PURIFICATION AND CHARACTERIZTION OF ENGINEERED HUMAN INTERFERON GAMMA

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Certificate of Examination

This is to certify that the dissertation titled "**Purification and Characterization of Engineered Human Interferon Gamma**" submitted by **Mr. Shahanaz Nazar** (Reg. No. MS10010) for the partial fulfillment of BS-MS dual degree programme of the Institute, has been examined by the thesis committee duly appointed by the Institute. The committee finds the work done by the candidate satisfactory and recommends that the report be accepted.

Dr. Kausik Chattopadhyay Dr. Samarjit Bhattacharyya Prof. Purnananda Guptasarma

(Supervisor)

Dated:_____

Declaration

The work presented in this dissertation has been carried out by me under the guidance of Prof. Purnananda Guptasarma at the Indian Institute of Science Education and Research Mohali. This work has not been submitted in part or full for a degree, a diploma, or a fellowship to any other university or institute. Whenever contributions of others are involved, every effort is made to indicate this clearly, with due acknowledgement of collaborative research and discussions. This thesis is a bonafide record of original work done by me and all sources listed within have been detailed in the bibliography.

Shahanaz Nazar

(Candidate)

Dated: _____

In my capacity as the supervisor of the candidate's project work, I certify that the above statements by the candidate are true to the best of my knowledge.

Prof. Purnananda Guptasarma

(Supervisor)

Acknowledgement

I am greatly indebted to my supervisor of my MS thesis, Prof. Purnananda Guptasarma for the successful completion of the projects, which needed constant help, discussion and inspiration from him. The long hours of discussion about science and life had opened new world to me and changed the paradigm of science for me. My very sincere thanks and gratitude goes to him for all the support he provided me.

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The role of family, where begin all kinds of our initial learning and development to become a good, kind and successful, can't be denied. I acknowledge with gratitude the support of my parents, my brother and all other family members for their encouragement. But above all I express my thanks to 'Allah' for showering his blessings all through my life.

Shahanaz Nazar

List of Figures

Notation

μ- Micro

APS- Ammonium persulphate

ATP- Adenosine triphosphate

bp- Base pair

DNA- Deoxyribonucleic acid

DW- Distilled water

EDTA- Ethylene diamine tetra acetate

kb- Kilo base pair

kDa- kilo Dalton

L-Litre

LB- Luria bertani

M- Molar

mg- Milligram

mL- Millilitre

mM- milli molar

^{0}C- Degree Celsius

PAGE- Polyacrylamide gel electrophoresis

rpm- Rotations per minute

SDS- Sodium dodecyl sulphate

TAE- Tris-acetate-EDTA

TEMED- Tetramethyl ethylene diamine

PMA- phorbol myristate acetate

HLA-DR - Human Leukocyte Antigen - antigen D Related

MHC-II - major histocompatibility complex class II

Contents

ABSTRACT

Human interferon gamma (IFN-γ) is a cytokine which is critical for innate and adaptive immunity during infection. It is a highly aggregation-prone protein and tends to accumulate in inclusion bodies upon overexpression, especially in heterologous systems. In the present study, we describe the use of an engineered version of IFN-γ which is cloned in *E.coli*. The expressed protein is produced in both insoluble and soluble forms, with the larger fraction of expressed protein going into inclusion bodies. Our first attempt to obtain functional protein relied on refolding techniques (stepwise dialysis and/or dilution of denaturant from solution) after solubilization of protein from inclusion bodies through the use of denaturant(s). Refolding attempts did not yield properly folded structure, and we turned our efforts towards improving the fraction of soluble expressed protein, through the inclusion of excipients in the growth medium which are likely to enter expressing cells, and found that this led to greater yields of solution protein with lower levels of aggregation-prone behavior. The identity of the recombinant engineered protein was established by mass-spectrometry and further confirmed through western blotting. Further, the engineered protein was characterized to determine its quaternary structural status (oligomerization), folding, chemical and thermal stability using various biophysical techniques. The protein was found to be dimeric. The biological activity of engineered IFN-γ was evaluated by monitoring the activation status of macrophages (HLA-DR expression) and it was found to be an active protein

1. INTRODUCTION

1.1General introduction to proteins.

Proteins are organic compounds made up of one or more chains of amino acid residues in which residues are joined to one another by peptide bonds that form through the condensation of carboxyl and amino groups of adjacent amino acid residues. Proteins are responsible for carrying out most of the functional activities of the living system and for the functioning of each protein it is necessary for it to achieve its desired, native conformation. The native conformation is described at four levels of structural classification; primary structure (describing the sequence of a linear chain of amino acids and any disulfide bonds), secondary structure (describing the existence of the folded polypeptide chain in α-helix, β-sheet, β turn or randomly coiled structures), tertiary structure (describing the three-dimensional fold or structural motif or super-secondary structure) and quaternary structure (describing the state and stoichiometry of association of multiply folded polypeptide chains). In terms of quaternary structure formation, the natively folded protein sometimes multimerizes to homo or hetero-multimers, depending upon various factors such as pH, ionic strength and physical/chemical factors, as well as any pre-existing tendencies to associate.

The recombinant proteins formed *in vivo* can have different folding tendencies, i.e. they can fold native conformation directly, without intermediates, or through the formation of intermediate folding states that can generate a propensity to misfold. This tendency to misfold results in the formation of inclusion bodies, which can be ordered or disordered aggregates, forming within cells upon overexpression of a protein, partly due to the high concentration of protein within the overexpressing cell and partly owing to the details of the molecular mechanisms underlying the folding of specific chains, and the availability or lack of environmental cues and factors necessary for folding, in different expression systems.

Protein aggregation can occur because of natural folding and solubility characteristics of a protein, or due to mutations, non-optimal physical and/or chemical environment within cells, and non-optimal conditions utilized during purification and storage of the protein. However, aggregation can be minimized if a protein is small, easy to fold, and reasonably hydrophilic. Aggregation is often accompanied by precipitation, owing to a general loss of solubility

through greater burial of surface areas of chain. Importantly though, precipitation can also occur in near-native conformation, e.g., when the pH of the protein's environment approaches the isoelectric point (or pI) of that protein, due to the neutralization of charge(s) and association through hydrophobic interactions. Different kinds of aggregates exist : (1) amorphous aggregates (stabilized predominantly through hydrophobic interactions), (2) amyloid aggregates (stabilized predominantly by hydrogen bonds) and (3) soluble aggregates (which are not large enough to have lost solubility altogether). Soluble aggregates that are formed in the initial stages of folding can also tend to reduce the quality of native proteins. Various aspects of aggregation are described pictorially in Figure 1.1.

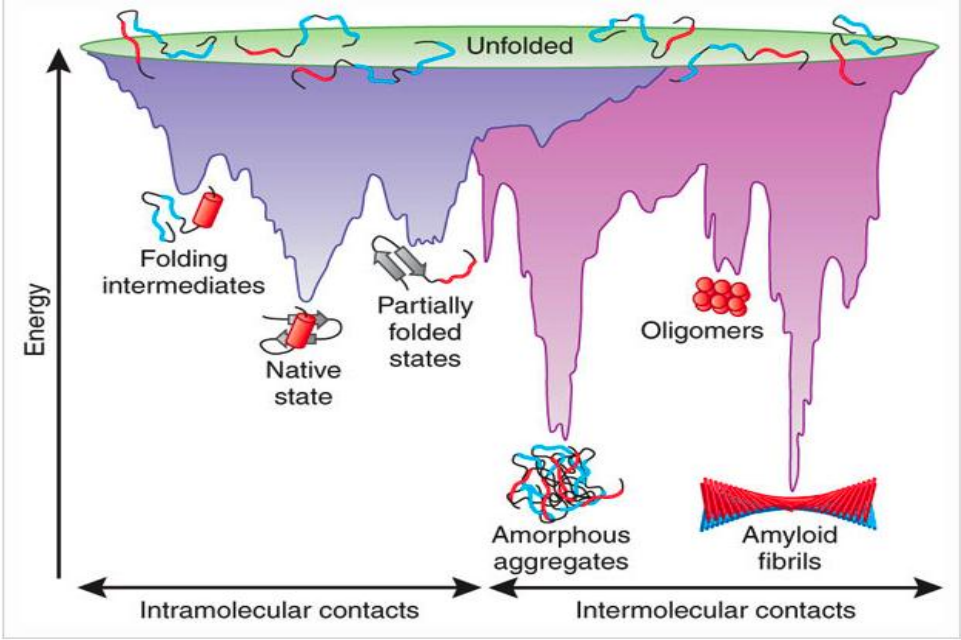


Fig 1.1 Energy landscape diagram of protein folding and misfolding where purple colored areas represents folding intermediate states and native states and the pink areas refers to conformations moving towards amorphous and amyloid aggregates via intermolecular contacts.(Hayer-Hatl, 2009).

Aggregation compromises the overall functioning of a protein and has been associated with many pathologies, including autoimmune conditions and and neurodegenerative diseases etc. In this thesis, we study the aggregation of biological effector molecules, known as cytokines which greatly tend to aggregate, particularly upon recombinant expression in a bacterial system. When overexpressed, these cytokines, IL-2, Il-6 etc. have been reported to aggregate and pose hindrance in use, in the lab or in therapy. We deal with one such aggregation-prone

cytokine, interferon gamma (IFN-γ), which is a member of type-II class interferon and has immunostimulatory and immunomodulatory activities

1.2 Interferon Gamma (IFN-γ)

IFN-γ is a glycoprotein cytokine, which is the only member of the type-II class of interferons. Interferon gamma (IFN-γ) is produced in functional dimerized form by different cells of the immune system: CD4 T-cells, CD8 T-cells and Natural Killer (NK) cells. The protein has a role in nearly all phases of immune and inflammatory responses, including growth, differentiation and activation of T-cells, B-cells and macrophages. It also has weak antiviral and anti-proliferative activity.

Any defects in the IFN-γ pathway can lead to a state of higher susceptibility to bacterial infections and diseases like multiple sclerosis. IFN-γ cytokine also stimulates a number of lymphoid cell functions including anti-microbial and anti-tumor responses of macrophages and NK cells. This protein, along with another cytokine, interferon-α, increases the production of antibodies in response to antigen by enhancing the antigen-presenting function of macrophages. Human IFN-γ is species-specific and is biologically active only in human and primate cells. Recombinant human interferon gamma is an 18.14 kDa protein containing 155 amino acid residues, which exists as a folded homo-dimer. The protein is largely alpha helical, as shown in Figure 1.2.

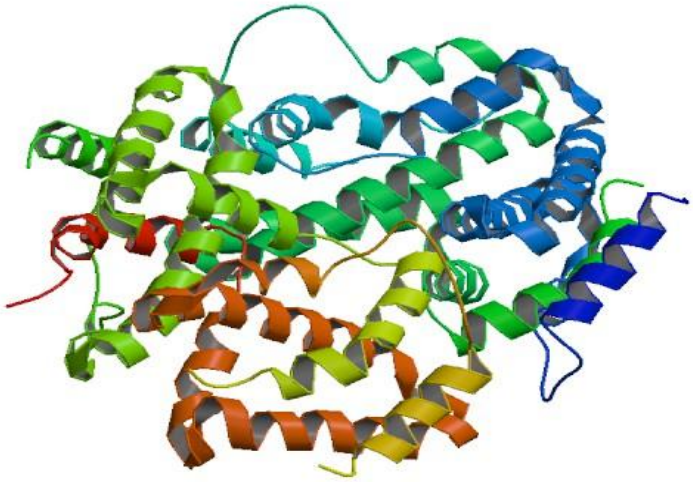


Fig 1.2 Crystal structure of human interferon gamma (PDBID 1HIG)

Here, we have used an engineered version of IFN-γ (cloned in our lab) in which the N-terminal stretch containing three cysteine residues was deleted, as shown in Figure 1.3. The engineered protein was expressed and purified, and studied for oligomeric status, secondary structure, chemical and thermal stability and biological activities.

```
1       10        20        30        40        50        60        70        80        90        100       110       120       130
|-------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------|
MKYTSYILAFQLCIYLGSLGCYCQDPYVKEAENLKKYFNAGHSDVADNGTLFLGILKNWKEESDRKIMQSQIVSFYFKLFKNFKDDQSIQKSVETIKEDMNVKFFNSNKKKRDDFEKLTNYSVTDLNVQR
          MRGSHHHHHHGSQDPYVKEAENLKKYFNAGHSDVADNGTLFLGILKNWKEESDRKIMQSQIVSFYFKLFKNFKDDQSIQKSVETIKEDMNVKFFNSNKKKRDDFEKLTNYSVTDLNVQR
..........$rgshghhgcgcQDPYVKEAENLKKYFNAGHSDVADNGTLFLGILKNWKEESDRKIMQSQIVSFYFKLFKNFKDDQSIQKSVETIKEDMNVKFFNSNKKKRDDFEKLTNYSVTDLNVQR

131   140       150       160   166
|-------+---------+---------+-----|
KAIHELIQVMAELSPAAKTGKRKRSQMLFRGRRASQ
KAIHELIQVMAELSPAAKTGKRKRSQMLFRGRRASQ
KAIHELIQVMAELSPAAKTGKRKRSQMLFRGRRASQ
```

Fig 1.3 Protein blast comparison between a full length wildtype IFN-γ sequence and engineered IFN-γ sequence in which first 23 amino acid residues from the N-terminus was removed. The first line shows the wildtype sequence, the second shows the sequence of our clone (including the N-terminal 6XHis affinity tag, and the third line shows the effective sequence obtained through sequencing, with the N-terminal methionine removed).

2. MATERIALS

2.1 Chemicals

2.1.1 Media for Bacterial growth

Luria broth

Component	**Amount (g/L)**
Tryptone	10
Yeast extract	5
NaCl	5

LB agar is prepared with adding 2 % agar into the above Luria broth solution.

Broth was autoclaved at 15 psi and 120 ^{o}C.

2.1.2 Antibiotics and chemicals

Antibiotic	**Stock concentration (1000X)**
Ampicillin	100 mg/mL in water
Kanamycin	25 mg/mL in water
Tetracycline	12.5 mg/mL in 70% ethanol
Chloramphenicol	35 mg/mL in methanol

Antibiotics were sterilized and stored at -20 ^{o}C and working concentration was 1X.

dNTP

Stock	**Working concentration**
100 μM	1 μM

Plasmids mini prep

Buffer	**Composition**
Buffer P1 (Resuspension buffer)	50 mM Tris-Cl, pH8.0, 10 mM EDTA, 100 µg/ml RNase A
Buffer P2 (Lysis buffer)	200 mM NaOH, 1% SDS (w/v)
Buffer P3 (Neutralization buffer)	3.0 M potassium acetate, pH 5.5
Buffer PE (Column Binding/ Wash buffer)	1.0 M NaCl, 50 mM MOPS, pH 7.0, 70% ethanol
Buffer EB (Elution buffer)	10 mM Tris, pH 8.0

Preparation of chemical competent cells

Calcium chloride	60 mM
Glycerol	15 % v/v
PIPES	10 mM

The pH of the buffer is 7.0 and sterilized after passing through 0.22 µm filter followed by autoclaving. Buffer was later stored at 4^{o}C.

2.1.3 Buffers and Solutions for recombinant DNA work

50X TAE Buffer

Tris-Cl	242 g
Glacial acetic acid	57.1 mL
0.5 M EDTA (pH 8.0)	100 mL

The final volume was 1L and pH of the solution is adjusted to 8.0

6X DNA gel loading buffer

Bromophenol blue	0.25 %
Glycerol	30 %

Ethidium bromide stock solution (1% w/v)

Ethidium bromide	0.1 g
Deionized water	10 mL

The solution is stored in amber color vial.

TE buffer

Tris-Cl (pH 8.0)	10 mM
EDTA	1 mM

Agarose gel (1%)

Agarose	1 g
Deionized water	100 mL

2.1.3 Buffers and Solutions for SDS-PAGE

Acrylamide

Acrylamide	30 g
N,N'-Methylene-bisacrylamide	0.8 g
Deionized Water	100 mL

Ammonium persulfate (APS, 10%)

APS	100 mg
Water (Deionized)	1 mL

Lower Tris (4X), pH 8.8

Tris	18.17 g
10 % SDS	4 mL
Water (Deionized)	100 mL

Upper Tris (4X), pH 6.8

Tris	6.06 g
10% SDS	4 mL
Water (Deionized)	100 mL

5X Sample loading buffer

Tris-Cl (pH 6.8)	0.15 M
SDS	5 %
Glycerol	25 %
β-mercaptoethanol	12.5 %
Bromophenol blue	0.06 %
Water (Deionized)	10 mL

Laemmli buffer

Tris buffer	3.0 g
SDS	1.0 g
Glycine	14.4 g

Water (Deionized)	Up to 1L

Gel Staining Solution

Methanol	40 %
Glacial acetic acid	10 %
Coomassie Brilliant Blue R-250	0.1 %
Water (Deionized)	50

Gel destaining Solution

Methanol	40 %
Glacial acetic acid	10 %
Water (Deionized)	50 %

SDS-PAGE Composition

Resolving gel 12%		Stacking gel 5%	
Lower tris	2.5 mL	Upper tris	0.5 mL
Acrylamide	4 mL	Acrylamide	0.333 mL
Water(Deionized)	3.4 mL	Water	1.167 mL
APS	50 µL	APS	12.5 µL
TEMED	10 µL	TEMED	5 µL

2.1.4 Buffers and solutions for protein purification

(i) Native purification of 6X His-tagged proteins

Native Lysis Buffer

NaH_2PO_4, pH 8.0	0.05 M

NaCl	0.3 M
Imidazole	0.01 M

Native Wash Buffer

NaH_2PO_4, pH 8.0	0.05 M
NaCl	0.3 M
Imidazole	0.02 M

Native Elution Buffer

NaH_2PO_4, pH 8.0	0.05 M
NaCl	0.3 M
Imidazole	0.25 M

Phosphate-Buffered Saline (PBS) buffer (10X, pH 7.4)

$Na_2HPO_4.7H_2O$	25.6 g
NaCl	80 g
KCl	2 g
KH_2PO4	2 g

Final volume is 1L. Into these components, Imidazole was added in varying concentration in order to purify 6X His-tagged proteins

(ii) Denaturing Purification of 6X His-tagged proteins

Lysis buffer, B

Urea/ GdmCl	8 M/6 M
Na_2HPO_4	0.1 M
Tris-Cl	0.01 M

pH is adjusted to 8.0

Wash buffer, C

Urea/ GdmCl	8 M/6 M
Na_2HPO_4	0.1 M
Tris-Cl	0.01 M

pH is adjusted to 6.3

Wash buffer, D

Urea/ GdmCl	8 M/6 M
Na_2HPO_4	0.1 M
Tris-Cl	0.01 M

pH is adjusted to 5.9

Elution buffer, E

Urea/ GdmCl	8 M/6 M
Na_2HPO_4	0.1 M
Tris-Cl	0.01 M

pH is adjusted to 4.5

2.2 Cloning

Bacterial strain used

Strains	**Genotype and phenotype**
***E. coli* XL1 Blue**	*endA1 gyrA96(nal$_R$) thi-1 recA1 relA1 lac glnV44 F'[Tn10proAB$_+$ lacI$_q$ Δ(acZ) M15] hsd17(r$_{K-}$ m$_{K-}$)* • Derived from *E. coli* K12 strain and it is resistant to nalidixic acid as well as tetracycline
***E. coli* BL21(DE3)**	F1 phage origin for ssDNA production, has T7 promoter, pBR322-type ColE1 origin , Multiple Cloning Sites (BamHI to XhoI), ampicillin resistance gene (beta-lactamase)

Plasmids used

For transformation of genes into *E.coli*, genes were inserted within plasmids having origin of replication, antibiotic selection, multiple cloning sites and compatible promoter.

- pQE30: has a size of 3.4 kbp, this plasmid contains ampicillin resistance gene as selection marker and T5 promoter. Helps in the expression proteins fused with a 6X His tag at N-terminal end.
- pET23a: has a size of 3.6 kbp, it contains ampicillin resistance gene as selection marker and T7 promoter. The vector expresses 6X C-terminal His tagged proteins.

2.3 Western blot

Transfer buffer

Tris	25 mM
Glycine	192 mM
Methanol	10 %

PBS Tween

Tween 20	0.01% (20 µL)
PBS buffer	Make up to 1L

Blocking Solution

Skim milk	5 % (20 µL)
PBST	Make up to 20 mL

Ponceau S Staining Solution (0.1 %(w/v))

Ponceau S	1 g
Acetic acid	50 mL
ddH_2O	Make up to 1L

3. METHODS

3.1 Expression of recombinant protein in *E.coli*.

3.1.1 Expression without any special treatment

Due to promoter compatibility, pQE30 plasmid was transformed into XL1-blue cells, in which the host is used for both cloning and expression. For the expression of engineered interferon gamma protein, overnight grown cultures were used for inoculation at final concentration of 1 % into LB containing requisite amounts of antibiotics. Culture of cells was allowed to grow for a period of 7 hrs/8 hrs at 37 ^{0}C since no induction is required in this system.

3.1.2 Expression of proteins at low temperatures.

To improve the solubility of the protein, expression was carried out at low temperature conditions (18 ^{0}C and 25 ^{0}C). After overnight grown cultures were added into LB broth (1 % of total volume of LB) containing ampicillin and tetracycline antibiotics, broth was allowed to grow to an OD_{600} of ~0.6 at 37^{0}C with shaking at 220 rpm. Then these cells were grown overnight at 18 ^{0}C and 25 ^{0}C respectively.

3.1.3 Expression of proteins in the presence of D-glucose.

In this case when the cell culture has reached an OD_{600} of ~0.6 (at 37 ^{0}C for 5:30 hrs), broth was treated with 1 % glucose and the culture was allowed to grow at 37 ^{0}C for the next 7 hrs.

3.2 Glycerol stock: To 1500 µL of overnight culture 500 µL of 60 % glycerol was added followed by storage at -80 ^{0}C.

3.3 Denaturing purification: After expressing the protein under the first condition described above, the cells were pelleted down at 8000 rpm for 6 min. The harvested cells were resuspended in 2-5 mL per gram wet weight using lysis buffer containing urea (Buffer B, pH 8) and incubated at RT overnight followed by sonication using a probe sonicator for alternating ‘on’ and ‘off’ pulses of 10 seconds for one hour. The cell debris was isolated from

the supernatant after centrifuging at 12000 rpm for 30 minutes. After equilibrating the Ni-NTA column with buffer B, supernatant was passed, and the column was washed with 15 mL of buffer C (pH 6.3) and finally the protein was eluted with Buffer E (pH 4.5), through a pH-based elution through changing the protonation states of histidine residues involved in the affinity to Ni-NTA.

3.4 Native purification:

The cells expressing protein at low temperature and after addition of glucose were harvested and resuspended in native lysis buffer (pH 8.0). To lyse the cells, the solution was sonicated at an amplitude setting of 20 for one hour with alternating on and off pulses of 10 seconds. The supernatant was separated from cell debris by centrifuging at 12000 rpm for 30 mins. This supernatant was passed through Ni-NTA column after equilibrating the column with Native lysis buffer. Washing was done with 50 mL of wash buffer in two separate steps (25 mL of 20 mM imidazole followed by 40 mM Imidazole) and finally the protein was eluted with elution buffer (250 mM Imidazole), through imidazole-based elution.

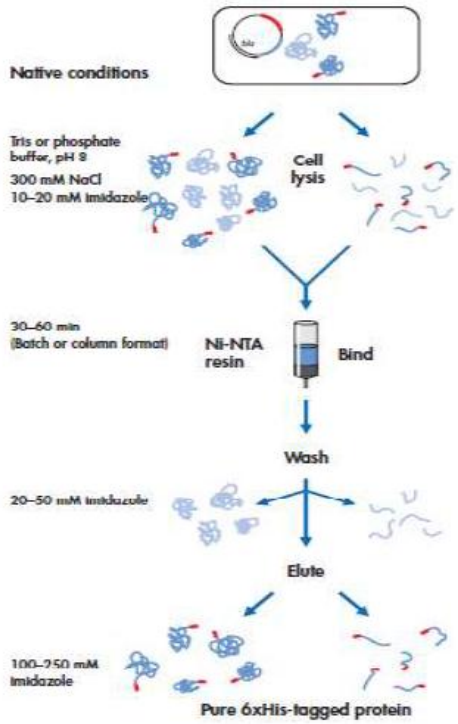


Fig 3.4 Protein purification (QIA expressionist)

3.5 PMF for unique identification of protein:

To identify the protein using mass spectrometry, samples were prepared in two ways:

For in-solution digestion, trypsin (proteomics grade) was prepared in 1 mM HCl at a concentration of 1 µg/mL (20 µL of 1 mM HCl for a 20 µg vial). So the final solution contains 1 mg/mL trypsin at pH 3.0.

For in-gel digestion, solution is.

Steps of In-gel digestion

1. The band of protein which is stained with Coomassie Brilliant Blue is excised from polyacrylamide gel using a scalpel.
2. This gel piece is kept in a siliconized **e**ppendorf tube which was already washed with 100 µL of a 0.1 % trifluoroacetic acid (TFA) in 50 % acetonitrile solution and allowed to dry before use. This siliconized tube helps in reduction of peptide binding to tube.

3. To destain this gel piece, 200 µL of 200 mM ammonium bicarbonate with 40% acetonitrile was added and incubated at 37 ^{0}C for 30 mins after which the supernatant was discarded. This step is repeated one more time. The gel piece was further dried in a rotary vacuum centrifuge for 15 to 30 mins.

4. 50 µL of trypsin (prepared by adding 100 µL of 1 mM HCl into trypsin and 900 µL of 40 mM ammonium bicarbonate in 9 % acetonitrile) was added. The final concentration of trypsin was 20 µg/mL. Incubation was done at 37 °C overnight to perform enzymatic digestion.
5. Gel pieces were settled at the bottom of the tube by centrifuging at 9500 rpm for 20 seconds. The supernatant having peptides was transferred to a new microcentrifuge tube.
6. For MALDI, samples were mixed with CHCA matrix (1:1) on the plate.

7. For ESI-MS analysis, the sample was diluted 10 times with MS grade water and subjected to LCMS.

b. In-Solution digestion

1. To one vial containing reconstituted trypsin (same as in the above procedure) 1 µl of protein sample (containing no more than 100 µg) was added and vortexed to mix.
2. This vial was incubated at 37 ^{0}C while being shaken overnight to digest the protein.

3.6 Dialysis

To bring the protein into a desired buffer for carrying out further studies it was dialyzed by two methods:

3.6.1 Stepwise dialysis

Protein eluted using pH-based elution method (after denaturing purification) was used for stepwise dialysis. The denaturant concentration was gradually reduced from 8 M to 4 M and then to 2 M using native lysis buffer. The final buffer contained no urea at all but native lysis buffer with 5 % glycerol at pH 7.4.

3.6.2 Dilution

After denaturing purification, 1 mL of 0.6 mg/mL of protein was added drop by drop into 40 mL of native lysis buffer containing 5 % glycerol at pH 11.55 and was continuously stirred at low rpm. The mixture was incubated overnight at RT. The mixture was then concentrated and analyzed.

3.7 UV-Vis Absorption spectroscopy

Cary50 UV-vis spectrophotometer was used for measuring protein concentration. The absorption was recorded at 280 nm and protein concentration was derived using the formula $A = \varepsilon cl$ (A= absorption, ε= extinction coefficient, c=protein concentration and l= path length).

OD_{280}=1= 0.634 mg/ml [ProtParam tool (ExPASY Bioinformatics resource tool)].

3.8 Gel filtration chromatography

The gel filtration elution profiles were studied on a GE (Pharmacia) Akta Purifier 10 system. After purifying protein using Ni-NTA affinity chromatography, size exclusion chromatography was used for re-purification of protein. The column and pumps were washed with filtered Millipore Elix-3 water and then equilibrated with respective, requisite buffers. Superdex75 column with a bed volume of 24 mL was used for the chromatography. For purification of protein, 500 µL of protein sample was loaded onto the column. The elution volumes were compared with the elution profiles of the standards run on the same column. The elution profile of proteins depends on the size (hydrodynamic volume) of the molecule.

3.9 Fluorescence spectroscopy

Fluorescence emission spectrum of protein was recorded after excitation wavelength was set at 295 nm. The emission spectrum was recorded from 300 nm to 400 nm. The slit width used for the experiment was set at 4 nm for both excitation and emission. All spectra were averages of 3 scans.

3.10 Circular Dichroism (CD) spectroscopy

Far UV CD spectra for the protein's secondary structures were collected using the MOS 500 CD spectrometer (Biologic). The data was collected in the 195/200-250 nm range with α-helix structure giving negative bands at 222 nm as well as 208 nm and β-sheet at 214-215 nm. For this study 0.1 cm path length cuvette was used and all spectra were averages of 3 scans. The obtained result in raw ellipticity was converted to Molar Residue ellipticity $[\theta]^+$ using the formula as follows:

$$[\theta]+ = \frac{\theta obs\ (mdeg) \times 100 \times MRW}{1000 \times concentration\left(\frac{mg}{ml}\right) \times pathlength\ (cm)}$$

Where, MRW = Mean Residue Weight (Total molecular weight of the protein/ Total number of amino acids).

θ_{obs} = Raw Ellipticity (millidegree)

3.11 Dynamic Light Scattering

Dynamic light scattering instrument [Zetasizer Nano ZS 90 (Malvern)] was used for measuring the hydrodynamic radius of the protein. Hydrodynamic radius distribution versus percent scattering and percent mass data were collected. DLS measures the changes in scattering of light from molecules with time in solution to determine the translational diffusion coefficient in order to find the hydrodynamic radius using the Stokes-Einstein equation. Since these measurements are directly proportional to size and shape of the molecule, a calibration curve can be created, depending upon which calculation of molecular weight of the desired molecule is possible.

3.12 FTIR

FTIR [Bruker (Tensor 27) with BioATR II] is another technique to collect information about secondary structure of protein. Data from this instrument details about two main regions,

- Amide I: absorption corresponding C=O bond having the 1500-1600 cm^{-1} range.
- Amide II: absorption corresponding N-H and C-N stretch having 1600-1700 cm^{-1}.

In terms of peaks, α-helix peaks at 1652 cm^{-1} (in amide I) and at 1548 cm^{-1} (in amide II), parallel β sheet have peaks at 1640 cm^{-1}, antiparallel β sheet peaks at 1615-1637 cm^{-1} and β turns show peak at 1680 cm^{-1}.

3.13 Electroblotting of proteins by semi-dry transfer

Size-separated proteins from the polyacrylamide gel were transferred to nitrocellulose or PVDF membrane using semi-dry electroblotting method in typical western blot experiments. Once proteins are transferred to the membrane, these are stained with antibodies which are specific to the target protein.

Steps

1. Separating the proteins in the sample using gel electrophoresis.

2. Preparing the transfer buffer (25mM Tris,192mM glycine and 10% methanol) and then making a sandwich including extra thick filter paper, acrylamide gel, membrane and filter paper stacked in the top-bottom manner (membrane should be on the anode side). Transfer buffer is filled between each of these layers.
3. Closing the semi-dry transfer apparatus and connecting the electrodes to the power supply, followed byapplication of continuous voltage of 30 V for 3 hours for efficient protein transfer. Placing of the membrane in a reversible stain (ponceau) to check the efficiency of protein transfer.

3.14 Immunoblot of IFN-γ

Once proteins are transferred to the membrane (PVDF or nitrocellulose), membrane was blocked, probed with primary antibody anti-IFN-γ antibody (1:200) followed by secondary antibodies HRP conjugated anti-goat antibody followed by detection using H_2O_2/Luminol.

3.14 Immunofluorescent staining

THP1 macrophages were resuspended in FACS buffer (FCS-2%, 2 mM sodium azide in PBS). To inhibit non-specific staining, cells were incubated with Fc binding inhibitor (eBiosciences, San Diego, CA) for 25 min at 4 ^{0}C. Later, cells were stained with fluorochrome-conjugated antibody (Ab) specific for human HLA-DR or isotype-matched control Abs, at a recommended concentration (0.5 μg/10^6 cells). The cells were fixed with 1X paraformaldehyde. Regular steps of washing were followed at each step. Data were collected using FACS Aria and analyzed with BD DIVA software.

4. RESULTS

4.1 Expression and Purification of Engineered Human Interferon Gamma

4.1.1 Denaturing Purification and Refolding efforts.

For the engineered Interferon gamma which was already cloned into pQE30 vector and transformed into XL1-Blue cells in our lab, the protein was firstly expressed without any special treatment i.e. LB broth containing bacterial culture was allowed to grow at 37 ^{0}C for 7 to 8 hrs with shaking at 220 rpm.

After expression, cells were lysed using native lysis buffer and purified using native Ni-NTA chromatography method (Fig 4.1.1 showing Imidazole based elution).

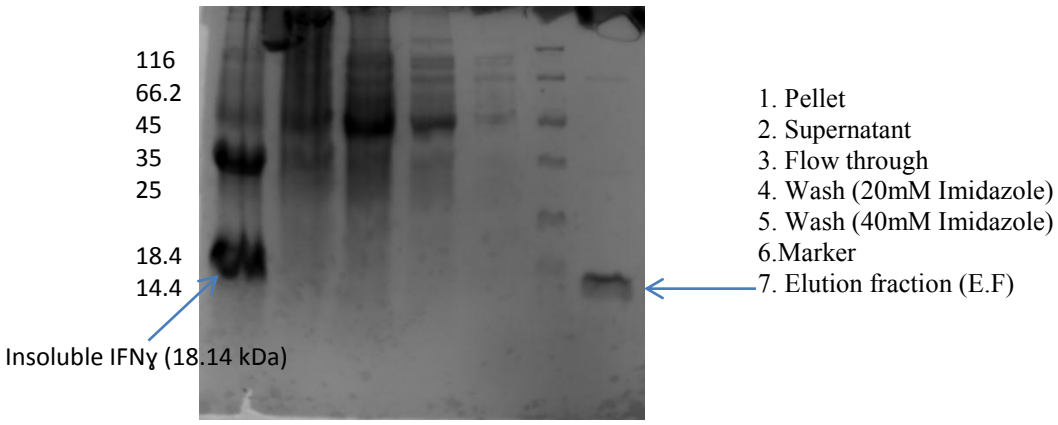


Fig 4.1.1 SDS-PAGE electrophoresis to analyze engineered IFN-γ after Native purification using Ni-NTA affinity chromatography.

From gel electrophoresis, major proportion of the desired protein is seen in insoluble form in pellet fraction. A small amount of the protein is also seen in the elution fraction. So, initially, emphasis was laid on solubilizing the insoluble protein fraction and various refolding techniques were tried.

In order to solubilize the protein from inclusion bodies, cells were lysed and contents denatured with 8 M urea and denaturing purification using Ni-NTA column was performed.(Fig 4.1.2)

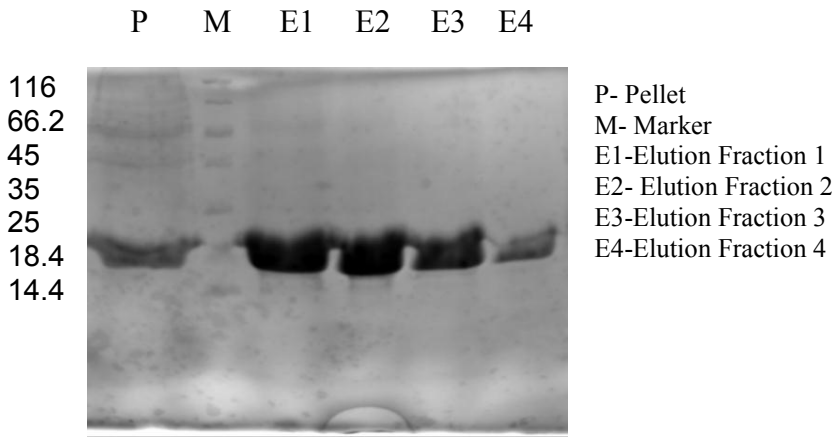


Fig 4.1.2 Denaturing purification of engineered IFN-γ after denaturing protein with 8M urea.

For refolding the protein, stepwise dialysis (method 3.6) was performed using elution fraction 4. As the concentration of denaturant (urea) was gradually reduced in each step, major portion of the protein precipitated. We could finally get 0.17 mg/ml of engineered IFN-γ protein in native lysis buffer containing 2 % of glycerol but no traces of urea. After fluorescence and CD analysis, it was realized that the protein could not acquire a native-folded structure (fig 4.1.3).

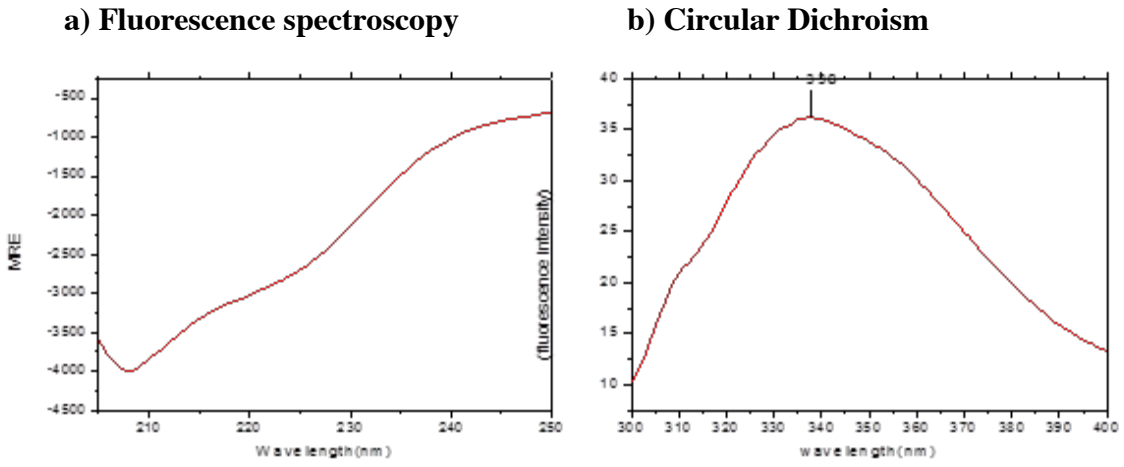


Fig 4.1.3 Analysis for checking the folded structure of engineered IFN-γ. a) Fluorescence spectroscopy showing a peak around 338 nm suggests protein after stepwise dialysis method was coming in folded structure. b) Circular dichroism of 0.17 mg/ml protein showing engineered IFN-γ protein was non-natively folded.

Next effort to refold this denatured protein was through dilution. The elution fraction (1 mL of 0.6 mg/mL protein in urea buffer at pH-4.5) was added drop-by-drop into 40 ml of refolding buffer (Tris-buffer containing 5 % glycerol at pH-11.5) with continuous stirring at RT. After an overnight incubation in this buffer containing protein, the solution was concentrated back to 1 mL and analyzed using fluorescence spectroscopy and circular dichroism (CD) (fig 4.1.4).

Fluorescence spectroscopy spectrum showed a peak at 343 nm whereas CD spectroscopy showed it to be helical in structure but with a fold that was still clearly non-native.

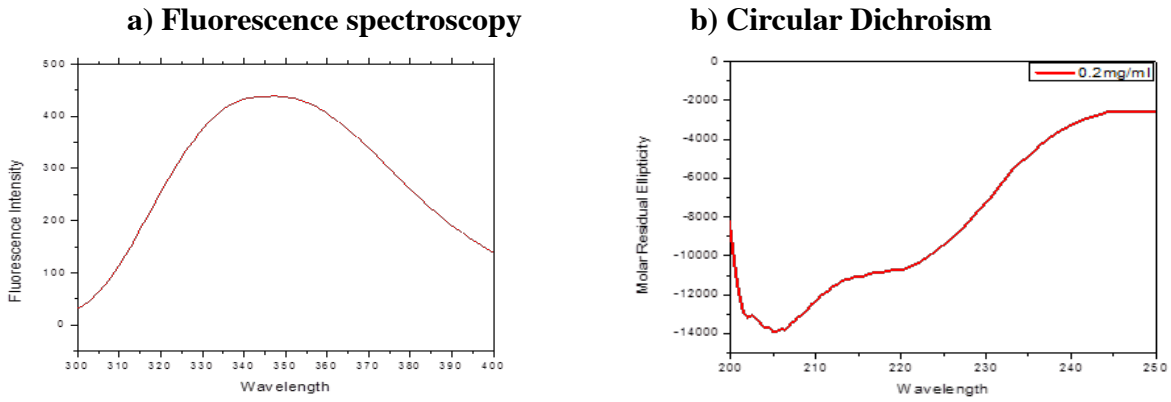
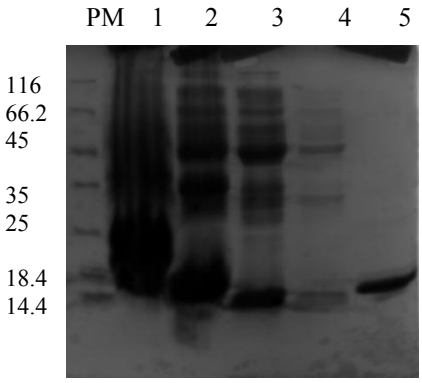


Fig 4.1.4 Analysis for checking the folded structure of engineered IFN-γ. a) Fluorescence spectroscopy showing a peak around343 nm suggests protein after dilution refolding method existed in folded structure. b) circular Dichroism of 0.2 mg/ml protein showing engineered IFN-γ protein was non-natively folded.

4.1.2 Expression of proteins at low temperatures.

Since the refolded protein was non-native in structure and most portion was lost in precipitation, the focus was shifted towards enhancing the yield of native portion of the protein, i.e., the yield of soluble protein produced in the bacterial cytoplasm. As mentioned before in the methods section, overnight grown culture was added into LB broth (1 % of total volume of LB) containing ampicillin and tetracycline antibiotics, The cells were then incubated at 18 ^{0}C and 25 ^{0}C respectively while on shaking at 220 rpm after the broth had reached an OD_{600} point ~0.6 at 37^{0}C.

Incubation of LB broth at 18 ^{0}C resulted in no protein in soluble as well as very little protein in insoluble fractions but after expressing at 25 ^{0}C soluble proteins were found to be produced in an amount of 0.8 mg of total yield from the culture (Fig. 4.1.5 lane 5 in the SDS-PAGE).



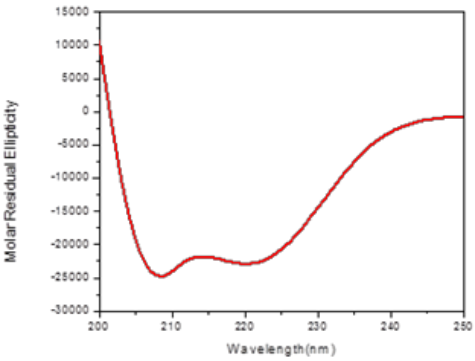
1. Pellet
2. Supernatant
3. Flow through
4. Wash
5. Elution

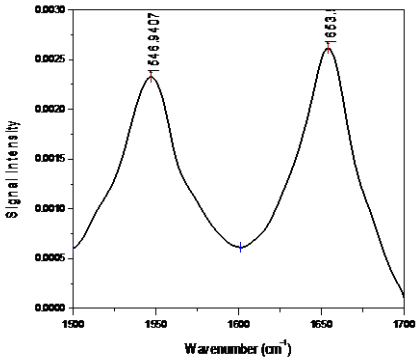
Fig 4.1.5 Native purification of engineered IFN-γ after expressing at 25 ^{0}C.

4.1.2.1 Structure analysis

The CD technique was used to check whether the protein now produced was folded in pure α-helical structure like full length native protein IFN-γ. The CD spectrum suggested it to be folded like the original full length IFN-γ (fig 4.1.6 a). But the amount of protein was too less to do any further experiments.

a) b)





Wave number (cm-1)	Secondary structure elements
1620-1640	β-sheets
1640-1650	Unordered
1650-1658	α-helices
1660-1680	Loops

Fig 4.1.6 a) Circular Dichroism of soluble engineered IFN-γ obtained from low-temperature method. b) FTIR spectrum of IFN-γ protein.

FTIR analysis also suggested a largely alpha helical fold. Amide I peaks at 1653 cm-1 shows IFN-γ is largely α-helix since 1653 & 1547 cm^{-1} correspond to Amide I and amide II respectively (fig 4.1.6 b).

4.1.3 Expression of protein in the presence of D-glucose.

Following similar conditions to express the bacterial cultures to reach OD_{600} of ~0.6, broth was treated with 1 % glucose and LB broth was allowed to grow at 37 ^{0}C for next 7 hrs. After that cells were lysed with native lysis buffer followed by native purification of solubly produced engineered IFN-γ. The Fig. 4.1.7a shows that with this expression method amount of insoluble protein was reduced and soluble fractions were considerably increased.

Since this native purification had some non-specific bands, washing step was modified with 20 mL of wash buffers containing 20mM and 40mM Imidazole was passed in two separate steps followed by passing of 10 mL of wash buffer which was supplemented with 1M NaCl. This approach had helped in removing endotoxins as well as further getting rid of non-specific proteins. Elution was carried out with elution buffer containing 250mM Imidazole (fig 4.1.7b)

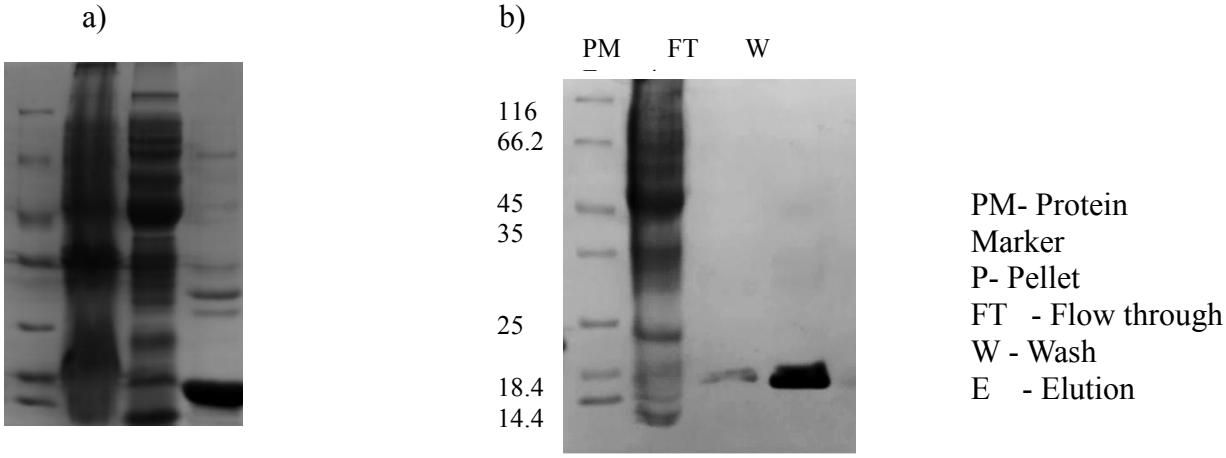


Fig 4.1.7 SDS-PAGE after engineered IFN-γ was expressed in presence of D-glucose and purified in native conditions. In the image b Lane-4, a single band is showing a very high amount of IFN-γ is present in a pure population.

The amount of protein finally obtained from a 1 L culture was 3.6 mg.

4.2 Size Exclusion Chromatography (Akta Purifier)

In order to analyse the oligomeric status of engineered IFN-γ and to remove imidazole from natively purified fraction gel filtration chromatography was carried out in PBS buffer (which is also a physiological buffer). The protein was passed through superdex75 column (fig 4.2.1a).

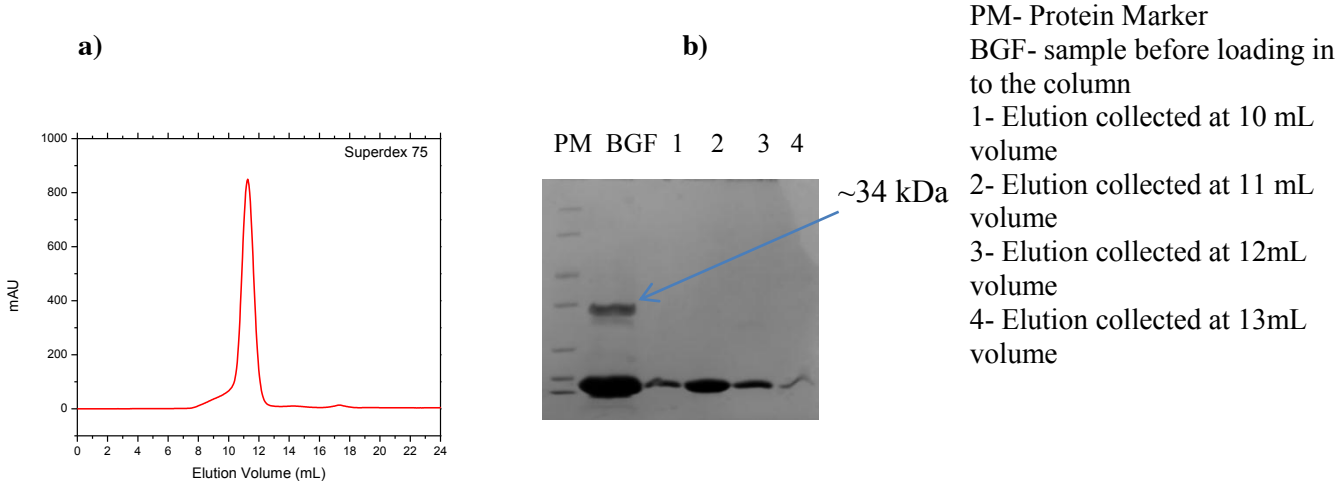
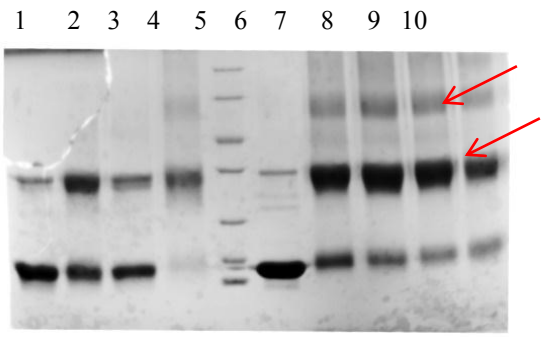


Fig 4.2.1 a) Gel filtration profile of engineered IFN-γ protein, which was eluted from Ni-NTA chromatography and run through Superdex75 column along with PBS buffer. b) Gel electrophoresis with the protein samples eluted at different elution volumes.

Before loading onto the column, the protein sample was highly concentrated (500 µL of 0.7 mg/mL) and it had also shown a band corresponding to dimer size (Fig. 4.2.1b). Glutaraldehyde cross linking was performed to further understand and confirm the oligomeric status of the protein.

4.3 Glutaraldehyde crosslinking to understand Oligomeric Status.



Engineered IFN-γ in

1. Tris + 0% Glutaraldehyde
2. Tris + 0.0 7 Glutaraldehyde
3. Tris + 0.1 Glutaraldehyde
4. Tris + 0.3 Glutaraldehyde
5. Protein marker
6. PBS + 0% Glutaraldehyde
7. PBS + 0.05Glutaradelhyde
8. PBS + 0.07 Glutaraldehyde
9. PBS + 0.1 Glutaraldehyde
10. PBS + 0.3 Glutaraldehyde

Fig 4.3 Protein was divided into two batches according to which buffer it was present in, and each group was similarly treated with different amount of glutaraldehyde and run them on SDS-PAGE.

Glutaraldehyde crosslinking experiment was conducted for two batches of protein in different buffer systems to understand the difference in oligomeric status of the protein if any. Out of these two batches, one was engineered IFN-γ in native lysis buffer and the other in PBS.

From the Figure 4.3 it is very evident that engineered IFN-γ in native lysis buffer exists in monomeric population largely, ratherthan dimeric population. But a large fraction of the protein in PBS buffer exits as dimeric population rather than monomeric population, and in addition some tetrameric population is also visible (marked with a red arrow). Since the native functional form of human IFN-γ is reported to be dimeric, it was concluded that PBS buffer is reliable for use for further functional studies.

4.4 Confirmation of Identity

To further confirm the identity of our protein and also examine its interaction with anti-IFN-γ antibodies (which are sequence specific antibodies) the below mentioned studies were carried out:

4.4.1 Western Blot

After 10 μg of engineered IFN-γ protein was separated using gel electrophoresis, the SDS-PAGE gel was used for transferring protein from gel to nitrocellulose membrane using semidry-transfer (Method 3.13). After successful transfer, the nitrocellulose membrane containing IFN-γ was incubated in blocking solution at 37 ^{0}C for 1 hour, and incubated in primary antibody (IFN-γ [C-19], sc-1377, Goat polyclonal IgG) at a ratio of 1:200 in 1% skimmed milk- PBS buffer at 37 ^{0}C for 2 hours. After washing (10 mins X 3) with PBS Tween, the membrane was incubated with secondary antibody (HRP conjugated Rabbit anti-Goat IgG) at a ratio of 1:5000 to the PBS buffer. Next, the membrane was incubated in H_2O_2/Luminal and the image was acquired using chemiluminescence LAS instrument.

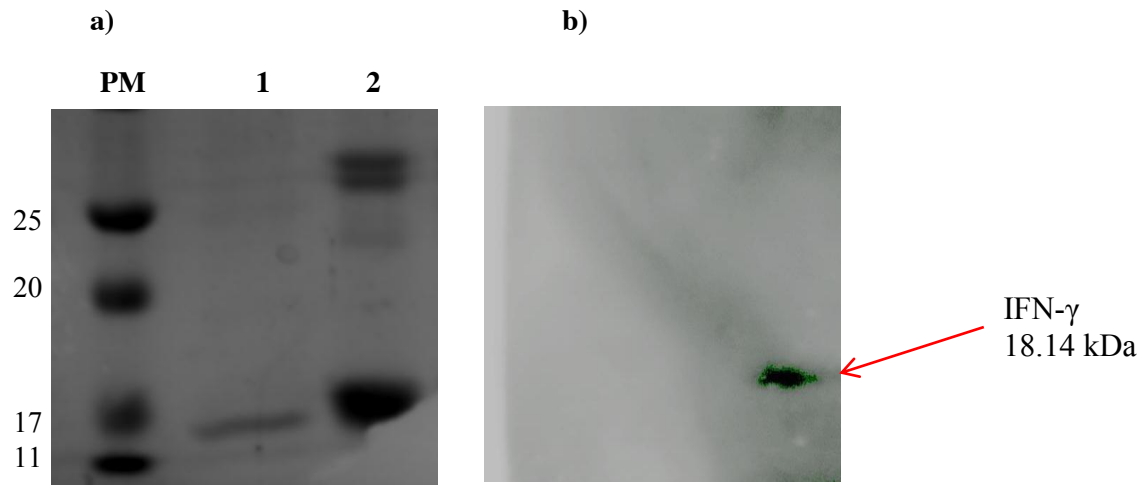


Fig 4.4.1 a) Gel electrophoresis of protein to separate IFN-γ from other proteins and also to be used in semi-dry transfer during electroblot. b) Nitrocellulose membrane containing antigen-primary & secondary antibody complex. Image taken using LAS instrument.

Image 4.4.1 shows our truncated engineered IFN-γ protein binds to anti-IFN-γ antibody (i.e. 10µg in Lane 2).

4.4.2 Peptide Mass Fingerprinting (PMF)

Trypsin digested samples (method 3.5) were subjected to liquid chromatography coupled with Synapt G2SHDMS Q-TOF mass spectrometer. Mobile phase A consisted of water+ 0.1% of formic acid and mobile phase B consists of ACN + 0.1% formic acid for elution of protein. Raw data was obtained from PLGS (Protein Lynx Global Server) software.

OK	Accession	Entry	Description	mW (Da)	pI (pH)	PLGS Score	Peptides
✓	P01579	ING_HUMAN	Interferon gamma precursor (IFN-gamma) (Immune int	19335	9.8	18591.2	91
✓	P42163	ING_MACMU	Interferon gamma precursor (IFN-gamma).	19319	9.8	5203.4	40
✓	P28341	ING_CALJA	Interferon gamma precursor (IFN-gamma).	19492	9.7	1389.3	17
✓	P28333	ING_CEREL	Interferon gamma precursor (IFN-gamma).	19408	9.7	847.7	16

ING_HUMAN Coverage Map

1 MKYTSYILAF QLCIVLGSLG CYCQDPYVKE AE[illegible]KYFNA GHSDVADNGT
51 [illegible]
101 [illegible]
151 KRKRSQML[illegible] GRRASQ

Fig 4.4.2 Wok pad of engineered IFN-γ between different protein sequences to identify best match using highest PLGS score obtained.

The maximum score matching was obtained with Human interferon gamma (ING_HUMAN) with 18591.2 PLGS score. The 'coverage map' showed that most parts of the engineered IFN-γ protein constituted a complete match with ING_HUMAN as blue shaded areas represents maximum match. Also, the region removed from the N-terminus to create the engineered form was completely absent in mass spec analysis.

4.5 Biophysical Characterization

To study the chemical and thermal stability of engineered IFN-γ, CD and fluorescence spectroscopic techniques were used, and to measure the size of the globular protein DLS was used.

4.5.1 Chemical Denaturation with Urea.

In order to understand the stability of the protein in presence of urea, engineered IFN-γ was chemically denatured with different molar concentrations of urea and the changes in secondary structure were studied using Far UV CD spectra.

a) **b)**

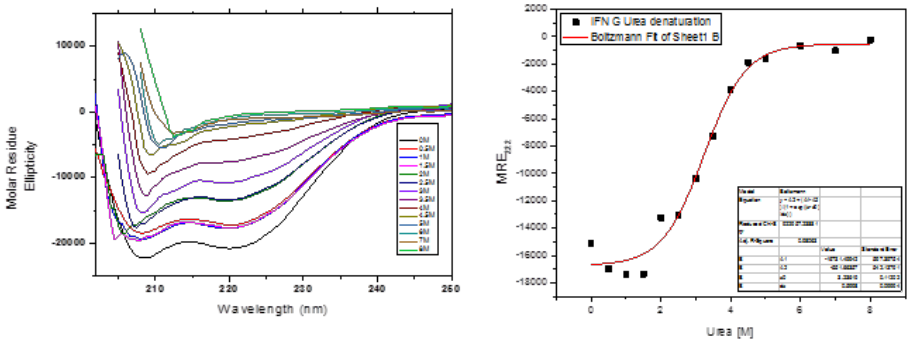


Fig 4.5.1 a) CD spectrum recorded after chemical denaturation of engineered IFN-γ with urea.
b) Sigmoidal plot drawn between different concentrations of urea versus the molar residual ellipticity values at 222nm. This plot gives the estimated value of Cm, i.e. at which concentration of urea half of the protein population is denatured.

From the figure 4.5.1 it can be noticed that the overall protein structure (observed at 222 nm) gradually decreases with increasing concentration of urea. The sigmoidal fit analysis (urea

concentration vs MRE at 22nm, fig4.5.1 b) shows this protein has an unfolding C_m of 3.2 M urea.

4.5.2 Chemical Denaturation with Guanidium Hydrochloride (GdmCl).

For checking the engineered IFN-γ protein's stability against GdmCl, protein was denatured with different molar concentrations of GdmCl and the changes in secondary structure were analyzed after recording far UV spectra.

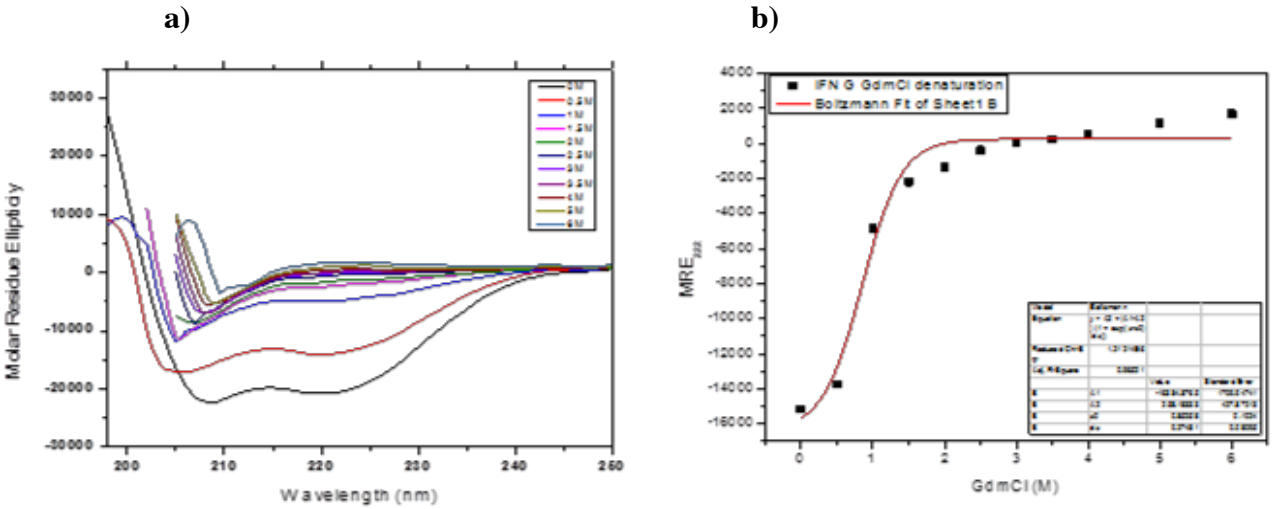


Fig 4.5.2 a) Far UV spectrum shows how the change in secondary structure (dip in 208nm and 222nm) after denaturing with GdmCl. b) sigmoidal graph plotted with the MRE values at different concentrations of GdmCl versus the concentration of GdmCl.

Sigmoidal plot analysis (Figure 4.5.2b) shows this engineered IFN-γ protein has and unfolding C_m of 0.8 M GdmCl.

4.5.3 Thermal Denaturation

The thermal stability of this α-helical engineered IFN-γ protein was checked after subjecting the protein to heating from 20 ^{0}C to 90 ^{0}C and then cooling to room temperature. The data was collected using CD spectra.

a) b)

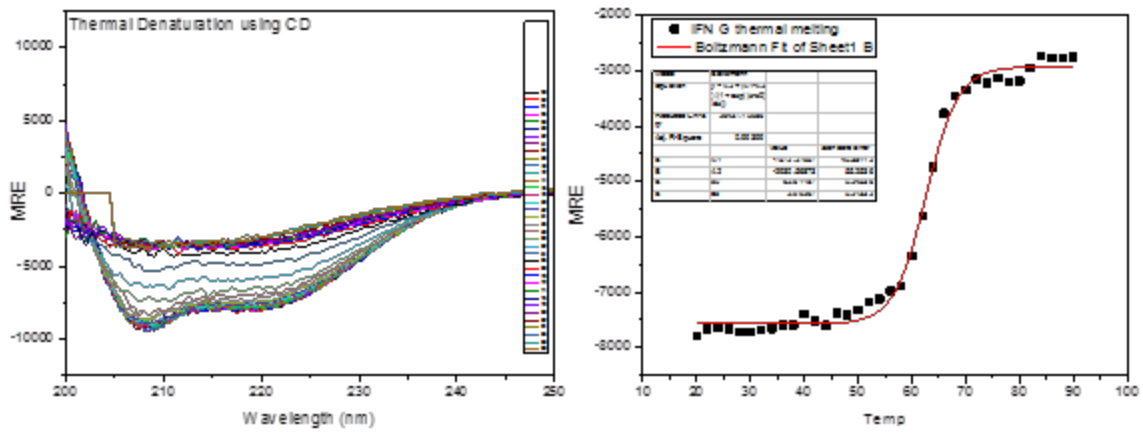


Fig 4.5.3 a) CD spectra recording the decrease in the MRE dip values at far UV wavelength range as engineered IFN-γ protein is thermally melted. b) Sigmoidal plot of Temperature melting using far UV CD spectra data.

Far UV CD spectrum suggests engineered IFN-γ has an unfolding, or melting temperature, or T_m, of 62 ^{0}C, and that it is thermally stable.

4.5.4 Differential Light Scattering (DLS)

To find out the hydrodynamic radius of the protein and thereby measuring the size, the engineered IFN-γ was subjected to DLS.

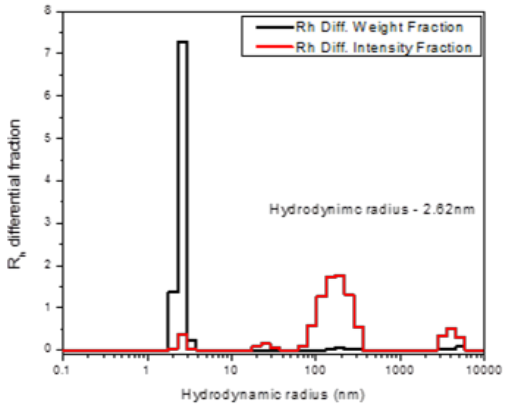


Fig 4.5.4 DLS profile of engineered IFN-γ, measuring scattering degrees in terms of weight fraction as well as intensity fraction

The hydrodynamic radius of the protein was measured to be 2.62 nm from the highest peak created due to weight fraction.

4.5.5 Immunostimulatory activity of engineered IFN-γ

THP-1 macrophages are sensitive to IFN-γ. It is well established that when THP-1 macrophages are treated with IFN-γ, it shows upregulation of MHC-II. To decipher the bio-activity of recombinant IFN-γ, we treated THP-1 macrophages with the same. To initiate the experiment THP-1 monocytes were stimulated to THP-1 macrophages after treatment with PMA (160 nM)

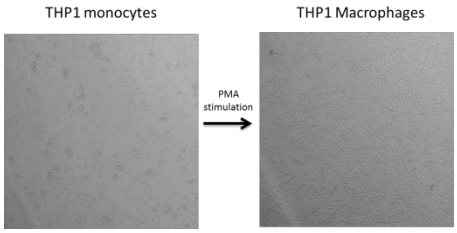


Fig 4.5.5.1 Stimulation of THP-1 monocytes into THP-1 macrophages after treating with 160nM PMA for 6h.

After 6 h IFN-γ (commercial and Recombinant) was added to the cultures and cells were stained for HLA-DR (MHC2) expression through Flow cytometry.(fig 4.5.5.2)

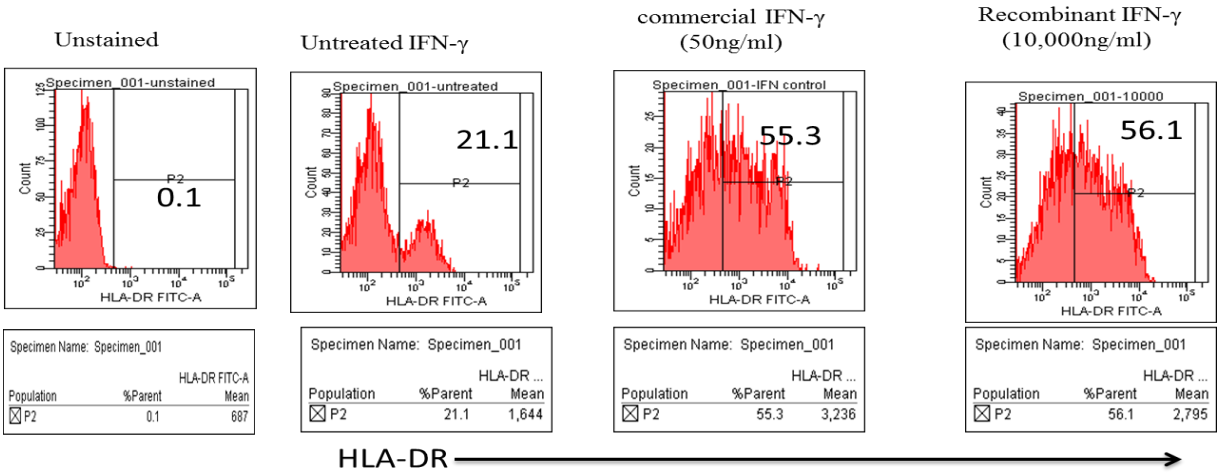


Fig 4.5.5.2 the level of expression of HLA-DR with and without treatment with IFN-γ (commercial and engineered)

Unstained represents the autoflourescenece by the cells only. Expression of HLA-DR was 21.1% in the absence of any IFN-γ stimulation (basal expression of HLA-DR) represented as untreated group. Upon stimulation with commercially available IFN-γ (50ng/ml), HLA-DR positive cells were 55.3%, whereas the same level of stimulation was achieved by using 10,000ng/ml (56.1%) of engineered form of IFN-γ.

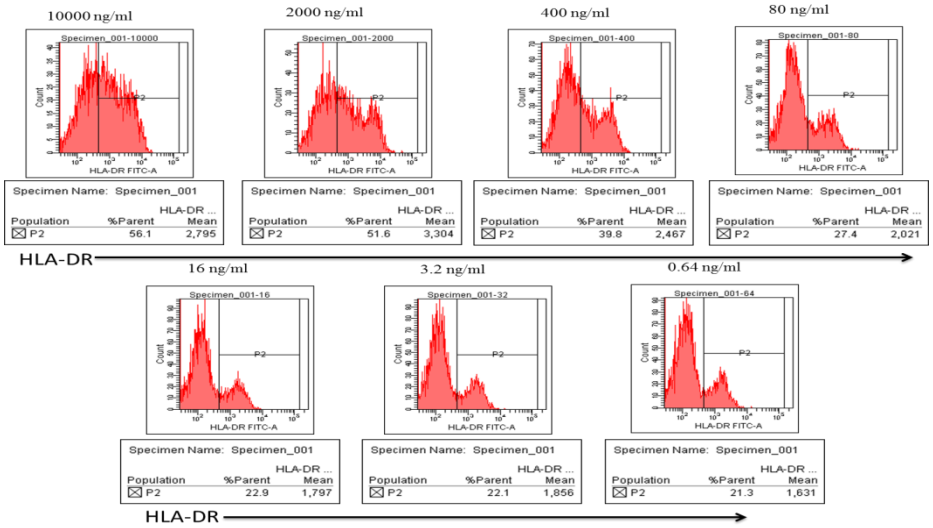


Fig 4.5.5.3 Dose-dependent response of IFN-γ on macrophage activation

After validating that the engineered form of IFN-γ was biologically active (evaluated from the expression of HLA-DR), next we looked for the dose dependent response of the engineered form of IFN-γ (fig 4.5.5.3). It is evident from the Figure 4.5.5.3, that till the concentration of 16 ng/ml (lower panel), there was no stimulation of macrophages upon IFN-γ treatment. Biological activity of engineered IFN-γ was evident from the concentration of 80 ng/ml and onwards. HLA-DR expression increases in dose dependent manner on macrophages upon stimulation with IFN-γ, 80 ng/ml (27%), 400 ng/ml (39.8%), 2000 ng/ml (51.6%) and 10000 ng/ml (56%).

THP-1 macrophages showed higher expression of MHC-II than the untreated cells. It shows that the recombinant IFN-γ is bioactive. However, we would like to make a note. The activity compares poorly with commercial sample. A possible reason was that the sample was stored in a deep freezer prior to addition to cells by the person helping with the experiment, and it was observed that there is lot of aggregation and precipitation of the sample upon storage in the deep freezer, particularly at concentrations approaching and exceeding 1 mg/ml. Thus, these experiments – done once – do not fully reflect the biological activity of the produced protein, and the experiment needs to be done again without any storage of the protein in the deep freezer.

5. Discussion

The most common complication associated with the recombinant expression of IFN-γ is its tendency to go into inclusion bodies. Denaturing purification followed by refolding is a common method to recover proteins that deposit in inclusion bodies. The common complication while purifying the proteins from the inclusion bodies is the tendency to aggregate upon refolding (due to improper and incomplete process), leading to structural as well as functional loss.

In our study, we have designed and engineered IFN-γ, in which a section of the chain consisting of 23 residues (signal peptide) from the N-terminal portion, containing 3 cysteine residues, was deleted. This engineered form of IFN-γ was expressed, and although most of the protein was still in the insoluble fraction, there was noticeable presence of solution protein. This result was not surprising as many reports support the expression of IFN-γ in insoluble fraction. Knowing that small amount of protein was expressed in soluble form, we explored various methods of expression, with the potential of improving the solubility of protein upon expression.

A recent article by Tileva et al; 2015, discussed that IFN-γ when expressed at low temperature (24^0C) with SUMO tag, results in soluble form of IFN-γ. In our study, when we expressed the engineered form of IFN-γ at low temperature of 25^oC, we were able to purify the protein in soluble form, but the yield was not sufficient enough as well as there was still too much aggregation. Further, to improve the soluble yield engineered IFN-γ was expressed in the presence of 1% glucose (described in methods section). Presence of glucose resulted in high yield of soluble protein (3.6 mg/L), which is in line with the previously published report with another protein. Identity of engineered IFN-γ was established by mass-spectrometry and was further confirmed by western-blotting using IFN-γ specific antibody (C-19).

We established that the engineered form of IFN-γ was stable as well as properly structured. We next evaluated the biological activity of the engineered IFN-γ by evaluating the expression of HLA-DR on macrophages in response to IFN-γ. Although the extent of stimulation was very less (200 times less) as compared to the commercial IFN-γ, we feel that this was because the protein happened to be stored in a deep freezer, with resultant

precipitation and loss of protein prior to addition to cells, to examine stimulation. Further standardization and optimization of the experimental condition associated with biological activity is needed to better characterize the immunostimulatory activity of engineered soluble IFN-γ.

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