



ESCHERICHIA COLI STRAIN BL21: CLONING AND EXPRESSION OF AN OPTIMIZED INTERFERON ALPHA 2B (DE3)

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several studies have demonstrated the involvement of this class of IFN in a wide range of non-viral pathogens [2]. The therapeutic potential of IFNs (e.g. hIFN α 2a and IFN α 2b) was recognized as drugs for the treatment of cancerous and viral diseases [7].

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Abstract: Interferon alpha 2b gene (INF α 2b) as a protein with antiviral and anti-tumor activities is potentially a valuable therapeutic protein to work on. Prior to having a large-scale production of the target protein, it is recommended to examine it on an experimental scale, so that a bacterial host could be a proper choice as it leads us to a deep insight into the subject. In this research, the INF α 2b sequence was obtained from the NCBI gene data bank, and after optimization, it was subjected to be cloned and expressed in pET28a+. In order to primary examination of the target protein, Escherichia coli was considered a prokaryotic expression system. IPTG induction of the protein in bacteria cells containing the construct pET: IFN, followed by resolving total proteins through SDS-PAGE. The expected size of the investigated protein, about 24kDa, was observed through gel separation. Further assessment via western blotting confirmed the successful expression of IFN α 2b.

Keywords: Recombinant Protein, Bacterial Expression System, IPTG Induction, Western Blotting.

1. Introduction

Interferons (IFNs), as cytokines, are among the molecules used for transmission signals between cells to induce the immune system's responses against pathogens [1], however, the specific mechanisms and physiological consequences are poorly understood [2]. In addition, they are known for their antiviral, anti-proliferative, anti-bacterial and immune-modulating activities [1], [3], [4] and affect the metabolism, growth and differentiation of cells [5].

More than twenty IFN genes has been classified among three classes named as type I, II, and III based on the specific receptor utilization for their signal transduction [1]–[3], [6]. Although, type I IFNs are classically known for antiviral im-

IFN α 2b, as a type I IFN, is a polypeptide containing 165 amino acids, in which four cysteine residues are involved in the formation of two disulfide bridges [8]. Based on crystal structure that is mediated by zinc dimer, each monomer of rhIFN α -2b consists of five alpha-helices [7]. IFN α 2b is being used extensively to treat chronic hepatitis B and C, [1], [4], [9], and several types of cancer due to its unspecific antiviral and anti-proliferative activities [1].

IFNs and specifically IFN α 2b, on the whole, are worthy proteins to be investigated for their applications in varieties of diseases. In order to produce recombinant IFNs, there are several host systems, among which *Escherichia coli* is the most broadly used host for the production of recombinant proteins [10]. Even though in the situations the bacteria host is not considered as the target host, it can be a proper host to start an investigation. Since *E. coli* can grow rapidly to high cell densities, and genetically modified strains utilized for recombinant protein production, so they are generally regarded as safe host for production of recombinant proteins [10]

In this study, our aimed to clone the Interferon- α 2b gene and subsequently its expression in the bacterial host, *E. coli*. Bacterial expression could be a start of examination of the potent therapeutic proteins like IFNs, so we designed a multi-optimized IFN- α 2b gene for expression first in a bacterial host, and then for further investigation in the plant (in our probable future work.

2. Materials and Methods

Bacteria strains and media: In different steps of cloning procedures, *E. coli* strain DH5 α was used as the cloning host. In addition, the strains BL21 (DE3) and BL21 (DE3) plus were applied for the production of the investigated recombinant proteins. LB media supplemented with appropriate antibiotics was used for culturing bacteria cells. The cultures were incubated at 37 °C with shaking at 180 rpm.

Plasmid constructions: Prior to cloning the gene into a T-vector, the optimum annealing temperature for amplification of IFN α 2b sequence was determined through gradient PCR, and according to the result, a specific sharp band obtained by using Taq DNA polymerase at 57 °C during 30 sec. Considering the capability of Pfu DNA polymerase to work with higher accuracy, the main amplification of the fragment for cloning purposes was done using Pfu DNA polymerase, while other PCR products in this study were carried out by using Taq DNA polymerase.

In this research, the commercially available vector pET28a+ (Novagen) was used for the expression of target gene. A 533 bp fragment containing appropriate restriction sites, besides an optimized sequence of interferon α 2b gene was designed. The primary IFN α 2b sequence was adopted from the NCBI data bank according to the accession number NG-029154 and was codon optimized for optimum gene expression in *E. coli*. The designed fragment was subjected to be synthesized via General Biosystem Company (USA). For PCR amplification, a specific pairs of primers designed as follow; IFN-For primer 5AACCATGGGCGGATCCTGTGATCTGCC and IFN-Rev primer 5 AAGAGCTCGAG-TGCGGCCGCGAATTCCTTAC. The optimum condition for amplification of IFN gene and screening colonies were obtained by Taq polymerase, however, amplification of IFN for TA-cloning was conducted by Pfu polymerase. The TA-cloned target sequence was sub-cloned in the expression vector pET28a+ via BamHI-XhoI restriction sites. Analysis of prepared plasmids were done by using restriction enzymes purchased from Thermo Science. All procedures of cloning, preparing competent cells and transformation methods were conducted according to the protocol [11] Totally, purification of DNA (e.g. PCR products or DNA fragments containing overhang nucleotides) from agarose gel was carried out using Glass milk method [12].

IPTG induction: Bacterial cells (BL21 (DE3) and BL21 (DE3) plysS) carrying the gene constructs were inoculated into the LB broth supplemented with ap-proprate antibiotics (100 mg/L kanamycin, 30 mg/L chloramphenicol). The overnight cultures were diluted in LB broth in the ratio 1:100 containing the half of antibiotics used for overnight cultures and they were then incubated at 37 °C until the OD600 reached 0.5. At this point,

2ml of each sample harvested as uninduced samples, and IPTG with final concentrations of 0.4 added to the culture to turn on the lac operon and induce protein synthesis [13].

Protein extraction: Bacterial protein extraction was done according to the protocols by Vaillancourt with some changes [13]. Accordingly, IPTG-induced recombinant proteins centrifuged at 5000×g for 5 min, and supernatant carefully decanted. For extraction of soluble proteins, 0.5 ml native lysis buffer (containing 50 mM NaH₂PO₄, 300 mM NaCl, pH=8) added to each sample, briefly vortexed, and were placed on ice for 15 min. Centrifugation was done at 8000 ×g at 4 °C for 10 min. Supernatant transferred to a new tube as soluble fraction. Afterwards, 0.4 ml of denaturing lysis buffer (containing 8M Urea, 100 mM NaH₂PO₄, 10 mM Tris-Cl, pH=8) added to the pellets of pervious step, they were vortexed and centrifuged at 8000 ×g for 10 min. Supernatant transferred to new tube as insoluble fraction.

SDS-PAGE and Western blotting: Bacterial total proteins were separated by 10% SDS-PAGE. After running the gel, one SDS-PAGE was transferred in staining solution and another gel was subjected for western blotting. Therefore, protein samples transferred from SDS-PAGE to Nitrocellulose membrane (GE Healthcare), at 50 V for 20 min and 250 mA for 30 min. This procedure was done using Mini-PROTEAN®Tetra Cell apparatus (Bio-Rad). Following transferring step, it was then overnight incubated in 5% skimmed milk. The next day the membrane was washed in appropriate amount of washing buffer (PBST) three times; each time 5 min. In the next step, the membrane was incubated in PBST containing mouse anti-His monoclonal antibody in the ratio 1:6000 at RT for 2 hours. Washing step carried out and then rabbit anti-mouse horseradish peroxidase secondary antibody added (1:4000) to each well. After 2 hours incubation, washing step carried out and DAB substrate added on the membrane. Stopping the reaction was done by adding distilled water.

3. Results and Discussion

In this study, we investigated the expression of interferon $\alpha 2b$ gene in bacteria. The sequence was multi-optimized for expression in *E. coli* (and also for expression in tobacco as a plant host for probable future work). Codon optimization is an option may lead to a more compatible expression in the hosts' expression systems. Given gene constructs made based on pET28 α +

Clones grown on the selective agar plates primarily were analyzed through colony PCR (Figure. 1A). In addition, initial confirmation of the T-vector harboring IFN $\alpha 2b$ (pTV:IFN) obtained by using restriction endonucleases (Figure. 1B). As, the pET System is a common developed system for cloning and expression of a gene in *E. coli*, the interested fragment sub-cloned in pET28a+. The obtained clones grown on kanamycin agar plates were confirmed by using colony PCR, followed by digestion of plasmids with restriction enzymes (Figure. 2). The given gene construct was named pET:IFN in which IFN was located between two histidine tags (His-IFN-His). Schematic map of the gene construct pET:IFN is shown in Figure. 2.

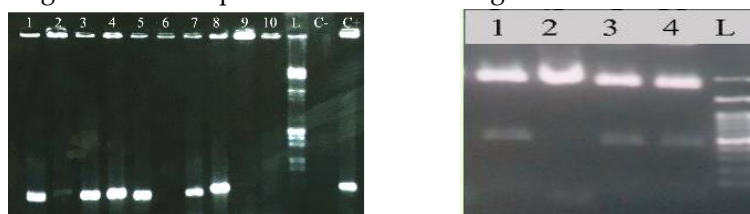


Figure 1. A Screening of putative clones harboring pTV:IFN via colony PCR. Numbers 1-10 indicate PCR products of putative clones harboring IFN $\alpha 2b$. A fragment of 533 bp observed in the lanes 1, 3, 4, 5, 7, 8 and C+. Letters C- and C+ indicate negative and positive controls, respectively. Letter L shows Lambda DNA/*EcoRI*+*HindIII* Marker,3 (Thermo Scientific). **B)** Confirmation of T-vector containing the optimized IFN $\alpha 2b$ gene. Number 1, 3 and 4 represent the same plasmid digested by *NcoI*-*EcoRI*, *NcoI*-*XhoI*, and *BamHI*-*XhoI*, releasing a fragment of 512, 527 and 519 bp, respectively. The gel-purified fragments were used in the subsequent cloning procedures. Number 2 shows uncut plasmid as control. Letter L indicates 100 bp plus DNA ladder (Sinaclon, Iran).

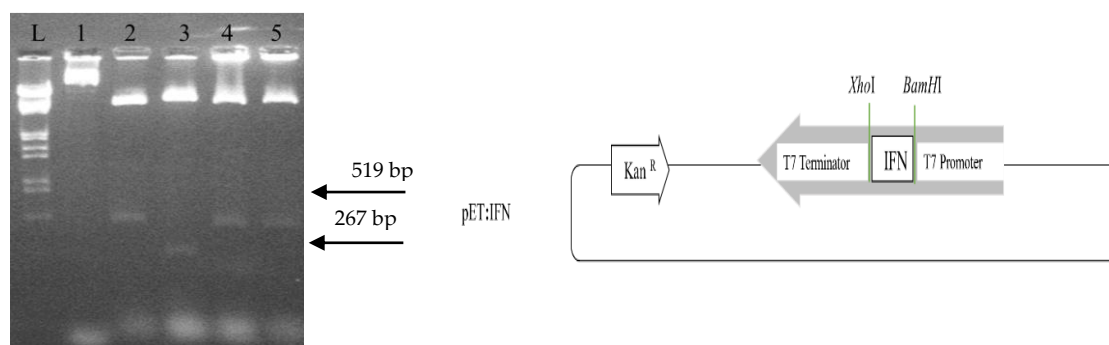


Figure 2. Confirmation of the gene construct pET:IFN. Number 1 indicates uncut pET28a+ plasmid as control. Number 2 and 3 indicate the same plasmid extracted from a positive clone harboring pET+IFN that was digested with restriction enzymes *BamHI-XhoI* (519 bp) and *PstI-XhoI* (267 bp). Number 4 and 5 indicate results of digestion reactions for two positive clones via *BamHI-XhoI* (519 bp). Letter L shows *Lambda EcoRI/HindIII* Marker,3 (Thermo Scientific).

Among *E. coli* strains, the expression host BL21 (DE3) and BL21 (DE3) *plysS* are commonly used to express target proteins (pET system manual); according to our results, there was no advantage for using one strain over the other (Figure. 3), thus BL21 (DE3) regarded to be good enough as the main host. As it is shown in the Figure. 3, the recombinant protein extraction from bacteria was conducted in two steps (as soluble and inclusion body fractions), showing that the most of the induced proteins obtained as inclusion bodies; lanes 1, 2, 3, 4 and 5 show the extracted total proteins by denaturing buffer and lanes 6 and 7 show proteins extracted under native condition. Getting the protein as inclusion bodies is a common observation in bacterial expression [13].

According to the result, lowering IPTG concentration (0.4 vs 0.8 mM), temperature (25 and 37°C) or increasing the time of harvesting (4h and overnight incubation) didn't improve the level of induced protein and its solubility in this research (data not shown). Therefore, the final expression was obtained by using 0.8 mM IPTG at 37 °C and 4h after induction. Theoretical molecular weight of recombinant protein His-IFN-His calculated 24.4 kDa and its pI estimated 6.97. Comparing profiles of total proteins that were electrophoresed through 10% SDS-PAGE showed several bands that were distinguished from control (Figure. 3).

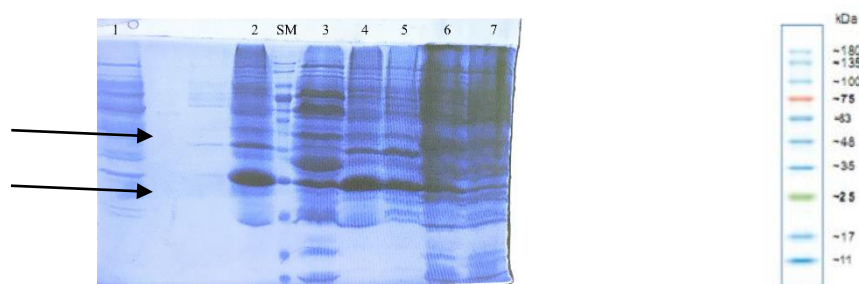


Figure 3. Bacterial expression of IFN by using *E. coli* BL21 (DE3) and BL21 (DE3) *plysS*. Number 1 shows total protein of control BL21 (DE3). Number 2 expression of IFN by BL21 (DE3) *plysS*. Number 3 expression of IFN by BL21 (DE3). Number 4 and 5 expression of IFN using BL21 (DE3) *plysS* and BL21 (DE3), respectively. Number 6 and 7 indicate soluble fraction of IFN expressed by BL21 (DE3) *plysS* and BL21 (DE3), respectively. SM indicates prestained protein size marker (Sinaclon, Iran).

Paying attention to the expected size of the investigated protein His-IFN-His (24.4 kDa), and as it is shown in the Figure, the lower band around 25 kDa appears to be monomer form of IFN, while the upper band with more intensity could be a doubled size of target protein, and this pattern of bands was repeated in several attempts. Apparently, the expressed target protein tend to be formed as dimers. The reliability of the expression confirmed by western blotting test (Figure. 4). There is a need to be mentioned that, the negligible size discrepancy that was observed via the gel separation and subsequently WB results, could be attributed to the different factors affecting the movement of proteins in SDS-PAGE (*i.e.* the monomer form of IFN was estimated about 24.4

kDa, while it was observed in the gel around 25-26 kDa). In addition, N-terminal polyhistidine tag affects the structure and function and stability of target protein [14]. In fact, polyhistidine tag, the number of histidine added to the recombinant protein, amino acid composition, glycosylation, phosphorylation, reducing agents and the effect of detergent on the proteins are among reasons explain size discrepancy [15]–[17].

For further analysis, western blotting test was carried out with single clones had already shown different profiles of expressed proteins. Interaction between the expressed recombinant proteins and antibody, besides positive and negative controls confirmed the presence of target protein (Figure 4 and 5).

When coming back to the SDS-PAGE result, the most remarkable difference between the profiles of proteins subjected to WB was the intensity of bands or in other words the level of protein expression. Probably, in the colonies with the low level of His-IFN-His expression a single band about 25 kDa has been detected (Figure. 4), while, the higher level of protein induction obtained from the other single clone, resulted in two bands corresponding to the monomer and dimer of the target protein (Figure. 5). In consistent with our observation, it has been reported that human IFN alpha 2b protein indicates a dimer form [18], [19]. The structure of human IFN- α 2b is considered an acceptable model for several interferon molecules. According to the numerous researches on the effects of mutations on biological activity of interleukin and interferons, it has been known that several protein surfaces are important in receptor activation. In addition it revealed the potential biological importance of the human IFN α 2b dimer [18]. On the whole, our results of protein gel electrophoresis besides western blotting tests that was carried out for two clones already shown different profile of proteins, successfully confirmed the expression of the investigated protein.

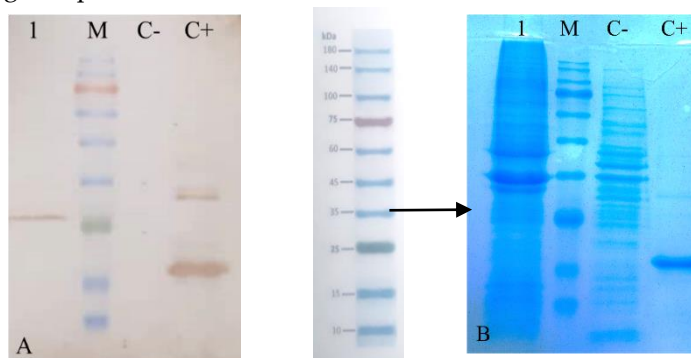


Figure 4. Confirmation of His-tagged IFN recombinant protein through western blotting. A) Number 1 shows the result of western blotting for bacterial total protein. Letter C- indicates negative control (*i. e.* total protein of uninduced bacteria). Letter C+ shows positive control of a known sample of two purified recombinant proteins with 21 and 33 kDa. B)

The SDS-PAGE corresponding to the membrane A. Letter M shows protein marker (Parstous, Iran).

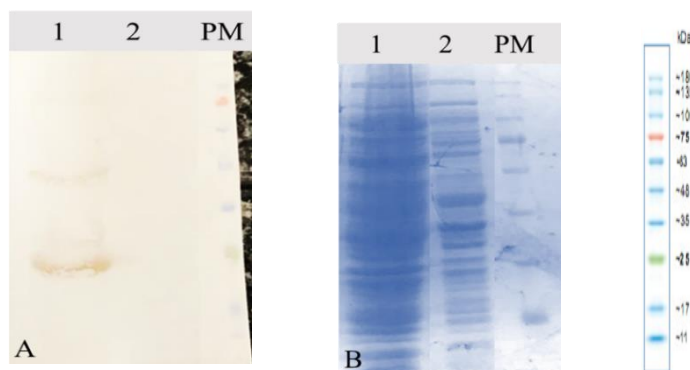


Figure 5. Western blotting test to confirm His-tagged IFN recombinant protein. A) Number 1 shows two bands (around 25 and 48 kDa) that are equivalent to monomer and dimer forms of target protein. Number 2 indicates negative control.

B) The SDS-PAGE corresponding to the membrane A. Letter PM shows protein marker (Sinaclon, Iran).

4. Conclusion

In conclusion, The INF-2b sequence used in this study was taken from the NCBI gene data library, and following optimization, it was cloned and expressed in pET28a+. *Escherichia coli* was chosen as the prokaryotic expression system for the target protein's initial analysis. Bacterial cells bearing the pET:IFN construct were incubated with IPTG to induce the protein, which was then resolved using SDS-PAGE. Through gel separation, the studied protein's predicted size of roughly 24 kDa was seen. The effective expression of IFN 2b was validated by further evaluation using western blotting.

Supplementary Materials:

No Supplementary Materials.

Author Contributions:

D. S. Alrseetmiwe and A. A. Almayah; methodology, writing—original draft preparation, A. A. Nasser and M. H. Alnussairi and H. Alizadeh; writing—review and editing, F. A. Mehrzi; paraphrasing. All authors have read and agreed to the published version of the manuscript.

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Informed Consent Statement:

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Data Availability Statement:

No Data Availability Statement.

Conflicts of Interest:

The authors declare no conflict of interest.

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