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Cloning, Expression, Purification, and Bio-functional Analysis of Human Interferon-γ

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Abstarct

Immunoregulatory and anticancer functions are only a few examples of the function that are attributed to Human interferon-γ (hIFN-γ). In this study, we extracted the hIFN-γ gene from the peripheral T-lymphocytes of individuals. Next, the desired gene fragment was inserted into the inducible bacterial plasmid vector pET28a+ and the vector was eventually transformed into E. coli strain BL21. The recombinant hIFN-γ protein was expressed in different expression conditions using IPTG as expression inducer and the resultant inclusion bodies were solubilized using a previously described method based on pH fluctuation shock. The protein solution was refolded using a solution containing Tris–HCl, EDTA, urea, glycerol, sucrose, and PMSF and later on purified using Vivaflow 200, SP Sepharose Fast Flow. The functionality assay was performed on the recombinant hIFN-γ which eventually confirmed the success of the whole process. Our results finally demonstrated that the experimental procedures performed in this study are capable of large-scale production of the pharmaceutical valuable cytokine of interferon-γ.

Key Words: *hIFN-γ, E. coli, Protein expression, Cytokine*

Introduction

Anti-viral defense, cell growth regulation, cytokine signaling, immunomodulation and hematopoietic development are all examples of important roles which are played by a large family of multifunctional proteins called *Interferons* (Mamane, Heylbroeck et al. 1999).

Human Interferon-γ (hIFN-γ) is homodimer protein composed of 143 amino acids with 2 N-linked glycosylation sites and a molecular weight of approximately 17 kDa at its natural state and is secreted by T-cells and NK cells and its secretion are majorly induced by antigens and mitogens. In human it is a single gene encoded protein whose gene is located on chromosome 12 with the presence of 3 introns (Farrar and Schreiber 1993).

Potential therapeutic efficacy of hIFN-γ has been evident through various clinical trials which explores various therapeutic fields, including renal and pulmonary cell carcinoma, melanoma, rheumatoid arthritis and even leukemia (Gohji, Murao et al. 1986, Krown 1986, Gohji, Maeda et al. 1987, Tamura, Makino et al. 1987, Shah, Van Der Meide et al. 1989).

So far, different expression systems including prokaryotic, protozoan, yeasts, plant, insect and mammalian cells have been investigated for the production of recombinant hIFN-γ. Generally, recombinant hIFN-γ expressed in *E. Coli* could be found in the market, but the process of its purification from bacteria is not very financially favorable. Furthermore, some expression systems such as yeasts (*Pichia pastoris*) are not capable of resulting in a significant amount of yield as compared to the other expression systems.

Well-characterized genetic information, the ability of rapid cell growth, and the vague need for expensive growth medium components, rapid biomass accumulation, compatibility with high-density fermentation as well as the feasibility for a scale-up process are all deemed as golden traits when considering a work-horse microorganism as a host for the production of heterologous proteins (Baneyx 1999, Baneyx and Mujacic 2004). To date, the gram-negative bacterium *Escherichia coli (E. Coli)* is considered one of the most salient microorganisms utilized as a factory-like system for genetic manipulation as well as industrial production of therapeutically or commercially beneficial proteins (Baneyx 1999, Baneyx and Mujacic 2004).

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Zhang et. Al described an optimized procedure for the expression of hIFN-γ in an intracellular manner in E. coli host which was claimed to be a process-scale operation that yields reproducible results. The hIFN-γ that is produced in E. coli is not glycosylated and except for one amino acid difference in its NH2-terminal, its amino acid sequence is completely identical with that of its natural occurring form which causes its immediate elimination from the bloodstream after its injection. It is evident that even beside this slight difference in its primary protein structure, it still fully retains its biological functionality (Zhang, Tong et al. 1992). Besides the facts that expression of foreign proteins in E. coli is intertwined with many problems because of their complexion and large molecular weight, this microorganism is still one of the most utilized and studied ones regarding industrial and therapeutic protein production (Lee 1996). One of the most encountered problems, while using the E. coli as the protein expression system, is the formation of inactive and insoluble aggregates recognized as inclusion bodies (Lee 1996, Lim, Jung et al. 2000) to overcome which many experimental methods and techniques have been proposed such as combined urea-ammonium chloride, chaperones, diafiltration, detergent-assisted refolding, dilution, and urea, which, besides their complex performance instructions, do not always result in high yield production of biologically active proteins (Jin, Guan et al. 2006, Petrov, Nacheva et al. 2010, Yan, Hu et al. 2012).

Various assessment methods are used to determine the functionality of the recombinant hIFN-γ usually produced in a non-traditional expression system. An antiviral assay previously described by Reddy et al. (Reddy, Reddy et al. 2007) is one of the methods used to evaluate the functionality of these recombinant proteins by challenging the human carcinoma cell line (HEP2C) with EMCV in the presence and absence of recombinant hIFN-γ. Furthermore, indirect methods are also used for functional evaluation as the one performed in our study using flow cytometry.

In this report, we describe the production of biologically functional hIFN-γ with the use of E. coli BL21 as our bacterial host, and the process of inclusion body solubilization as well as the purification process developed to achieve a therapeutically beneficial hIFN-γ.

Materials and methods

1. Materials

1.1. Microorganism and plasmid vector

In order to express hIFN-γ, we chose our biological host to be *E. coli* strain BL21 (DE3) (Novagen, UK). hIFN-γ gene was inserted into the commercial expression-inducible plasmid vector pET28a (Novagen, UK) using the bamHI and XhoI insertion sites.

1.2. Media and solution

The buffers used for cell disruption, renaturation, elution and chemicals were all purchased from Merck Co. (Frankfurt, Germany). All other chemicals and reagents were analytical grade and purchased from Sigma Chemical Co. (USA).

2. Methods

2.1. Extraction and amplification of hIFN-γ gene

Blood samples were extracted from healthy individuals to obtain human interferon γ (hIFN-γ) gene from the peripheral T-lymphocytes after isolation of lymphocytes by Ficoll-Paque PLUS density gradient media according to the manufacturer's protocol (GE Lifesciences). Subsequent RNA extraction from activated T-lymphocytes were performed and hIFN-γ mRNAs were extracted and used for further steps. hIFN-γ mRNAs were reverse transcribed into cDNA using RT-PCR and were further confirmed by gel electrophoresis as forming a band of around 500bp. Later our hIFN-γ cDNAs were amplified by PCR using a set of two specific primers. The resultant PCR products then were used for further steps.

2.2. Cloning into pET28a+

The amplified hIFN-γ cDNA were enzymatically digested using the BamHI and XhoI restriction enzymes and eventually ligated into the commercially inducible plasmid vector pET28a+ (Novagen, UK) which was previously digested by the same restriction enzymes. The presence of hIFN-γ gene in our plasmid vector was further confirmed by gel electrophoresis (Nikkhoi, Rahbarizadeh et al. 2017).

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2.3. Plasmid Vector Transformation

The recombinant vector containing hIFN-γ gene was later transformed into *E. coli* strain BL21 (Novagen, UK) using the chemical method of $CaCl_2$ as well as electroporation. The resultant transformed cells were subsequently cultured on LB agar plates containing 50 μg/ml kanamycin. The resultant colonies were randomly selected and the presence of the hIFN-γ gene in them was confirmed by perfuming colony PCR.

2.4. Expression of hIFN-γ

In order to optimize the production of the hIFN-γ protein, various expression conditions were applied. The colonies containing our desired gene fragment were cultured in 5ml of LB medium which contained 50 μg/ml kanamycin. Further on, the culture media were incubated for 4-24 hours in different temperatures ranging between 12-37°C and 0.1-1 mM isopropyl-β-D-thio-galactoside (IPTG) was used for expression induction of the hIFN-γ protein (Nikkhoi, Rahbarizadeh et al. 2018). Table 1 represents the cultural conditions in a detailed manner.

2.5. Cell lysis and preparation of inclusion bodies

In an attempt to harvest the cells from the LB broth medium, they were centrifuged at 5000g for 10 minutes at 4C and the resulting supernatant was discarded. Subsequent pellet resuspension in water and further centrifugation at 5000g for 10 minutes at 4 C was performed to achieve a biomass free of any residual salts. Later on, the resultant pellet was resuspended in lysis buffer (50 mM NaH2PO4, 300 mM NaCl, 10 mM Imidazole, 1 mM PMSF, pH 8.0) and the cells were disrupted by the use of a sonication process (Eppendorf, 2500 v, 5 msec). The suspension of the disrupted cells was centrifuged at 10000g for 10 minutes at 4 C and subsequent washing was also performed on the resultant pellet using sodium phosphate buffer (10mM). Further centrifugation of the suspension at 10000g for 20 minutes at 4C was performed to achieve cell pellet. Small fractions of the eventual suspension were used to further confirm the presence of the hIFN-γ protein via sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDSPAGE) (15% polyacrylamide gel followed by staining with Coomassie brilliant blue). The rest of the protein solutions were stored for further steps of purification.

2.6. Solubilization of inclusion bodies

In the next step, we attempted to solubilize the hIFN-γ proteins which were obtained as inclusion bodies throughout the previous step with a method previously described by Singh and Panda (Singh and Panda 2005). Our solubilization method was based on giving the protein aggregates a pH shock that is distant from their isoelectric point which eventually leads to their solubilization. By the use of such mild solubilization methods for such bioactive proteins, the further refolding and purification steps are performed in a much easier manner. Different pH ranges from 3 to 13 in 100 mM Tris buffer and the percent of the solubilized inclusion bodies were reported.

2.7. Purification and refolding (Chromatography procedures)

In order to ensure that our suspension is free of any residual cell debris or other unwanted insoluble materials, 15000 g centrifugation for 15 minutes at 4 °C was performed on the suspension. The resultant solubilized hIFN-γ containing supernatant was stored at 4C for further steps. The suspension was later loaded on Ni^{2+} chromatography column that was pre-equilibrated with 0.05M ammonium acetate buffer containing 6M urea, 0.002% PMSF (pH 7.2). The eluting process was performed using a gradient concentration of imidazole ranging from 50 to 500 and the consequent effluent was collected for further steps (Nikkhoi, Rahbarizadeh et al. 2018).

The refolding step was performed on the fractions which were obtained from the chromatography process using a peculiar refolding solution containing 8M urea, 10mM Tris-HCl, 100mM NaH2PO4, pH 8.0 followed by a 2 h incubation at 37 °C while shaking at 60 rpm. After centrifugation at 10,000 g for 2 minutes at 4 °C, the supernatants were loaded on Ni-NTA chromatography columns preequilibrated with 8M urea, 10mM Tris-HCl, 100mM NaH2PO4, pH 8.0. The chromatography columns were then washed with denaturation wash buffer (8M urea, pH 6.3), and later on washed with renaturation wash buffer 1 (8M urea, pH 8.0), renaturation wash buffer 2 (6M urea, pH 8.0), renaturation wash buffer 3 (4M urea, pH

8.0), renaturation washes buffer 4 (2M urea, pH 8.0), renaturation wash buffer 5 (1M urea, pH 8.0), renaturation wash buffer 6 (0M urea, pH 8.0) and a native wash buffer containing 40mM imidazole. Eventually, the chromatography columns were eluted using 450mM imidazole.

2.8. Analytical procedures

The method of cell culture turbidity was used as a means of measuring cell growth and also optical density (OD) was determined at a wavelength of 600nm. Sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) with 12.5% polyacrylamide gel was used for the determination of hIFN-γ expression levels and Coomassie brilliant blue R250 was utilized in order to stain the gels. In an attempt to determine the purity of the expressed hIFN-γ, the Coomassie brilliant blue R250 stained gels were scanned using a densitometer. Colony PCR and SDS PAGE techniques were used in order to screen for plasmid stability in the recombinant strain of E. coli. The efficiency of cell lysis by sonication was quantitatively measured by culturing the resulting disrupted cell suspension on LB agar culture plates and counting subsequent colonies.

2.9. Bioassay test for functional characterization of hIFN-γ

Mouse myeloma cell line B16-F10 and human myeloma cell line SK-MEL-37 normally depends on the presence of IFN-γ in their culture medium to upregulate the expression of PDL-1 which is normally expressed at lower levels in normal cells. IFN-γ is the principal stimulator of PDL-1 expression and enhances its expression in various cancer cell lines through the activation of downstream signaling cascades. The expression of PDL-1 was induced in the above mentioned cell lines. 6-well culture plates were filled with glutamax RPMI alongside 10% FBS and each well was used for the culturing of around 10^6 cells until reaching a confluence of 80%. The culture media of the standard wells were exchanged eventually and for the evaluation of our expressed IFN-γ, two concentrations of purified IFN-γ were added to different wells (25 and 50 µg/ml) alongside 200 µg/ml of commercial IFN-γ as positive control. The treatment was proceeded for 12 and 24 hours after induction with IFN-γ to evaluate the transcription level of PDL-1 expression and separately for 24 and 48 hours after induction with IFN-γ to evaluate the protein level of PDL-1 expression. The cells were separated using trypsin treatment and for the evaluation of PDL-1 expression, we added PD-1-FITC to each well.

The cells were washed twice with 500 µl of FACS buffer. Eventually, the cells were resuspended in 300 µl of FACS buffer and the binding of PD-1 was evaluated through the measuring of mean fluorescence intensity (MFI) using flow cytometry.

Results

1. Cloning into pET28a+

The amplified hIFN-γ cDNA and the commercially inducible plasmid vector pET28a+ were enzymatically digested using the BamHI and XhoI restriction enzymes. Figure 1 represents a gel electrophoresis image of the digested vector alongside the amplified hIFN-γ gene fragments. The hIFN-γ and the digested vector were successfully ligated and the results were confirmed by PCR on gel electrophoresis.

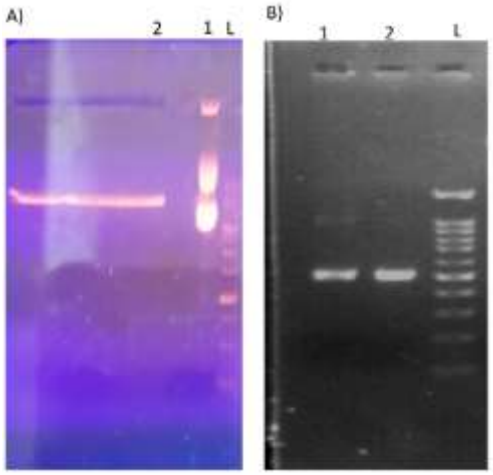

Figure 1 – A) gel electrophoresis picture of the undigested and digested pET28a+ plasmid vector. L: Ladder, 1: undigested vector, 2: digested vector. B) Amplified Human Interferon γ gene fragment. L: ladder, 1: undigested DNA fragment, 2: digested DNA fragment.

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2. Expression of hIFN-γ

In order to induce the expression of hIFN-γ in our recombinant strain, 0.1-1 mM IPTG was added onto the culture medium. The different mass of the produced recombinant hIFN-γ protein in comparison to all of the bacteria proteins in different conditions after expression induction with IPTG is represented in Figure 2. The final specific yield was assessed by scanning of SDS-PAGE gels.

Table 1 – Representation of different bacterial culture conditions

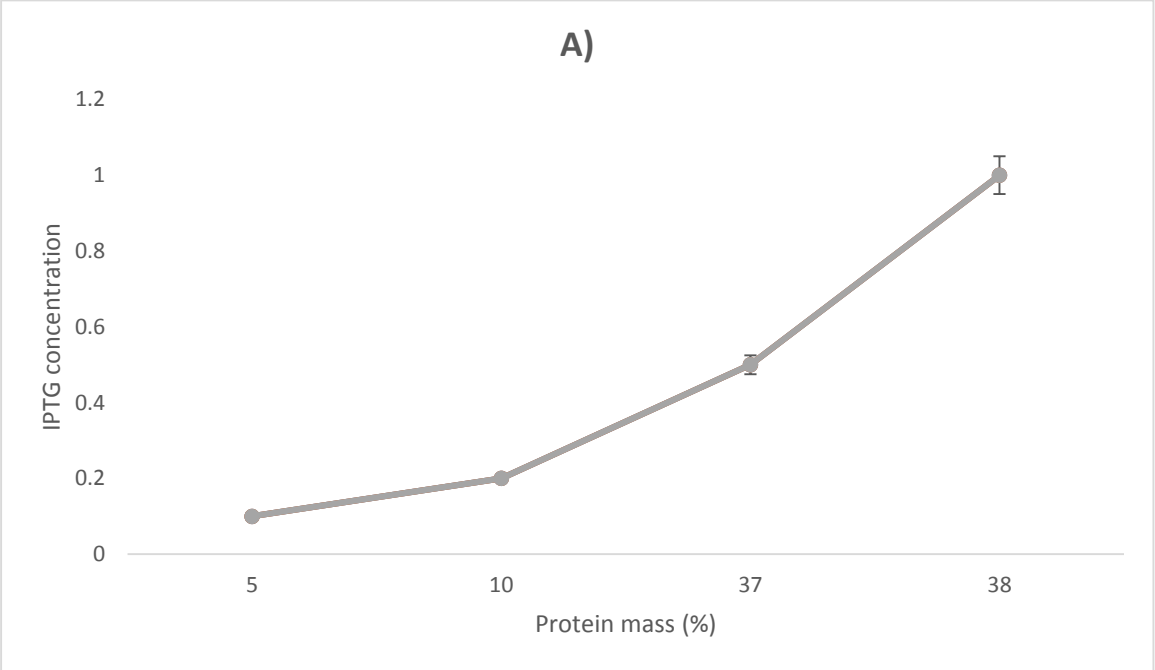
Conditions				
Temperature (°C)	18	28	32	37
Time (hours)	8	16	24	48
IPTG concentration (mM)	0.1	0.2	0.5	1

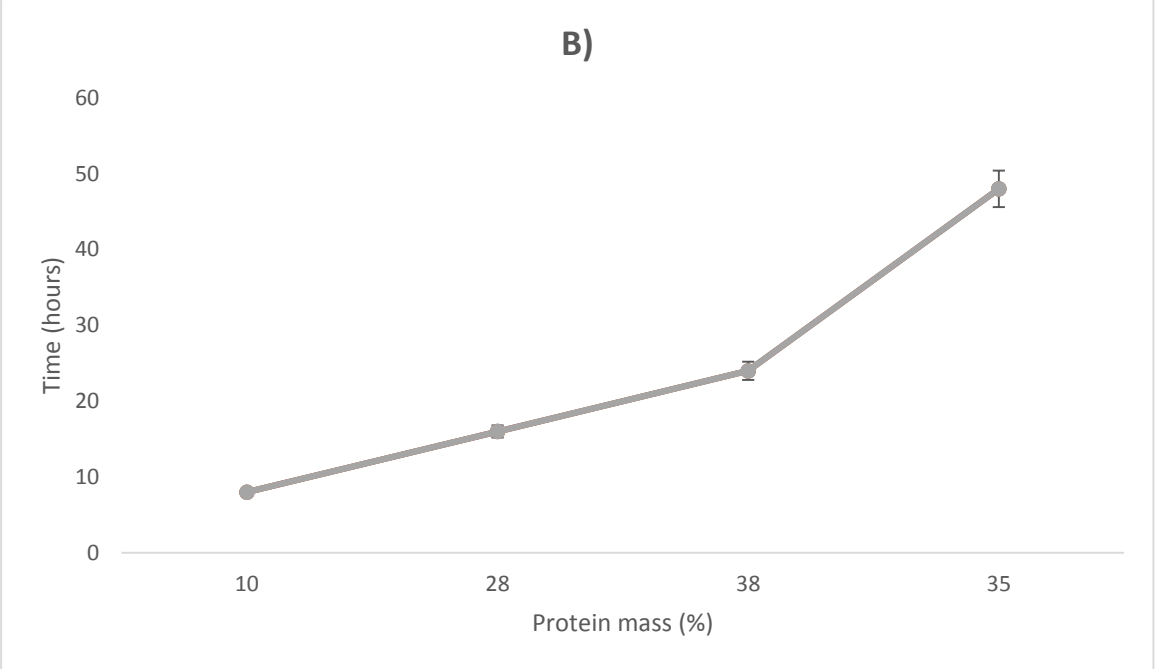
3. Cell lysis and preparation of inclusion bodies

Unwanted proteolysis of our desired proteins may happen due to the release of some enzymes capable of such activities from the non-disrupted cells, which happen to be sedimented alongside the inclusion bodies while the pellet is being formed during the centrifugation process. So, in order to verify the accuracy of the sonication-related cell disruption step, the resultant cell lysis suspension was further on cultured on LB agar medium plates and the colonies formed were counted. The results indicated that most of the bacteria were lysed. After the previous step, the inclusion bodies suspension was centrifuged at 10000 g for 25 minutes at 4C, the resulting supernatant underwent various analytical analysis including Braford method.

4. Solubilization of inclusion bodies

In the next step, the solubilization process was performed as previously described. As the results indicated an incremental pattern was observed during the process of solubilization as pH was increased from 6 to 13. Also, using urea alongside Tris buffer was considered as helpful for a more efficient solubilization process. Table 2 represents the percent of solubilization at different pHs with and without urea. It is also worth mentioning that urea did not disrupt the native secondary structure of the protein.





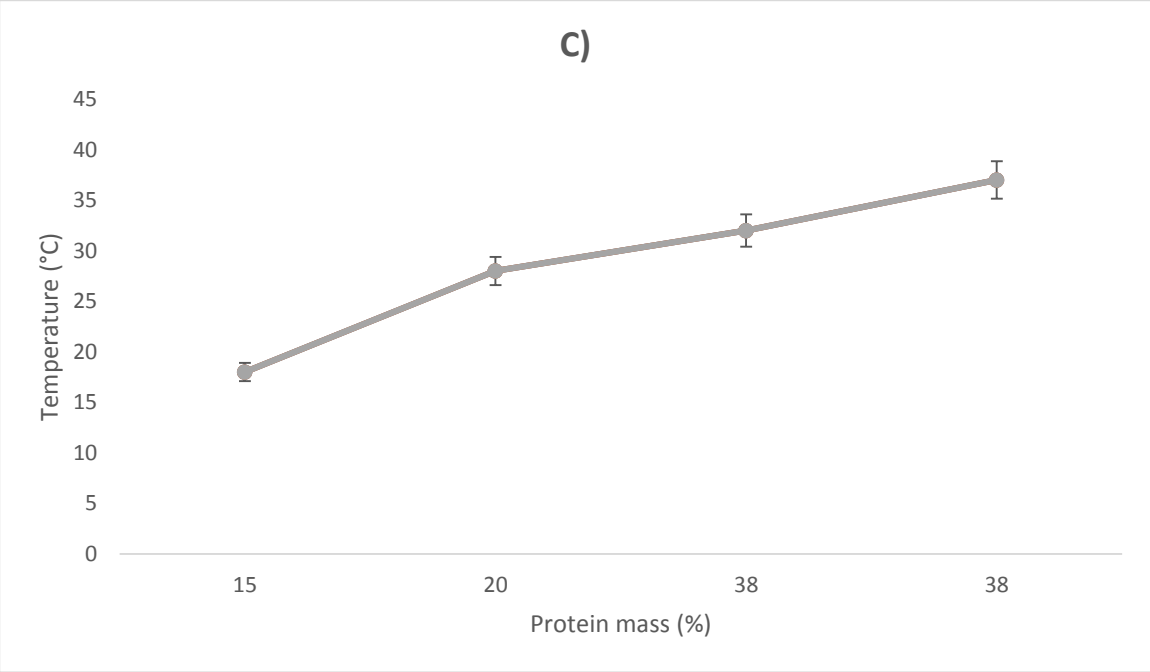


Figure 2 – Representation of the effects of different conditions for the expression of hIFN-γ. A) Effects of the different concentrations of IPTG, B) effects of different culture times, C) effect of different temperatures of incubation.

Table 2 - Solubilization of purified hIFN inclusion bodies at different pHs

pH	**Percent solubilization of hIFN-γ inclusion bodies**	
	Tris buffer (no urea)	Tris buffer (2 M urea)
6	-	5
7	-	5
8	5	10
9	5	10

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10	12	15
11	20	25
12	40	85
12.5	50	95

Inclusion body proteins were solubilized at different pHs and the percent of solubility was calculated by measuring the percent of the solubilized protein concentration by Bradford assay.

5. Purification and refolding

The solubilized hIFN-γ proteins were loaded on Ni^{2+} chromatography column pre-equilibrated with 0.05M ammonium acetate buffer containing 6M urea, 0.002% PMSF (pH 7.2) after the solubilization process of the inclusion bodies and the effluent was eluted using different concentrations of imidazole. The obtained fractions were later on 5-fold diluted by refolding solutions (8M urea, 10mM Tris-HCl, 100mM NaH2PO4, pH 8.0) and stored in refrigerator for further steps. The refolded solution was later loaded on Ni^{2+} chromatography column which was pre-equilibrated with 0.05M ammonium acetate buffer containing 6M urea, 0.002% PMSF (pH 7.2) and the washing process was performed using the previously mentioned buffers and the resulting refolded hIFN-γ proteins were eluted as previously detailed. Our results substantiated that the non-inclusion body method was the more efficient one and since the results from the inclusion body method was not favorable, we managed the perform our functional assays on the proteins obtained from the non-inclusion body method.

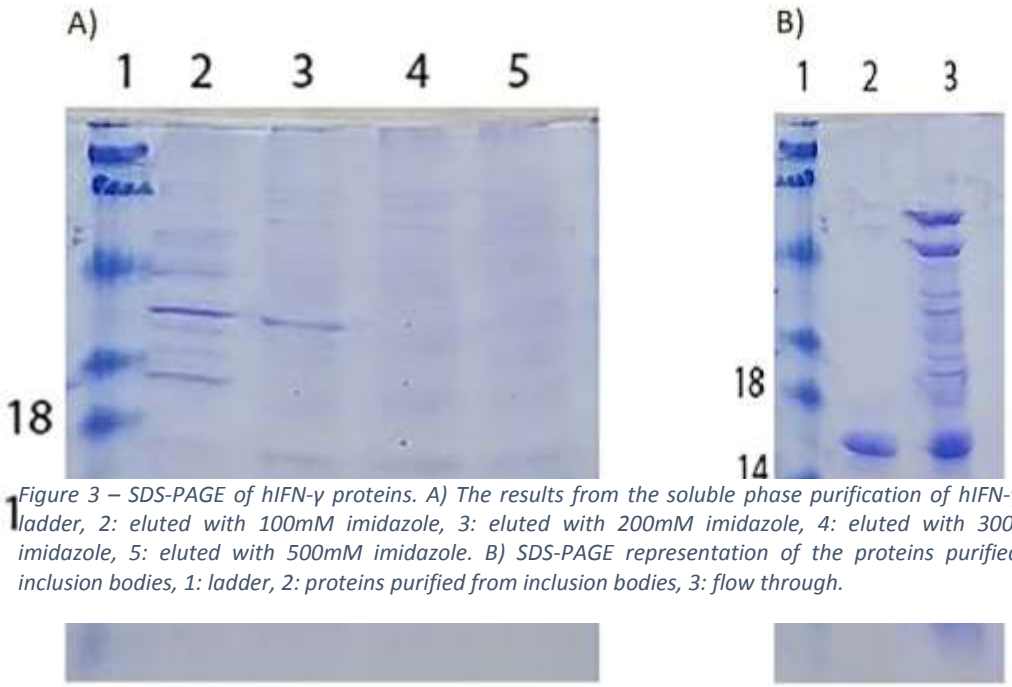
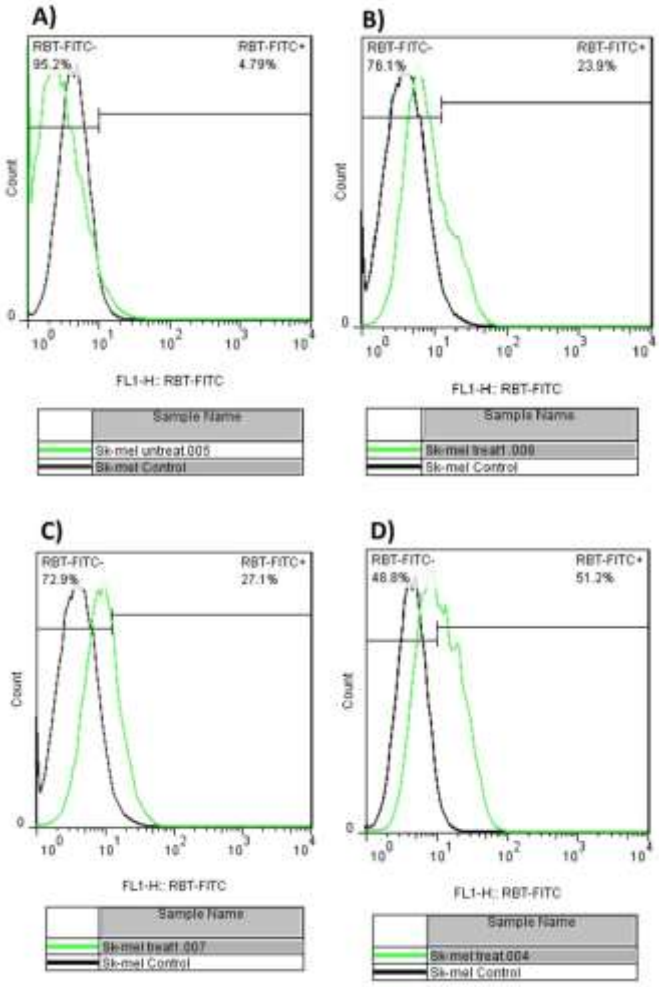


Figure 3 – SDS-PAGE of hIFN-γ proteins. A) The results from the soluble phase purification of hIFN-γ, 1: ladder, 2: eluted with 100mM imidazole, 3: eluted with 200mM imidazole, 4: eluted with 300mM imidazole, 5: eluted with 500mM imidazole. B) SDS-PAGE representation of the proteins purified as inclusion bodies, 1: ladder, 2: proteins purified from inclusion bodies, 3: flow through.

6. Bioassay test for functional characterization of hIFN-γ

Flow cytometry results indicated that our hIFN-γ proteins were functional as compared with standard hIFN-γ. Figure 4 represents the results of the flow cytometry assay.

Figure 4 – Flow cytometry results of hIFN-γ proteins indicated their biological functionality in comparison to commercially available hIFN-γ. A) non-treated, B) treated with 25 µg/ml of hIFN-γ, C) treated with 50 µg/ml of hIFN-γ proteins, and D) treated with 200 µg/ml of commercial hIFN-γ.



Discussion

hIFN-γ is a biologically and pharmaceutically precious cytokine (Chen and Liu 2009, Zaidi and Merlino 2011) which makes it a particularly interesting protein as an industrial and pharmaceutical point of view, thus there had been plenty of researchers and pharmaceutical companies attempting to produce it in large industrial scales using different types of biological host such as E. coli and CHO cells. One of the disadvantages of E. coli in biosynthesis of bioactive proteins with eukaryotic sources is the formation of inactive and insoluble aggregates known as inclusion bodies. To this date, researchers all over the world have developed various different techniques to have a better yield of recombinant proteins while using biological expression hosts that are correlated with the formation of inclusion bodies. Techniques such as urea, urea-ammonium chloride, chaperones, and dilution which all casually lowers the final yield of the bioactive product (Yan, Hu et al. 2012). Low cost, high productivity, easy cultivation methods, and high cell density, concentration are all among the reasons that led to choosing E. coli as our protein expression host in this study. It is also worth mentioning that the low productivity of the recombinant hIFN-γ in CHO cells is considered as one of the disadvantages of producing these proteins in eukaryotic expression hosts (Fukuta, Yokomatsu et al. 2000).

In this study, the biologically active hIFN-γ, which is a homodimer that has interactions with INF-γ receptor (Ealick, Cook et al. 1991, Sadir, Forest et al. 1998), was successfully expressed in E. coli with a reasonable production yield and biological functionality in comparison to various other related research papers published in this field using the commercially inducible pET28a+ inserted into the BamHI and XhoI restriction sites. In the current study, we performed both the inclusion and non-inclusion body method of purification, but the results from the inclusion body method was not favorable. Besides the final production yield of the hIFN-γ protein, its functionality as a cytokine is an important factor throughout the process of its production. In this study, the functionality of the E. coli-

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produced hIFN-γ was evaluated via flow cytometry technique and the encouraging results further confirmed the success achieved thorough this study. Although some study have reported lower biological activity for recombinant hIFN-γ in comparison to the standard which could potentially result from the formation of complex structures or monomers instead of homodimers (Wang, Ren et al. 2014).

There are different methods utilized to assess the bio-functionality of the recombinant hIFN-γ produced by different expression systems. As we mentioned earlier, it has been proven that IFN-γ induces an up-regulation in the expression level of PDL-1 in several mouse and human cell lines through the activation of downstream signaling cascades (Mouse myeloma cell line B16-F10 and human myeloma cell line SK-MEL-37). We used this potential to indirectly evaluate the functionality of the recombinant hIFN-γ produced in this study which indicated highly encouraging results. On the other hand, similar studies have also used a method named "Antiviral assay" to evaluate the bio-functionality attributed to the recombinant hIFN-γ.

Also, further scientific investigations need to be conducted to better the productivity of the recombinant hIFN-γ as well as increasing their biological functionality through circumventing the formation of inclusion bodies alongside other multimeric complexes which are considered detrimental to the bioactivity of hIFN-γ.

References

[1] Baneyx, F. (1999). "Recombinant protein expression in Escherichia coli." Current opinion in biotechnology **10** (5): 411-421.

[2] Baneyx, F. and M. Mujacic (2004). "Recombinant protein folding and misfolding in Escherichia coli." Nature biotechnology **22**(11): 1399.

[3] Chen, J. and X. Liu (2009). "The role of interferon γ in regulation of CD4+ T-cells and its clinical implications." Cellular immunology **254**(2): 85-90.

[4] Ealick, S. E., et al. (1991). "Three-dimensional structure of recombinant human interferon-gamma." Science **252**(5006): 698-702.

[5] Farrar, M. A. and R. D. Schreiber (1993). "The molecular cell biology of interferon-gamma and its receptor." Annual review of immunology **11**(1): 571-611.

[6] Fukuta, K., et al. (2000). "Genetic engineering of CHO cells producing human interferon-γ by transfection of sialyltransferases." Glycoconjugate journal **17**(12): 895-904.

[7] Gohji, K., et al. (1987). "Enhanced inhibition of anticancer drugs by human recombinant gamma-interferon for human renal cell carcinoma in vitro." The Journal of urology **137**(3): 539-543.

[8] Gohji, K., et al. (1986). "Enhanced inhibition of colony formation of human renal cell carcinoma in soft agar by the combination of α-difluoromethylornithine and recombinant γ-interferon." Cancer research **46**(12 Part 1): 6264-6268.

[9] Jin, T., et al. (2006). "On- column refolding of recombinant human interferon- γ inclusion bodies by expanded bed adsorption chromatography." Biotechnology and bioengineering **93**(4): 755-760.

[10] Krown, S. (1986). "AIDS and Kaposi's sarcoma: interferons in pathogenesis and treatment." Interferon **7**: 185-211.

[11] Lee, S. Y. (1996). "High cell-density culture of Escherichia coli." Trends in biotechnology **14**(3): 98-105.

[12] Lim, H.-K., et al. (2000). "Production characteristics of interferon-α using an L-arabinose promoter system in a high-cell-density culture." Applied microbiology and biotechnology **53**(2): 201-208.

[13] Mamane, Y., et al. (1999). "Interferon regulatory factors: the next generation." Gene **237**(1): 1-14.

[14] Nikkhoi, S. K., et al. (2017). "Oligo-clonal nanobodies as an innovative targeting agent for cancer therapy: new biology and novel targeting systems." Protein expression and purification **129**: 115-121.

[15] Nikkhoi, S. K., et al. (2018). "Multivalent targeting and killing of HER2 overexpressing breast carcinoma cells with methotrexate-encapsulated tetra-specific non-overlapping variable domain heavy chain anti-HER2 antibody-PEG-liposomes: in vitro proof-of-concept." European Journal of Pharmaceutical Sciences **122**: 42-50.

[16] Nikkhoi, S. K., et al. (2018). "Liposomal nanoparticle armed with bivalent bispecific single-domain antibodies, novel weapon in HER2 positive cancerous cell lines targeting." Molecular immunology **96**: 98-109.

[17] Petrov, S., et al. (2010). "Purification and refolding of recombinant human interferon-gamma in urea–ammonium chloride solution." Protein expression and purification **73**(1): 70-73.

[18] Reddy, P. K., et al. (2007). "Increased yield of high purity recombinant human interferon-γ utilizing reversed phase column chromatography." Protein expression and purification **52**(1): 123-130.

[19] Sadir, R., et al. (1998). "The heparan sulfate binding sequence of interferon-γ increased the on rate of the interferon-γ-interferon-γ receptor complex formation." Journal of Biological Chemistry **273**(18): 10919-10925.

[20] Shah, P., et al. (1989). "Effect of human recombinant tumour necrosis factor and rat gamma interferon on nitrosomethylurea-induced mammary tumours." British journal of cancer **59**(2): 206.

[21] Singh, S. M. and A. K. Panda (2005). "Solubilization and refolding of bacterial inclusion body proteins." Journal of bioscience and bioengineering **99**(4): 303-310.

[22] Tamura, K., et al. (1987). "Recombinant interferon beta and gamma in the treatment of adult T- cell leukemia." Cancer **59**(6): 1059-1062.

[23] Wang, D., et al. (2014). "Expression, purification and characterization of human interferon-γ in Pichia pastoris." Molecular medicine reports **9**(2): 715-719.

[24] Yan, X., et al. (2012). "Coexpression of chaperonin GroEL/GroES markedly enhanced soluble and functional expression of recombinant human interferon-gamma in Escherichia coli." Applied microbiology and biotechnology **93**(3): 1065-1074.

[25] Zaidi, M. R. and G. Merlino (2011). "The two faces of interferon-γ in cancer." Clinical cancer research **17**(19): 6118-6124.

[26] Zhang, Z., et al. (1992). "Production, purification and characterization of recombinant human interferon γ." Journal of Chromatography A **604**(1): 143-155.