 EU/mL. Each dilution was vortexed for 60 seconds prior to proceeding to the next dilution.

Using the 1 EU/mL endotoxin solution, a serial two-fold dilution series was prepared according to the following Chart:

Dilution Series for Use With Lysate of 0.125 EU/mL Endpoint

Tube no.	Water (mL)	Volume Added to Water	Endotoxin Concentration
1	1.0	1.0 mL from 1 EU/ mL	0.5 EU/ mL
2	1.0	1.0 mL from Tube 1	0.25 EU/ mL
3	1.0	1.0 mL from Tube 2	0.12 EU/ mL
4	1.0	1.0 mL from Tube 3	0.06 EU/ mL
5	1.0	1.0 mL from Tube 4	0.03 EU/ mL

0.10 mL of standard was transferred into the appropriate 10 X 75mm reaction tube. Then, 0.10 mL of the reconstituted lysate was added to each tube beginning with the highest concentration of endotoxins. Immediately following the addition of the lysate to each tube, the contents were mixed gently and the tube was placed in a 37° C water bath. After one hour ($\pm$ 2 minutes) of incubation, each tube was examined for gelation. A firm gel that remains intact momentarily when the tube was inverted 180° indicated a positive reaction.

For the sample two sets of experiments were set. In the first set 50 μ L of neat Heat Shock and the Non-Heat Shock $\text{rhIFN}\alpha 2\text{a}$ samples were transferred in different reaction tubes and 50 μ L of LAL Reagent was added into each tubes. The tubes were vortexed for 2-5 minutes and incubated in a 37° C water bath. After one hour ($\pm$ 2 minutes) of incubation, each tube was examined for gelation. The amount or concentration of the gelation were compared qualitatively with the Standard vials with the known amount of Endotoxin in it.

In the second set of experiment, 1: 2 dilution of the Heat Shock and the Non-Heat Shock $\text{rhIFN}\alpha 2\text{a}$ samples were made for checking the Endotoxin content in it. To achieve this 50 μ L of neat Heat Shock and the Non-Heat Shock $\text{rhIFN}\alpha 2\text{a}$ samples were transferred in different reaction tubes and 50 μ L of LAL water was added into each tube. After that 50 μ L of the content from each tube were transferred into pre-marked Heat Shock and the Non-Heat Shock $\text{rhIFN}\alpha 2\text{a}$ glass tubes and then 50 μ L of LAL reagent was added into the each tube. The tubes were vortexed for 2-5 minutes and incubated in a 37° C water bath. After one hour ($\pm$ 2 minutes) of incubation, each tube was examined for gelation. The amount or concentration of the gelation were compared qualitatively with the Standard vials with the known amount of Endotoxin in it.

2.9 IFN α ELISA of the elctroeluted protein sample:

Materials:

- HRP-Conjugate.
- IFN α standard
- Wash Buffer Concentrate 20x (phosphate-buffered saline with 1% Tween 20)
(Wash Buffer Concentrate 20 x (25 ml) was added in to 475 mL distilled water.
Stored at 2° to 25°C.)
- Substrate Solution I (tetramethyl-benzidine)
- Substrate Solution II (0.02% buffered hydrogen peroxide)
- Assay Buffer Concentrate 20x. (Assay Buffer Concentrate 20 x (5 mL) was added to 95 mL distilled water.)
- Stop Solution (1M Phosphoric acid)

To quantify the amount of rhIFN α 2a Electroeluted from the SDS-PAGE gels, IFN α ELISA of the elctroeluted protein sample was performed with the Bender Med Systems ELISA kit (Cat No.BMS216). In this ELISA an anti-IFN α monoclonal-coated antibody was adsorbed onto microwells. IFN α present in the samples or standard binds to antibodies adsorbed to the microwells; an HRP-conjugated monoclonal anti- IFN α antibody was added which binds to the captured IFN α by the first antibody. Following incubation, unbound enzyme conjugated anti- IFN α was removed during a wash step and substrate solution reactive with HRP was added to the wells. A colored product was formed in proportion to the amount of IFN α present in the sample. The reaction was terminated by addition of acid and absorbance was measured at 450_{nm}.

The reagents (Wash Buffer, Assay Buffer, HRP-Conjugate, and TMB Substrate Solution) provided with the kit were prepared according to the kit insert instructions. All the reagents were thoroughly mixed without foaming before use. The microwell strips provided with the kit were washed twice with 300 μ L Wash Buffer per well with thorough aspiration of microwell contents before washes. After the last wash, the microwell strips were tapped on filter paper to remove excess Wash Buffer. 100 μ L of Assay Buffer was added in the Standard wells, leaving the first wells (500pg/mL) empty. Standard dilutions were prepared by pipetting 200 μ L of IFN- α Standard into the first wells and Standard dilutions created ranging from 500 to 8 pg/mL (500pg/mL, 250pg/mL, 125pg/mL, 63pg/mL, 32pg/mL, 16pg/mL, 8pg/mL) by transferring 100 μ L from well to well. Finally 100 μ L was discarded from the last well. 100 μ L Assay Buffer was added to the blank wells and 80 μ L of Assay Buffer into the sample wells. After that 20 μ L of Electroeluted rhIFN α 2a protein samples were added into the wells. HRP-conjugate was prepared and 50 μ L of diluted HRP-conjugate was added to all the wells. The microwell strips were covered and incubated for 2 hours at room temperature (18-25°C). The microwell strips were then washed three times with Wash Buffer. After that, fresh TMB-Substrate Solution was prepared and 100 μ L was added to all the wells including the Blank wells. The strips were again incubated for about 15 minutes at room temperature and after that finally 100 μ L Stop Solutions were added to all the wells including the Blank wells. Color intensity was measured at 450_{nm}.

2.10 IFN Bioassay on WISH Human Amnion Cell lines by the Cytopathic Effect

(CPE) reduction technique:

Materials:

- WISH Human Amnion Cell lines from National Institute of Virology (NIV); Pune.
- Encephalomyocarditis Virus (EMC) from NIV, Pune
- Standard (Reference) IFN (Natural Human Leukocyte IFN) from NIV
- MEM (E) [Sigma M0769] with
 - 2% Foetal Calf serum
 - P&S (Penicillin and Streptomycin)

Penicillium G Sodium 500,000 units was dissolved in 2.5 mL D/W and Streptomycin 1 gm was dissolved in 5mL D/W. Equal volume of Penicillin and Streptomycin were mixed and stored in 1mL aliquots. 1mL of the antibiotic solution was added to 500mL of the medium. The final concentration of Penicillin was 200U/mL and Streptomycin 200 µg/ mL.
 - Nystatin

500,000 units / vial was dissolved in absolute alcohol and added to the culture medium to give a final concentration of 50U/mL.
 - CMC (Carboxymethyl cellulose) at a final concentration of 0.5%
- Amido Black 100mL
 - Amido Black 1 gm
 - Glacial Acetic Acid 60mL

- Sodium Acetate 13.6gm

- D/W 40mL

- Trypan Blue

The assays [124,125] were performed in 96 wells flat-bottomed (micro titer) plates using WISH Human Amnion Cell lines $2-3 \times 10^4$ cells were added to each micro titer plate in 0.1 mL of MEM (E) with 2% serum with a multichannel pipette. For testing the antiviral activity, the rhuIFN α 2a samples were added after the cells had attached on the next day. The samples were diluted with MEM and 2% serum in another sterile micro titer plate in 0.12 mL volumes using multichannel pipette and 0.1 mL was transferred to the plate with cell monolayers. The cell monolayer plate was then incubated at 37° C in a 5% CO₂ atmosphere for 18 hrs. Encephalomyocarditis Virus (EMC) was diluted to 10^{-3} in MEM (E) with 2% serum to yield a moi of 1-5 pfu/mL. 0.1 ml of the virus suspension was added and kept for incubation for 15-16 hours at 37° C in a 5% CO₂ atmosphere. Cytopathic effect (CPE) readings were taken after the incubation when the controls showed 4+ CPE. The plates were then washed under tap water and stained with Amido Black for 1hour at room temperature to confirm the subjective CPE readings. Dilution of sample showing protection of 50% of cell sheet was taken as endpoint. Standard (Reference) IFN (Natural Human Leukocyte IFN) was included in the assay. The titer was converted to IU/mL by multiplying with the factor 1 obtained by the formula:

$$\text{Factor} = \frac{\text{Expected titer of Standard IFN}}{\text{Observed titer of Standard IFN}} \dots\dots\dots[1]$$

2.11 HIV-1 RT Inhibition Assay of the eluted rhuIFN α 2a protein by Thymidine

Uptake Inhibition determination:

Materials:

- 5% Na₂HPO₄
- RT assay mixture: 1000 μ L
 - Thymidine 5 μ L
 - Glutathione 20 μ L
 - BSA 20 μ L
 - 50 mM Tris-HCL pH-7.8, 100 μ L
 - 75 mM KCL, 150 μ L
 - 2 mM DTT (Dithiothreitol) 100 μ L
 - 5 mM MgCl₂ 300 μ L
 - 5 μ g / ml poly (A), 1.6 μ g / mL oligo (dT), 40 μ L
 - 0.05 % NP-40, 30 μ L
 - 1 mM EDTA, 60 μ L
 - EGTA(Ethylene Glycol Tetra Acetic) 25 μ L
 - D/W 150 μ L

The assay was performed with the Electroeluted rhuIFN α 2a molecules using the Scintillation Counter (Liquid Scintillation Analyzer; Model No. Tri-Carb 2100TR; Packard Bioscience, USA.) [66]. Reverse Transcriptase Percentage Inhibition in the presence of the rhuIFN α 2a molecule was also calculated [66,118,119].

In Reverse Transcriptase (RT) assay, the template/primer hybrid poly (A) × oligo (dT) are incorporated into the DNA strand, which is newly synthesized by the RT. The RT inhibition is inversely proportional to the amount of DNA synthesized, that is, more the polymerization / DNA synthesis the less is the percentage inhibition of Reverse Transcriptase enzyme by the antiviral molecule. The oligo (dT) primes the incorporation of [3H] dTTP by RT using a poly (A) template, and the labeled reaction products are bound to a DEAE membrane. The amount of RT activity is proportional to the intensity of the spots after exposure to X-ray film. Excising the DEAE paper spots and counting them in a Liquid Scintillation Counter obtains quantitative results.

100 µL of complete RT mixture, 20 µL of the neat HIV suspension (105 cpm) and 5 µL of the Electroeluted rhuIFNα2a molecule (Heat Shock and Non Heat Shock rhuIFNα2a samples) were added into an eppendorf tube. 5 µL of Phosphate Buffer Saline (PBS) together with 100 µL of complete RT mixture and 20 µL of the neat HIV suspension (105 cpm) were taken in another eppendorf tube and were kept as a control. Eppendorf tubes were incubated at 37° C for 3 hours. The reaction was stopped by adding 25 µL of EDTA into each eppendorf tubes. 100 µL of the reaction mixture was spotted on to DEAE membranes for the Scintillation Counting. DEAE membranes were allowed to dry in a laminar flow hood (approx. 30-min); the membrane was then removed from the Containment Laboratory. The membranes were washed four times in 5% Na₂HPO₄ and then two times in D/W. The DEAE membrane was transferred into a Scintillation counting container containing the Cocktail mixture. The containers were then kept in the

Scintillation Counting instrument for quantifying the Thymidine uptake. The counts were utilized to assess the inhibitory effects of the rhIFN α 2a molecule against HIV- RT.

$$\text{RT \% Inhibition} = 100 - \frac{\text{SAMPLE cpm} \times 100}{\text{PBS cpm}} \dots\dots\dots[2]$$

[Note: PBS = Phosphate Buffer saline (Control), cpm = Counts per minute]

2.12 Anti-HIV and Cell Cytotoxicity Assay of rhIFN α 2a on MT-2 cell line:

Materials:

- **T lymphoid cell line -MT-2 Cell line** (obtained from NIH AIDS Research and Reference Programme, USA by National AIDS Research Institute (NARI), Pune, India.) The cell line was maintained in growth medium (RPMI containing 10% heat inactivated FCS) and 100 IU/mL Penicillin, 100 μ g/mL Streptomycin, 80 μ L/mL Gentamycin. The cell line was maintained by incubating at 37 $^{\circ}$ C in 5% CO $_2$ and humidified atmosphere.
- **HIV I – III B Strain** was obtained from NIH AIDS Research and Reference Programme, USA by NARI, Pune, India. The Virus stock was prepared by passaging the viruses in PHA-P activated Peripheral Blood Mononuclear cells (PBMCs) of healthy, HIV negative individuals. The virus stock was filtered through 0.22 μ m syringe filter and maintained at -70 $^{\circ}$ C prior to use. The virus was propagated in MT-2 cells and the infectivity titre of the stock solution was determined (50% tissue culture infectivity dose).

- RPMI-1640 (Hi Media) w/ L-Glutamine, w/o Sodium bicarbonate - (AT028)
- RPMI growth medium
 - RPMI 10.3gm
 - L – Glutamine 0.59gm
 - Sodium Bicarbonate 2gm
 - Penicillin 100 U/mL
 - Gentamycin 80µg/mL
 - Streptomycin 100µg/mL
 - D/W 1000mL
 - pH 7.0-7.2
- RPMI-10% growth medium
 - RPMI growth medium 490mL
 - Fetal Calf Serum 10mL
- RPMI-2% growth medium
 - RPMI growth medium 450mL
 - Fetal Calf Serum 50mL

(Moregate, Australia)

 - Trypan blue dye 0.4% (w/v) aqueous solution

Procedure for the preparation of RPMI Growth medium:

The content of one unit vial was suspended in 500mL of tissue culture grade water at room temperature with constant, gentle stirring until the medium was completely dissolved. The container was rinsed with tissue culture grade water to

remove all the traces of powder and was added to the above solution. 2 gm of sodium bicarbonate and 0.59 gm of L- Glutamine were added and stirred until dissolved. The pH of the medium was adjusted to 7.0 - 7.2 using 1N HCl. The final volume was made to 1000 mL with tissue culture grade water. The medium was sterilized immediately by filtering through a sterile membrane filter with porosity of 0.22-micron or less using positive pressure. Sterile supplements i.e. Penicillin, Gentamycin, Streptomycin and Fetal Calf Serum were added as required and the desired amount of sterile medium was dispensed into sterile containers and was stored at 2-6°C till use [219].

- Standard (Reference) IFN (Roferon) obtained from Roche Diagnostics, Singapore.

Cytotoxicity and potential anti-HIV Evaluation of $\text{rhuIFN}\alpha 2\text{a}$ using HIV-1 IIIB treated MT-2 cells :

The assay was performed in the 96 well 'U' bottom tissue culture plate [219]. The number of MT-2 cells per well was adjusted to 4×10^4 cells to 100 μL of growth medium. 100 μL of 1:5, 1:10, 1:20, 1:40, 1:80 and 1:160 dilutions of Heat Shock $\text{rhuIFN}\alpha 2\text{a}$ and the Non-Heat Shock $\text{rhuIFN}\alpha 2\text{a}$ in duplicate were added to wells seeded with MT-2 cells. 100 μL of the Standard Reference IFN (Roferon – A; 10^5 6MIU/ mL) was also maintained simultaneously as a positive control. 100 μL growth medium were added into each Cell Control and Virus Control wells. The above layout of the sample and the standard distributions in the micro titer plate were done according to the Figure 7a for the Ist set of experiment and Figure 7b for the IInd set of experiment.

The culture plate was then kept for 18 hrs incubation at 37° C in a 5% CO₂ atmosphere. After the 18 hrs incubation, 50 µL of 200 TCID₅₀ HIV-1 IIIB was added to each of the wells except the cell control and cytotoxicity wells. The virus was allowed to adsorb to the MT-2 cells for 2 hours. After virus adsorption, to remove the residual virus, the cells were washed thrice by spinning cells at 1500 rpm for 10 mins. For each wash the supernatant was aspirated out carefully from each well without disturbing the cell pellet. Precautions were taken to avoid cell loss after each washing steps. Approximately 200 µL of supernatant were harvested from the last wash for P-24 antigen detection (0 PID –0 Post infection day). The pellet was re suspended in 100µL of growth medium and to this respective dilution of 100µL rhuIFNα2a (Heat Shock and Non Heat Shock) was added into each well as per the protocol except the Virus Control and Cell Control wells where only 100µL of growth medium was added. Cytotoxicity wells were also maintained simultaneously for all the dilutions with out virus addition. The plate was then incubated for 5 days at 37° C in a 5% CO₂ atmosphere. On day 5, approximately 200 µL of the supernatants from the test wells were harvested (and pooled together) and tested for P-24 antigen detection. Viability of the cells in the cytotoxicity wells were determined by using Trypan blue dye exclusion method and compared with that of the cell control.

Figure 7 a. Layout of the sample and the standard distributions in the micro titer plate for the anti-HIV Assay. Ist Experiment

Conc.	1	2	3	4	5	6	7	8	9	10	11	12
A Std IFN	10 ⁵ of IFN	10 ⁵ of IFN	10 ⁵ of IFN	1:5 of 10 ⁵ IFN	1:5 of 10 ⁵ IFN	1:5 of 10 ⁵ IFN	CC	CC	VC	VC	VC	VC
B HS IFN	1:5	1:10	1:20	1:40	1:80	1:160	HS 1:5 Cyto	HS 1:10 Cyto	HS 1:20 Cyto	HS 1:40 Cyto	HS 1:80 Cyto	HS 1:160 Cyto
C HS IFN	→						NHS 1:5 Cyto	NHS 1:10 Cyto	NHS 1:20 Cyto	NHS 1:40 Cyto	NHS 1:80 Cyto	NHS 1:160 Cyto
D HS IFN	→						X	X	X	X	X	X
E NHS IFN	→						X	X	X	X	X	X
F NHS IFN	→						X	X	X	X	X	X
G NHS IFN	→						X	X	X	X	X	X
H	X	X	X	X	X	X	X	X	X	X	X	X

Key: HS – Heat Shock; NHS – Non-Heat Shock; IFN – Interferon; Std.-Standard; CC – Cell Control; VC – Virus Control; Cyto – Cytotoxicity; X – Wells not used

Figure 7 b. Layout of the sample and the standard distributions in the micro titer plate for the anti-HIV Assay. IInd Experiment

Conc.	1	2	3	4	5	6	7	8	9	10	11	12
A Std IFN	X	X	IFN 10 ⁵	IFN 10 ⁵	X	X	CC	CC	X	X	VC	VC
B HS IFN	X	X	X	X	X	X	1:5	1:10	1:20	1:40	1:80	1:160
C HS IFN	X	X	X	X	X	X						
D NHS IFN	X	X	X	X	X	X						
E NHS IFN	X	X	X	X	X	X						
F HS Cyto	1:5	1:10	1:20	1:40	1:80	1:160	X	X	X	X	X	X
G NHS Cyto							X	X	X	X	X	X
H	X	X	X	X	X	X	X	X	X	X	X	X

Key: HS – Heat Shock; NHS – Non-Heat Shock; IFN – Interferon; Std.-Standard; CC – Cell Control; VC – Virus Control; Cyto – Cytotoxicity; X – Wells not used

HIV-1 P-24 Antigen ELISA:

Materials:

- Antibody to HIV-1 P-24 Antigen (Murine Monoclonal) Coated Microwell Plates (96 wells)
- HIV-1 P-24 Antigen Reagent – contains inactivated, purified HIV-1 P-24 antigen from an HIV-1 LAV –infected human T-cell line
- Anti-HIV-1 (human) Biotin Reagent – lyophilized. (21mL of d/w was added to the Biotin Reagent vial and mixed thoroughly and stored at 2 -8°C)
- Negative control (human) – human serum found non reactive when tested
- Positive control – A 1: 5 dilution of the HIV – 1 P-24 Antigen Reagent in Negative Control.
- SA-HRPO - Streptavidin – horseradish peroxidase conjugate. (21 µL of SA-HRPO was added to 21mL of SA-Buffer just before use to make the SA-HRPO Working Solution.)
- SA-Buffer- Tris buffer for Streptavidin – horseradish peroxidase
- TMB Reagent (in DMSO) -Tetramethylbenzidine in Dimethylsulfoxide. (210 µL of TMB Reagent was added to 21mL of TMB Diluent just before usage.)
- TMB Diluent (in H₂O₂) – contains citrate phosphate buffer, 0.0045 %H₂O₂
- Lyse Buffer – contains Triton X- 100, dipotassium EDTA, Tween – 20
- 20X Wash Buffer Concentrate – phosphate buffer with sodium chloride and detergent. (50mL of Wash Buffer was mixed with 950mL of d/w and stored at 2 - 8°C)
- 4N Sulphuric Acid

HIV-1 P-24 Antigen ELISA Test System is a qualitative in vitro enzyme-linked immunosorbent assay (ELISA) for the detection of uncomplexed P-24 antigen of the HIV-1 in human plasma or serum. The kit provides an enzyme-linked immunoassay to compare the effects of experimental treatments on HIV-1 replication *in vitro* and compare them to untreated HIV-1 infected cells.

HIV-1 is recognized as the etiological agent of acquired immunodeficiency syndrome (AIDS). After exposure to the virus, HIV-1 infection is characterized by an early period of antigenemia in which HIV-1 antigens are detectable in blood. One of the viral antigens present in the blood during antigenemia is the core protein, P-24 the major internal structure protein of HIV-1.

HIV-1 P-24 Antigen ELISA Test System (Product Code 933085; Beckman Coulter) uses a murine monoclonal antibody to HIV-1 P-24 antigen coated onto microwells. A specimen of serum or plasma and lyse buffer are added to the coated microwell and incubated. Lyse buffer disrupts virus particles in the specimen and HIV-1 P-24 antigen, if present, binds to the monoclonal antibody on the microwell. Following a wash step, biotinylated human anti-HIV-1 IgG is added to the microwells and during incubation binds to any HIV-1 P-24 antigen bound to the microwells. Following another wash step, streptavidin-horseradish peroxidase is added which complexes with the biotinylated antibodies. After a final wash step, a substrate reagent containing tetramethylbenzidine (TMB) and hydrogen peroxide is added which reacts with complexed peroxidase to form

a blue color. The enzyme reaction is stopped by addition of acid. Color absorbance is measured using a spectrophotometer. The intensity of the color developed is directly proportional to the amount of uncomplexed HIV-1 P-24 antigen present in the plasma or serum. The presence of HIV-1 P-24 antigen is qualitatively indicated if the assay absorbance is greater than or equal to the assay cutoff value.

Approximately 30 minutes prior to the beginning of the procedure all kit components were brought to room temperature (15-30°C). In addition to specimens, one substrate blank, three negative controls and two positive controls were used on the plate. Controls, specimens and Lyse Buffer were added to the designated microwells. 200 µL of Negative Control or specimen was added to appropriate wells. In the next step, 250 µL of Positive Control was added to appropriate wells. 20 µL of Lyse Buffer was added to all wells except to the Substrate Blank well. The contents of the microwells were thoroughly mixed. The microwell plate was covered with a plate sealer. The plate was incubated at 37°C ±2°C for 60 minutes ±5 minutes. With a ELISA washer device, all the wells were aspirated and washed according to the following procedure:

The sample solution from the microwells were completely aspirated and then filled with Wash Buffer (1X). The aspiration/filling sequence was completed for five additional times. Wells were completely aspirated to remove excess Wash Buffer.

200 µL of reconstituted Biotin Reagent was then added to all wells except the Substrate Blank. The plate was then again incubated at 37°C ±2°C for 60 minutes ±5 minutes. After the second incubation, the wells were washed as described above. 200 µL of SA-HRPO Working Solution was added to all wells except substrate Blank. The plate

was then again incubated at $37^{\circ}\text{C} \pm 2^{\circ}\text{C}$ for 30 minutes ± 2 minutes. After the third incubation, the wells were washed as described above. 200 μL of TMB Substrate Solution was then added to all wells, including Substrate Blank. The plate was then incubated at room temperature in the dark, for 30 minutes ± 2 minutes. After the incubation, 50 μL of 4N Sulfuric Acid (H_2SO_4) was added to all the wells, including Substrate Blank. The plate was read at a wavelength of 450 nm, with a reference set to 570 nm, within 30 minutes following the addition of 4N Sulfuric Acid (H_2SO_4). Specimens with absorbance less than the Cutoff Value (calculated according to the kit insert) are considered nonreactive by the criteria of HIV-1 P-24 Antigen ELISA Test System and may be considered negative for HIV-1 P-24 antigen.

2.13 Amino acid composition determination of the Electroeluted rhulFN α 2a sample:

Materials:

Loading Buffer - 0.2 M Sodium citrate (pH 2.2)

Tri-sodium citrate	9.8 gm
Conc. HCl	8.25 mL
Thiodiglycol	10 mL
Phenol (100%)	0.48 mL

Buffer A (pH 3.2)

Sodium Citrate $2\text{H}_2\text{O}$	39.2gm
Distilled Alcohol	14.6mL
Perchloric Acid (70%)	34mL

Buffer B (pH 10)

Sodium Citrate 2H₂O 58.8gm

Boric Acid 12.4 gm

4 (M) Na OH 30mL

Reagent Stock (1 litre)

Na₂CO₃ 40.7g

H₃BO₃ 13.6g

K₂SO₄ 18.8g

Reagent A

Reagent Stock 500 mL

Na-hypochloride 200 µL

Reagent B

Reagent Stock 500 mL

O-phthalaldehyde 400µg

Distilled Alcohol 7.3 mL

Mercaptoethanol 1 mL

30% Brij 2 mL

For Peptide hydrolysis

6 N HCl with 0.5% phenol

Water (Milli Q) 25 mL

Conc. HCl 25 mL

Phenol 250 μ L

Amino acid analyzer:

Amino acid analysis is the determination of what types of amino acids and how many of each composes a protein. Only the amino acid composition is determined, not the order or sequence of amino acid residues. Amino acid analysis involves four basic steps:

1. Hydrolyze a protein to individual constituent amino acids
2. Label amino acids with a detectable UV-absorbing or fluorescent marker
3. Separate different types of amino acids by chromatography
4. Measure relative amounts of each amino acid type based on intensity of the detectable marker associated with the emergence of each type of amino acid from the chromatographic system

Sample Preparation and Hydrolysis Methods:

Isolating a pure protein is an important first step for determining amino acid content. Purification methods are based on one or more physical characteristics of proteins, such as solubility, molecular size, charge, polarity and specific interactions. The techniques

commonly used to separate proteins and peptides include gel filtration, ion exchange chromatography, affinity chromatography and polyacrylamide gel electrophoresis.

After purification, protein and peptide samples must be hydrolyzed to free amino acids. The most widely used agents for hydrolyzing proteins are acids, usually HCl. Hydrolysis with 10 N HCl is performed at 110° C for 24 hours, with the temperature being accurately controlled. Formation of cysteic acid, methionine sulfoxide, and chlorotyrosine can be avoided if all air is removed from the sample before hydrolysis. The HCl hydrolysis procedure also minimizes decomposition of reduced S-carboxymethylcysteine and preserves S-carboxymethylated amino acids. After hydrolysis, residual HCl is removed in a rotary evaporator and the residue dissolved in water or buffer, depending on whether the separation method will be ion exchange chromatography or high-pressure liquid chromatography (HPLC).

Amino Acid Derivatization:

Derivatization or chemical labeling of amino acids for detection can be performed either pre- or post-column separation. Ninhydrin is used routinely for pre-column derivatization, whereas o-phthalaldehyde (OPA) are post-column derivatization reagents, used often when greater analytical sensitivity is required. Derivatization can also be done with phenylisothiocyanate (PITC), also called Edman's Reagent.

Buffer System:

Separation of amino acid analysis products by ion exchange chromatography depends on the buffer pH and ionic strength. The counter ion typically is either sodium or lithium,

and buffer changes occur either in a step-wise or gradient manner. Sodium is used for acid-hydrolyzed samples, and lithium is used for isotonic samples. Citrate is the most commonly used buffer component for systems using a $\text{pH} < 7$. Unfortunately, citrate is not suitable for high sensitivity applications because of inherent amino acid contamination. For consistent analyses, high-purity reagents must be used for buffer preparation.

Principle of the Ion-Exchange Chromatography Method:

A cation exchange chromatography process using the Shimadzu LC10AS Amino Acid Analyser Japan, achieves amino acid analysis. The resin has a negative charge and the amino acids are added at a low pH, which ensures they are all positively charged. The conditions on the column are then altered to increase the pH, the temperature and the concentration of the buffer counter ions. These changes result in the isoionic point of an amino acid being reached, at which point the ionic attraction to the resin is lost and the amino acid elutes from the column. The conditions are adjusted so that the isoionic points for all the different amino acids are reached at different times. This affects the separation.

The resin used in modern amino acid analyzers consists of spherical beads of polystyrene. The polystyrene chains are cross-linked by the incorporation of divinylbenzene groups (DVB) charge. The cross-linked, charged resin structure is called the resin matrix. The passages inside the skeleton of the matrix are the pores, and the charged groups ($-\text{SO}_3^-$) are termed the fixed ions. Counter-ions are the interchangeable ions, which are attached to the matrix by heteropolar bonds (the positively charged groups in the buffers or on the amino acids). Ion exchange takes place by the counter-ions contained in the buffer

diffusing into the pores of the matrix and changing places with the counter-ions which are loosely attached there. Instrument in the chromatographic system, which senses the presence of a compound passing through, and provides an electronic signal to a recorder or computer data station. Output is usually peaks, that is, the chromatogram.

The sample containing a mixture of amino acids is stored in an autoloader then loaded onto a column of cation-exchange resin. Buffers of varying pH and ionic strength are then pumped through the column to separate the various amino acids. The column temperature is accurately controlled, this being varied as necessary to produce the required separation. The column eluate is mixed with the O-phthalaldehyde reagent, this mixture being passed through the high temperature reaction coil. In the reaction coil, the O-phthalaldehyde reacts with the amino acids present in the eluate to form coloured compounds. The amount of coloured compound produced is directly proportional to the quantity of amino acid present in the eluate. From the reaction coil the eluate/ O-phthalaldehyde mixture is fed to the photometer unit where the amount of each coloured compound is determined by measuring the amount of light absorbed at two wavelengths, 570 nm and 440 nm. The photometer output is connected to a two-channel chart recorder, one channel for the 570nm output, the other channel for the 440nm output. The amino acid concentrations are recorded as a series of peaks. The retention time of the peak on the chart identifies the amino acid; the area under the peak indicates the quantity of amino acid present. As the amino acid analyser is a comparative instrument, a calibration analysis is performed before commencing a series of analyses thus producing a standard trace for comparison purposes.

Hydrochloric acid hydrolysis:

Known amount of sample was placed into a thick walled glass hydrolysis tube. Aliquot of constant boiling (10N) hydrochloric acid was then added to the hydrolysis tube in the ratio of 1 mL of hydrochloric acid to 2 mg of nitrogen. Hydrolysis tubes were then attached to a system, which allowed the connection of nitrogen and vacuum lines without disturbing the sample. The sample was purged with oxygen free nitrogen. This step was repeated 2 or 3 times before sealing the tube. The sealed tube was heated in an oven to 110°C for 24 hours. The tube was then removed from the oven and the acid was extracted from the sample by neutralizing with (7.5N) Sodium hydroxide. The sample was diluted to the required final volume using a known amount of pH 2.2 loading buffer and filtered using a 0.22µm membrane.

In the present analysis, approximately 50µL of sample was added to 1mL of conc. HCl for the protein hydrolysis. The hydrolysate was added to 500 µL of the Loading Buffer and 100 µL of it was loaded into the autoloader of the analyser. Required amounts of Buffer A & B were then pumped automatically through the column in a pre-programmed interval of time to separate the various amino acids from the column. During the process the column temperature was maintained to 55°C. Column eluate was then automatically mixed with the O-phthalaldehyde Reagent and was allowed to pass through the high temperature reaction coil. The absorbance readings were then recorded. Instrument in the chromatographic system senses the presence of a compound passing through, and provides an electronic signal to a recorder or computer data station. Output is usually peaks, that is, the chromatogram.

3. FLOW CHART OF THE WORK DONE

Preparation of competent *E.coli* BL21 (DE3) cells and transformation of rhuIFN α 2a

clone into the competent *E.coli* BL21 (DE3) cells



Standardization of the Growth curves of the *E.coli* strain



IPTG induction of the rhuIFN α 2a gene



Estimation of rhuIFN α 2a protein production level by SDS-PAGE and IFN α ELISA



Electroelution of the rhuIFN α 2a protein of specific growth hours from the SDS-PAGE gels



Refolding of the Electroeluted rhuIFN α 2a molecule by Dialysis



Total protein estimation of the elctroeluted rhuIFN α 2a and of the *E.coli* growth hour cell pellet by Miller's method.



Test for Endotoxin in the Electroeluted rhuIFN α 2a sample



IFN α ELISA of the elctroeluted protein sample was performed



Cell Cytotoxicity Assay of rhuIFN α 2a on MT-2 cell line



IFN Bioassay of the electroeluted rhuIFN α 2a proteins together with sonicated cell pellet samples of *E.coli* cells on WISH Human Amnion Cell lines by the Cytopathic

Effect (CPE) reduction technique



Anti-HIV Assays by

- 1. HIV-1 RT Inhibition Assay of the eluted rhuIFN α 2a protein by Thymidine Uptake Inhibition determination**
- 2. HIV-1 P-24 Inhibition Assay with the Electroeluted rhuIFN α 2a sample**



Primary Structure Analysis by Amino acid composition determination of the Electroeluted rhuIFN α 2a sample

RESULTS

4. RESULTS

4.1 Effect of Heat Shock on the recombinant *E.coli* growth and on the rhuIFN α 2a production:

The effect of a mild heat shock upon the growth and recombinant protein production was determined for the *E.coli* strain, containing a plasmid for the production of rhuIFN α 2a. To establish the positive effects, if any, of a mild stress response on recombinant protein production the cells were exposed to a minimum heat stress of 50°C for 5-10 minutes in a water bath. The growth of the *E.coli* strain (Experiment A) is shown in Table 3 and plotted in Figure 8.

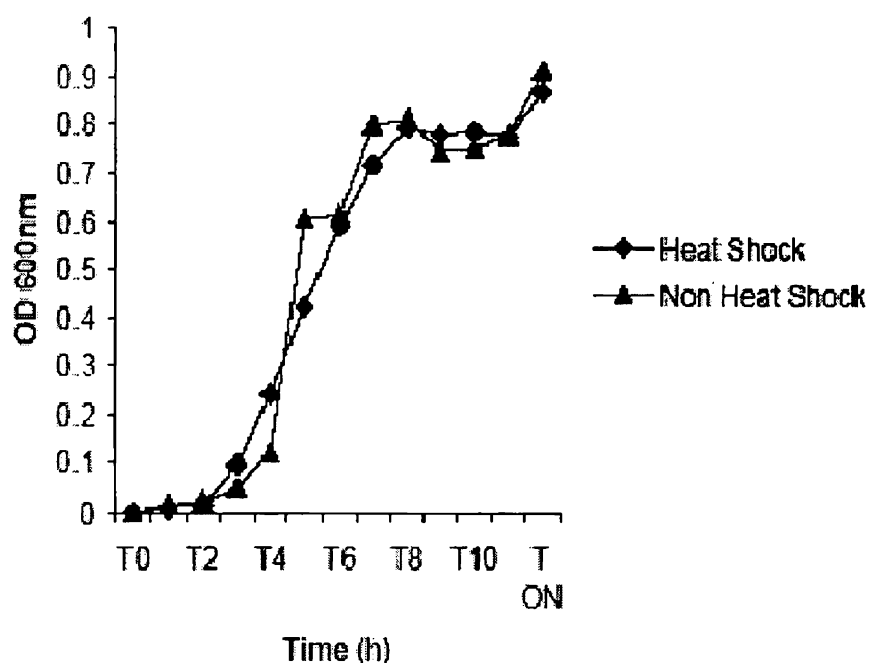
Table 3. O.D_{600nm} values of *E.coli* strain BL21 (DE3) producing rhuIFN α 2a

O.D _{600nm}								
	Experiment I		Experiment II		Experiment III		Mean O.D _{600nm}	
Time in Hours	HS	NHS	HS	NHS	HS	NHS	HS	NHS
T0	0	0	0	0	0	0	0	0
T1	0.007	0.002	0.012	0.007	0.001	0.03	0.006	0.013
T2	0.016	0.005	0.011	0.046	0.010	0.010	0.012	0.020
T3	0.192	0.015	0.054	0.075	0.049	0.053	0.098	0.048
=>T4	0.377	0.067	0.153	0.274	0.201	0.018	0.244	0.120
* T5	0.406	0.651	0.360	0.638	0.507	0.524	0.424	0.604
T6	0.733	0.561	0.350	0.473	0.687	0.815	0.59	0.616
T7	1.124	0.853	0.320	0.309	0.711	0.877	0.718	0.8
T8	1.190	1.005	0.230	0.371	0.968	1.059	0.796	0.812
T9	1.226	1.142	0.335	0.350	-	-	0.781	0.746
T10	1.281	1.153	0.295	0.353	-	-	0.788	0.753
T11	1.251	1.216	0.300	0.348	-	-	0.776	0.782
(ON)	1.188	1.282	0.291	0.286	1.125	1.170	0.868	0.913

Growth with and without Heat Shock in three duplicate experiments I, II & III. (Culture Volume is 50mL at the time of Heat Shock)

Key: => Heat Shock applied, * IPTG induction, HS = Heat Shock, NHS = Non Heat Shock, ON = Over night

Figure 8. Growth of *E.coli* strain with and without Heat Shock



Culture Volume is 50mL at the time of Heat Shock). The mean of the O.D_{600nm} Values of the Experiments I, II & III were taken from the Table 6 to plot the growth curve.

Key: ON = Over night

The *E.coli* strain was grown in shake flasks in three separate experiments under duplicate conditions. The flasks were inoculated with a predefined amount of biomass (equivalent of 100μL of culture at OD_{600nm} 1.0). Growth was followed by sampling at 1 hourly intervals and the optical density (OD_{600nm}) determined. The duplicate flasks for the strain were grown to T4 hours (4 hours) (OD_{600nm} of approximately 0.1 – 0.3) and then one of the flask was subjected to Heat Shock by heating in a 50°C water bath for 5-10 minutes. The Heat Shocked culture was returned to the incubator and grown with its Non

Heat Shocked matched flask. Unfortunately, the *E.coli* growth hour samples of T9 (9 hours growth) – T11 (11 hours growth) from the third set of the experiments were lost

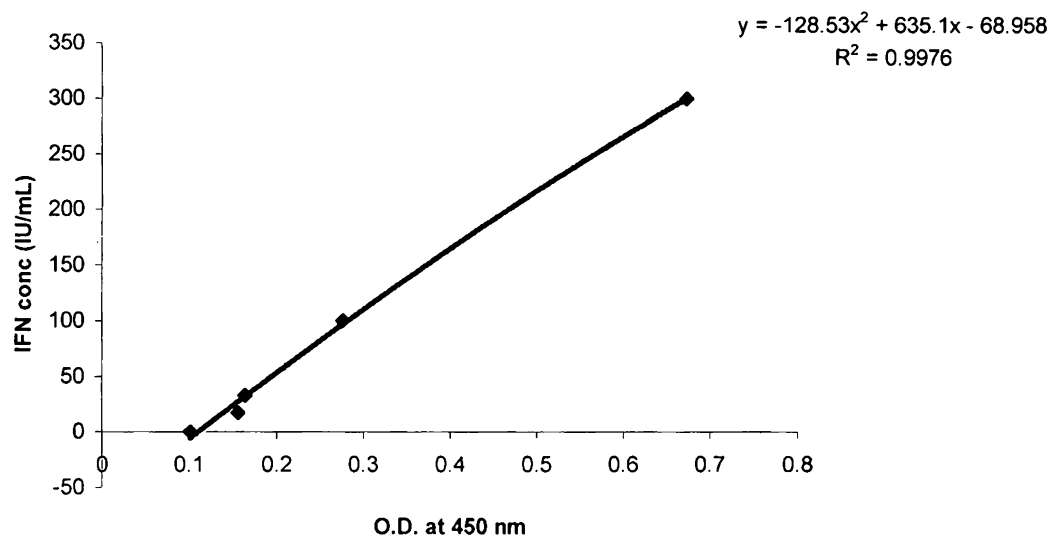
Table 4. O.D._{450 nm} values for the IFN ELISA of the *E.coli* growth hour samples from the Experiments I, II, & III

Standard		Time in Hours	O.D. _{450nm}							
			Experiment I		Experiment II		Experiment III		Mean O.D. _{450nm}	
			HS	NHS	HS	NHS	HS	NHS	HS	NHS
Conc. IU/mL	O.D. _{450nm}									
0	0.102	T5	0.386	0.298	0.219	0.338	0.294	0.239	0.3	0.29
17	0.156	T6	0.301	0.260	0.198	0.253	0.200	0.191	0.23	0.23
33	0.164	T7	0.296	0.327	0.215	0.265	0.217	0.220	0.24	0.27
100	0.276	T8	0.386	0.376	0.224	0.229	0.216	0.149	0.27	0.25
300	0.673	T9	0.368	0.257	0.212	0.223	-	-	0.28	0.22
		T10	0.310	0.321	0.202	0.201	-	-	0.25	0.26
		T11	0.389	0.306	0.226	0.323	-	-	0.3	0.31
		(ON)	0.349	0.281	0.217	0.202	0.168	0.146	0.24	0.21

The values in the table are the O.D._{450 nm} IFN ELISA End Point results, together with the Standard IFN concentrations with their O.D._{450nm} IFN ELISA End Point Results.

Key: ON = Over night, HS = Heat Shock, NHS = Non Heat Shock

due to experimental error. There is no difference in the growth curve during growth preceding the Heat Shock. Immediately after the Heat Shock there is no evidence that growth has slowed in the Heat Shocked flask, at sample T5 (5 hours growth) following Heat Shock at T4 (4 hours). Growth pattern is similar in all the three experiments and differences are probably experimental error.

Figure 9. Standard Curve for IFN Alpha ELISA

The effect of the heat stress on the quantity of the rhuIFN α 2a production is estimated by the IFN ELISA. As the rhuIFN α 2a gene was induced at the T5 hours, duplicate *E.coli* growth hour samples from both the Heat Shocked and the Non Heat Shocked flasks were collected from all the three experiments from T5 hours (5 hours growth) to over night growth. rhuIFN α 2a concentration in these growth hour samples were estimated by the IFN ELISA. The IFN ELISA O.D. _{450nm} End Point results of the *E.coli* growth hour samples were shown in the Table 4 and the amount of rhuIFN α 2a present in each of these growth hour samples were calculated from the formula derived by plotting the IFN ELISA standard curve (Figure 9). rhuIFN α 2a concentration (IU/mL) in each of the *E.coli* growth hour samples from T5 hours (5 hours growth) to Over night growth were shown in the Table 5 and in the bar diagram Figure 10.

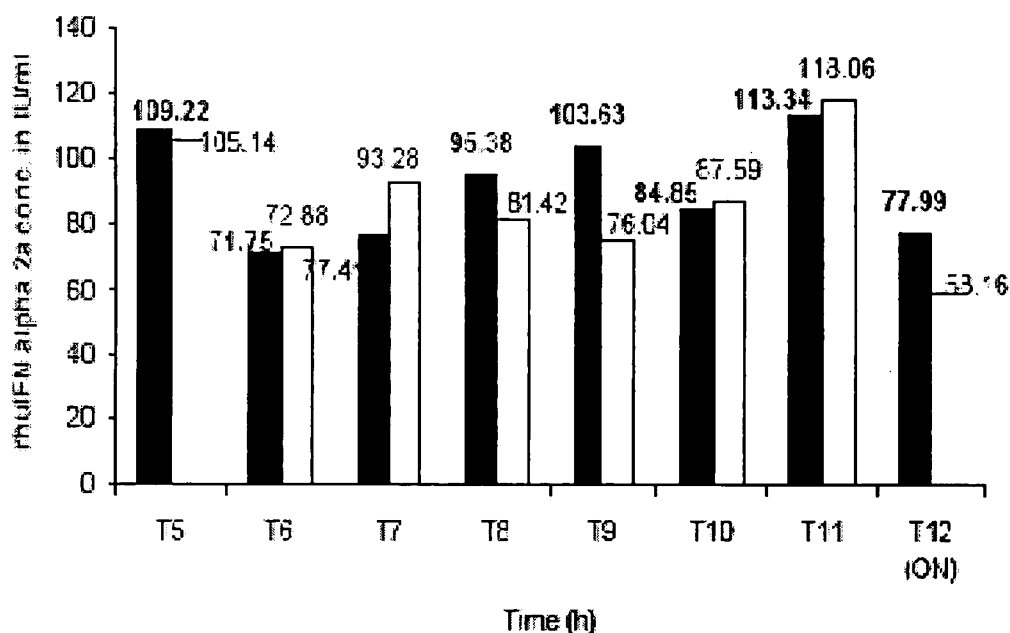
Table 5. rhuIFN α 2a (IU/mL) concentration in the *E.coli* growth hour samples from the Experiments I, II, & III

rhuIFNα2a (IU/mL)								
Time in Hours	Experiment I		Experiment II		Experiment III		Mean	
	HS	NHS	HS	NHS	HS	NHS	HS	NHS
T5	157.04	108.9	63.97	131.03	106.66	75.5	109.22	105.14
T6	110.57	87.48	51.76	83.5	52.93	47.66	71.75	72.88
T7	107.78	124.98	61.65	90.32	62.81	64.55	77.41	93.28
T8	157.04	151.67	66.86	67.95	62.23	22.83	95.38	81.42
T9	147.35	85.78	59.91	66.29	-	-	103.63	76.04
T10	115.57	121.67	54.10	53.51	-	-	84.84	87.59
T11	158.66	113.35	68.02	122.77	-	-	113.34	118.06
(ON)	137.03	99.36	62.81	54.1	34.12	21.03	77.99	58.16

$P > 0.05$ (rhuIFN α 2a production for each growth hours were compared between the Heat Shock and the Non Heat Shock culture condition.)

Key: ON = Over night, HS = Heat Shock, NHS = Non Heat Shock

Figure 10. rhuIFN α 2a conc. at different growth hours of *E.coli* strain



The mean of rhuIFN α 2a conc. values of the Experiments I, II & III was taken from the Table 8 to plot the bar diagram.

Heat Shock



Non Heat Shock



(Culture volume is 50 ml at the time of Heat Shock) ON = Over night

The T5 (5 hours growth), T8 (8 hours growth), T9 (9 hours growth), and the Over night *E.coli* growth hour samples show an increase in the rhuIFN α 2a production due to Heat Shock as compared to the Non Heat Shocked one. Though the difference in the rhuIFN α 2a concentration between the Heat Shocked and the Non Heat Shocked T5, T8, T9 and Over night *E.coli* growth hour samples were not significant, but still the benefits of such increase of rhuIFN α 2a concentration in an Industrial scale could not be ignored. Complying with the results of the growth curve in Figure 8 the bar diagram also shows a statistically non-significant effect of the Heat Shock on the *E.coli* growth pattern and on

the $\text{rhuIFN}\alpha 2\text{a}$ production. For example, the amount of $\text{rhuIFN}\alpha 2\text{a}$ produced at the T5 hours under the Heat Shocked condition was 109.22 IU/mL where as, under the Non Heat Shocked condition was 105.14 IU/mL. At the T5 hours (5 hours growth), *E.coli* growth hour samples were collected before the IPTG induction. Thus, the production of $\text{rhuIFN}\alpha 2\text{a}$ at this growth hour confirms a leaky expression of the $\text{rhuIFN}\alpha 2\text{a}$ gene.

Table 6. O.D_{600nm} values for the *E.coli* strain BL21 (DE3) producing $\text{rhuIFN}\alpha 2\text{a}$

O.D _{600nm}		
Time in Hours	HS	NHS
T0	0	0
T1	0.013	0.011
T2	0.067	0.026
T3	0.109	0.120
=>T4	0.378	0.420
* T5	0.434	0.813
T6	0.399	0.830
T7	0.455	0.869
T8	0.329	0.822
T9	0.384	0.802
T10	0.300	0.950
T11	0.336	0.710
(ON)	0.290	0.628

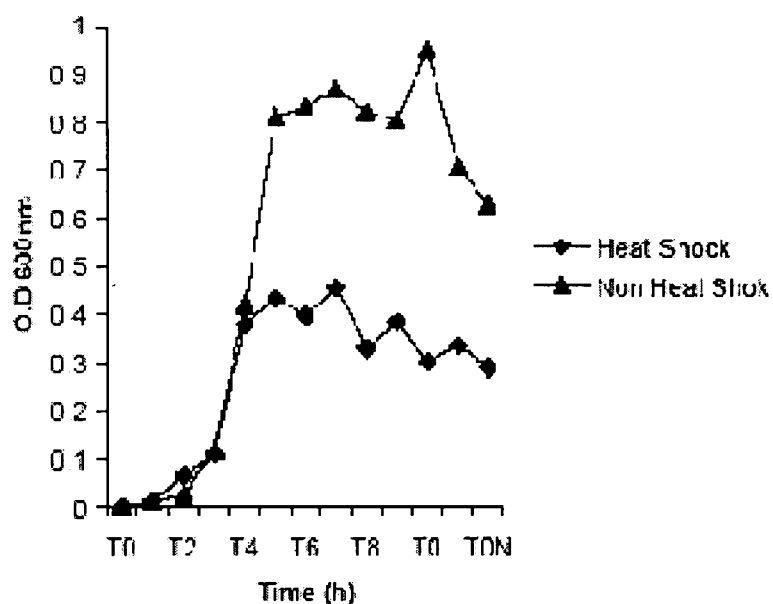
Growth with and without Heat Shock (Culture Volume is 40mL at the time of Heat Shock)

Key: => Heat Shock applied, * IPTG induction, HS = Heat Shock, NHS = Non Heat Shock, ON = Over night

In another set of experiment (Experiment B) the duplicate flasks were inoculated with a predefined amount of biomass (equivalent of 100 μ L of culture at OD_{600nm} 1.0) where the culture volume was maintained to approximately 40mL. This was done to induce a greater heat stress to the culture. Like the first set of experiments, the *E.coli* strain was

subjected to 50°C for 5-10 minutes in a water bath. The growth of the *E.coli* strain is shown in Table 6 and plotted in Figure 11.

Figure 11. Growth of *E.coli* strain with and without Heat Shock



Culture Volume is 40mL at the time of Heat Shock

Key: ON = Over night

Growth was followed by sampling at 1 hourly intervals and the optical density (OD_{600nm}) determined. The duplicate flasks for the strain were grown till T4 hours (4 hours) (OD_{600nm} of approximately 0.3 – 0.4) and then one of the flask was subjected to Heat Shock by heating in a 50°C water bath for 5-10 minutes. The Heat Shocked culture was returned to the incubator and grown with its non Heat Shocked matched flask.

Unlike the first set of experiments (Experiment A), the *E. coli* growth curve of the Figure 11 shows a definite effect of the applied Heat Shock. Immediately after the Heat Shock

there is evidence that growth has slowed in the Heat Shocked flask, at sample T5 (5 hours growth) following Heat Shock at T4 (4 hours).

Table 7. O.D_{450nm} values for the IFN ELISA of the *E.coli* growth hour samples

Standard		O.D _{450nm}		
Conc. IU/mL	O.D _{450nm}	Time in Hours	HS	NHS
0	0.102	T5	0.261	2.459
17	0.156	T6	0.777	1.055
33	0.164	T7	1.267	1.055
100	0.276	T8	0.375	0.782
300	0.673	T9	0.477	0.946
		T10	0.355	1.297
		T11	0.713	0.623
		(ON)	0.793	0.936

Culture Volume is 40mL at the time of Heat Shock. The values in the table are the O.D_{450nm} IFN ELISA End Point results, together with the Standard IFN concentrations with their O.D_{450nm} IFN ELISA End Point Results.

Key: ON = Over night, HS = Heat Shock, NHS = Non Heat Shock

The effect of the heat stress on the quantity of the $\text{rhuIFN}\alpha 2\text{a}$ production (for the Experiment B) is estimated by the IFN ELISA methods. The IFN ELISA O.D_{450nm} End Point results of the *E.coli* growth hour samples are shown in the Table 7. $\text{rhuIFN}\alpha 2\text{a}$ concentration (IU/mL) in each of the *E.coli* growth hour samples from T5 hours (5 hours growth) to Over night growth is shown in the Table 8 and in the bar diagram Figure 12.

Table 8. rhuIFN α 2a (IU/mL) concentration in the *E.coli* growth hour Samples

rhuIFNα2a (IU/mL)		
Time in Hours	HS	NHS
T5	713.04	715.56
T6	346.91	458.01
T7	529.38	458.01
T8	151.14	349.09
T9	204.75	416.83
T10	140.31	538.55
T11	318.53	276.82
(ON)	353.85	412.89

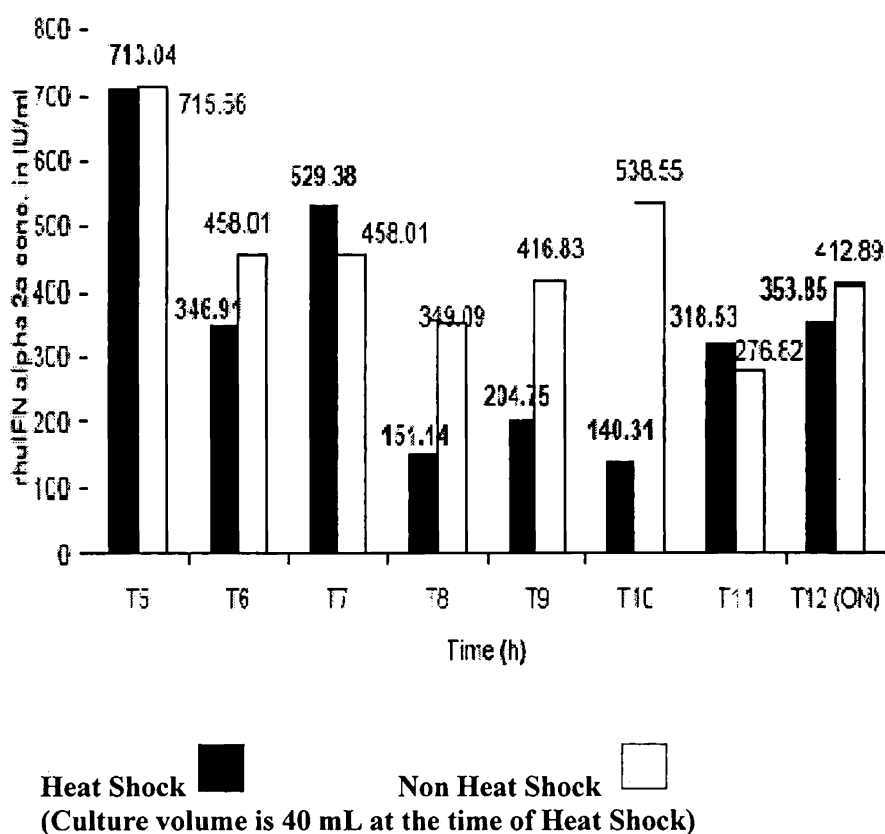
Culture Volume is 40mL at the time of Heat Shock

Key: ON = Over night, HS = Heat Shock, NHS = Non Heat Shock

The T7 (7 hours growth), and T11 (11 hours growth) *E.coli* growth hour samples show an increase in the rhuIFN α 2a production due to Heat Shock as compared to the Non Heat Shocked one. In this experiment the culture volume was reduced to 40mL to induce a greater heat stress effect on the *E.coli* cells. It should be noted that in this case, the OD_{600nm} of the culture during the Heat Shock was approximately 0.3 – 0.4 unlike to the previous sets of experiments (Experiment A) where the OD_{600nm} of the culture during the Heat Shock was approximately 0.1 – 0.3. Since the cell density is more in the second experiment, it is assumed that it can withstand a greater heat stress as compared to the previous set of three experiments, but since the culture volume was less, the *E.coli* cells might have experienced a greater heat stress thus failing to overcome the effect in the subsequent growth hours. Though the intention of reducing the culture volume was to create an environment so that the cells undergoes a greater heat stress, leading to an

increase in the overall production of the $\text{rhuIFN}\alpha 2\text{a}$ protein, it has been found that the $\text{rhuIFN}\alpha 2\text{a}$ protein production increases at the cost of the over all cell density in the Heat Shocked flask. Like the previous set of experiments (Experiment A), the difference in the $\text{rhuIFN}\alpha 2\text{a}$ production between the Heat Shocked and the Non Heat Shocked has been found minimal but nevertheless, the concentration of the $\text{rhuIFN}\alpha 2\text{a}$ produced in each of the collected *E.coli* growth hour samples has increased dramatically to many folds as compared to the 50mL culture in the earlier set of experiments.

Figure 12. $\text{rhuIFN}\alpha 2\text{a}$ conc. at different growth hours of *E.coli* strain



Key: ON = Over night

In another set of experiment (Experiment C), three set of cultures were grown and Heat Shocked following the same protocol of Experiment A. The production of $\text{rhuIFN}\alpha 2\text{a}$

was determined by SDS gel electrophoresis and quantification of the Coomassie blue stained protein bands (Figure 13) using the Universal Software (Zeineh Programs) from Advanced American Biotechnology (AAB) was performed. Since, initially in the Experiment A the T5 hours, T8 hours, T9 hours and the Over night growth hour samples showed a maximum $\text{rhuIFN}\alpha 2\text{a}$ production under the Heat Shock condition, it was decided to harvest the $\text{rhuIFN}\alpha 2\text{a}$ subsequently from those growth hours for further analysis. Since Electroelution was adopted for isolating the $\text{rhuIFN}\alpha 2\text{a}$ molecule from the T5 hours, T8 hours, T9 hours and the Over night growth hours, the SDS-PAGE gels ran for Electroelution was also utilized for quantifying the production of $\text{rhuIFN}\alpha 2\text{a}$ using the Universal Software. The IFN band was identified by comparison with the standard molecular weight markers run in the last track of each gel (Figure 13 & 14) from its known molecular weight. The results are shown in Table 9.

Table 9. Amounts of $\text{rhuIFN}\alpha 2\text{a}$ produced in Heat Shocked and Non-Heat Shocked cultures of *E.coli* strain taken at 5,8.9 and. over night (ON) Hour's growth determined by SDS-PAGE Analysis

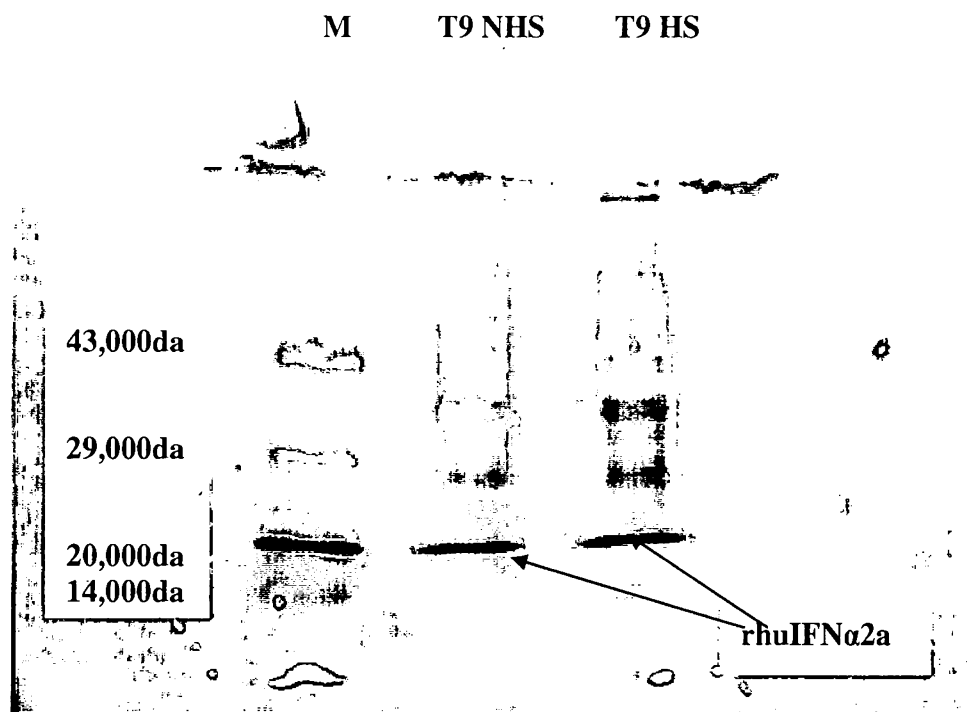
Growth Hours	Heat Shock			Non Heat Shock			Mean Value	
	Exp I	Exp II	Exp III	Exp I	Exp II	Exp III	HS	NHS
T5	79877	160313	51545	61181	219536	93194	97245	124637
T8	98188	249416	38931	113507	242986	127145	128845	161212
T9	91958	232245	55156	84870	243236	51711	126453	126605
ON	109135	138975	161480	95817	122665	167735	136530	128739

P > 0.05 ($\text{rhuIFN}\alpha 2\text{a}$ production for each growth hours were compared between the Heat Shock and the Non Heat Shock culture condition.)

Key: HS = Heat Shock, NHS = Non Heat Shock

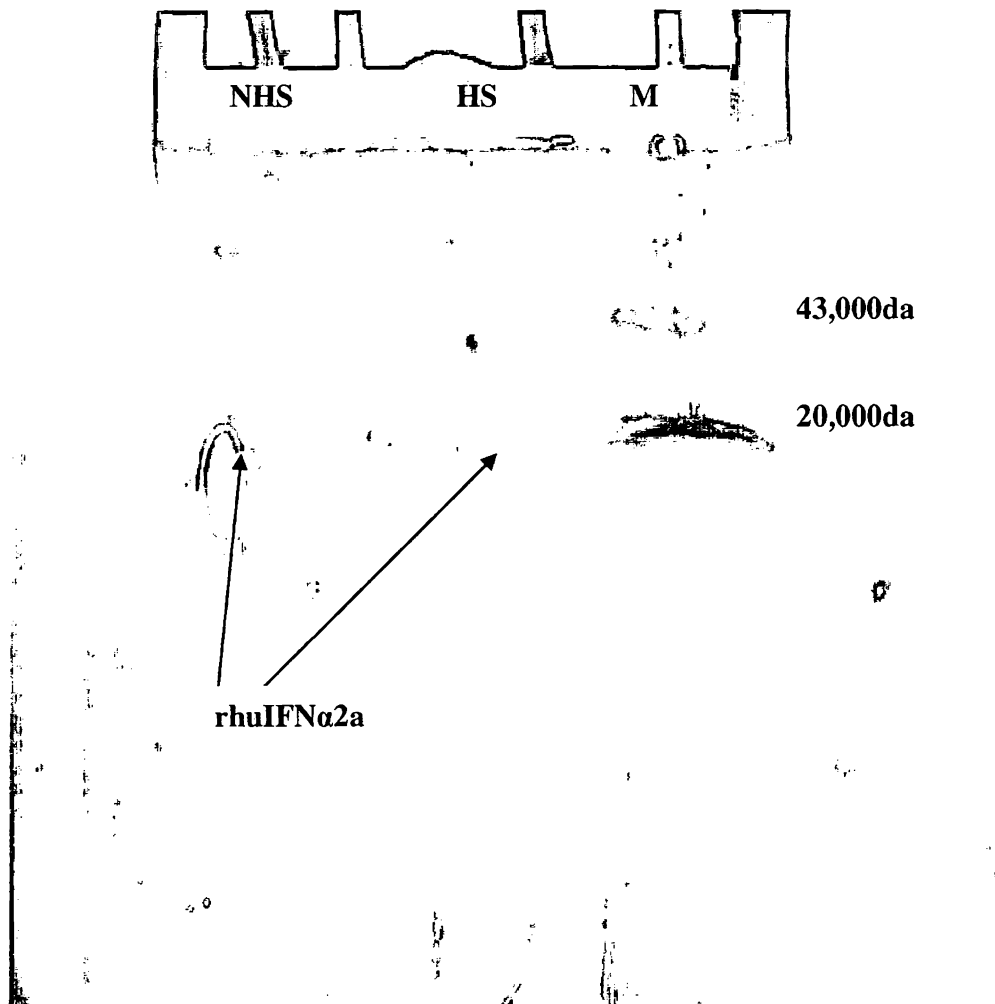
The mean value of the amount of rhulFN α 2a produced in Heat Shocked cultures of *E.coli* strain (from the Experiments C) taken at 5,8.9 and. over night (ON) growth hours sample were found less than the Non Heat Shocked growth hour samples except of the over night growth hour sample.

Fig. 13. SDS-PAGE of (T9) nine hours



Key: Non Heat Shock (NHS) and Heat Shock (HS) growth hour samples.
M = Molecular weight Ladder.

Fig. 14. SDS-PAGE of the Electroeluted rhuIFN α 2a sample



Key: Non Heat Shock (NHS) and Heat Shock (HS), M = Molecular weight Ladder.

4.2 Endotoxin estimation of the Electroeluted rhuIFN α 2a by the LAL Method:

For the neat samples, the Endotoxin amounts are as follows:

Heat Shock Electroeluted rhuIFN α 2a: 0.25 EU/mL (Approximate)

Non Heat Shock Electroeluted rhuIFN α 2a: greater than 0.25 EU/mL (Approximate)

For the 1: 2 dilutions of the samples, the Endotoxin amounts are as follows:

Heat Shock Electroeluted rhuIFN α 2a: 0.125 EU/mL (Approximate)

Non Heat Shock Electroeluted rhuIFN α 2a: greater than 0.125 EU/mL (Approximate) but less than 0.25 EU/mL (Approximate).

According to the Indian Pharmacopoeia, the permissible Endotoxin limit for the injectible drugs is approximately 0.25 EU/mL. Thus, neat Heat Shock Electroeluted rhuIFN α 2a sample, the 1: 2 dilutions of the Heat Shock and the Non Heat Shock Electroeluted rhuIFN α 2a sample samples were found to have a permissible Endotoxin limit.

4.3 IFN-ELISA of the Electroeluted rhuIFN α 2a:

rhuIFN α 2a was Electroeluted from the SDS-PAGE gels. The Electroeluted rhuIFN α 2a was run together with the Molecular Weight marker in the SDS-PAGE gels to determine the purity of the Eluted rhuIFN α 2a protein molecule (Figure 14). The Electroeluted rhuIFN α 2a was comparatively found free from other proteins and cellular debris/contamination. The samples were divided mainly into two parts i.e. rhuIFN α 2a harvested / Electroeluted from the T5 hours, T8 hours, T9 hours and Over night *E.coli* growth hour samples of the Heat Shocked culture and rhuIFN α 2a harvested / Electroeluted from the

Table 10. O.D_{450nm} values for the IFN ELISA of the Standard (for the Electroeluted rhuIFN α 2a).

Standard	
Pg/mL	O.D _{450nm}
0	0.036
8	0.073
16	0.129
32	0.235
63	0.42
125	0.828
250	1.655
500	2.676

Figure 15. Standard Curve for IFN Alpha ELISA (for the Electroeluted rhuIFN α 2a)

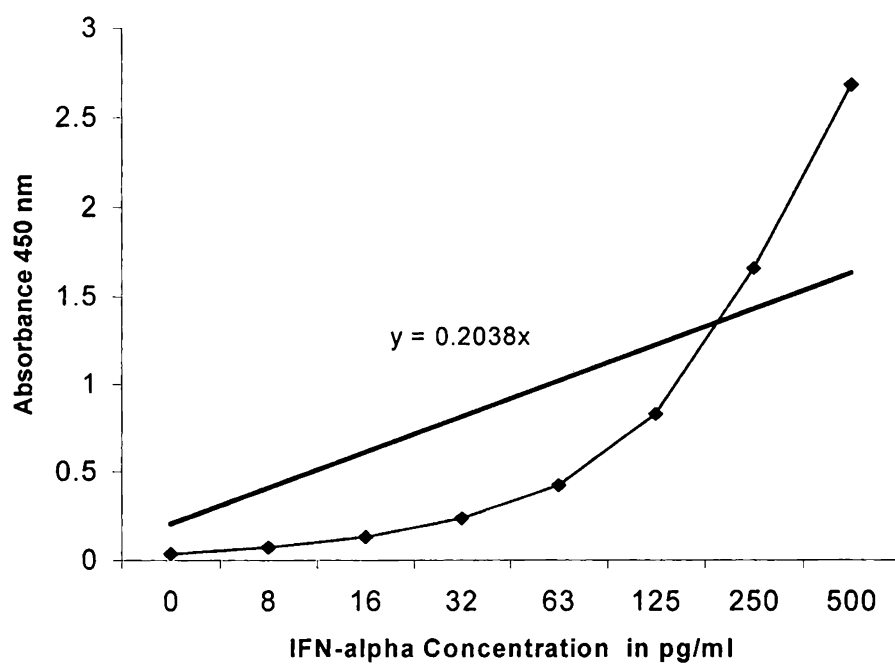


Table 11. O.D_{450nm} values for the IFN ELISA of the Electroeluted rhuIFN α 2a Samples from the 1st & 2nd Batch and the *E.coli* Cell Pellet Sample

O.D. 450nm	
1st SET	
Heat Shock (1:20) Electroeluted sample	Non Heat Shock (1:20) Electroeluted sample
1.666	2.054
2nd SET	
Heat Shock (1:5) Electroeluted sample	Non Heat Shock (1:5) Electroeluted sample
1.089	1.280
Heat Shock (1:20) Cell Pellet	Non Heat Shock (1:20) Cell Pellet
5.930	3.798

T5 hours, T8 hours, T9 hours and Over night *E.coli* growth hour samples of the Non Heat Shocked culture. rhuIFN α 2a was Electroeluted in two batches. The Electroeluted rhuIFN α 2a from the first batch is termed as 1st Set and the Electroeluted rhuIFN α 2a from the second batch is termed as 2nd Set. The Electroeluted rhuIFN α 2a from the 1st Set was diluted to 1:5, 1:10, & 1:20 and the concentration of the rhuIFN α 2a in each of the dilutions were determined by IFN-ELISA. Since the O.D_{450nm} values for both the 1:5 & 1:10 were much higher than the maximum IFN Standard value, the O.D_{450nm} readings for the 1:20 dilution of the Electroeluted rhuIFN α 2a sample was considered appropriate and tabulated in the Table 11. Similarly, the O.D_{450nm} values of the 1:5 dilution of the

Electroeluted rhuIFN α 2a from the 2nd Set was considered appropriate and tabulated in the Table 11. rhuIFN α 2a concentration (pg/mL) in the Electroeluted samples is shown in the Table 12. The amount of rhuIFN α 2a present in each of the Electroeluted samples was calculated from the formula derived by plotting the IFN ELISA standard curve (Figure 15). The concentration of rhuIFN α 2a in the T5 hour's *E.coli* growth hour sample was also determined by IFN-ELISA in the same experiments and the O.D_{450nm} values IFN-ELISA End Point values and the concentration in pg/mL are shown in the Table12.

Table 12. rhuIFN α 2a (pg/ mL) concentration in the T5 hour's *E.coli* Cell Pellet samples and in the Electroeluted rhuIFN α 2a samples from the 1st and the 2nd set

rhuIFN α 2a (pg/ mL)	
1 st SET	
Heat Shock Electroeluted sample (1:20 diln.)	Non Heat Shock Electroeluted sample (1:20 diln.)
163.49	201.57
2 nd SET	
Heat Shock Electroeluted sample (1:5 diln.)	Non Heat Shock Electroeluted sample (1:5 diln.)
27.25	32.5
Heat Shock Cell Pellet (1:20 diln.)	Non Heat Shock Cell Pellet (1:20 diln.)
581.94	372.72

Table 13. Miller's standard End Point Results

Standard	
Conc. $\mu\text{g/mL}$	O.D. 660 nm
0	0
0.2	0.005
0.4	0.008
0.6	0.008
0.8	0.008
1	0.009
2	0.025
4	0.032
6	0.038
8	0.047
10	0.06
12	0.067
14	0.08
16	0.083
20	0.136
40	0.294
60	0.363
80	0.352
100	0.413

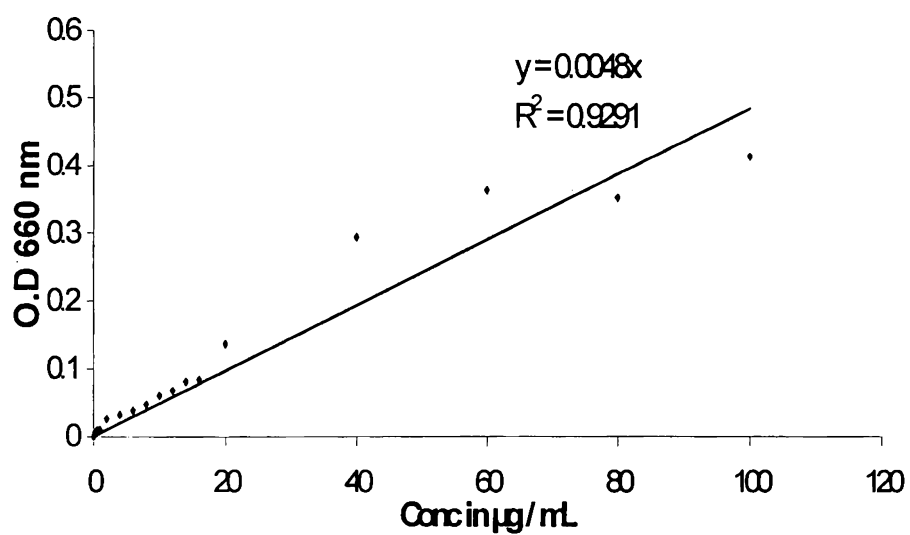
Figure 16. Miller's Standard Graph

Table 14. O.D_{660nm} values (of the Electroeluted rhIFN α 2a samples and the *E.coli* Growth hour cell pellet Heat Shock and the Non Heat Shock Samples) of the Miller's End Point results and the Total protein amounts (of the Electroeluted rhIFN α 2a samples and the *E.coli* cell pellet heat shock and the Non Heat Shock samples) estimated by the Miller's Method.

O.D _{660nm}	
Heat Shock (1:100 diln.) Electroeluted sample of I st SET	Non Heat Shock (1:100 diln.) Electroeluted sample of I st SET
0.016	0.008
Heat Shock (1:50 diln.) Cell Pellet	Non Heat Shock (1:50 diln.) Cell Pellet
0.3075	0.093
Total protein $\mu\text{g}/\text{mL}$	
Heat Shock (1:100 diln.) Electroeluted sample of I st SET	Non Heat Shock (1:100 diln.) Electroeluted sample of I st SET
333	166
Heat Shock (1:50 diln.) Cell Pellet	Non Heat Shock (1:50 diln.) Cell Pellet
3203	9685

4.4 Total protein estimation of the Electroeluted rhIFN α 2a by Miller's Method:

The total protein concentration in the *E.coli* T5 hours growth hour sample and in the Electroeluted rhIFN α 2a protein sample was quantitated by the Miller's method. The results were used to determine the Specific Activity, Purification factor and the Percentage yield of the SDS-PAGE purified rhIFN α 2a protein samples according to the following formulae:

$$\text{Specific activity} = \frac{\text{Number of protein units}}{\text{Total protein}} \dots\dots\dots[3]$$

$$\text{Percentage yield} = \frac{\text{Proteins units recovered}}{\text{Proteins units at start}} \times 100 \dots\dots\dots [4]$$

$$\text{Purification Factor} = \frac{\text{Specific activity of purified material}}{\text{Specific activity of original material}} \dots\dots\dots [5]$$

For the Total protein estimation by the Miller's method, both the Heat Shock and the Non Heat Shock Electroeluted rhuIFN α 2a samples (from the Ist SET) were diluted to 1:100 with D/W and the T5 hours growth hour cell pellet from both the Heat Shocked and the Non Heat Shocked cultures were diluted to 1:50 with D/W. O.D_{660nm} values (of the Electroeluted rhuIFN α 2a samples and the *E.coli* growth hour cell pellet Heat Shock and the Non Heat Shock samples) of the Miller's End Point results and the Total protein amounts (of the Electroeluted rhuIFN α 2a samples and the *E.coli* cell pellet Heat Shock and the Non Heat Shock samples) estimated by the Miller's Method is shown in Table 14.

The amount of Total protein in 20 μ L of Heat Shock (1:100 diln.) Electroeluted sample (of Ist SET) = 333 μ g/ mL $\sim$ 3.33 x 10⁸ pg/ mL

Therefore, The amount of Total protein in 5 μ L of Heat Shock (1:100 diln.) Electroeluted sample (of Ist SET) = 0.8325 x 10⁸ pg/ mL

The amount of Total protein in 20 μ L of Non Heat Shock (1:100 diln.) Electroeluted sample (of Ist SET) = 166 μ g/ mL $\sim$ 1.66 x 10⁸ pg/ mL

Therefore, The amount of Total protein in 5 μ L of Heat Shock (1:100 diln.) Electroeluted sample (of Ist SET) = 0.415 x 10⁸ pg/ mL

The amount of Total protein in 20 μL of Heat Shock (1:50 diln.) T5 hours *E.coli* growth hour cell pellet = $3203\mu\text{g/ mL} \sim 3203 \times 10^6 \text{ pg/ mL}$

Therefore, The amount of Total protein in 5 μL of Heat Shock (1:50 diln.) T5 hours *E.coli* growth hour cell pellet = $8.0075 \times 10^8 \text{ pg/ mL}$

The amount of Total protein in 20 μL of Non Heat Shock (1:50 diln.) T5 hours *E.coli* growth hour cell pellet = $968.5\mu\text{g/ mL} \sim 968.5 \times 10^6 \text{ pg/ mL}$

Therefore, The amount of Total protein in 5 μL of Non Heat Shock (1:50 diln.) T5 hours *E.coli* growth hour cell pellet = $2.42125 \times 10^8 \text{ pg/ mL}$

Therefore, according to the above formulae,

$$\text{Specific activity of the Heat Shock } E.coli \text{ 5 hours growth cell pellet sample of OD}_{600\text{nm}} 0.5 \text{ (appx)} = \frac{581.94}{8.0075 \times 10^8} = 72.67 \times 10^{-8}$$

$$\text{Specific activity of the Non Heat Shock } E.coli \text{ 5 hours growth cell pellet sample of OD}_{600\text{nm}} 0.5 \text{ (appx)} = \frac{372.72}{2.42125 \times 10^8} = 153.03 \times 10^{-8}$$

$$\text{Specific activity of the Heat Shock } \rho\text{hIFN } \alpha 2\text{a protein sample} = \frac{163.49}{0.8325 \times 10^8} = 196.38 \times 10^{-8}$$

$$\text{Specific activity of the Non Heat Shock } \rho\text{hIFN } \alpha 2\text{a protein sample} = \frac{201.57}{0.415 \times 10^8} = 485.71 \times 10^{-8}$$

Percentage yield of the Heat Shock rhuIFN α 2a protein sample

$$= \frac{163.49}{581.94} = 28.09 \%$$

Percentage yield of the Non Heat Shock rhuIFN α 2a protein sample

$$= \frac{201.57}{372.72} = 54.08 \%$$

Purification Factor for the Heat Shock rhuIFN α 2a protein sample

$$= \frac{196.38 \times 10^{-8}}{72.67 \times 10^{-8}} = 2.7$$

Purification Factor for the Non Heat Shock rhuIFN α 2a protein sample

$$= \frac{485.71 \times 10^{-8}}{153.03 \times 10^{-8}} = 3.1$$

Therefore the results are;

Specific activity of the Heat Shock *E.coli* 5 hours growth cell pellet sample of OD_{600nm}

$$0.5 \text{ (appx)} = 72.67 \times 10^{-8}$$

Specific activity of the Non Heat Shock *E.coli* 5 hours growth cell pellet sample of

$$\text{OD}_{600\text{nm}} 0.5 \text{ (appx)} = 153.03 \times 10^{-8}$$

Specific activity of the Heat Shock rhuIFN α 2a protein sample = 196.38×10^{-8}

Specific activity of the Non Heat Shock rhuIFN α 2a protein sample = 485.71×10^{-8}

Percentage yield of the Heat Shock rhuIFN α 2a protein sample = 28.09 %

Percentage yield of the Non Heat Shock rhuIFN α 2a protein sample = 54.08 %

Purification Factor for the Heat Shock rhuIFN α 2a protein sample = 2.7

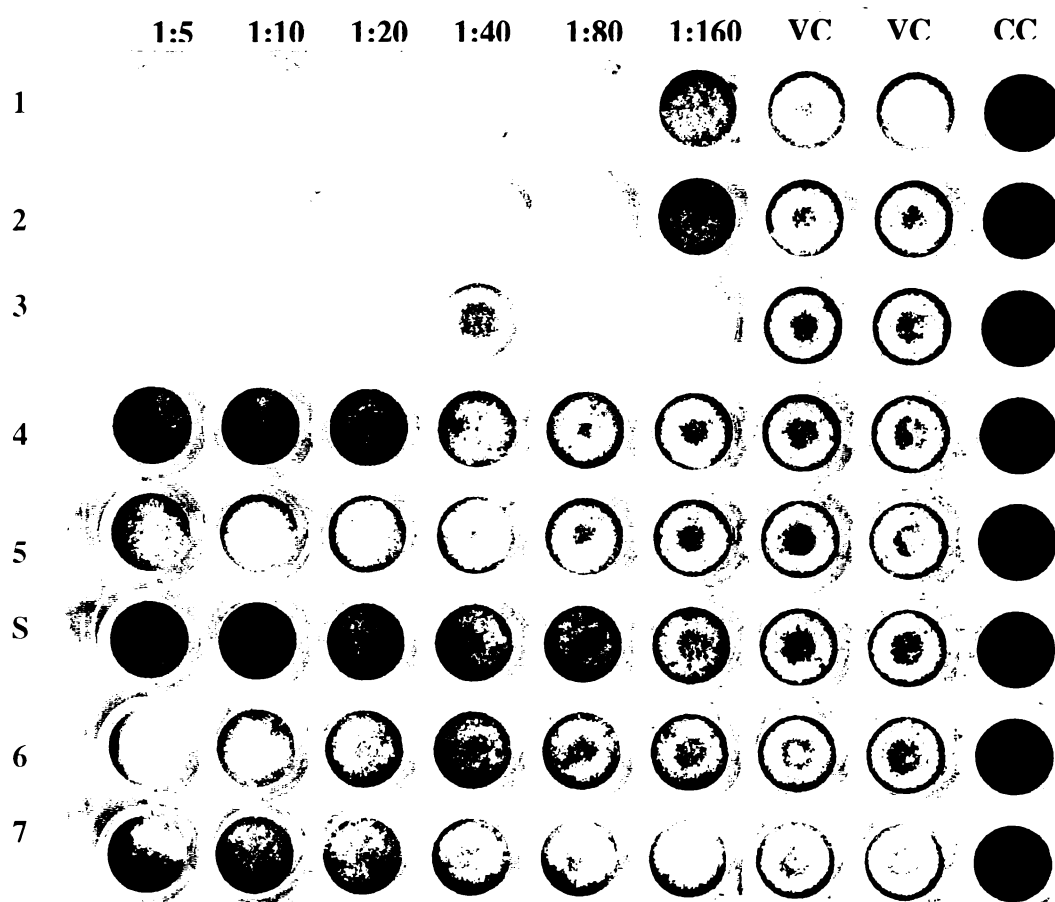
Purification Factor for the Non Heat Shock rhuIFN α 2a protein sample = 3.1

4.5 IFN Bioassay of Heat Shock and Non Heat Shock Electroeluted rhIFN α 2a:

IFN Bioassay of the electroeluted Heat Shock and Non Heat Shock rhIFN α 2a proteins together with sonicated growth hour cell pellet samples of Heat Shock and Non Heat Shock *E.coli* cells were performed on WISH Human Amnion Cell lines. The IFN activity against the Encephalomyocarditis Virus (EMC) was determined by the Cytopathic Effect (CPE) reduction technique. The results are shown in Figure 17.

In case of the IFN Standard, the IFN titer was at the IFN concentration of 1 Unit. Thus, according to the formula No. [1] the Factor was 1. Sonicated cell pellet samples showed cell cytotoxicity for most of the dilutions. In case of one set Heat Shock rhIFN α 2a, the IFN titer or the End Point is between 1:20 & 1:40. Thus, the titer was taken as 1:30 or 300 Units/mL or 300×1 (Factor) = 300 IU/mL. In case of one set of Non Heat Shock, the IFN titer was at 1:10. Thus, the titer is 100 Units/mL or 100×1 (Factor) = 100 IU/mL. Similarly, for another set of Heat Shock rhIFN α 2a Sample, the IFN titer is at 1:40 dilution, therefore 400 IU/mL and for another set of Non Heat Shock rhIFN α 2a sample, the IFN titer was at 1:20 dilution, therefore 200 IU/mL. The Heat Shock eluted rhIFN α 2a protein samples showed a higher IFN titer than the Non Heat Shock eluted rhIFN α 2a protein samples indicating a possible beneficial effect of Heat Shock on the rhIFN α 2a protein production with respect to its antiviral activity.

Figure 17. IFN Bioassay of the Electroeluted Heat Shock and Non Heat shock *rh*IFN α 2a proteins together with sonicated cell pellet samples of Heat Shock and Non Heat Shock *E.coli* cells on WISH Human Amnion Cell lines by the Cytopathic Effect (CPE) reduction technique.



Samples: 1. Heat Shocked IPTG Induced *E.coli* growth hour pellets, 2. Non-Heat Shocked IPTG Induced *E.coli* growth hour pellets, 3. Non-Heat Shocked Non IPTG Induced *E.coli* growth hour pellets, 4. Heat Shocked *rh*IFN α 2a eluted proteins, 5. Non Heat Shocked *rh*IFN α 2a eluted proteins, 6. IFN standard, 7. Heat Shocked *rh*IFN α 2a eluted proteins, 8. Non Heat Shocked *rh*IFN α 2a eluted proteins. CC = Cell control, VC = Virus control, S = Standard IFN (From Left: 8 Units, 4 Units, 2 Units, 1 Unit) [Note: The dilution of Sample 5 has been started from left 1:10, 1:20, 1:40, 1:80, 1:160 and 1:320]

4.6 Reverse Transcriptase / Thymidine Uptake Assay of the Heat Shock and Non Heat Shock Electroeluted rhulFN α 2a:

Table 15. Reverse Transcriptase / Thymidine Uptake Assay of the Electroeluted rhulFN α 2a.

Reverse Transcriptase / Thymidine Uptake Assay of the Electroeluted rhulFN α2a							
Experiment	Counts/minute			RT% Inhibition		Mean % Inhibition	
	Samples			Samples		Samples	
	PBS	HS	NHS	HS	NHS	HS	NHS
1 st	668	-	273	-	59.13	33.33	28.11
2 nd	60	46	38	28.33	36.67		
3 rd	60	37	52	38.33	13.33		
4 th	60	-	58	-	3.33		

Key: PBS = Phosphate Buffer Saline HS = Heat Shock, NHS = Non Heat Shock, RT = Reverse Transcriptase

To check the NNI activity of the Electroeluted rhulFN α 2a against the HIV – RT enzyme, Reverse Transcriptase / Thymidine Uptake Assay of the Electroeluted rhulFN α 2a was performed. Four experiments were performed with the 1st SET of the Electroeluted rhulFN α 2a and PBS as control. The results of the four experiments are shown in the Table 15. The counts / minute for the Heat Shock Electroeluted rhulFN α 2a in the 1st and the 4th experiments were not considered due to experimental error. Both the Heat Shock and the Non Heat Shock Electroeluted rhulFN α 2a show RT inhibition. In the mean RT inhibition results (of the four experiments) Heat Shock Electroeluted rhulFN α 2a show a greater RT inhibition than the Non Heat Shock Electroeluted rhulFN α 2a.

4.7 Anti-HIV testing of Heat Shock and Non Heat Shock Electroeluted rhIFN α 2a

on MT-2 cells lines:

Anti-HIV testing of Heat Shock and Non Heat Shock Electroeluted rhIFN α 2a was performed on MT-2 cells lines. The MT-2 cell line was pretreated with the Heat Shock and Non Heat Shock Electroeluted rhIFN α 2a before the cell line was infected with 200 TCID₅₀ HIV-1 III. This was done to induce/ prime the antiviral state to the MT-2 cell lines so that the cell succeeds in blocking viral entry or multiplication within the cell. After the virus infection, fresh dilutions of both the Heat Shock and Non Heat Shock Electroeluted rhIFN α 2a were again put into the virus infected MT-2 cell wells and cells were incubated for 5 days. The P-24 Assay with the 0PID and the 5PID sample was performed to understand the level of viral multiplication in the rhIFN α 2a treated MT-2 cell lines. The MT-2 cell count was monitored to see the cytotoxic effect of the Electroeluted rhIFN α 2a on the MT-2 cell lines. The result of the Anti-HIV testing of Heat Shock and Non Heat Shock Electroeluted rhIFN α 2a is shown in Table 16 and Table 17.

The results show considerable anti HIV effect of the IFN standard, Heat Shock and Non Heat Shock Electroeluted rhIFN α 2a on the MT-2 cell lines. The 0 PID sample was expected to show no viral multiplication and the P-24 values of the samples for the 1st experiment are also found to be less than the cut off value of 0.155. Both the 1:5 and 1:10 dilution of Heat Shock and Non Heat Shock Electroeluted rhIFN α 2a show a decrease in the P-24 value on the 5th day when compared to the P-24 value of the Virus Control sample. The 1:5 dilutions in both the cases show Cell Cytotoxicity as the cell counts in

both the wells were found to be less than 0.77×10^6 cells of the Cell Control well. The 1:20 - 1:160 dilutions of both the Heat Shock and Non Heat Shock Electroeluted rhuIFN α 2a show no effect on the virus infection of the MT-2 cells. The 0 PID sample showed no viral multiplication for the IInd experiment and the P-24 values are found to be less than the cut off value of 0.134. Only the 1:5 dilution of Heat Shock and Non Heat Shock Electroeluted rhuIFN α 2a show a decrease in the P-24 value on the 5th day when compared to the P-24 value of the Virus Control sample. The 1:5 dilutions in both the cases show Cell Cytotoxicity as the cell counts in both the wells were found to be less than 0.86×10^6 cells of the Cell control well. But when compared with the Ist experiment the cell cytotoxicity for the 1:5 dilutions were found to be less in the IInd experiment. The 1:10 - 1:160 dilutions (in the IInd experiment) of both the Heat Shock and Non Heat Shock Electroeluted rhuIFN α 2a show no effect on the virus infection of the MT-2 cells.

Table 16. Anti-HIV testing of Heat Shock and Non Heat Shock Electroeluted rhuIFN α 2a. [Cut off Value: 0.155] Ist Experiment

S r. No	Concentration	P-24 Results			Cell Count 5 PID
		0 PID	4 PID	5 PID	
1	Cell Control	ND	ND	0.086	0.77×10^6
2	Virus Control	0.071	0.424	0.380	-----
3	Std IFN 10^5	0.105	0.116	0.102	0.78×10^6
	Std IFN 1:5 of 10^5	0.091	0.113	0.278	0.79×10^6
4	Heat Shock IFN				
	1:5	0.093	ND	0.088	0.41×10^6
	1:10	0.081	ND	0.122	0.79×10^6
	1:20	0.108	ND	0.252	0.77×10^6
	1:40	0.106	ND	1.789	0.81×10^6
	1:80	0.086	ND	0.207	0.83×10^6
	1:160	0.081	ND	0.308	0.83×10^6
5	Non-Heat Shock IFN				
	1:5	0.119	ND	0.079	0.45×10^6
	1:10	0.091	ND	0.117	0.80×10^6
	1:20	0.098	ND	0.176	0.78×10^6
	1:40	0.079	ND	0.193	0.81×10^6
	1:80	0.123	ND	0.848	0.83×10^6
	1:160	0.084	ND	1.043	0.79×10^6

**Key: 0 PID = Zero Post Infection Day,
4 PID = Fourth Post Infection Day.
5 PID = Fifth Post Infection Day.
ND = Not detected**

Table 17. Anti-HIV testing of Heat Shock and Non Heat Shock Electroeluted rhuIFN α 2a. [Cut off Value: 0.134] IInd Experiment

Sr. No	Concentration	P- 24 Results		MT-2 Cell Count
		0 PID	5 PID	
1	Cell Control	ND	0.071	0.86 x10 ⁶
2	Virus Control	0.060	1.608	----
3	Std IFN 10 ⁵	ND	0.069	0.88 x10 ⁶
4	Heat Shock IFN			
	1:5	0.060	0.076	0.62 x10 ⁶
	1:10	0.060	2.140	0.88 x10 ⁶
	1:20	0.060	3.500	0.86 x10 ⁶
	1:40	0.073	2.861	0.86 x10 ⁶
	1:80	0.072	3.500	0.88 x10 ⁶
	1:160	0.072	1.246	0.82 x10 ⁶
5	Non Heat Shock IFN			
	1:5	0.065	0.075	0.64 x10 ⁶
	1:10	0.065	3.500	0.86 x10 ⁶
	1:20	0.069	3.500	0.86 x10 ⁶
	1:40	0.069	3.500	0.88 x10 ⁶
	1:80	0.067	3.500	0.84 x10 ⁶
	1:160	0.072	3.500	0.88 x10 ⁶

**Key: 0 PID = Zero Post Infection Day,
5 PID = Fifth Post Infection Day.
ND = Not detected**

4.8 Amino acid composition determination of Heat Shock and Non Heat Shock

Electroeluted rhuIFN α 2a:

The cation exchange chromatography process using the Amino Acid Analyser determined the amino acid composition of the electroeluted Heat Shock and the Non Heat Shock rhuIFN α 2a protein sample. The amino acid composition of both the Heat Shock and the Non Heat Shock electroeluted rhuIFN α 2a protein are shown in the Table 18.

Although small peaks could be observed (Figure 19 & 20) for the Tyr, Pro, Cys and Phe in the chromatograms for the Heat Shock and the Non Heat Shock rhuIFN α 2a samples the Analyzer failed to show any definite value in nano moles as the concentration of the rhuIFN α 2a protein samples used for the analysis was less. The Amide, Asparagine [Asn] and Glutamine [Glu] are converted to their acids, Aspartate [Asp] and Glutamate [Glu] and the Tryptophan [Trp] is simply unstable under Acid Hydrolysis condition and is destroyed.

Both the Heat Shock and the Non Heat Shock Electroeluted protein samples shows approximately equal amount of amino acid concentrations except the concentration of Aspartate in the Non Heat Shock samples are found to be more than the Heat Shock samples and the concentration of the Glutamate are more in the Heat Shock samples than the Non Heat Shock samples.

The three main domains necessary for IFN bioactivity includes the Leucine, Isoleucine, Phenylalanine, Tyrosine, Serine, Proline, Threonine, Cysteine, Asparagine, Glutamine, Lysine, Histidine, Arginine, Aspartate and Glutamate [286]. Thus, all the amino acids

required for the bioactivity of the IFN molecule are found to be present in an adequate concentration in the Electroeluted $\text{rhuIFN}\alpha 2\text{a}$ samples except the Phenylalanine, Tyrosine, Proline and Cysteine. Although the peaks for the amino acids could be seen in the chromatogram the Analyzer failed to show any definite value in nano moles as the concentration of the $\text{rhuIFN}\alpha 2\text{a}$ protein samples used for the analysis was less, and generally peak height of these amino acids were much less as compared to the other amino acids both in the sample and in the standard. The results also shows that, the amount of the detected amino acids (except Aspartate and Glutamate) present in both the Heat Shock and Non Heat Shock electroeluted $\text{rhuIFN}\alpha 2\text{a}$ samples are almost same as expected.

Figure 18. Chromatogram of the Standard

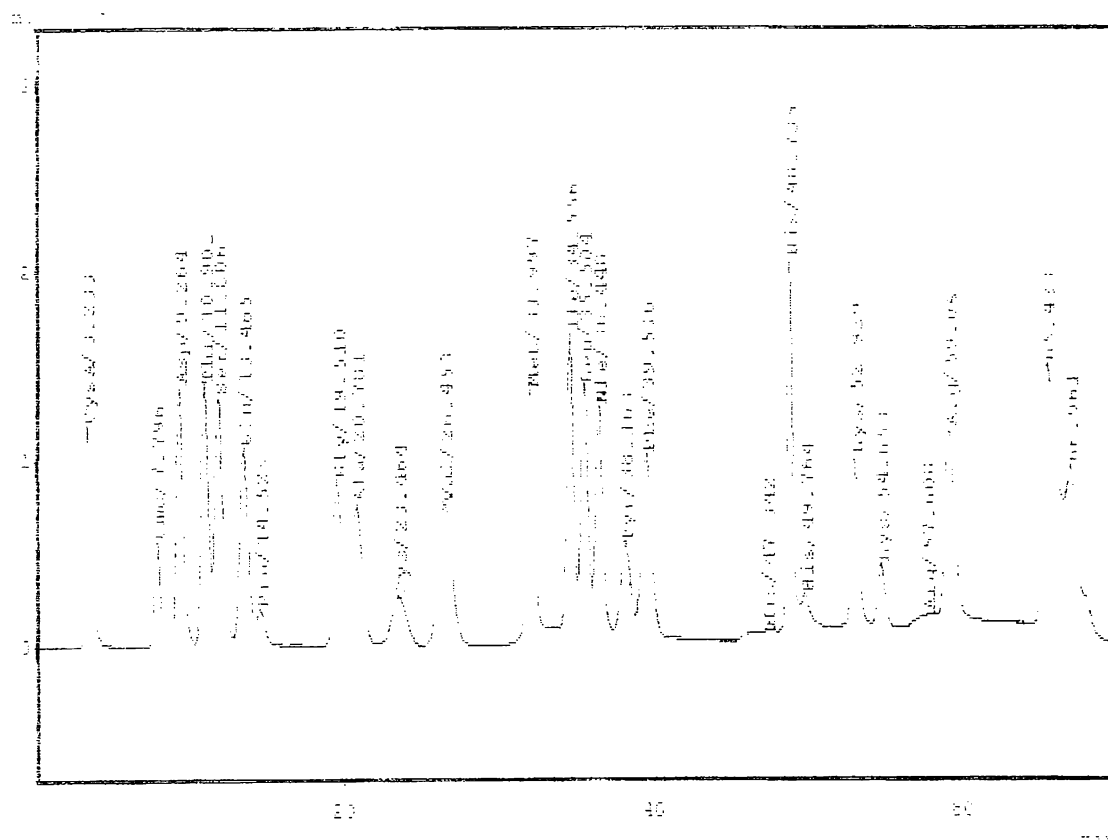


Figure 19. Chromatogram of the Non Heat Shock Electroeluted rhuIFN α 2a sample

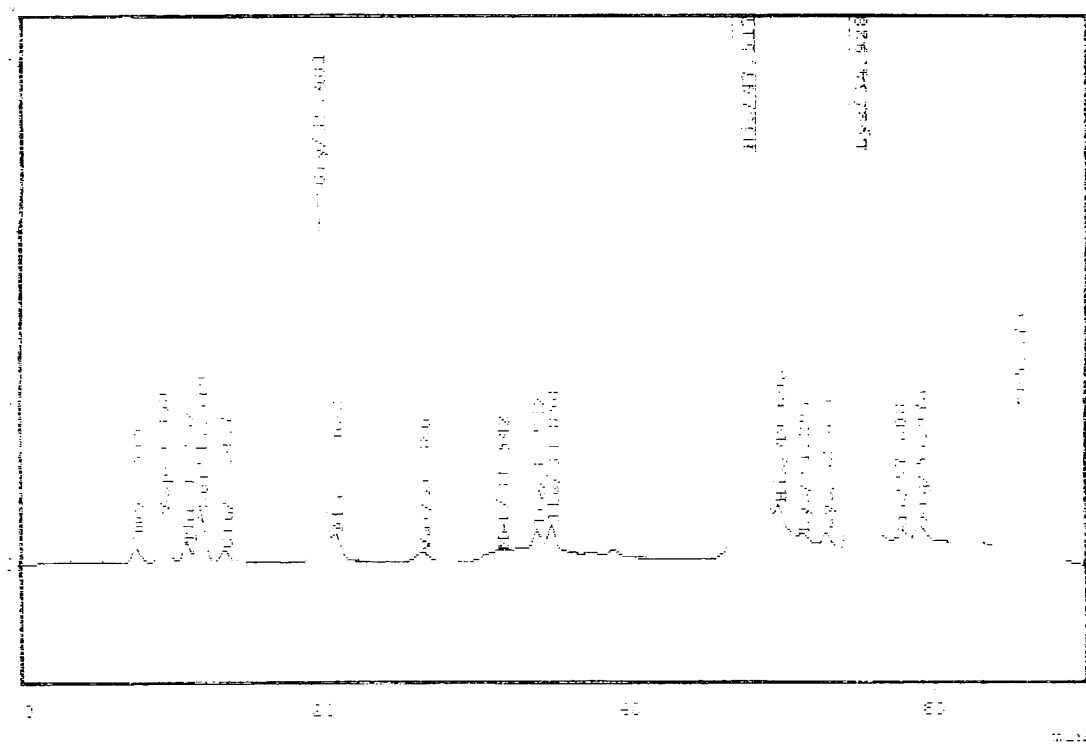


Figure 20. Chromatogram of the Heat Shock Electroeluted rhuIFN α 2a sample

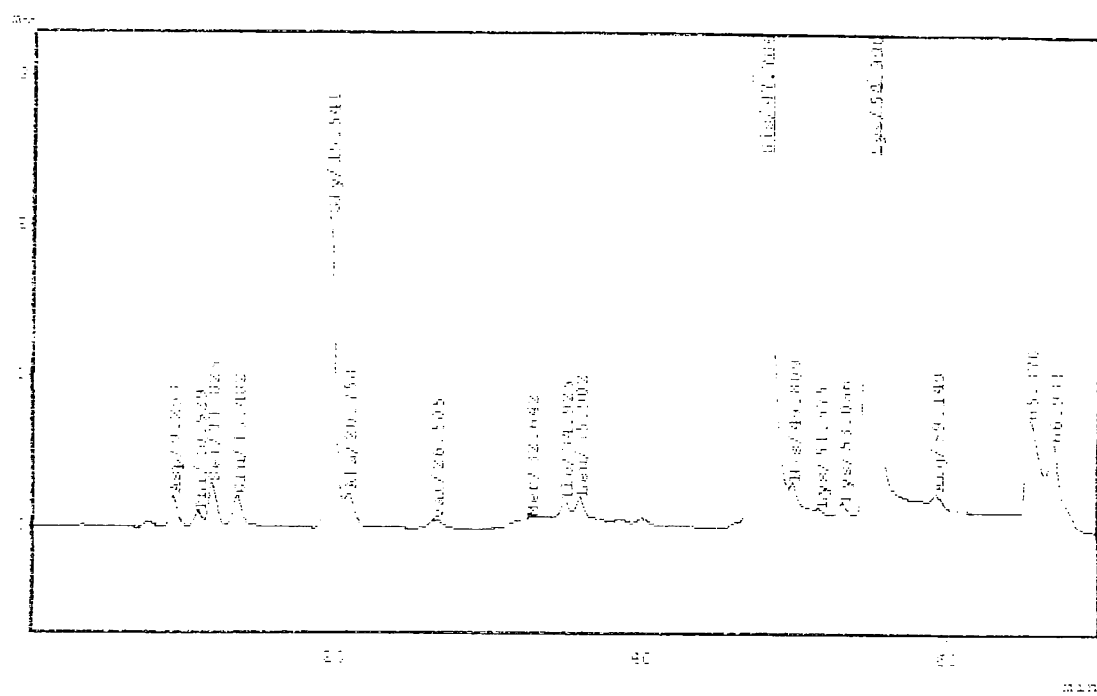


Table 18. Amino acid composition of the Electroeluted rhuIFN α 2a protein Sample

Amino acids present in rhuIFN α 2a	No. Of Amino Acids residues present in rhuIFN α 2a	Amino acid composition Determination [Conc. in nano moles]	
		Non Heat Shock	Heat Shock
Glycine [Gly]	5	2.5703	2.4295
Alanine [Ala]	8	0.1971	0.2947
Valine [Val]	7	0.0670	0.0620
Leucine [Leu]	21	0.1758	0.1789
Isoleucine [Ile]	8	0.1230	0.1183
Methonine [Met]	5	0.1659	0.1484
Tyrosine [Tyr]	10	-	-
Tryptophan [Trp]	5	-	-
Serine [Ser]	2	0.2289	0.2111
Proline [Pro]	14	-	-
Threonine [Thr]	5	0.0765	0.0691
Cysteine [Cys]	10	-	-
Asparagine [Asn]	4	-	-
Glutamine [Glu]	4	-	-
Lysine [Lys]	11	0.0842	0.1153
Histidine [His]	11	0.2318	0.1950
Arginine [Arg]	3	0.0526	0.1225
Aspartate [Asp]	9	0.2564	0.1566
Glutamate [Glu]	10	0.0547	0.1773
Phenylalanine [Phe]	14	-	-

DISCUSSIONS

5. DISCUSSIONS

5.1 Investigation of the production pattern of the rhuIFN α 2a protein molecule:

To scale up a technology from laboratory to the industrial scale it's of absolute necessity to understand the growth profile of the host microorganism. Numerous bioproducts including primary and secondary metabolites, recombinant proteins, and other biopolymers are being produced by microbial fermentation. However, natural microorganisms are not optimized for this task, and therefore microorganisms are often engineered to enhance their metabolic capacities. One such application is the improvement of general cellular properties to improve mid-stream and down-stream processes. Since bioproducts are formed by the operation of metabolic pathways, understanding metabolism during cell growth, as well as product formation, is essential to design a strategy for its enhanced production.

Operation of a metabolic network in a given microorganism is the consequence of the coordinated function of enzymes through the reaction pathways. Therefore, protein production analysis can be used to examine changes in protein expression level, and consequently to develop a systematic strategy for designing optimal fermentation process. Production analysis allows profiling of the specific over expressed proteins in a given organism on a two-dimensional polyacrylamide gel or by specific quantitative ELISA. The analysis involves the systematic separation, identification, and quantification of the specific over expressed proteins by the host organism. By such action, the changes in the levels of protein expression under different environmental conditions can be

determined. The information obtained by this analysis can be used to identify specific fermentation condition and time to be manipulated to achieve the desired goals.

Commercial production of recombinant protein produced by genetically engineered organisms requires two distinct body of knowledge, namely, molecular biology and process engineering. Background in molecular biology will enable us to create effectively expressed genes in bacteria or cells of animal origin for manufacturing. Background in process engineering principles will enable us to design and operate large-scale plants for growing genetically engineered organisms and for the subsequent processing of purification and formulation of product. In the early days, it was thought that scale-up was simply a matter of using larger volumes. That is, conditions that were found to be good at a small-scale would be equally effective on a larger scale and that to achieve this it was merely necessary to use a larger fermentor vessel with a larger medium volume. Such an approach resulted in product variability, both in terms of yield and quality, but also expensive operating costs. Hence, a systematic study of process engineering principles is needed for scaling up and operation of biotechnological processes for manufacture.

Thus, in the present experiments an attempt has been made for a study to understand and standardize the growth of the genetically modified *E.coli* strain both under Heat Shock and Non Heat Shock condition.

The aim of the project was to identify any potential beneficial affect of mild Heat Shock on recombinant protein production by *E.coli*. As application of Heat Shock at high

temperature for a long time has a deleterious affect on recombinant protein production, the cultures were Heat Shocked in 50° C water bath for a period of 5-10 minutes. Part of the heat stress response is induction of non-specific proteases [86], which can lead to degradation of the recombinant proteins. In addition there is a stringent response, which leads to decreased growth of the cells. But these responses are likely to lead to reduce recombinant protein expression, as well as degradation of protein quality. However, part of the stress response exhibited by *E.coli* leads to increased production of protein chaperones and transcription factors thus increasing the ease of protein folding and also affecting the overall transcription and translation mechanism with potentially beneficial effects. Therefore a Heat Shock effect, which leads to decreased growth of *E.coli*, was unlikely to exhibit improved recombinant protein production.

The effect of Heat Shock on the *E.coli* growth curves was distinct and found to be effective. When the culture volume was reduced to 40 mL, the effect of Heat Shock was found to be stringent and the culture failed to overcome the stress response but nevertheless showed a prominent lag, log and death phase (Figure 11). Figure 8 also indicates a distinct lag, log and a death phase. The heat response was mild when the culture volume was 50mL and the culture was found to overcome the stress response (Figure 8). The overall IFN secretion by the *E.coli* strain was increased (> 300 IU/ mL) when the culture volume was reduced to 40mL, maintaining the Heat Shock effect at 50°C for 5-10 minutes (Figure 12). The main objective of reducing the culture volume was to apply a greater thermal stress to the cells in the flask. In this experiment the culture showed an increase in the IFN production under the Heat Shock condition only at

7 and 11 hours, unlike the second experiment where the culture volume was maintained to 50 mL and was Heat Shocked at 50°C for 5 minutes. The growth hour samples (5,8,9 and Over night growth hour samples collected from three fresh set of reproducible *E.coli* growth experiments) were analyzed and rhuIFN α 2a protein production level has been studied by SDS-PAGE. Universal Software (Zeineh Programs) from Advanced American Biotechnology (AAB) was used to quantitate the rhuIFN α 2a production (Table 9). According to the values in the Table 9, in the Ist set of experiment except the 8 hours growth, all the other growth hours showed an increase in the rhuIFN α 2a production under the Heat Shock condition. In case of the II experiment, only 8 hours and Over Night growth hour's samples showed an increase in the rhuIFN α 2a production and in the III set of experiment only 9 hours growth sample showed an increase in the rhuIFN α 2a production under the Heat Shock condition. An IFN α ELISA was performed with the growth hour samples to further confirm the results of the SDS-PAGE, as quantitative ELISA is more accurate than the SDS-PAGE analysis system. Although the culture showed a leaky expression of the rhuIFN α 2a gene at 5 hours, nevertheless the 5, 8, 9 and the 12-hour growth samples (Over Night) showed an increase in the rhuIFN α 2a production in the Heat-Shocked cultures (Figure 10).

It has been found that, over expression of recombinant proteins interferes with cellular processes in many ways [56,60-64]. Drainage of precursors and energy urges the cell to readjust metabolic fluxes and enzyme composition, when stress responses are induced, and hence the cellular activity is shifted from growth to reorganization of biomass. This may result in inhibition of growth or low level of product accumulation [56,60-64]. In the

present experiments apart from the cellular inbuilt stress of the *E.coli* strains due to over expression of the recombinant protein, an external thermal stress is applied. Though, the effect is more distinct when the culture volume was reduced to 40mL nevertheless, in both the growth curves a slight inhibition of growth could be seen immediately after the application of Heat Shock. Bacteria are known to respond to unfavorable conditions, e.g., exposure to toxic chemicals and physical agents, nutrient limitation, or sudden increase in growth temperature, by rapid expression of regulons related to the Heat Shock, SOS, and oxidative stress responses [57,62-64]. The SOS responses allow the bacteria to overcome the detrimental condition and helps in adapting to the stressful situation. Recent investigations have revealed that the cellular content of many direct participants in protein synthesis (e.g., initiation factors, elongation factors, even RNA polymerase) are decreased when *E. coli* enters stationary phase as the result of nutrient and energy starvation. In particular, the amount of active ribosome is drastically different between fast- and slow- growing cultures [58]. This could indicate, that at that condition the cells might lead to lesser production of the desired recombinant protein contradictory to the findings that simultaneous expression of Heat Shock protein genes increases the expression of recombinant protein [59]. The results from the present study also indicates that the maximum production of the rhuIFN α 2a by the *E.coli* strain could be seen between the 7 hours and the Over Night growth hours agreeing to the findings that simultaneous expression of Heat Shock proteins might contribute to the increased accumulation of recombinant proteins in the *E.coli* expression system. Under conditions of stress, Heat Shock proteins constitute a major portion of prokaryotic cellular proteins [56,57]. Thus, in the present study, the role of Heat Shock in *E.coli* leading to an

increased accumulation of rhuIFN α 2a molecule at the different growth hours is an important finding. Though the quantity of rhuIFN α 2a produced in all the *E.coli* growth experiments (Figure 10 & 12) are not consistent but it has been observed that in each growth experiments at certain hours with elevated temperature, quantity of rhuIFN α 2a protein molecule is increased. The crucial finding is, when the culture volume is reduced to 40mL, the production of the rhuIFN α 2a protein molecule has been found to increase approximately more than three folds than the rhuIFN α 2a protein production level in the 50mL culture. As more than 50°C might be detrimental for the *E.coli* strains, it could be assumed that a Heat Shock more than 5-10 minutes might be beneficial in terms of increasing the rhuIFN α 2a protein production level. The effect of Heat Shock on the viability of the 50mL cultures was found almost negligible as compared to the 40mL culture. The growth curves in the Figure 8 have been found to overcome the effect of heat stress rapidly and no difference in the growth process is evident. To argue regarding the significance of the difference in the rhuIFN α 2a production between the Heat Shock and the Non-Heat shock results it could be considered that the experiments were performed at the laboratory scale and the Heat Shock was applied only for 5-10 minutes. Considering the fact, that a Heat Shock increases the recombinant protein production level [67-81], optimum condition could be maintained for a larger scale operation, where a significant difference in the rhuIFN α 2a protein production level could be achieved. Besides, the quantity of rhuIFN α 2a formed in the laboratory conditions could easily be scaled up many more folds at the Industry level. Ways of increasing the rhuIFN α 2a production level even slightly, under the normal fermentation condition would be a welcome option for the industries. As seen in the previous experiments [65,88] that irrespective of the

genetic modifications of the host strain and the usage of different synthetic promoters in the expression of the rhuIFN α 2a in the *E.coli* expression system, an increase in the temperature in the main fermentation system during the log phase of the culture could definitely lead to an increase expression of the gene resulting to an increase intracellular accumulation of rhuIFN α 2a protein molecule. Thus the above approach could be a solution for the development of a cost effective rhuIFN α 2a molecule leading to an affordable interferon biomolecule for the drug market.

It has been mentioned earlier that, Stress induced proteases in recombinant *E. coli* can degrade the recombinant protein, which is the product. The present study could have been given a more specific angle by following some of the recent work on analysis of the exact change in the amino acid composition in the *E.coli* during the Stress response. A structured kinetic model that incorporates detailed amino acid biosynthesis information for *E. coli* could be used to predict amino acid depletion (stress) for batch and continuous fermentations [220-233]. This could examine the fate of the recombinant protein under typical fermentation conditions and quantifies protease induction. Also, in some of the recent publications, it has been demonstrated that DNA microarrays could be employed to investigate the relationship between protease activity and gene expression [220-233].

Similarly, Glycosylation heterogeneity due to bioprocess conditions is an important pharmaceutical problem since correct glycosylation is required for the recombinant proteins to be biologically effective. It is well documented that bioprocess changes, such as temperature, pH, dissolved oxygen, dissolved CO₂, and nutrient levels, can adversely

effect the glycosylation of a recombinant protein. What is not well understood is how these environmental changes result in glycosylation heterogeneity. Recent advances in gene expression quantification (DNA microarrays and quantitative reverse transcription-polymerase chain reaction (QRT-PCR)) make it possible to observe the changes in the gene expression levels [220-233].

E. coli is the most intensively studied microorganism. For this reason, it is the workhorse for recombinant protein production. Yet, *E. coli* does not represent an ideal recombinant host, in part because it expends significant material and energy resources on non-essential cell components, with respect to recombinant protein production and growth in a bioreactor. Removal of these non-essential genes, metabolic engineering, might potentially improve the efficiency of *E. coli* as a recombinant host. The ideal engineered minimal genome *E. coli* cell has the potential to synthesize only the desired products, and little else [220-233].

Thus, monitoring the changes in the amino acid composition in the *E. coli* under stress, analyzing the changes in the Glycosylation pattern of the rhuIFN α 2a protein molecule and genetically modifying the *E. coli* strain with a minimal genome content could be an extension of the present study.

5.2 Electroelution and an attempt to retain the small Heat Shock proteins:

The conventional purification step for the rhuIFN α 2a produced has not been followed and in spite of a simple or rather crude Electroelution process of the rhuIFN α 2a protein

bands directly from the SDS-PAGE has been performed. Although Electroelution is a well-described method, it usually requires appropriate apparatus. This method is efficient and reproducible enough to be used as a routine method for eluting multiple protein bands, allowing considerable time saving. It is simple, fast, and cost-effective and does not require prior knowledge of the protein characteristics. However it is not possible to predict protein recovery, because this parameter varies according to the particular amino acid studied. Clearly, when a certain protein is excreted in very small quantities, its visual identification could be difficult despite high activity levels. On the other hand, for proteins with high expression levels, this method offers various advantages.

Researchers often use sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) as an analytical tool to assess protein purification. Because of its excellent ability to resolve individual components of complex mixtures, SDS-PAGE may be used not only for evaluating purity but also as an active step in the purification process. Protein purification by gel electrophoresis has been used in various applications such as antigen preparation for antibody generation and isolation of proteins for identification by N-terminal sequencing and mass spectrometry.

Polyacrylamide Gel Electrophoresis (PAGE) is both an analytical and preparative tool widely used in protein analysis. Proteins are separated by PAGE in an electric field according to their size, shape and charge both in their native configuration (native PAGE) or in the presence of denaturing agents such as the ionic detergent sodium dodecyl sulfate (SDS-PAGE).

Elution is accomplished in an electric field that moves the protein out of the gel slice into a gel zone made of agarose, polyacrylamide or polypropylene, depending on the specific electroelution device used. The eluted fraction is finally collected in reservoirs. The eluted protein is diluted significantly during the process and often requires further concentration and desalting. Although, elution times can be long for some protein preparations, leading to protein denaturation.

Thus, Electroelution has been tried in this study to minimize subsequent loss of proteins in different conventional purification steps. This was also to ensure that the aim of keeping the small Heat Shock proteins like the IbpA and IbpB etc. together with the expressed $\text{rhuIFN}\alpha 2\text{a}$ protein molecule be fulfilled. Following transcription and translation, the fate of any recombinant protein within *E. coli* is typically one of the following: its proper folding to a native biologically active form, its proteolytic degradation, or its accumulation in the form of biologically inactive inclusion bodies (IBs). Therefore, depending on the characteristics of the protein to be produced, one should decide how to produce the protein. For example, if a protein of interest contains a number of disulfide bonds, it will most likely be difficult to refold to a functional form. On the other hand, if the protein is relatively easy to refold, one can produce it in an inclusion body form, which has several advantages, such as easy recovery of protein and less contamination by other cellular proteins, and less degradation by proteases. Considering the above facts, the strategy of the electroeluting the over expressed $\text{rhuIFN}\alpha 2\text{a}$ protein band from the SDS-PAGE gels has been considered. This would give the total $\text{rhuIFN}\alpha 2\text{a}$ produced in the *E. coli* cells irrespectively of its soluble and

insoluble form, and would definitely minimize the chances of losing the proteins in each purification steps of chromatography purification techniques. Besides, its also been hypothesized that electroeluting the rhuIFN α 2a protein would help in retaining the small Heat Shock proteins with it which is otherwise not possible.

It is assumed that such simple techniques of purification could not only be cost effective compared to the modern chromatographic techniques but at the same time could be useful to procure the rhuIFN α 2a protein molecule together with the small Heat Shock proteins which otherwise gets unutilized in the protein specific column purification techniques.

The main aim of Electroeluting the rhuIFN α 2a protein was to minimize the loss of associated small Heat Shock proteins if any from the rhuIFN α 2a protein produced. It is mentioned earlier that, Heat Shock proteins chaperone antigenic peptides. In addition, Heat Shock proteins have a remarkably strong and unusual adjuvant property. They direct the immune system to mount both antibody and cytotoxic T cell responses to the tumor-specific peptides, which they carry, and the latter response is highly prized in cancer vaccines. They are the first adjuvants of mammalian origin to be discovered [236]. Professional antigen-presenting cells usually present antigens taken up by endocytosis, the anticipated route of uptake for Heat Shock protein-peptide complexes, in the context of Major Histocompatibility Complex (MHC) Class II receptors, which elicits an antibody response. The unusual aspect is that these complexes somehow cross over into the MHC Class I pathway to elicit strong T-cell responses against the peptides, and this was unexpected [242]. Receptors for Heat Shock proteins on the surface of antigen

presenting cells have been reported [235], and these may target the Heat Shock protein complex uniquely in the endocytic pathway.

The first formal prediction and indeed the first indication that Heat Shock protein-specific receptors exist came from the work of Srivastava *et al* in 1994 [241]. Further indications that Heat Shock protein-specific receptors exist came originally from studies demonstrating a correlation between Hsp70 on the surface of tumor cells and increased sensitivity to cell-killing by allogeneic natural killer cells [237], and the demonstration that Hsp70 binds to the cell surface and is internalized by human promonocytes [237]. Heat Shock proteins also stimulate antigen-presenting cells to secrete pro-inflammatory cytokines and they are players in the maturation of dendritic cells. In recognition of this unique role, Calderwood and colleagues have coined the term 'chaperokine' to highlight the dual functions of Hsp70 as both cytokine and molecular chaperone [234].

Some further examples include the demonstration that heat and arsenite activate the nonclassical histocompatibility class I gene encoding HLA-G, a potential signalling molecule in the immune response to stress [239]. Another signalling story involves the Hsp90-specific inhibitor geldanamycin. Treatment of T-lymphocytes with this compound blocks interleukin-2 secretion, IL-2 receptor expression, and T-cell proliferation, implicating Hsp90 in the kinase-mediated signalling events of T-cell activation [240].

Thus, the above findings definitely justifies the intention of isolating the $\text{rhuIFN}\alpha 2\text{a}$ protein molecule in a crude form in order to retain the small Heat Shock proteins expressed together with the $\text{rhuIFN}\alpha 2\text{a}$ protein under the applied heat stress. Whether, the approach was successful or not could be analyzed further in this section.

The total protein concentration in the *E.coli* growth hour samples and in the Electroeluted rhuIFN α 2a protein samples were quantitated by the Miller's method. The results indicate an increase in the Specific activity of both the Heat Shock and the Non Heat Shock rhuIFN α 2a protein molecule from the original *E.coli* growth hour samples after the SDS-PAGE purification process. Though the Percentage yield and the Purification factor for both the Heat Shock and the Non Heat Shock rhuIFN α 2a protein molecule was found to be low nevertheless the objective of purifying the rhuIFN α 2a protein molecule from the original *E.coli* growth hour samples in a crude form (to retain the maximum number of small Heat Shock proteins with the Eluted rhuIFN α 2a protein molecule) is successful. Advanced purification process like the Chromatography techniques could have given a more purified form of the rhuIFN α 2a protein molecule without the small Heat Shock proteins. The SDS-PAGE of the Electroeluted rhuIFN α 2a protein molecule (Figure 14) shows that the protein was managed to purify to a decent state.

5.3 IFN Bioassay:

Both the Heat Shock and the Non Heat Shock Electroeluted rhuIFN α 2a protein molecule was utilized for checking the difference in their antiviral potency against the Encephalomyocarditis Virus (EMC) on WISH Human Amnion Cell lines.

WISH cells are transformed human cell line with epitheloid morphology originally established from amnion epithelium. This cell line is used to assay human IFN-alpha and IFN-gamma . The interferons are quantified by performing a cytopathic effect inhibition assay of Encephalomyocarditis Virus (EMC) .The cells are significantly more sensitive

for both IFN-alpha and IFN-gamma when EMCV (Encephalomyocarditis virus) is used instead of Vesicular Stomatitis Virus (VSV) [124,125,139-162].

The antiviral state of these cells is enhanced in the presence of mixtures of human interferons . IFN-gamma inhibits the rise in Transferrin receptor mRNA level, which is normally observed when stationary WISH cells are stimulated to proliferate.

Pretreatment of WISH cells with TNF-alpha , IL1 , and TNF-beta induces 2-5A synthetase activity, protects them from EMCV effect, and markedly reduces virus yields. Inclusion of polyclonal antibodies to IFN-beta during cytokine pretreatment abrogates the antiviral state elicited by these cytokines . The antiviral activity of TNF can be attributed to the induction of IFN-beta [124,125,139-162].

Alpha-type antiviral protein is a factor, presumably protein in nature that mediates specific interferon inhibition of virus replication. According to studies of mouse-man hybrid clones, the locus determining this protein is carried on chromosome 21. This character was also called interferon sensitivity (IS) [124,125,139-162]. It has been showed that trisomy-21 cells (e.g. WISH cells) have increased IFN sensitivity. Trisomy-16 cells have reduced sensitivity [139]. This might suggest the presence on chromosome 16 of a regulator of mouse antiviral protein. Antibody to a cell surface component coded by human chromosome 21 inhibits the action of IFN [153]. This suggested that antiviral protein is an interferon receptor. In trisomy-21 fibroblasts, an exaggerated response to both classic (virus-induced) and immune (phytohemagglutinin-induced) forms of IFN has also been demonstrated [142].

Commonly used methods for the detection of IFN- α and IFN- β include immunometric assays, such as ELISA. These binding assays require the use of (mono) specific anti-human IFN- α and anti-human IFN- β antibodies and do not inform the bioactivity of the interferons tested. Thus, measurement of the bioactivity of IFN- α and IFN- β is often desirable.

The rhuIFN α 2a protein produced under the Heat Shock condition and purified by the SDS-PAGE system was found to be biologically active and the hypothesis set at the beginning of the work that Heat Shock might influence the overall antiviral activity of the rhuIFN α 2a protein produced was also confirmed. In the IFN bioassay experiment (Figure 17) the Heat Shock Eluted rhuIFN α 2a protein samples showed a higher IFN titer than the Non Heat Shock Eluted rhuIFN α 2a protein samples indicating a possible beneficial effect of Heat Shock on the rhuIFN α 2a protein production with respect to its antiviral activity.

5.4 Anti-HIV activity of the Electroeluted Heat Shock and Non Heat Shock Electroeluted rhuIFN α 2a molecule:

The next section in this work was focused on the anti-HIV activity of the Electroeluted Heat Shock and Non Heat Shock Electroeluted rhuIFN α 2a molecule. As Heat Shock and the Heat Shock proteins are the core point in this work, from the very beginning it has been tried to analyze the results of this whole research keeping the effect of Heat Shock on the rhuIFN α 2a molecule in mind. Thus, before discussing results of the Anti-HIV testing of Heat Shock and Non Heat Shock Electroeluted rhuIFN α 2a it also becomes important to highlight the negative effects of the Heat Shock proteins which could contribute to any unexpected results of the Anti-viral testing.

Molecular chaperones (Heat Shock proteins) are important components of cellular networks, such as protein–protein and gene regulatory networks. As discussed earlier, chaperones participate in the folding of immunologically important proteins, presentation of antigens and activation of the immune system. It has also been proposed lately that, chaperone-related immune dysfunction might be more general than was previously thought [245-279].

Mutations and polymorphism of chaperones and the regulators of their synthesis, Heat Shock factor-1, chaperone diseases, sick chaperones and chaperone overload might all affect (mostly impairing) immune responses. The contribution of chaperones to immune dysfunction is larger than previously thought. Deleterious mutations of chaperones, chaperone polymorphisms, impaired chaperone production, chaperone damage and chaperone overload might all lead to chaperone-related immune dysfunction [245-279].

Reverse Transcriptase Assay:

The mode of action of interferon has already been discussed in the earlier paragraphs. IFN α mediates its signal transduction by binding to its receptor on the cell surface, which initiates a cascade of other systems, which are involved in the IFN mode of action. Those are 2-5A oligosynthetase / RNase L, The Mx system, Guanylate Binding Protein (GBP), 9-27 and nitric oxide synthase (iNOS) systems. But, whether interferon also acts as a Non Nucleoside Inhibitors (NNIs) or not was an interest to find out.

Thus, in the present work an attempt has also been made to see the antiretroviral affect of IFN alpha 2a molecule as a NNI for the HIV-RT enzyme. The elctroeluted rhuIFN α 2a has been targeted directly against the HIV-RT enzyme too see its affect on the viral replicon system. IFN is already in use as an alternative antiretroviral therapy but the effect could be made very specific if the IFN protein molecule is made to target directly to the RT enzyme during the treatment procedures. By developing an adequate drug delivery system, targeting the IFN molecule directly to the HIV-RT enzyme could be achieved. Till now, there is no published data on such attempts, but the possibility of using IFN as a NNRTI also could not be ruled out.

As a result of intense efforts to identify inhibitors of viral replication, three classes of drugs are available to treat patients infected with HIV: the nucleoside reverse transcriptase inhibitors (NRTI), the non-nucleoside reverse transcriptase inhibitors (NNRTI), and the protease inhibitors (PI). The currently accepted standard of medical treatment of HIV infection involves the use of a combination of at least three drugs that includes members from at least two of the three classes of drugs [120-123,189-193].

The HIV-1 genome contains at least nine different genes, of which the major three, *gag*, *env*, and *pol*, have homologues in all retroviruses. The mature HIV-1 core particle is made up of four polypeptides encoded in the *gag* gene [120-123,189-193]. These four polypeptides designated p17 (matrix, MA), P-24 (capsid, CA), p7 (nucleocapsid, NC) and p6 are formed by cleavage of the 55 KD *gag* precursor protein by the virally encoded protease. The *env* coding sequence gives rise to two glycoproteins, gp120 and gp41, that are incorporated into the membrane envelope of the HIV-1 virion. Target cell recognition

is mediated by gp120; gp41 is required for fusion of viral and target cell membranes. The *pol* gene encodes a large polyprotein that is cleaved to yield the viral protease (PR), reverse transcriptase (RT) and integrase (IN) [120-123,189-193]. The active form of the PR is a homodimer formed from two copies of the p10 protein encoded at the 5' end of the *pol* gene. The functional reverse transcriptase is also a heterodimer formed from the p66 translated from the *pol* mRNA and a fragment of p66, p51, formed by PR mediated cleavage of p66. All of the *pol* gene products are localized within the capsid of the virion.

The currently approved therapies for the treatment of HIV infection act on two targets encoded on the *pol* gene, the viral reverse transcriptase (RT) or the viral protease (PR).

The use of reverse transcriptase is a unique characteristic of retroviruses such as HIV. After HIV has attached itself to a human cell and released its genetic material in the form of RNA into the cell, reverse transcriptase converts the viral RNA into a piece of DNA using the 'nucleotide' building blocks in the cell. This process is known as reverse transcription. Reverse transcriptase works by looking in turn at each of the nucleoside blocks that make up the viral RNA and then using it as a blueprint to assemble a matching DNA chain of nucleotides from the supply in the cell. This chain is held together by chemical bonds between the individual component nucleotides. The piece of viral DNA is called the provirus [120-123,189-193]. Another HIV enzyme called integrase then inserts this piece of viral DNA into the human DNA of the cell.

The main class of reverse transcriptase inhibitors is the nucleoside reverse transcriptase inhibitor (NRTI) drugs. They include 3TC (lamivudine, *Epivir*), abacavir (*Ziagen*), AZT (zidovudine, *Retrovir*), d4T (stavudine, *Zerit*), ddC (zalcitabine, *Hivid*), ddI (didanosine, *Videx / VidexEC*) and FTC (emtricitabine, *Emtriva*). NRTIs must be converted into their active 'triphosphate' forms within cells by a process known as phosphorylation. These NRTI triphosphates resemble the nucleotides found in the human cell [120-123,189-193].

Mutations in human immunodeficiency virus type 1 (HIV-1) are a major impediment to successful highly active antiretroviral therapy (HAART) and the design of anti-HIV vaccines [120-123,189-193]. Although HAART has made long-term suppression of HIV a reality, drug resistance, drug toxicity, drug penetration, adherence to therapy, low levels of continued viral replication in cellular reservoirs and augmentation of host immune responses are some of the most important challenges that remain to be sorted out. Continuing viral replication in the face of HAART leads to the accumulation of drug resistance mutations, increase in viral loads and eventual disease progression. Thus, it could be said that even if IFN fails to act as a NNRTI due to mutations in the HIV strain, it could still act as an antiretroviral drug independent of the consequences of the mutations unlike of the other antiretroviral drugs, which depends on either the structure of the HIV-RT enzyme or the viral capsid.

According to the Guidance for Industry; Antiviral Drug Development of the May 2005 report from the U.S. Department of Health and Human Services, Food and Drug Administration, Center for Drug Evaluation and Research (CDER) [194] a Phase I

clinical studies of an Antiviral drug could be initiated depending on the Nonclinical virology reports on the mechanism of action of the drug. One of the recommended mechanisms of action studies could include the investigational drug's ability to specifically inhibit viral replication or a virus-specific function or establishing the site of the drug's action (e.g., viral replicase, integrase, protease). Thus, the results of the RT assay to demonstrate the possible NNRTI activity of IFN could be justified as a significant data to support the alternative mechanism of action of IFN against the HIV.

Protein transduction and similar methods are promising techniques for delivering a wide variety of drugs directly into cells [195].

Most small-molecule drugs reach their targets because they are able to passively diffuse through the cell membrane. But reliance on passive diffusion limits the universe of drugs to those that are soluble in both the polar extracellular environment and the nonpolar cell membrane. Researchers in academia and start-up companies are working on ways to deliver all types of drugs--small molecules, peptides, proteins, and nucleic acids--without relying on passive diffusion.

A promising method is known as protein transduction. Despite the name, protein transduction can be used to deliver practically every type of molecule [195].

Several naturally occurring proteins have been found to enter cells easily, including the TAT protein from HIV, the antennapedia protein from *Drosophila*, and the VP22 protein from the herpes simplex virus. Specific short sequences within the larger molecule

account for the transduction abilities of these proteins. These peptides can be used to deliver a variety of molecules by covalently linking to them.

The process is not receptor mediated. Protein transduction can be used to target all cell types and that the process works regardless of the size of the cargo. In addition, protein transduction appears able to deliver cargo regardless of its composition. Everything from small molecules to oligonucleotides to peptides to proteins to iron beads to liposomes can be delivered using protein transduction. The ability to deliver larger molecules could lead to drugs with catalytic activity rather than simply inhibitors [195].

Though, most protein transduction work is still in the preclinical phase. However, two companies have candidates in clinical trials. One of Syntem's compounds is in Phase I clinical trial. CellGate has a product formulated for topical application in Phase II trials for psoriasis [195]. Thus applying recombinant IFN molecule directly against the HIV-RT enzyme in a HIV infected cell could not be a distant approach any longer.

Broadly speaking, the pharmacokinetic (PK) of a given antiretroviral drug are considered satisfactory when levels are maintained above the IC₅₀ of the virus (the concentration of the drug required to inhibit *in vitro* growth by 50%) [120-123]. It has been found that a new NNRTI, S-1153 has a 50% effective concentration for its inhibitory action in the range of 0.3 to 7 ng/mL [120-123]. Various Pyridine Oxide derivatives (e.g. JPL-133, JPL-71, JPL-10, JPL-30, JPL-44, JPL-88 and JPL-153) were explored for their potential anti HIV1-RT activity and it was demonstrated that the most active HIV1-RT inhibitors were JPL-133 and JPL-71 which inhibited HIV-1 RT, with IC₅₀s of 2.4 and 5.2 µg/mL,

respectively [120-123]. In the present study, the mean values of HIV1-RT percentage inhibition, obtained from the three sets of the experiments, shows a overall 33.33 % inhibition of HIV1-RT enzyme by 163.49 pg/mL of Heat Shocked rhuIFN α 2a protein molecule and a 28.11 % inhibition of HIV1-RT enzyme by 201.57 pg/mL of Non Heat Shocked rhuIFN α 2a protein molecule. Though the Heat Shocked rhuIFN α 2a protein molecule has showed a greater HIV-RT inhibition compared to the Non Heat Shocked rhuIFN α 2a protein molecule, it cannot be assumed that the difference between the two is due to the effect of Heat Shock since HIV-RT inhibition difference is not significant. But, compared to the above findings [120-123], it could be argued that the concentration of the rhuIFN α 2a protein molecule in the Electroeluted samples were relatively less to show a significant *in vitro* inhibition of the HIV1-RT enzyme. But, nevertheless the results shows that rhuIFN α 2a protein molecule could be used as NNRTIs provided adequate concentration of the molecule are targeted against the HIV1-RT enzyme.

P-24 Assay:

Since, P-24 Assay is considered as a sensitive test for HIV detection, both the Electroeluted Heat Shock and the Non Heat Shock samples were tested for the Anti – HIV I IIIB activity. In the assay the MT-2 cells lines were infected with the HIV I IIIB strain and simultaneously treated with the rhuIFN α 2a sample. The cell lysates from the experiment were then used as the sample for the P-24 Assay to understand the Ant-HIV activity of the Elctroeluted rhuIFN α 2a sample.

Viral isolation through viral culture, nucleic acid tests to detect viral RNA, and tests to detect P-24 antigen can be used to demonstrate virus or viral components in blood, or any other specific samples e.g. HIV infected experimental cell lysates, thereby verifying infection. These methods are highly specific, and a positive result confirms infection [196-217]. The P-24 antigen test is most useful in detecting antigen in supernatants from cultures that have been inoculated with cells from a patient suspected of being infected (viral culture and isolation). The antigen test is the method of choice for detecting the presence of free antigen in culture, and it is two orders of magnitude more sensitive than the reverse transcriptase assay. The test is most useful for detecting viral antigen in culture fluids because large quantities of antigen are exuded in the supernatant fluid during viral replication [196-217].

Interferons (IFNs) encode a family of secreted proteins that provide the front-line defence against viral infections [282]. It was recently shown that ISG20, a new 3'-->5' exonuclease member, represents a novel antiviral pathway in the mechanism of IFN action. In this report, it was shown that ISG20 expression is rapidly and strongly induced during human immunodeficiency virus type 1 (HIV-1) infection. In addition, it was demonstrated that the replication kinetics of an HIV-1-derived virus expressing the ISG20 protein (HIV-1 (NL4-3ISG20)) was delayed in both CEM cells and peripheral blood mononuclear cells. No antiviral effect was observed in cells over expressing a mutated ISG20 protein defective in exonuclease activity, suggesting that the antiviral effect was due to the exonuclease activity of ISG20 [282].

Levels of the antiviral factor type I interferon are dramatically reduced during primary HIV infection, but can be restored by early Anti-HIV therapy. These results suggest that HIV treatment early in infection, or use of artificial interferon therapy, may help the body to keep HIV under control [283].

In the first few weeks after HIV infection, the body attempts to attack the virus by producing antiviral factors as part of the 'innate' immune response. Immune cells called 'plasmacytoid dendritic cells' (PDCs) are responsible for the production of factors such as type I interferon, which co-ordinate the immune system's attack on HIV. Levels of type I interferon and PDCs are known to be reduced during long-term HIV infection, as the body loses the ability to keep HIV under control. However, few studies have examined innate immunity during the early stages of HIV infection, when a balance between the virus and the body's anti-HIV defences is set up.

Pharmaceutical versions of type I interferon, known as interferon alfa, are available for use in the treatment of viral infections such as hepatitis C infection. It has been speculated that interferon alfa treatment may be beneficial in helping the body to maintain its anti-HIV activity during primary HIV infection [283].

Unlike many anti-HIV drugs, which target the functions of HIV proteins, the antiviral activity of interferon-a is achieved by its ability to alter interactions between the host and virus in a complex manner.

The field of immune-based therapies (IBTs) is still in its infancy, but not so new that the reality of IBTs is outside the grasp of day-to-day use in the practice of medicine. There are currently approved and proven cytokine therapies that are routinely used by people living with HIV. These include cytokines like interferon-alpha, granulocyte colony stimulating factor and erythropoietin-alpha [283].

In the first set of experiment (the Anti-HIV testing of Heat Shock and Non Heat Shock Electroeluted $\text{rhIFN}\alpha 2\text{a}$), the result show significant anti HIV effect of the IFN standard, Heat Shock and Non Heat Shock Electroeluted $\text{rhIFN}\alpha 2\text{a}$ on the MT-2 cell lines. The 1:5 & 1:10 dilutions (5 PID sample) of Heat Shock and Non Heat Shock Electroeluted $\text{rhIFN}\alpha 2\text{a}$ and the IFN standard show a P-24 value less than the cut off value of 0.155 indicating a positive effect of the IFN standard and the Electroeluted $\text{rhIFN}\alpha 2\text{a}$ molecule on the MT-2 cell lines against the HIV 1-III strain. Both the Heat Shock and Non Heat Shock Electroeluted $\text{rhIFN}\alpha 2\text{a}$ has given a similar pattern of result where the 1:5 & 1:10 dilutions of both the samples found effective against the virus. This also shows the consistency of the test. The second experiment was performed to cross check the results for the first experiment, as the virus growth in the Virus Control well in the first experiment was relatively low. The Virus Control well in the second experiment showed a higher virus titer and still the 1: 5 dilution of the $\text{rhIFN}\alpha 2\text{a}$ samples showed an inhibitory activity against the HIV 1-III B strain. As assumed that, the Heat Shock Electroeluted $\text{rhIFN}\alpha 2\text{a}$ molecule might show a greater inhibition of the virus could not be seen from this result but nevertheless, the $\text{rhIFN}\alpha 2\text{a}$ biomolecule produced in this work was found to be active against the HIV 1-III strain. The study was indeed unique in

its way that till now there is no published data available showing the antiviral activity of Electroeluted rhuIFN α 2a biomolecule against the HIV 1-III B strain. The results are in contrast to the findings by David *et al* that IFN loses its bioactivity *in vitro* after 3-4 days [285].

Although the microtiter infection assay for HIV described in this report or by David *et al* should prove to be a powerful tool to the investigator, it probably falls short of measuring the full potential of at least one class of Anti-HIV agents, the biological response modifiers. These agents (i.e., IFNs etc.) have the potential to elicit pleiotropic responses *in vivo* beyond those, which can be measured by *in vitro* cell systems [285]. *In vitro* comparisons of these agents with other Anti-HIV drugs should always take this into consideration.

The progression toward AIDS of HIV type 1 (HIV-1)-infected individuals is marked by a progressive decline of CD4⁺ T cells caused not only by lysis of infected cells after their activation but also and more significantly by loss of the cellular immune response as evidenced by anergy and apoptosis of uninfected T cells. The view of some investigators is that the CD4⁺ T cell decline is caused chiefly, if not solely, by extensive HIV-1 replication with direct lysis of infected cells followed by a rapid CD4⁺ T cell replacement. But, recent findings showed that the decline in CD4⁺ T cells also importantly involves immunosuppression and apoptosis of uninfected T cells [287].

Immunosuppression of uninfected cells may be initiated *in vitro* by CD4⁺ T cell anergy, which in HIV-1 infection may be caused by an effect of the HIV-1 gp120 on these cells. Anergy is associated with dysregulation of cytokines, including lack of interleukin (IL) 2

and IL-12 and overproduction of interferon (IFN) γ and IFN α occurring at an early stage of HIV-1 infection. Chiefly macrophages and dendritic cells produce IFN α , and although it may exhibit antiviral effects, it also acts as a cytokine during cell activation; at low concentrations, it induces T cell proliferation and differentiation, whereas at higher concentrations it induces an antiproliferative effect [287]. It could be because of this reason that in the first and the second set of experiment in this work for the Anti-HIV testing of Heat Shock and Non Heat Shock Electroeluted rhuIFN α 2a an increase in the MT-2 cells were found in the 1:20 to 1:160 dilution wells of the IFN samples. Whereas the higher concentration like the 1:5 and 1:10 were found to be toxic for the MT-2 cells. In immunodeficient patients, serum levels of IFN α are elevated, although the half-life of this cytokine is only a few minutes.

Like gp120, the HIV-1 transactivator Tat protein is produced by infected cells and released into the extracellular compartment during acute infection. There is now evidence for extracellular Tat involvement in HIV-induced immune suppression. Thus, extracellular Tat may act as a viral toxin on uninfected T cells and trigger immunosuppression and apoptosis of these uninfected cells [287].

It has been reported that, during acute HIV-1 infection, immunosuppression of uninfected T cells is governed by the cytokine IFN α and by the HIV-1 Tat protein and that the early production of C-C chemokines by immune cells in response to activation depends on IFN α and Tat and markedly declines as T cell immunosuppression progresses in patients at an advanced stage of infection [287]. These findings are indeed contradictory to the present study where the IFN treated MT-2 cells were challenged with

the HIV 1 IIB strain, and although the higher concentration were found to be cytotoxic, it definitely inhibits the multiplication of the virus within the cell unlike the above findings where the IFN production leads to immunosuppression of the adjacent cells.

The permissible Endotoxin level in the Heat Shock Electroeluted $\text{rhIFN}\alpha 2\text{a}$ was found lesser than in the Non Heat Shock Electroeluted $\text{rhIFN}\alpha 2\text{a}$. Whether this is due to the application of heat shock or not could be an interest for further research.

5.5 Primary Structure Analysis:

As discussed in the previous sections, the Heat Shock-like response during heterologous protein synthesis may also enhance intracellular proteolytic activities. Furthermore, the host-vector system and the characteristics of recombinant protein to be produced affect the quality and quantity of recombinant protein obtainable. Therefore, amino acid composition determination of both the Heat Shock and the Non Heat Shock Electroeluted $\text{rhIFN}\alpha 2\text{a}$ protein molecule will make it possible to identify the exact changes in the amino acid composition if any, in the Electroeluted $\text{rhIFN}\alpha 2\text{a}$ protein samples. This might consequently allow the development of a custom-made production system.

One rational approach of exploiting the amino acid composition determination results in enhancing recombinant protein production is to identify potentially beneficial or deleterious effect of the Heat shock on the $\text{rhIFN}\alpha 2\text{a}$ protein production.

Determining a protein's structure involves first protein sequencing - determining the amino acid sequences of its constituent peptides; and also determining what conformation

it adopts and whether it is complexed with any non-peptide molecules. Discovering the structures and functions of proteins in living organisms is an important tool for understanding cellular processes, and allows drugs that target specific metabolic pathways to be invented more easily.

The two major direct methods of protein sequencing are mass spectrometry and the Edman degradation reaction. It is also possible to generate an amino acid sequence from the DNA or mRNA sequence encoding the protein, if this is known. However, there are a number of other reactions which can be used to gain more limited information about protein sequences and can be used as preliminaries to the aforementioned methods of sequencing or to overcome specific inadequacies within them.

But it is often desirable to know the unordered amino acid composition of a protein, prior to attempting to find the ordered sequence, as this knowledge can be used to facilitate the discovery of errors in the sequencing process or to distinguish between ambiguous results.

At times, the alteration of a manufacturing process includes the benefits of better yields, scale-up, new technologies and increased purity, as well as new formulations or product delivery systems. These alterations often lead to the variability of the therapeutic proteins or vaccines produced.

Variability of proteins can be localized to the protein backbone or to the carbohydrate moiety of glycosylated proteins. In monoclonal antibodies (mAbs), for example, protein

heterogeneity can be attributed to the formation of alternative disulfide pairings, deamidation and the formation of isoaspartyl residues, methionine oxidation, cyclization of N-terminal glutamine residues to pyroglutamate and partial enzymatic cleavage of C-terminal lysines by mammalian carboxypeptidases. Carbohydrate heterogeneity introduced during fermentation of mAbs can include differential addition of fucose, alternative mannose branching linkages, differential presence of terminal galactose residues (termed G0, G1, G2 molecular species) and sialylation. Heterogeneity analysis of therapeutic mAb glycoforms can be further complicated by differential carbohydrate occupancy at multiple sites. In addition, variations in manufacturing conditions can lead to alterations in glycosylation [163]. Glycosylation heterogeneity is not isolated to mAbs. It can occur in any glycosylated therapeutic protein, including cytokines (e.g., erythropoietin and granulocyte-macrophage colony-stimulating factor) and can affect the potency of the product. This most often occurs through differences in the pharmacokinetics and stability of the different isoforms.

One must also consider changes to the secondary, tertiary or quaternary structure of protein products that can be induced by any chemical changes, or under the influence of other factors (e.g., temperature and pH). Protein stability can be altered and can affect aggregation depending on formulation and the physical environment.

Changes in potency can occur through a variety of physicochemical or structural changes. Such changes can occur directly through altering the biological activity of the protein, or indirectly by altering the pharmacokinetics or bioavailability of the protein, often through

changes in glycosylation. For example, removing 20% of the total sialic acid content of a glycoprotein can result in a 50% loss in the amount of circulating protein [164]. Direct changes in potency can occur if the protein aggregates or is misfolded.

A change in immunogenicity profile due to a manufacturing change is a critical assessment for a protein therapeutic or vaccine. Immune responses can lead to anaphylaxis, cause neutralization of the product alone or of an endogenous counterpart, alter pharmacokinetics/pharmacodynamics by forming immune complexes, or have no effect. If an immune response is elicited, the resulting clinical sequelae can range from benign to severe. At one extreme, some therapeutics elicit neutralizing antibodies that cross-react with endogenous proteins (e.g., megakaryocyte growth and development factor [165] and epoetin [166]) and represent the 'worst-case' scenario, whereas other nonvaccine therapeutics produce tolerable immunogenicity without significantly affecting efficacy or safety [167].

Few common patterns emerge regarding the manufacturing changes that affect immunogenicity the most, other than perhaps those that alter aggregation. Changes in formulation can both increase and decrease immunogenicity; in some cases, this has been traced to changes in levels of aggregates. Product aggregation has been shown to induce immune responses for products, such as IFN [170,171], interleukin-2 and certain monoclonal antibodies [167,168]). Therefore, it is now considered very important to assess the status of aggregates in biological products.

Even though there are examples of altered immunogenicity following process changes, many process changes, including major ones involving changes in cell line, culturing conditions and purification steps, have had no effect.

rhIFN α 2 exists in different subtypes. rhIFN α 2a and rhIFN α 2c both differ in one amino acid from rhIFN α 2b. The majority of the human population expresses the gene encoding rhIFN α 2b [174]. Differences in immunogenicity seen in patients could not be related to differences in primary structure. Antibodies from patients treated with either rhIFN α 2 showed complete cross-reactivity [174].

As discussed above, several therapeutic proteins are glycosylated. Glycosylation is one of the most common posttranslational modifications. Glycosylation may differ by sequence, chain length, and position of linkage to the polypeptide chain and of branching sites [179]. Glycosylation is species- and cell-specific. Moreover, the culture conditions of production cells have direct effects on N-linked glycosylation patterns [180]. Thus, recombinant human glycoproteins will never have exactly the same glycosylation patterns as their endogenous counterparts. Naturally occurring human IFN α 2 is O-glycosylated at Thr106 [181]. No significant antigenic differences between glycosylated natural and nonglycosylated rhIFN α 2 proteins have been identified [182]. So, glycosylation probably does not affect the immunogenicity of human IFN α 2.

Recombinant human interferon beta (nonglycosylated) expressed in *E. coli* (*E. coli*-rhIFN β), and CHO-cells (CHO-rhIFN β , glycosylated) as well as (nonrecombinant human, glycosylated) interferon beta obtained from human fibroblasts (huIFN β) were compared for their immunogenicity. The three forms possess equal antiviral activities and a mouse monoclonal anti-huIFN β antibody had the same affinity for the three variants. However, the antiviral activity of CHO-rhIFN β was neutralized more effectively by mouse monoclonal anti-huIFN β than were huIFN β or *E. coli*-rhIFN β , which may be explained by glycosylation differences.

Physical degradation processes such as unfolding, misfolding, and aggregation of the protein can result in an immune response. Physical degradation of the protein can occur during the production and purification but can also result from improper formulation or storage or handling conditions. The choice of the formulation and the dosage form requires extensive research to guarantee the physical stability of the protein and thus minimize immunogenicity.

Oxidation and deamidation, sometimes followed by isomerization, are the major causes of chemical degradation of proteins (e.g., during storage or handling). One of the impurities found in rhIFN α 2a formulations because of improper storage was an oxidized form [175]. The oxidation sites were not determined. The oxidation product was isolated and compared with nonoxidized rhIFN α 2a for its immunogenicity in wild-type mice. The oxidized form was more immunogenic than the nonoxidized rhIFN α 2a [175,183]. Deamidation mainly occurs at asparagine residues. In this reaction, the asparagine residue

is, via a succinimide intermediate, converted into an aspartate or an iso-aspartate residue [184-186]. Also, glutamine residues can undergo deamidation resulting in glutamate residues. The rate of deamidation is dependent on the type of nearby amino acids and on the conformation [184,187]. Not much is known about the immunogenicity of deamidated proteins. It has been showed that, antibodies could be raised specifically against the succinimide derivative of peptides, showing weak cross-reactivity with the parent peptide [188].

The Primary structure analysis of the Electroeluted rhuIFN α 2a protein molecule was attempted by determining its amino acid composition. It was mainly done to find out any structural or residual differences between the Heat Shock and the Non Heat Shock Electroeluted rhuIFN α 2a protein molecule. The IFN bioassay results have already confirmed a sharp distinction between the IFN titer of the Heat Shock and the Non Heat Shock Electroeluted rhuIFN α 2a protein molecule. In this step, it was decided to pursue the idea of exploring the amino acid compositions of the Electroeluted rhuIFN α 2a protein molecule. As expected the anomalies between the Heat Shock and the Non Heat Shock Electroeluted rhuIFN α 2a protein molecule was confirmed from the results that, both the Heat Shock and the Non Heat Shock Electroeluted protein samples shows approximately equal amount of amino acid concentrations except the concentration of Aspartate in the Non Heat Shock samples are more than the Heat Shock samples and the concentration of the Glutamate are more in the Heat Shock samples than the Non Heat Shock samples. Now, whether these differences could be the reason for the differences in the antiviral property of the Heat Shock and the Non Heat Shock Electroeluted rhuIFN α 2a protein

molecule or not, is a matter of further research. Human IFN- α is a family of structurally related proteins that exhibit a wide range of antiproliferative activities and it could definitely be said that such differences do lead to differences in the immunological property of the IFN species.

For example, to understand the structural basis for these different antiproliferative activities, eight recombinant human IFN- α hybrids (HY) of α 21a/ α 2c (HY-4, HY-5) and mutants were expressed, purified, and characterized [244]. The data showed that the amino acid region 81–95 is important for antiproliferative activity [244]. Site-directed mutagenesis and cassette mutagenesis studies showed that if serine (S) 86 and asparagine (N) 90 were replaced by tyrosine (Y), the antiproliferative activity was increased. It has also been observed that if Y86 was replaced by isoleucine (I), the antiproliferative activity was comparable [244]. However, if Y86 was replaced by aspartic acid (D), lysine (K), or alanine (A), the antiproliferative activity was substantially decreased [244]. The results indicated that Y and/or I at position 86 and Y at position 90 are very important in antiproliferative activity of human IFN- α . Circular dichroism spectra showed that the amino acid replacements at position 86 did not change the secondary structure. Thus the biological activity changes among those mutants do not appear to be due to conformational changes [244]. The results also suggest that hydrophobic residue(s) at position 86 may be important for the interaction of the molecule with its receptor. The competitive binding data correlated with the antiproliferative activity. The N-terminal region of the molecule and the hydrophobic residues (including Y and I) on the C-helix

region at positions 86 and/or 90 are important for binding and antiproliferative activities of human IFN- α [244].

In the present work it has been found that the three main domains necessary for IFN bioactivity is present in an adequate concentration in the Electroeluted rhuIFN α 2a samples. The results also shows that, the amount of the detected amino acids (except Aspartate and Glutamate) present in both the Heat Shock and Non Heat Shock elctroeluted rhuIFN α 2a samples are almost same as expected. This might have been the reason of getting a positive biological activity of the rhuIFN α 2a protein molecule in the IFN Bioassay. But as expected the primary structure of the Heat Shock and Non Heat Shock Electroeluted rhuIFN α 2a protein molecule has not revealed much of difference. Most probably the structural anomalies might have occurred in the secondary structural level, which could have been analyzed by the advanced technologies discussed in the Introduction section. But the difference in the Aspartate and Glutamate concentration in both the Heat Shock and Non Heat Shock Elctroeluted rhuIFN α 2a molecule could be considered as an important point in context to future research. Like Renqiu *et al* (2001) [244], site directed mutagenesis of Asparagine / Aspartate and Glutamine / Glutamate on the rhuIFN α 2a molecule to see the change in the biological activity, could be one of the options among many.

SUMMARY

6. SUMMARY

From the experiments, it could be inferred that, the Heat Shock effect of 50°C for 5-10 minutes was probably too short to observe a definite effect in terms of production. However to the extent that an effect was observed it appears to be beneficial in terms of rhuIFN α 2a production. In the case of Heat Shock experiment by Harcum and Bentley (1999) [63], a beneficial and a definite effect of Heat Shock was only observed in the *E.coli* JM105 (pSH101) mutant. This mutant was defective in the stringent response. Therefore probably it will be only by genetically engineered strains of *E.coli* to have deleterious processes, such as stringent response inactivated, that beneficial stress can be applied to these proteins. The results indicated that Heat Shock and alternatively the use of the stringent response mutant host could both be used to facilitate increased recombinant protein yields in *E.coli* expression system (Harcum and Bantley, 1999) [63] and to increase its antiviral activity.

Also in the present study, the possibility of the presence of small Heat Shock proteins [75,89] with the Eluted rhuIFN α 2a protein molecule cannot be ignored. The results from the IFN Bioassay clearly shows that, the Heat Shock Eluted rhuIFN α 2a protein samples has a higher IFN titer than the Non Heat shock Eluted rhuIFN α 2a protein samples indicating a possible beneficial effect of Heat Shock (\ small Heat Shock proteins) on the rhuIFN α 2a protein production with respect to its antiviral activity.

Both the Heat Shock and the Non Heat Shock Elctroeluted protein samples shows approximately equal amount of amino acid concentrations except the concentration of

Aspartate in the Non Heat Shock samples are found to be more than the Heat Shock samples and the concentration of the Glutamate are more in the Heat Shock samples than the Non Heat Shock samples. It could be possible that increase in the Glutamate concentration might have triggered the increased antiviral activity of the Heat Shock rhuIFN α 2a against the Encephalomyocarditis Virus. As, a single deletion or substitution of an amino acid in a therapeutical protein greatly affects their biological property [172-174,176-178]; the increased concentration of Glutamate in the Heat Shock rhuIFN α 2a could be an important finding for the future research in the field of IFN.

As discussed earlier in the introductory chapters that, oxidation and deamidation, sometimes leads to chemical degradation of proteins (e.g., during storage or handling) [175,183,184-187], nothing could be concluded definitely at this stage, regarding the altered behavior of the Heat Shock rhuIFN α 2a. Deamidation mainly occurs at Asparagine and Glutamine residues to Aspartate and Glutamate [175,183,184-187]. The rate of deamidation is dependent on the type of nearby amino acids and on the conformation [184,187]. Thus, it can be stated that the application of Heat Shock might have lead to the difference in the Aspartate and Glutamate concentration in the Electroeluted Heat Shock and Non Heat Shock rhuIFN α 2a protein samples.

The biological activity of the Electroeluted Heat Shock and Non Heat Shock rhuIFN α 2a protein samples against the HIV I is indeed an important findings in the field of antiretroviral. The production and purification procedure of the rhuIFN α 2a protein molecule followed in the present study and studying its antiviral activity against the HIV

I strain by the reduction in the P-24 quantity and percentage inhibition of the Reverse Transcriptase enzyme are the new approaches in the field of IFN research. The data and the results could definitely be useful to understand more on the antiviral activity of the IFN against HIV. It gives a picture that IFN might have the ability to act as an immunomodulatory & NNRTI as described by Daniel *et al*, 1998 that, IFN in higher doses might lead to immunosuppression but in lower doses could remain effective in inhibiting the virus binding to the cells or the virus multiplication in an infected cell.

Thus, the goal ahead is to optimize the Heat Shock effect during the main fermentation procedure so that it not only helps in increasing the rhuIFN α 2a protein production but also at the same time enhances its antiviral and other immunomodulatory properties. The main aim of the work to indigenously produce a bioactive rhuIFN α 2a protein molecule was successful. The findings from the study could contribute towards a cost effective process of indigenous production, purification and application of the rhuIFN α 2a protein molecule.

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7. REFERENCES

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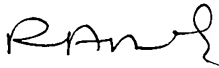
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**SYNOPSIS OF THE THESIS TO BE SUBMITTED TO THE UNIVERSITY OF
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Indigenous production and application of recombinant Interferon alpha using Recombinant DNA technology

INTRODUCTION

Since the discovery of interferon (IFNs) by Isaacs and Lindenmann and the elucidation of their biological actions, they have long been regarded as potentially very efficient antiviral and antitumor agents. The interferons represent proteins with antiviral activity secreted from cells in response to a variety of stimuli. In mammals, class I IFN (IFN) genes form a super family consisting of three gene families, the alpha interferon (IFN- α), the beta interferon (IFN- β) and the omega interferon (IFN- ω) genes [35]. In humans the IFN- α family comprises more than 20 genes and pseudogenes [40] giving rise to 15 different functional gene products. The various species of human IFN- α are closely related in amino acid sequences with homologies in the range of 80 to 100 %. The molecular weight of the recombinant human IFN- α species is about 19 kDa consisting of 166 (165 for IFN- α 2) amino acid residues lacking any N-glycosylation (α 14 has N-glycosylation). The cysteine mediated disulphide bonds are essential for the biological activity of IFN- α . The secondary structure of IFN- α was determined to be mainly α -helical. Target analysis of human IFN- α suggests that the functional unit is a monomer [36]. The genes coding for all known class I interferons have been located to chromosome 9, the coding sequences (cDNAs) are sub cloned and characterized. High-level expression of the interferons was achieved in *E. coli* giving rise to a protein essentially identical to the natural protein [1,2,5,6,42]. The interferons exhibit a huge number of biological effects. The antiviral activity led to the name interferon and serves

to define the unit of interferon activity. On purification of the natural human leukocyte interferons (IFN- α), it was found that all fractions that exhibited antiviral activity also exhibited anti-growth activity. This observation was confirmed with purified recombinant interferons and extended to other activities like: stimulation of cytotoxic activities of lymphocytes and macrophages, natural killer cell activity as well as increase in expression of some tumor-associated antigens [36]. The antiproliferative and antitumor activities of interferon have led to the application as an antitumor agent. The interferons also modulate cellular differentiation. A major effect of interferons is their modulation of antigens of the major histocompatibility complex (MHC). All interferons induce an increase in surface expression of class I MHC antigens. Expression of the *Fc* receptors is also stimulated by interferon. Alterations in surface antigens may be an important mechanism by which interferon can modulate cellular interactions. The interaction of the interferons with their receptors determines the biochemical events and their modulation of cellular functions. This is a complex process just in the beginning to be dissected [42]. The role of IFN- α as a disease marker and marker for immunotherapeutic approaches has been demonstrated for a number of different indications and pathological situations:

- During the acute phase of a viral infection IFN- α levels are significantly elevated in the majority of patients. IFN- α levels fall significantly during the period of convalescence at the time viral infection is indicated by seroconversion tests [37].
- Increased levels of IFN- α were found in the majority of patients suffering from inflammatory arthropathies like juvenile polyarthritis, rheumatoid arthritis, ankylosing spondylitis, polychondritis, psoriatic arthritis, polymyalgia rheumatica and scleroderma. Elevated IFN- α levels have also been shown for other

autoimmune disorders like systemic lupus erythematosus and systemic vasculitis [39].

- Serum IFN- α levels can help to distinguish between children with nonspecific abdominal pain or mesenteric adenitis, and those with acute appendicitis [38].

The Biotechnology Industry has invested heavily in these agents and the worldwide market for interferons continues to increase. Recent clinical trials have shown that IFNs have limited but nevertheless clinically important role in cancer therapy and the treatment of viral diseases. Alpha Interferon (IFN- α) is a synthetic version of a protein, which occurs naturally in the body and plays an important role in the functioning of the immune system. In the test tube, it inhibits HIV by interrupting the last stage of viral reproduction. IFN- α treatments have previously been shown effective against a rare leukemia and genital warts. It has also been proved that IFN- α is an effective anti-Kaposi's Sarcoma agent in-patient with less advanced stages of immunologic decline.

Currently, IFNs for therapeutic use are produced from two major sources, natural IFNs from human leukocytes or leukocyte cell lines and recombinant IFNs produced in bacterial cells. Natural IFNs are considered to be superior as they consist of the entire complement of IFNs and have the proper structure, but they are expensive and time-consuming to produce. Bacterially produced recombinant IFN is cheaper and more efficient to make. Thus, a method for enhancing the production of recombinant IFN to make it less expensive to produce would be advantageous.

An inevitable result of high- level protein expression in *E.coli* is that the cells amino acid pools, energy supply and normal growth processes are stressed [1,2,5,6,8]. This can lead to increased production of protein chaperones [1-8]. The chaperones in the cytoplasm of *E. coli* like the ATP-dependent DnaK-DnaJ-GrpE and GroEL-GroES systems [12,13] are found to increase the fidelity of protein folding. Few other *E.coli* heat shock proteins include ClpB, HtpG and IbpA/B, belonging to the σ^{32} stress regulons. Thioredoxins 1 and 2, and glutaredoxins 1, 2 and 3 are involved in the reduction of disulfide bridges that transiently arise in cytoplasmic enzymes [24]. At the same time the stress can also lead to the induction of proteases, which would normally degrade misfolded, or denatured proteins but which may also degrade recombinant proteins [1,5,7,8]. Studies have shown that removing the stringent response (by using a ppGpp-deficient strain of *E.coli*) can increase the production of recombinant protein five folds [7]. In recombinant protein systems with high productivity expression systems the stress response generated by over expression is rapid and complete so that any beneficial affects at times may be masked [1- 10]. Thus the objective could be to strike a balance between the positive and negative effects of stress generates due to over expression of proteins. Heat shock protein e.g. HSP70 have been implicated in MHC class II [27] and in a transporter associated with antigen processing (TAP)-independent way MHC class I antigen presentation pathways [28,29]. Thus, this property would allow peptides bound to HSP70 to be presented more efficiently and would thus promote stronger stimulation of antigen-specific T cells [27- 29].

Heat shock proteins are extremely potent molecules [8,13,20,23,27], the importance of which to physiological and immunological processes is indicated by the high degree to

which their structure and function are phylogenetically conserved. Enhanced understanding of the various immunoregulatory mechanisms in which heat shock proteins are involved could help us to harness the power of these molecules for the control of inflammatory processes and the treatment of human disease.

The production of recombinant proteins rapidly and efficiently is of great importance in the Biotechnology Industry as a whole [5]. The development of fermentation processes for recombinant protein production can involve several time consuming and costly stages. Following shake flask studies the process is “scaled up” stepwise from small-scale fermenters to pilot plant production and can culminate in the definition of a large scale manufacturing process. The time to progress from small to large-scale production can be a limiting step in the introduction of a new product to the commercial market. The reduction of this time can thus be seen as a major objective. The optimization of protein production under fermentation conditions can be very time consuming and would be greatly facilitated by the use of accurate mathematical and computer models, which can predict the process of protein expression, by the organisms. Developments of such models require an accurate understanding of the biological process involved in the expression of foreign proteins by organisms such as *Eschericia coli*.

Production of Heat Shock proteins together with the recombinant proteins has already been found to increase the folding and thus the solubilization of the recombinant proteins [10,12-16]. Hence, the present study was designed to standardize an indigenous approach towards the $\text{rhuIFN}\alpha 2\text{a}$ production and to observe the potentially beneficial effects of Heat Shock on the $\text{rhuIFN}\alpha 2\text{a}$ protein production by the *E.coli* strain and also to observe

the change in the biological property or the antiviral activity of the rhuIFN α 2a protein molecule when expressed under the Heat Shock condition.

MATERIALS AND METHODS

- **Preparation of competent *E.coli* BL21 (DE3) cells and transformation of rhuIFN α 2a clone into the competent *E.coli* BL21 (DE3) cells:**

In the recombinant *E.coli* strain used in this study, the rhuIFN α 2a coding sequence was transformed into the *E.coli* BL21 (DE3) cells (Invitrogen). The protein expression system was under the control of an IPTG inducible *T7* promoter.

- **Culturing the transformed *E.coli* BL21 (DE3) cells on Ampicillin Luria Agar plates.**
- **Growth of the transformed *E.coli* BL21 (DE3) cells in the Luria Broth by the Scale-up process:**

Inoculum: For each growth a loopful of the strain grown overnight on ampicillin Luria Agar plate was used to inoculate 10mL of Luria Broth medium supplemented with ampicillin in each 50mL flask. The cultures were grown at 37°C for approximately 15-16 hrs at 150 rpm in an Orbital Incubator Shaker. After incubation, OD_{600nm} was measured and a volume calculated to give the equivalent of 100 μ L of culture at OD_{600nm} 1.0 and this was used for inoculation of the growth experiments.

Growth: The inoculated 250mL culture flasks containing 50mL culture broth were incubated at 37°C/150 rpm and samples were taken every 1 hr. for 11-13

hrs. A sample of the Inoculum was taken at inoculation and a sample was taken after growth overnight. OD_{600nm} was measured for each sample.

- **Standardization of the Growth curves of the transformed *E.coli* BL21 (DE3) cells under Heat Shock and Non Heat Shock condition:**

Heat shock: The cultures were given the heat shock of 50° C for 5 minutes when the OD_{600nm} reaches 0.4. This step was performed to generate heat stress response within the strains. In another experiment the culture volume was reduced to 40mL to induce a greater heat stress to the cells.

- **IPTG induction of the *rhulFNα2a* gene cloned into the competent *E.coli* BL21 (DE3) cells:**

rhulFNα2a gene was induced [33] by 100 μL IPTG at the culture biomass level of approximately 0.5 OD_{600nm} after the heat shock was applied to the culture.

- ***rhulFNα2a* protein production level under Heat shock and Non heat shock condition was estimated by SDS-PAGE and IFNα ELISA:**

SDS-PAGE: Hand cast gels were used to assess the protein production [33]. The gels were used in Vertical Gel Electrophoresis systems Mini Model (Bangalore Genei Pvt. Ltd. India) and run at 150 V for 2 hours. 50μL β-Mercaptoethanol was added to 950μL of sample buffer prior to use. The sample [biomass equivalent to 1.0 mL of O.D_{600nm} 0.3-1 (appx.)] was resuspended in 20μL of distilled water; 5 μL was used for SDS gel electrophoresis. The sample (5,8,9,Over night *E.coli* growth hour samples) was diluted to 50 μL with sample buffer and heated at 95 ° C for 10 minutes to lyse the cells and dissolve the sample proteins, before loading 40 μL on to the gels. Protein standard markers were loaded alongside the protein

samples to allow determination of the apparent molecular weight of the proteins of interest. Gels were run until the dye front reached the bottom. Gels were washed three times for 5 minutes in 200 mL – 300 mL distilled water and 300 mL Coomassie Stain was added to the gels. The gels were kept shaking for overnight on a regular Gel Rocker. It was then rinsed with 200 mL – 300 mL of distilled water. Digital images of the gels were taken using the UV-Visible Transilluminator for Gel Documentation and analyzed by the Universal Software (Zeineh Programs) from Advanced American Biotechnology (AAB).

IFN α ELISA: Interferon α enzyme immunoassay has been used (using the IFN α ELISA kit of Immunotech France; Cat No. IM3193 as per their insert) to assess the protein production (Fig. 1 & 2). The sample results were calculated by interpolation from a standard curve that is performed in the same assay as that of the sample. The curve was drawn, plotting on the horizontal axis the IFN α concentration of the standards and on the vertical axis the corresponding absorbance. The absorbance for each sample was located on the vertical axis and the corresponding IFN α concentration was read off on the horizontal axis.

- **Purification of the rhuIFN α 2a protein molecule by Electroelution of the rhuIFN α 2a protein from the cut bands of specific growth hours (both from Heat Shock and Non Heat Shock cultures) from the SDS-PAGE gels:**

The rhuIFN α 2a bands obtained from the SDS-PAGE gels were electroeluted using the Bio- Rad's Electro-Eluter (Model No.422) as per the company's protocol. To increase sample recovery, several bands (Bands of the 5, 8, 9 and the Over night growth hour samples of the Heat Shocked and the Non Heat

Shocked *E.coli* Strain were excised and put into two different Electroeluter tubes, one for Heat Shocked samples and another for the Non Heat shocked samples) were excised from the gel and minced and placed into the tubes. The entire module was placed into the buffer chamber. The lower buffer chamber was filled with ~600 mL of elution buffer. The upper buffer chamber was filled with ~100 mL of elution buffer. Elution is done at 8-10 mA/glass tube for 3 hours.

- **Dialysis of the Electroeluted rhuIFN α 2a molecule:**

The electroeluted IFN molecules were dialyzed against the refolding buffer [9,32,33]. The activity of the eluted protein is recovered by unfolding the protein with denaturants urea and then refolded by removing the denaturants by dialysis or dilution allowing the formation of native structure of protein. The dialysis bag (Dialysis Membrane – 50, Hi-Media Cat No.: LA 387) was dipped in distilled water for sometime and tied from one end. Approximately 500 μ l of eluted protein was suspended into the bag. The bag was tied from the other end. The bag was then dipped in about 25 mL of renaturation buffer containing urea (6 M) and kept overnight at 4-5 ° C. The renaturation buffer was replaced by a fresh 25mL of renaturation buffer without β -Mercaptoethanol containing (3 M) urea and kept for 8 hours at 4-5 ° C. After 8 hours, the renaturation buffer was further replaced with a fresh renaturation buffer without β -Mercaptoethanol containing (1.5 M) urea and kept overnight at 4-5 ° C. The renaturation buffer was then again replaced with a fresh 25 mL of renaturation buffer without urea and β -Mercaptoethanol kept for 8 h at 4-5 ° C. After 8 hours, the renaturation buffer is discarded and the renatured rhuIFN α 2a protein molecule from the dialysis bag

was collected in an eppendorf tube and stored at -20°C . The whole process was performed with intermittent stirring of the renaturation buffer in the beaker containing the dialysis bag.

- **Total protein estimation of the electroeluted rhuIFN α 2a and of the cell pellet obtained from both Heat Shock and Non Heat Shock cultures of transformed *E.coli* BL21 (DE3) cells by Miller's method:**

In this method, soluble proteins are treated with NaOH- Na_2CO_3 - CuSO_4 tartarate reagent before adding the Folin-ciocaltu reagent. The phenol reagent of Folin ciocaltu is essentially a phosphotungstate-phosphomolybdenum blue, which can be estimated calorimetrically. Proteins also reduce it and therefore it can be used for their estimation through the amount of color produced. The color development varies greatly with different proteins since it is almost entirely due to their content of tyrosine and tryptophan and the effect is presumably due to Cu complex of other amino acid. This increases sensitivity and reduces unequal chromogenicities of different proteins. Insoluble proteins are dissolved in NaOH and then treated with Na_2CO_3 CuSO_4 tartarate reagent. Various dilution (0-100 $\mu\text{g/ml}$) of the given standard BSA were made together with specific dilutions of the Heat Shock and Non Heat shock electroeluted rhuIFN α 2a protein molecule and the lysed growth hour cell pellets. .1 ml of Folin's solution was added, mixed well and kept at room temperature for 10 minutes. 1ml of Folin's Ciocaltu reagent was added to each test tube. The tubes were kept in water bath at 50°C for 10 min. Optical density of the test tube were taken at 660 nm. The sample results were calculated by interpolation from the BSA standard curve that is performed in the same assay

as that of the sample. The curve was drawn, plotting on the horizontal axis the BSA standard concentration and on the vertical axis the corresponding absorbance. The absorbance for each rhuIFN α 2a and lysed cell pellet sample was located on the vertical axis and the corresponding total protein concentration was read off on the horizontal axis.

- **To quantify the amount of rhuIFN α 2a electroeluted from the SDS-PAGE gels, IFN α ELISA of the electroeluted protein sample was performed with the Bender Med Systems ELISA kit (Cat No.BMS216).**
- **HIV-1 RT Inhibition Assay of the eluted rhuIFN α 2a protein by Thymidine uptake inhibition determination:**

The assay was performed with the electroeluted rhuIFN α 2a molecules using the Scintillation counter (Liquid Scintillation Analyzer; Model No. Tri-Carb 2100TR; Packard Bioscience, USA.) [11]. Reverse Transcriptase Percentage Inhibition in the presence of the rhIFN α 2a molecule was also calculated [42,43]. In this assay, oligo (dT) primes the incorporation of [32 P] dTTP by RT using a poly (A) template, and the labeled reaction products were bound to a DEAE membrane. The amount of RT activity was proportional to the intensity of the spots after exposure to X-ray film. Excising the DEAE paper spots and counting them in a Liquid Scintillation Counter obtained quantitative results. 100 μ L of complete RT mixture, 20 μ L of the neat HIV suspension (105 cpm) and 5 μ L of the electroeluted rhuIFN α 2a molecule (heat shock and non heat shock rhuIFN α 2a samples) were added into an eppendorf tube. 5 μ L of Phosphate Buffer Saline (PBS) together with 100 μ L of complete RT mixture and 20 μ L of the neat HIV

suspension (105 cpm) were taken in another eppendrof tube and were kept as a control. Eppendrof tubes were incubated at 37° C for 3 hours. The reaction was stopped by adding 25 µL of EDTA into each eppendrof tubes. 100 µL of the reaction mixture was spotted on to DEAE membranes for the Scintillation Counting. DEAE membranes were allowed to dry in a laminar flow hood (approx. 30-min); the membrane was then removed from the Containment Laboratory. The membranes were washed four times in 5% Na₂HPO₄ and then two times in D/W. The DEAE membrane was transferred into a Scintillation counting container containing the Cocktail mixture. The containers were then kept in the Scintillation Counting instrument for quantifying the Thymidine uptake. The counts were utilized to asses the inhibitory effects of the rhuIFNα2a molecule against HIV-RT.

$$\text{RT \% Inhibition} = \frac{\text{SAMPLE cpm} \times 100}{\text{PBS cpm}}$$

[Note: PBS = Phosphate Buffer saline (Control), cpm = Counts per minute]

- **IFN Bioassay of the elctroeluted Heat Shock and Non Heat Shock rhuIFNα2a proteins together with sonicated cell pellet samples of Heat Shock and Non heat shock transformed *E.coli* BL21 (DE3) cells on WISH Human Amnion Cell lines by the Cytopathic Effect (CPE) reduction technique:**

The assays [48,49] were performed in 96 wells flat-bottomed (micro titer) plates using WISH Human Amnion Cell lines 2-3 × 10⁴ cells were added to each micro titer plate in 0.1 ml of MEM (E) with 2% serum with a multichannel pipette. For

testing the antiviral activity, the rhIFN α 2a samples were added after the cells had attached on the next day. The samples were diluted with MEM and 2% serum in another sterile micro titer plate in 0.12 ml volumes using multichannel pipette and 0.1 ml was transferred to the plate with cell monolayers. The cell monolayer plate was then incubated at 37° C in a 5% CO₂ atmosphere for 18 hrs. Encephalomyocarditis Virus (EMC) was diluted to 10⁻³ in MEM (E) with 2% serum to yield a moi of 1-5 pfu/ml. 0.1 ml of the virus suspension was added and kept for incubation for 15-16 hours at 37° C in a 5% CO₂ atmosphere. Cytopathic effect (CPE) readings were taken after the incubation when the controls showed 4+ CPE. The plates were then washed under tap water and stained with Amido Black for 1hour at RT to confirm the subjective CPE readings. Dilution of sample showing protection of 50% of cell sheet was taken as endpoint. Standard (Reference) IFN (Natural Human Leukocyte IFN) was included in the assay. The titer was converted to IU/ml by multiplying with the factor 1 obtained by the formula:

$$\text{Factor} = \frac{\text{Expected titer of Standard IFN}}{\text{Observed titer of Standard IFN}}$$

- **Work to be done:**

- **HIV p24 Inhibition Assay with the electroeluted rhuIFN α 2a sample.**
- **Test for Endotoxin in the electroeluted rhuIFN α 2a sample.**

RESULTS AND DISCUSSIONS

Heat Shock effect and *rhulFN α 2a* protein production studies by SDS-PAGE and ELISA:

The effect of Heat Shock on the *E.coli* growth curves was distinct and found to be effective. When the culture volume was reduced to 40 mL, the effect of Heat Shock was found to be stringent and the culture failed to overcome the stress response but nevertheless showed a prominent lag, log and death phase (Figure 3). Figure 4 also indicates a distinct lag, log and a death phase. The heat response was mild when the culture volume was 50mL and the culture was found to overcome the stress response (Figure 5). Both the growth curves (Figure 3 & 4) are plotted with the mean O.D 600_{nm} values of three sets of reproducible *E.coli* growth experiments. The overall IFN secretion by the *E.coli* strain was increased (> 300 IU/ mL) when the culture volume was reduced to 40mL, maintaining the Heat Shock effect at 50°C for 5 minutes (Figure 1). The main objective of reducing the culture volume was to apply a greater thermal stress to the cells in the flask. In this experiment the culture showed an increase in the IFN production under the Heat Shock condition only at 7 and 11 hours, unlike the second experiment where the culture volume was maintained to 50 mL and was Heat Shocked at 50°C for 5 minutes. The growth hour samples (5,8,9 and Over night growth hour samples collected from three fresh set of reproducible *E.coli* growth experiments) were analyzed and *rhulFN α 2a* gene expression level has been studied by SDS-PAGE. Universal Software (Zeineh Programs) from Advanced American Biotechnology (AAB) was used to quantitate the *rhulFN α 2a* production (Table 1). According to the values in the Table 1, in the I set of experiment except the 8 hours growth, all the other growth hours showed an

increase in the rhuIFN α 2a production under the Heat Shock condition. In case of the II experiment, only 8 hours and over night growth hours samples showed an increase in the rhuIFN α 2a production and in the III set of experiment only 9 hours growth sample showed an increase in the rhuIFN α 2a production under the Heat Shock condition. An IFN α ELISA was performed with the growth hour samples to further confirm the results of the SDS-PAGE, as quantitative ELISA will be more accurate than the SDS-PAGE analysis system. Although the culture showed a leaky expression of the rhuIFN α 2a gene at 5 hours, nevertheless the 5, 8, 9 and the 12-hour growth samples (Over night) showed an increase in the rhuIFN α 2a production in the Heat-Shocked cultures (Figure 2). The ELISA of the *E.coli* growth hour samples (Figure 1 & 2) is the results of the mean values of three duplicate ELISA experiments with the growth hour samples (Samples for the ELISA have been taken from the three reproducible growth curves, Figure 4 is the result of the mean O.D_{600nm} values of those three sets reproducible growth experiments).

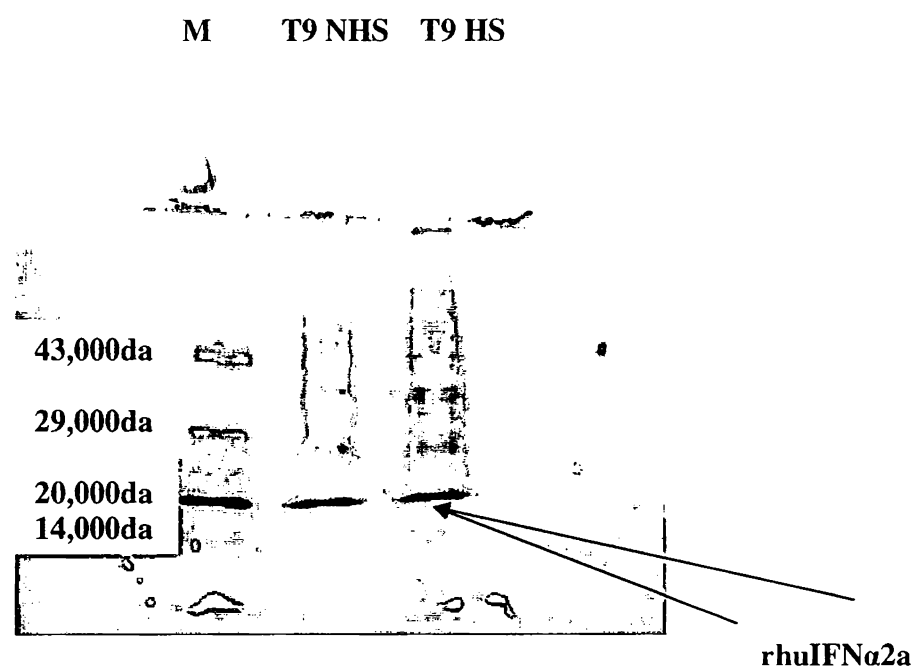


Fig.6. SDS-PAGE of nine hours Non Heat Shock (NHS) and Heat Shock (HS) growth hour samples. M = Molecular weight ladder.

Growth Hours	Heat Shock			Non Heat Shock		
	I Exp.	II Exp.	III Exp.	I Exp.	II Exp.	III Exp.
T5	79877	160313	51545	61181	219536	93194
T8	99188	249416	38931	113507	242986	127145
T9	91958	232245	55156	84870	243236	51711
Over night	109135	138975	161480	95817	122665	167735

Table 1

Amounts of rhuIFN α 2a produced in Heat Shocked and Non-Heat Shocked cultures of *E.coli* strain taken at 5,8,9 and over night (ON) hour's growth.

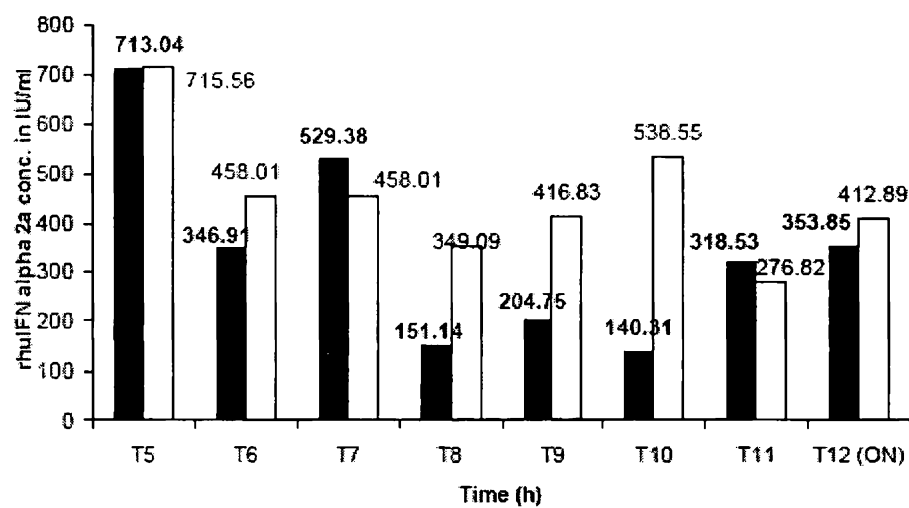


Figure1

rhuIFN α 2a conc. at different growth hours of *E.coli* strain.

Heat Shock  **Non Heat Shock** 

(Culture volume is 40 ml at the time of heat shock) ON = Over night



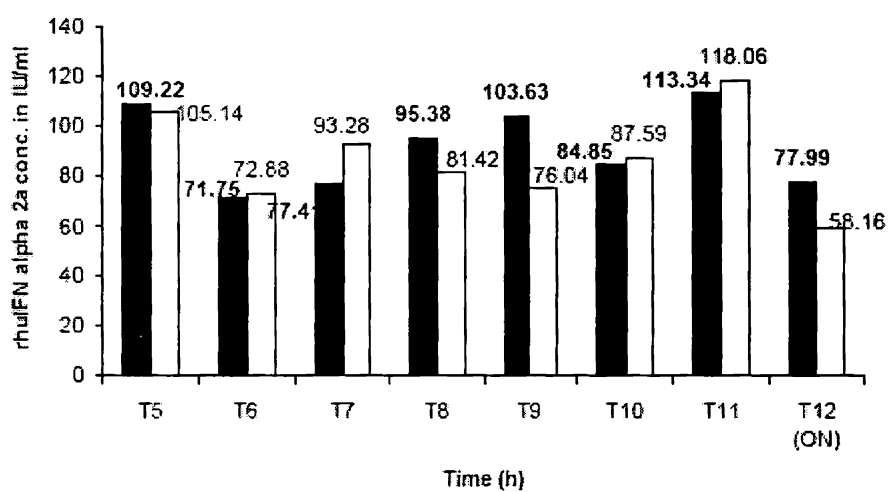


Figure 2

rhuIFN α 2a conc. at different growth hours of *E.coli* strain.

Heat Shock **Non Heat Shock**

(Culture volume is 50 ml at the time of Heat Shock) ON = Over night

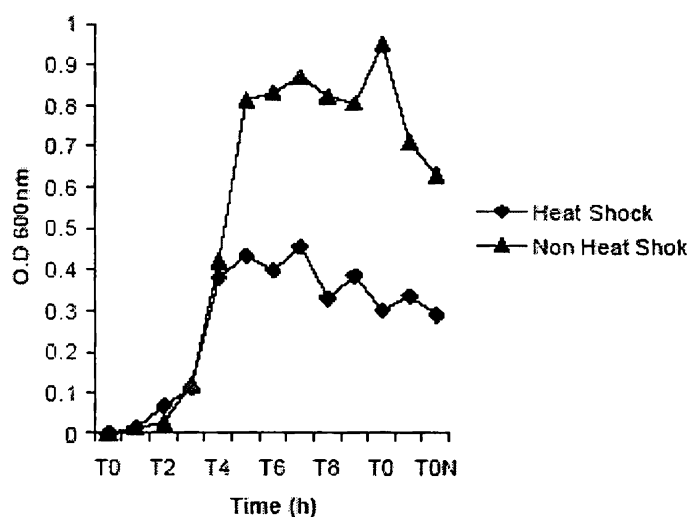


Figure 3

Growth of *E. coli* strain with and without Heat Shock (Culture Volume is 40mL at the time of Heat Shock) ON = Over night

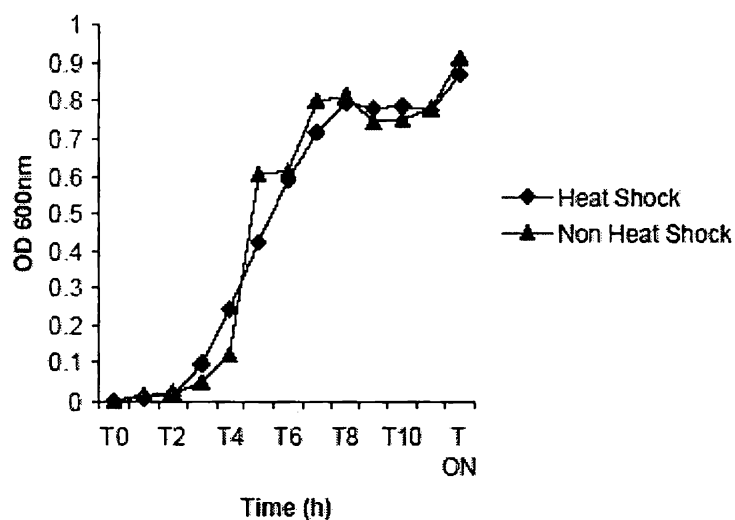


Figure 4

Growth of *E. coli* strain with and without Heat Shock (Culture Volume is 50mL at the time of Heat Shock) ON = Over night

It has been found that, over expression of recombinant proteins interferes with cellular processes in many ways [1,5,6,7-9]. Drainage of precursors and energy urges the cell to readjust metabolic fluxes and enzyme composition, when stress responses are induced, and hence the cellular activity is shifted from growth to reorganization of biomass. This may result in inhibition of growth or low level of product accumulation [1,5,6,7-9]. In the present experiments apart from the cellular inbuilt stress of the *E.coli* strains due to over expression of the recombinant protein, an external thermal stress is applied. Though, the effect is more distinct when the culture volume was reduced to 40mL nevertheless, in both the growth curves a slight inhibition of growth could be seen immediately after the application of Heat Shock. Bacteria are known to respond to unfavorable conditions, e.g., exposure to toxic chemicals and physical agents, nutrient limitation, or sudden increase in growth temperature, by rapid expression of regulons related to the Heat Shock, SOS, and oxidative stress responses [2,7-9]. The SOS responses allow the bacteria to overcome the detrimental condition and helps in adapting to the stressful situation. Recent investigations have revealed that the cellular content of many direct participants in protein synthesis (e.g., initiation factors, elongation factors, even RNA polymerase) are decreased when *E. coli* enters stationary phase as the result of nutrient and energy starvation. In particular, the amount of active ribosome is drastically different between fast- and slow- growing cultures [3]. This could indicate, that at that condition the cells might lead to lesser production of the desired recombinant protein contradictory to the findings that simultaneous expression of Heat Shock protein genes increases the expression of recombinant protein [4]. The results from the present study also indicates that the maximum production of the $\text{rhuIFN}\alpha 2\text{a}$ by the *E.coli* strain could be seen

between the 7 hours and the over night growth hours agreeing to the findings that simultaneous expression of Heat Shock proteins might contribute to the increased accumulation of recombinant proteins in the *E.coli* expression system. Under conditions of stress Heat Shock proteins constitute a major portion of prokaryotic cellular proteins [1,2]. Thus, in the present study, the role of Heat Shock in *E.coli* leading to an increased accumulation of rhuIFN α 2a molecule at the different growth hours is an important finding. Though the quantity of rhuIFN α 2a expressed in all the *E.coli* growth experiments (Figure 1 & 2) is not consistent but it has been observed that in each growth experiments at certain hours with elevated temperature, quantity of rhuIFN α 2a protein molecule is increased. The crucial finding is, when the culture volume is reduced to 40mL, the gene expression of the rhuIFN α 2a protein molecule has been found to increase approximately more than three folds than the rhuIFN α 2a protein production level in the 50mL culture. As more than 50°C might be detrimental for the *E.coli* strains, it could be assumed that a heat shock more than 5 minutes might be beneficial in terms of increasing the rhuIFN α 2a protein production level. The effect of Heat Shock on the viability of the 50mL cultures was found almost negligible as compared to the 40mL culture. The growth curves in the Figure 4 have been found to overcome the effect of heat stress rapidly and no difference in the growth process is evident. To argue regarding the significance of the difference in the rhuIFN α 2a production between the Heat Shock and the Non-Heat shock results it could be considered that the experiments were performed at the laboratory scale and the Heat Shock was applied only for 5 minutes. Considering the fact, that a Heat Shock increases the recombinant protein production level [12-26], optimum condition could be maintained for a larger scale operation, where a significant difference in the

rhIFN α 2a protein production level could be achieved. Besides, the quantity of rhIFN α 2a formed in the laboratory conditions could easily be scaled up many more folds at the Industry level. Ways of increasing the rhIFN α 2a production level even slightly, under the normal fermentation condition would be a welcome option for the industries. As seen in the previous experiments [34,10] that irrespective of the genetic modifications of the host strain and the usage of different synthetic promoters in the expression of the rhIFN α 2a in the *E.coli* expression system, an increase in the temperature in the main fermentation system during the log phase of the culture could definitely lead to an increase expression of the gene resulting to an increase intracellular accumulation of rhIFN α 2a protein molecule. Thus the above approach could be a solution for the development of a cost effective rhIFN α 2a molecule leading to an affordable interferon biomolecule for the drug market.

Total protein estimation:

The total protein concentration in the BL21 (DE3) *E.coli* growth hour samples and in the elctroeluted rhIFN α 2a protein samples were quantitated by the Miller's method. The results were used to determine the Specific Activity, Purification and the Percentage yield [52,53,54] of the SDS-PAGE purified rhIFN α 2a protein samples according to the following formulas:

$$\text{Specific activity} = \frac{\text{Number of protein units}}{\text{Total protein}}$$

$$\text{Percentage yield} = \frac{\text{Proteins units recovered}}{\text{Proteins units at start}} \times 100$$

$$\text{Purification Factor} = \frac{\text{Specific activity of purified material}}{\text{Specific activity of original material}}$$

Specific activity of the Heat Shock BL21 (DE3) *E.coli* 5 hours growth cell pellet sample of OD_{600nm} 0.5 (appx) = 72.67×10^{-9}

Specific activity of the Non Heat Shock BL21 (DE3) *E.coli* 5 hours growth cell pellet sample of OD_{600nm} 0.5 (appx) = 153.03×10^{-9}

Specific activity of the Heat Shock rhuIFN α2a protein sample = 1963.8×10^{-9}

Specific activity of the Non Heat Shock rhuIFN α2a protein sample = 4857.1×10^{-9}

Percentage yield of the Heat Shock rhuIFN α2a protein sample = 28.09 %

Percentage yield of the Non Heat Shock rhuIFN α2a protein sample = 54.08 %

Purification Factor for the Heat Shock rhuIFN α2a protein sample = 27.02

Purification Factor for the Non Heat Shock rhuIFN α2a protein sample = 31.55

The results indicates an increase in the Specific activity of both the Heat Shock and the Non Heat Shock rhuIFN α 2a protein molecule from the original BL21 (DE3) *E.coli* growth hour samples after the SDS-PAGE purification process. Though the Percentage yield and the Purification factor for both the Heat Shock and the Non Heat Shock rhuIFN α 2a protein molecule was found to be low nevertheless the objective of purifying the rhuIFN α 2a protein molecule from the original BL21 (DE3) *E.coli* growth hour samples in a crude form (to retain the maximum number of small Heat Shock proteins with the eluted rhuIFN α 2a protein molecule) is successful. Advanced purification process like the Chromatography techniques could have given a more purified form of the rhuIFN α 2a protein molecule without the small Heat Shock proteins.

In vitro inhibitory effects of rhuIFN α 2a against HIV-1 RTs:

Broadly speaking, the pharmacokinetic (PK) of a given antiretroviral drug are considered satisfactory when levels are maintained above the IC₅₀ of the virus (the concentration of the drug required to inhibit *in vitro* growth by 50%) [44-47]. It has been found that a new NNRTI, S-1153 has a 50% effective concentration for its inhibitory action in the range of 0.3 to 7 ng/ml [44-47]. Various Pyridine Oxide derivatives (e.g. JPL-133, JPL-71, JPL-10, JPL-30, JPL-44, JPL-88 and JPL-153) were explored for their potential anti HIV1-RT activity and it was demonstrated that the most active HIV1-RT inhibitors were JPL-133 and JPL-71 which inhibited HIV-1 RT, with IC₅₀s of 2.4 and 5.2 μ g/ml, respectively [44-47]. In our study, the mean values of HIV1-RT percentage inhibition, obtained from the three sets of the experiments, shows a overall 22.33 % inhibition of HIV1-RT enzyme by 163.49 pg/mL of heat shocked rhuIFN α 2a protein molecule and a 36.37 % inhibition of HIV1-RT enzyme by 201.57 pg/mL of non heat shocked rhuIFN α 2a protein

molecule. Compared to the above findings [44-47] it could be argued that the concentration of the rhuIFN α 2a protein molecule in the electroeluted samples were relatively less to show a significant *in vitro* inhibition of the HIV1-RT enzyme. But, nevertheless the results shows that rhuIFN α 2a protein molecule could be used as NNRTIs provided adequate concentration of the molecule are targeted against the HIV1-RT enzyme. The future research in this field could be an extensive pharmacokinetic study with the protein molecule. Thus, the project was having three objectives; one to find out that whether Heat Shock helps in increasing the quantity of the rhuIFN α 2a protein molecule or not, secondly to find out whether rhuIFN α 2a protein molecule could be used as a NNRTIs or not and third was to find out that whether the Heat Shock leads to the increase in the antiviral property of the expressed rhuIFN α 2a protein molecule or not due to the possible expression of the Heat Shock proteins with the rhuIFN α 2a protein molecule. An increase of temperature in the main fermentation system could lead to an increased expression of the rhuIFN α 2a protein resulting in an increased intracellular accumulation of rhuIFN α 2a protein molecule. The results also indicates that though an elevated temperature condition might be useful for increasing the rhuIFN α 2a protein production level it has no beneficiary effect on the non nucleoside inhibitory property of the rhuIFN α 2a protein molecule. Finally, rhuIFN α 2a protein molecule could be opted as a NNRTI.

IFN Bioassay of the electroeluted Heat Shock and Non Heat Shock rhIFN α 2a proteins together with sonicated cell pellet samples of Heat Shock and Non heat shock transformed *E.coli* BL21 (DE3) cells on WISH Human Amnion Cell lines by the Cytopathic Effect (CPE) reduction technique:

In case of the IFN Standard, the IFN titer was at the IFN concentration of 1 Unit. Thus, according to the above formula the Factor was 1. Sonicated cell pellet samples showed cell cytotoxicity for most of the dilutions. In case of one set Heat Shock rhIFN α 2a, the IFN titer or the End Point is between 1:20 & 1:40. Thus, the titer was taken as 1:30 or 300 Units/ml or 300×1 (Factor) = 300 IU/ml. In case of one set of Non Heat Shock, the IFN titer was at 1:10. Thus, the titer is 100 Units/ml or 100×1 (Factor) = 100 IU/ml. Similarly, for another set of Heat Shock rhIFN α 2a Sample, the IFN titer is at 1:40 dilution, therefore 400 IU/ml and for another set of Non Heat Shock rhIFN α 2a sample, the IFN titer was at 1:20 dilution, therefore 200 IU/ml. The Heat Shock eluted rhIFN α 2a protein samples showed a higher IFN titer than the Non Heat shock eluted rhIFN α 2a protein samples indicating a possible beneficial effect of Heat Shock on the rhIFN α 2a protein production with respect to its antiviral activity.

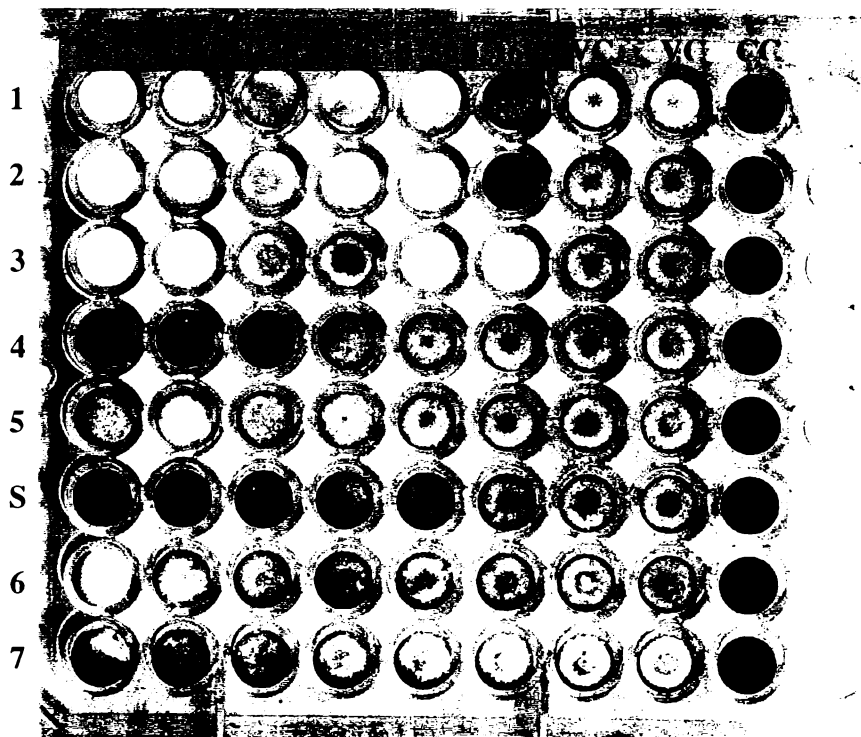


Figure 5. IFN Bioassay of the electroeluted Heat Shock and Non Heat Shock

rhuIFN α 2a proteins together with sonicated cell pellet samples of Heat Shock and Non Heat shock transformed *E.coli* BL21 (DE3) cells on WISH Human Amnion Cell lines by the Cytopathic Effect (CPE) reduction technique.

Samples: 1. Heat Shocked IPTG Induced *E.coli* growth hour pellets, 2. Non-Heat Shocked IPTG Induced *E.coli* growth hour pellets, 3. Non-Heat Shocked Non IPTG Induced *E.coli* growth hour pellets, 4. Heat Shocked rhuIFN α 2a eluted proteins, 5. Non Heat Shocked rhuIFN α 2a eluted proteins, 6. IFN standards, 7. Heat Shocked rhuIFN α 2a eluted proteins, 8. Non Heat Shocked rhuIFN α 2a eluted proteins. CC = Cell control, VC = Virus control, S = Standard IFN (From Left: 8 Units, 4 Units, 2 Units, 1 Unit) [Note: The dilution of Sample 5 has been started from left 1:10, 1:20, 1:40, 1:80, 1:160 and 1:320]

Thus, from the experiments done till now, it could be inferred that, the Heat Shock effect of 50°C for 5 minutes was probably too short to observe a definite effect in terms of production. However to the extent that an effect was observed it appears to be beneficial in terms of $\text{rhuIFN}\alpha 2\text{a}$ production. In the case of Heat Shock experiment by Harcum and Bentley (1999) [8], a beneficial and a definite effect of Heat Shock was only observed in the *E.coli* JM105 (pSH101) mutant. This mutant was defective in the stringent response. Therefore probably it will be only by genetically engineered strains of *E.coli* to have deleterious processes, such as stringent response inactivated, that beneficial stress can be applied to these proteins. The results indicated that Heat Shock and alternatively the use of the stringent response mutant host could both be used to facilitate increased recombinant protein yields in *E.coli* expression system (Harcum and Bantley, 1999) [8] and to increase its antiviral activity. Also in the present study, the possibility of the presence of small Heat Shock proteins [50,51] with the eluted $\text{rhuIFN}\alpha 2\text{a}$ protein molecule cannot be ignored. The results from the IFN Bioassay clearly shows that, the Heat Shock eluted $\text{rhuIFN}\alpha 2\text{a}$ protein samples has a higher IFN titer than the Non Heat shock eluted $\text{rhuIFN}\alpha 2\text{a}$ protein samples indicating a possible beneficial effect of Heat Shock on the $\text{rhuIFN}\alpha 2\text{a}$ protein production with respect to its antiviral activity.

Thus the goal ahead is to optimizing the Heat Shock effect during the main fermentation procedure so that it not only helps in increasing the $\text{rhuIFN } \alpha 2\text{a}$ protein production but also at the same time enhances its antiviral and other immunomodulatory properties. The findings from the study could contribute towards a cost effective process of indigenous production, purification and application of the $\text{rhuIFN}\alpha 2\text{a}$ protein molecule.

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