Research in Molecular Medicine

Cloning, Expression, and Purification of Recombinant Mouse Interferon-γ

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ABSTRACT

Background: Interferon-gamma (IFN-γ) is the most important cytokine in the immune system. This protein has been expressed in bacterial cells. However, bacterial cloning is not an easy task. We aimed to clone, express, and purify recombinant mouse IFN-γ and overcome problems in favor of commercial purposes.

Materials and Methods: To amplify the gene product for cloning, we primarily designed two specific primers for the target gene. Following PCR amplification, the amplicon was inserted into the pET-21b(+) vector. The *E. coli* BL21 (DE3) CodonPlus strain was chosen for the expression of the target gene. Finally, the expressed recombinant mouse IFN-γ was assessed through the western blotting method.

Results: We performed a cloning process and produced recombinant mouse IFN-γ in an optimal condition. We also noticed that monomeric protein could be transformed to a homodimeric structure which can be observed using the SDS PAGE (SDS-polyacrylamide gel electrophoresis) and western blotting.

Conclusion: Experimental conditions strongly affect the large-scale cloning procedures required to be optimized in each laboratory. The expressed recombinant mouse IFN-γ described here is appropriate for commercial purposes.

1. Introduction

Interferons (IFNs) are secretory proteins produced by lymphocytes and fibroblasts. They are a family of cytokines consisting of three major types based on antigenicity, chemical traits, and biological activity: Type I (IFN-α, -β, -ε, -ω), Type II (IFN-γ), and Type III (IFN-λ1, IFN-λ2, and IFN-λ3) [1]. Among them, IFN-γ is mostly produced by immune cells and serves different biological activities in the immune system and autoimmune disorders [2-4].

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With the advent of genetic engineering technologies, many proteins have been experimentally expressed in Escherichia coli (*E. coli*) with high efficiency. Because of its short life cycle, easy genetic manipulation, and simple culture conditions, *E. coli* is a preferable host to produce many recombinant proteins [5, 6]. *E. coli* was the first host utilized by Eli Lilly in 1982 to produce recombinant insulin [7]. Although all bacteria could be used for recombinant protein technologies, *E. coli* is still the preferable host for the industrial production of recombinant proteins [8]. For cloning purposes in *E. coli* species, researchers are faced with two main obstacles: solubility of recombinant proteins for high expression and low and complicated expression of a foreign gene. Accordingly, the *E. coli* BL21 (DE3) CodonPlus strain is a good host for large-scale protein expression. The expression of a foreign protein in *E. coli* is influenced by a range of factors, and there is no standard strategy to overcome these issues.

Overall, five variabilities affect the solubility and expression of proteins in the cloning process, which might help to proper folding of desired protein and increase the expression level: 1- using proper host, 2- proper vector, 3- co-expression with other genes, 4- changing the gene sequences, and 5- variations of culture parameters of the recombinant host strains [9]. Using an appropriate expression host and vector significantly increase the activity and solubility of target proteins. A suitable vector can enhance expression, folding, and solubility in different ways. The folding can be improved in hosts that allow the formation of disulfide bonds in the cytoplasm when using vectors designed for cytoplasmic expression [10-13]. The pET-21b(+) vector has a T7 RNA promoter and 6 His-tag DNA sequence, which facilitates the purification and detection of protein or may increase the probability of biological activities by affecting solubility in the cytoplasm or exportation to the periplasmic area.

Since the development of recombinant DNA and protein technology, cDNA sequences of IFN-γ have been recognized and cloned into prokaryotic hosts and vectors. It was then verified that the produced recombinant IFN-γ has the same chemical properties and immunological activities as natural IFN-γ. It was supposed that recombinant technology would be the most effective way of providing larger quantities of IFNs. It was then shown that the produced materials exhibit acceptable bioactivity, suggesting their clinical values in the treatment of a wide range of viral, neoplastic, and immunological disorders [14-16]. This study aimed at cloning the mouse IFN-γ in pET-21b(+) vector and *E. coli* BL21 (DE3) CodonPlus strain to overcome protein expression in prokaryotic hosts and produce recombinant IFN-γ for diagnostic and commercial purposes.

2. Materials and Methods

Solubility Prediction of Recombinant IFN-γ

When a target protein is cloned in an expression vector, unwanted sequences may be added to it, affecting the functional properties of the protein. In this study, we used the pET-21b(+) vector from Novagen company. To clarify the solubility of the target protein, we applied predictive tools using bioinformatics analysis. To this effect, we used Swiss-PDB viewer software (v. 4.1.0). The full length of our recombinant target is shown in Figure 1. According to Novagen instruction, these unwanted sequences do not affect the target, for example, changing its antigenicity or solubility. In this study, we used the extracellular chain mouse IFN-γ with 133 amino acids with 343 bp length (Query P01580 on Uniprot database).

Expression and Cloning of Recombinant Mouse IFN-γ

Total RNA of BALB/c mouse spleen was extracted by RNA extraction kit (Parstous, Iran). To determine the RNA extraction quality, the isolated RNA was electrophoresed with 1% agarose gel. The cDNA (complementary DNA) was synthesized using oligo-dT primers and RT (Reverse Transcriptase) enzyme according to the manufacturer's instructions (Parstous kit, Iran). Then, the mouse IFN-γ cDNA was amplified, without signal peptide sequence, using specific primers comprising *EcoRI* and XhoI restriction sites. These primers were designed by Gene Runner software (v.5.2) as follows: Forward *5'-GATCCGAATTCGCACGGCACAGTCATTGAAAGC-3'* with *EcoRI* restriction site and reverse *5'-GTGGTGCTCGAGCTGGTGGACCACTCGGATGAG-3'* with XhoI restriction site. The PCR was then performed using KlenTaq 2x Premix kit (Parstous kit, Iran).

The expected length of the amplified sequence was determined by gel electrophoresis with 1% agarose gel. To eliminate the impurities and nonspecific bands, the desired bands were purified by a DNA gel extraction kit (Parstous, Iran). The PCR product was digested at 37°C for 2 hours and then digested PCR products were extracted with a liquid DNA extraction kit (Parstous kit, Iran) and cloned into the pET-21b(+) vector, which was cut with the same restriction enzymes at 22°C for 4 hours by T4 DNA ligase and ligase buffer. Subsequently, the recombinant plasmid was transformed by heat-shock method (From 0°C to 42°C) into the competent *E. coli* Top10 strain (OD600: 0.5-0.8 in logarithmic phase),

which was prepared through Inoue method [17], and cultured on LB Agar (containing 100 µg/mL ampicillin) at 37°C for 19 hours. Screening of transformed colonies with recombinant plasmid was performed by electrophoresis of PCR product using T7 primers. The recombinant plasmids of selected colonies were extracted by plasmid extraction kit (Parstous, Iran) and confirmed by sequencing (Macrogen Company). To express the recombinant IFN-γ, *E. coli* BL21 (DE3) CodonPlus strain competent with Inoue method was transformed by recombinant plasmid using heat-shock process. Then, these bacteria were cultured in 4 mL LB broth (containing 100 µg/mL ampicillin). After overnight incubation at 37°C, 0.5 mL of culture was added to 100 mL fresh LB broth with the same condition for growing bacteria to obtain OD600: 0.5-0.8. The 0.2 mM Isopropyl-β-D-thiogalactopyranoside (IPTG) was used to induce and express recombinant protein after OD600: 0.5-0.8 and incubated at 37°C with 150 RPM shaking for 19 hours. Consequently, the bacteria were lysed with sonication in lysis buffer (50 mM KH2PO4, pH:7.8, 10% glycerol, 0.5% Triton), and the expressed protein was assessed by SDS-PAGE (SDS-polyacrylamide gel electrophoresis) in supernatant and bacteria pellet.

Metal affinity purification and dialysis

Because of the presence of a His-tag in the recombinant IFN-γ, metal affinity chromatography using Ni-IDA column (Amersham Pharmacia Biotech) was applied to purify the target protein. Initially, the column was washed using 25 mL distilled water and equilibrated with 25 mL start buffer (50 mM Tris-HCl, 100 mM NaCl, pH:8.0). Then, the protein supernatant from a cell lysate, which had been previously treated with 100 mM NaCl, was added to the column at a low flow rate. After that, to remove the unbound or weakly bonded components, the washing was performed using 40 mL starting buffer and 100 mL washing buffer (50 mM Tris-HCl, pH:8.0, 100 mM NaCl, and 25 mM imidazole), respectively. Finally, the recombinant protein was eluted by starting buffer containing 250 mM imidazole. The SDS-PAGE was performed to determine product purity. Finally, the collected fractions were dialyzed against buffer containing 150 mM PBS and 25% glycerol overnight at 4°C by dialysis tubing with a 12 kDa cutoff point.

The SDS-PAGE and Western blotting

After dialysis, the solubility of recombinant proteins was evaluated by electrophoresis of the dialyzed product on a 12.5% SDS-PAGE. Next, the proteins were transferred to the PVDF (polyvinylidene difluoride) membrane by electroblotting. This membrane was blocked using 2% BSA (bovine serum albumin) in PBS at 4°C overnight. Subsequently, the polyclonal anti-mouse IFN-γ biotin-conjugated antibody (1:50 and 1:500 diluted in 1% BSA) was added at room temperature for 2 hours and detected with a 1:20000 diluted horseradish peroxidase-streptavidin (HRP) in 1% BSA solution. Chemiluminescent substrate (GE Healthcare, Buckinghamshire, UK) was used to visualize the reactivity of a specific antibody with the target protein.

Protein assay

The bicinchoninic acid (BCA) method was applied to measure the protein concentration. First, reagents and standards were prepared according to the kit procedure (Parstous, Iran), and the sample was diluted. Then, 25 µL of each sample and 85 µL of working reagent were added into duplicate wells of the micro-titration plate. The mixture was incubated at 60°C for 60 minutes. The OD (optical density) was measured at 630 nm, and the concentration of samples was determined using a standard curve.

3. Results

Prediction of solubility and antigenicity

Using Swiss-PDB viewer software (v. 4.1.0), we predicted antigenicity and solubility of full-length recombinant IFN-γ. The result revealed that the first 16 amino acids and 6 His-tag added to our target had not affected the protein antigenicity and solubility. In this study, we used the extracellular chain mouse IFN-γ with 133 amino acids and 343 bp length (Query P01580 on Uniprot database).

Figure 1. The full length of IFN-γ target protein

Recombinant mouse IFN-γ is constructed of 3 parts: the first part consists of 16 amino acid belonging to pET-21b(+) with green color; the seconds part comprises 133 amino acids (Query P01580 from Uniprot database) that belonged to extracellular mouse IFN-γ chain with blue color, and the third part belonged to pET-21b(+) 6 histidine-tag (6 His-tag) with red color.

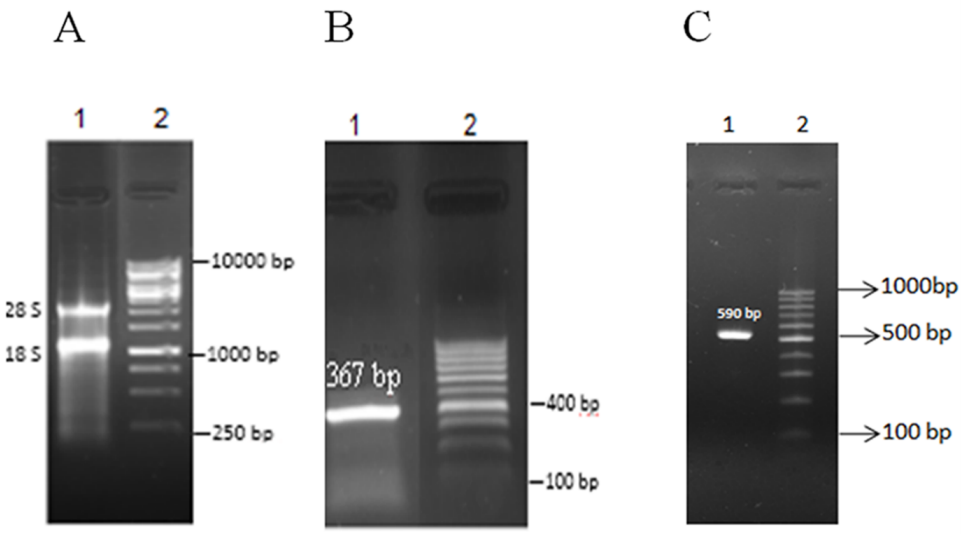

Figure 2. Gel electrophoresis results

A: The quality of extracted RNA from mouse spleen, Lane 1; 28s and 18s band, Lane 2; 10000 bp ladder. B: Specific amplified IFN-γ gene, Lane 1; 1000 bp ladder, Lane 2; a specific 367 bp band of IFN-γ gene. C: Gel electrophoresis of PCR product with T7 primers, Lane 1; verification of mouse IFN-γ gene cloning into the recombinant plasmid, Lanes 2; 100 bp ladder.

IFN-γ Gene Cloning in pET21-b vector

To determine RNA quality, we visualized the 28s and 18s band on 1% agarose gel (Figure 2A). Then, 367 bp IFN-γ gene was amplified with specific primers observed on 1.5% agarose gel (Figure 2B). Since the fragment had been inserted inside the pET-21b(+) 's T7 coding region, 223 bp was added to the amplified sequence of the transformed *E. coli* Top10 strain by recombinant vectors. So, the correct ligation of recombinant plasmid was demonstrated by observing the 590 bp band after PCR with T7 primers (Figure 2C). Furthermore, NCBI BLAST following DNA sequencing of PCR fragment indicated 100% homology with IFN-γ precursor.

IFN-γ Expression and SDS-PAGE Analysis

To evaluate the low (Figure 3A) and high (Figure 3B) expression levels of the recombinant IFN-γ by transformed *E. coli* BL21(DE3) CodonPlus strain, we performed electrophoresis using SDS–PAGE after centrifugation of bacterial lysates on supernatant and cell pellet. Our results showed that more protein fractions were soluble in the supernatant (Figure 3B). However, the results demonstrated the expression of a 16.42 kDa protein to be equal with the molecular weight of the recombinant IFN-γ. The supernatant was passed through the Ni-IDA column to eliminate nonspecific protein fractions. The SDS–PAGE results are shown in Figure 3C. The SDS–PAGE was also performed on dialyzed recombinant IFN-γ, showing a 16.42 kDa specific band (Figure 3D).

Western blotting and protein assay

The SDS-PAGE outcome indicated the presence of a 16.42 kDa soluble protein. Then, the western blot analysis confirmed the biological activity of the target protein, interacted with specific anti-mouse IFN-γ antibody (Figure 4). Additionally, we observed a nonspecific band that, according to its molecular weight, is probably related to IFN-γ homodimeric form. The final concentration of recombinant protein was measured by the BCA method showing 79.2 µg/mL (OD630: 0.414).

4. Discussion

We performed a cloning process to produce recombinant mouse IFN-γ in an optimal condition. We also noticed that monomeric protein could be transformed to a homodimeric structure which can be observed using the SDS-PAGE and western blotting. The mouse IFN-γ is a 17.907-kDa homodimeric soluble cytokine having the same activity as human IFN-γ but lacking intra-molecular disulfide bonds. Patrick W. Gray et al. were the first who performed the expression and production of a recombinant IFN-γ and revealed the characteristics of human IFN-γ [18]. A critical point for any expression of recombinant protein is to primarily predict the solubility and cellular localization of the recombinant target.

In many cases, it is desirable to express proteins in their soluble and active forms. The solubility of a recombinant protein is predictable by various factors, including the

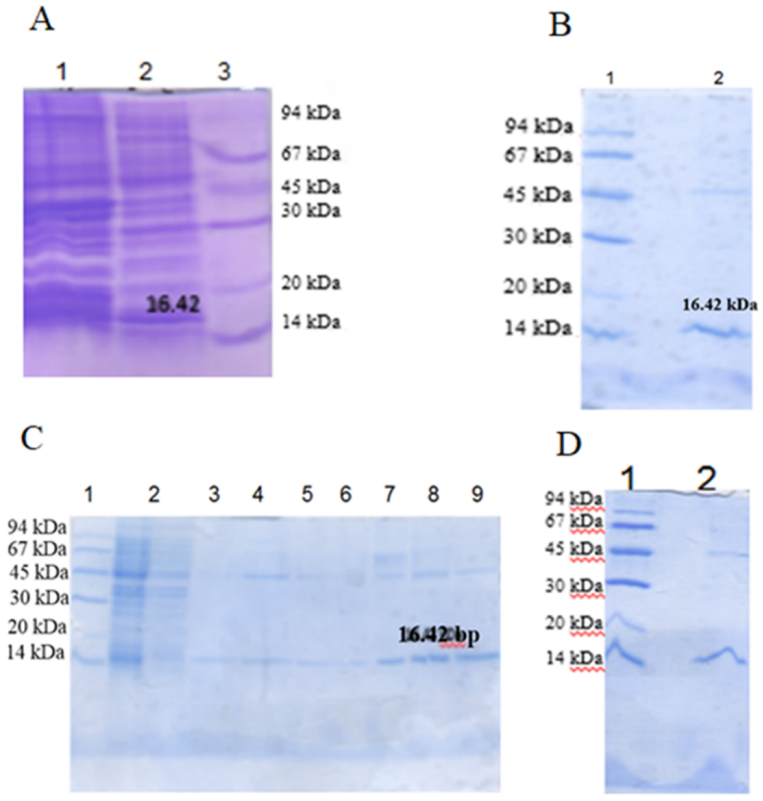

Figure 3. SDS-PAGE with Coomassie blue staining results

A: The low-level expression of the recombinant IFN-γ, Lane 1; bacterial lysates cell pellet protein mixture containing bacterial protein and the cloned target, Lane 2; bacterial lysates supernatant protein mixture that contains bacterial protein and 16.42 kDa of the target protein, Lane 3; protein marker. B: The high-level expression of the recombinant IFN-γ, Lane 1; protein marker, Lane 2; bacterial lysates supernatant protein mixture comprising 16.42 kDa recombinant IFN-γ. C: Metal affinity purification, Lane 1; protein marker, Lane 2; the supernatant protein mixture before purification that contains bacterial protein and our target, Lane 3-9; the supernatant protein mixture after purification that contains recombinant IFN-γ 16.42 kDa. D: SDS–PAGE performed on dialyzed recombinant IFN-γ, Lane 1; protein marker, Lane 2; desalted and purified recombinant protein after dialysis.

number of disulfide bonds, glycosylation, and protein sequence. The host, vector, and cell culture conditions can be considered to decrease or increase the amount of insoluble and soluble forms [8, 9, 19, 20].

In this study, based on the mouse IFN-γ DNA and protein sequences in the Uniprot database, two specific primers were designed and used in the PCR test. The PCR conditions for the amplification of the IFN-γ gene were adjusted, and finally, a fragment of the expected size (367 bp) was obtained. To confirm the cloned IFN-γ cDNA, sequencing of the plasmid was performed. The results of sequencing data exhibited that the correct cDNA fragment has been cloned. To express, translate, and produce the recombinant IFN-γ, we used the *E. coli* BL21 strain. For general expression of recombinant proteins, *E. coli* BL21, K12, and their other strains are most commonly used [8]. The *E. coli* BL21 strain is a common choice for the expression of recombinant proteins due to the lack of two major proteases (Lon and OmpT protease) genes. The *E. coli* strains that are deficient in OmpT and Lon proteases cannot degrade inserted foreign proteins. The T7 RNA polymerase system is one of the most widely-used expression systems and elongates the mRNAs about five folds faster than *E. coli* RNA polymerase. In other hand, T7 RNA and *E. coli* RNA polymerase recognizes different promoters and could be controlled selectively. The T7 RNA polymerase gene is located in the bacterial chromosome under the control of a lac promoter in *E. coli* BL21 (DE3) strain. For increasing the production of recombinant proteins, the lac promoter is rarely used since it is a weak promoter [21-24]. Over-induction of lac promoter can be achieved by adding IPTG to fully activate the *E. coli* BL21 (DE3) strain. In many experiences, protein expression does not occur in the *E. coli* host due to the differences in preferential codon use by the *E. coli* strains and interesting gene codons. To overcome this problem, a few biotechnologies

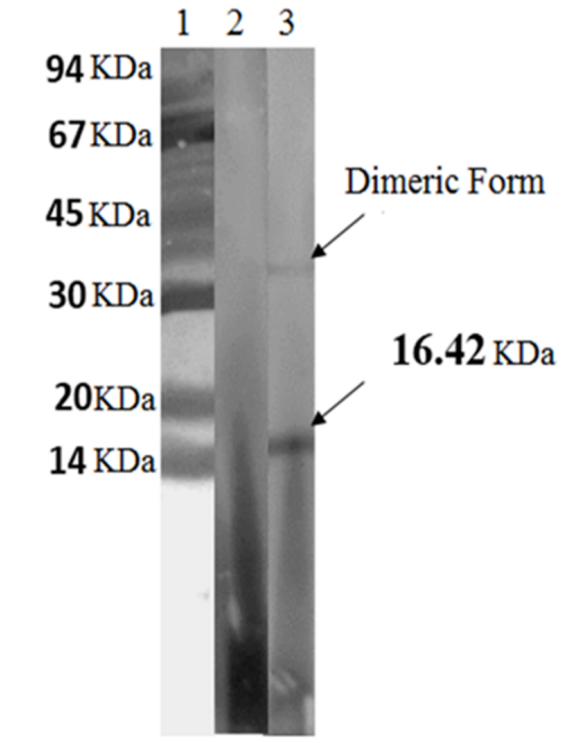

Figure 4. Western blot analysis of purified recombinant IFN-γ

Lane 1: Protein marker; Lane 2: Negative control to determined nonspecific interaction antibody; Lane 3: IFN-γ dimeric form and 16.42 kDa mi-IFN-γ that reacted with +a specific antibody.

provide modified *E. coli* hosts that have extra tRNA coding genes (AGA, CCC, CUA, GGA, AUA, and AGG) to compensate for the infrequency of rare tRNA. The bacterial host strain was designed as *E. coli* BL21 (DE3) CodonPlus strain that we applied in our experiments. Thousands of heterologous and homologous recombinant proteins have been successfully expressed in high levels using *E. coli* BL21 (DE3) CodonPlus strain like our previous studies [5, 6].

Although *E. coli* and its derivative strains have extended into a common host organism for molecular cloning, the transformation technique of *E. coli* is highly inefficient even using competent bacterial cells [25]. An essential step in molecular cloning is the transformation of the plasmid into bacterial competent cells. The best time for transformation is when the bacterial cells are in an early logarithmic growth phase. Ryu. J. et al. have noticed that the early logarithmic phase is crucial for plasmid transformation [26]. Based on their findings, we transformed competent bacterial in OD600:0.5-0.8 in which they were in the logarithmic phase.

Tsen. S. D. et al. have shown that certain strains of *E. coli* could naturally incorporate extracellular plasmid vectors into cytoplasm area at low frequencies [27]. Many genera performed natural transformation, such as Streptococcus, Bacillus, Haemophilus, and Micrococcus with their several proteins on their outer cell membrane that can be attached to DNA and carry it inside. However, it is still an uncommon feature for most bacteria, such as *E. coli*, to naturally take up external DNA from the surrounding environment [25]. In this context, Cohen et al. presented a method to successfully transform R-factor and recombinant plasmids into *E. coli* cells using CaCl2 [28]. To transform the plasmids with high efficiency, there are many different methodologies to improve bacterial competent cell preparation. The transformation efficiency is a critical event in molecular cloning experiments which could be influenced by many factors [25, 29]. Some of these factors include the nature of cations in diverse buffers, transformation procedure, treating the cells with reducing agents, altering the temperature of the condition media, harvesting time at stages of the growth cycle, chemical agents in media, condition of freezing and thawing cells, environment temperature, time and duration of heat-shock, and so on [30-34]. More recently, Chen et al. have suggested a quick and alternative way for the plasmid transformation in *E. coli* cells using a method called "plate transformation." It is performed on selective medium plates containing Ca2+ cations using mixing the host recipient

cells with plasmid DNA as the classical transformation process with calcium; the whole procedure takes only two minutes [32]. Generally, this method is widely used for competent bacterial cells due to its convenience; however, it is very variable based on experimental conditions. Hence, we used an optimal Inoue method. The reproducibly of this protocol creates competent cultures of *E. coli* cells that yield 108 to 3.1×108 transformed colonies/µg of the plasmid. This protocol works optimally when the bacterial culture is grown at 20°C. If a suitable incubator is not available, a standard bacterial shaker could be set up in a 4°C cold room and regulated to 20°C [17]. Electroporation and chemical transformation are two ways used to plasmid transformation into the *E. coli* cells. Electroporation provides transformation efficiency at least 10 times greater than other methods such as chemical ones. According to these findings, we used the Inoue method using transformation buffer and a short-heat pulse at 42.1°C.

Solubility, antigenicity, and other characteristics of recombinant protein in *E. coli* host can be predicted using available software by adding the sequence of the fusion tags with the target protein sequence. This application may be helpful before cloning and inserted target gene in vectors. It is also vital to search common cloning sites for different vectors before designing the primers. For this purpose, we used Swiss-PDB viewer analysis (v. 4.1.0), and we noticed that 16 first amino acids have no effects on the solubility and antigenicity of our recombinant IFN-γ.

Finally, the IFN-γ cDNA gene was sub-cloned into pET-21b(+) vector that was under the control of T7 RNA promoter and also was linked to a 6 His-tag DNA sequence which facilitates the purification and detection of recombinant protein or may increase the solubility and biological activity by protein expression in the cytoplasm or exportation to the periplasmic area [35, 36]. A suitable vector should be considered that the tags increase solubility but do not interrupt the activity of the target protein. Also, it has been observed that the incorporation of the stop codon is occurred due to altering the reading frame. To avoid this event, we added a G nucleotide to the beginning of the IFN-γ cDNA gene. Choosing a pET vector for expression is usually influenced by several factors such as information about the target protein, the application of expressed protein, and cloning strategy. The pET-21b(+) is a translation vector with high efficient ribosome binding site for T7 major capsid protein, which is conducted to express desired genes without their ribosomal binding site [37, 38]. The pET vector with 6 histidine tag is normally the first choice for purification of recombinant protein duo to His-tag is strong purification system such as Ni-NTA or Ni-IDA column. In two studies by Abdi. A. et al. and Maleki. F. et al. to cytokine cloning, they used pET-21b(+) to improve protein expression [5, 6].

5. Conclusion

The exact information of the target protein is essential for selecting the proper vector and host. Experimental conditions also strongly affect the large-scale cloning procedures required to be optimized in each laboratory. If a high yield of an active form of the target protein is desired on a repetitive basis, it is worth testing a matrix of host, vector, and culture combinations to describe the optimal conditions. The expressed recombinant mouse IFN-γ described here is appropriate for commercial purposes. Biochemical characterization of the recombinant mouse IFN-γ, methods for large-scale expression and purification, and finally, studying its biological activities might be the further goals.

Ethical Considerations

Compliance with ethical guidelines

This article is a meta-analysis with no human or animal sample. There were no ethical considerations to be considered in this research.

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Authors' contribution's

Conceptualization and supervision: Mojtaba Sankian; Methodology, data analysis: Seyedeh Elham Badiee Kheirabadi and Kazem Mashayekhi; Data collection: Malihe Moghadam; Data analysis, writing – review & editing: Mohammad Javad Mousavi; Final approval: All author.

Conflict of interest

The authors declared no conflicts of interest.

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