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# Two Step Production of Optimized Interferon Beta 1b; A Way to Overcome its Toxicity

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**Interferons are a large group of biological substances with many important functions like immunomodulatory, antiviral and anti inflammatory activities. There are so many clinical applications for IFN β for example in treatment of multiple sclerosis (MS), hepatitis, genital condylomata acuminate and arthritis. In this study the optimized IFN β 1b for expression in *E.coli* BL21 was designed and coloned in pET15b. In general, because of its toxicity and instability the yield of expressed IFN β 1b is low, so the amounts of expression and the effect of co products on production of IFN β 1b were evaluated and a two step production condition was designed for more efficacy in expression of this important biological recombinant product.**

**Key words:** Interferon, Optimization, cloning, Multiple sclerosis, *E. coli*.

Interferons are a major group of biological substances which are classified as cytokines subgroup[1]. There are two types of interferons (I and II)[2]. IFN α, β, τ and ω are subgrouped in type I and IFN γ in type II[3]. Human interferon beta [hIFN β] is a glycoprotein with 166 amino acids that naturally can be produced by a wide range of cells in body in response to some inducers, diseases and ribonucleic acids[4]. When IFN β binds to its surface receptor, a combination of cascade reactions take place, leading to immunomodulatory, antiviral, anti inflammatory and anti proliferative activities[4]. There are so many clinical applications for hIFN β in the treatment of hepatitis[1], genital condylomata acuminate, arthritis[5] and multiple sclerosis [MS][6]. IFN β has a single gene without any introns[7]. There are several brands for recombinant IFN β, like AVONEX® which is IFN β 1a. For keeping its glycosylated structure it is produced in Chinese Hamster ovary [CHO][4]. But this method has some disadvantages, like its expense[1]. Studies show that in the case of IFN β both the glycosylated (IFN β 1a) and unglycosylated (IFN β 1b) forms have similar specific activities and the glycosyl chain do not help to improve its biological activity[8]. *Escherichia coli* is the most common and favorable host for expression of many types of recombinant proteins[1];

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Some of its features like, easy growth, short growth time, low expense and knowing its whole genome map make this bacterium the host of choice for many biotechnological and pharmaceutical purposes[1]. So many methods for production of non glycosylated forms of IFN β (IFN β 1b) in *E.coli* have been developed like BETASERON® with the same biological activity to IFN β 1a which its cysteine amino acid in position 17 has been changed to serine in order to its lack of glycosyl chain[4]. In comparison between AVONEX® and BETASERON® both of them have similar biological activities while AVONEX® is more potent (about 10 times) than BETASERON®[4]. During production of recombinant IFN α, the solubility of expressed protein is highly relative to the temperature of the induction; decrease of the temperature will result in the production of more soluble forms instead of inclusion bodies[9].

## MATERIALS AND METHODS

**Design and optimization of synthetic gene**

The human IFN β gene was studied by NCBI (pubmed) search and cysteine in position 17 was replaced to serine. Then all codons of amino acids were optimized for expression and production in *E. coli* BL21 (DE3). The optimization analysis was done through Genscript website. The IFN β 1b gene was synthesized with *Nde*I cut site at 5' end and *Bam*HI at its 3' end.

**Bacterial strains and vector**

Strains of DH5α and *E. coli* BL21 (DE3) were selected as carrier host and expression host that were prepared from Persian type culture collection [PTCC]. pET15b was purchased from novagen (USA) that has a gene sequence for ampicillin resistance .

**Construction of recombinant IFN β 1b-pET15b**

The optimized IFN β 1b gene was cloned in expression plasmid pET15b between *Nde*I and *Bam*HI cut sites during ligation reaction with T4 DNA ligase (Fig. 1). Then the recombinant IFN β 1b pET15b was transformed into DH5α and recombinant colonies were cultured in LB medium. After 6 hours, medium was centrifuged and bacterial pellets were used for plasmid preparation. Then recombinant plasmids were transformed into *E.coli* (DE3) for expression.

**Media and culture conditions**

The LB medium consists of: 10 g/ml tryptone, 10 g/l NaCl and 5 g/l yeast extract, its pH was adjusted around 7.2. The modified terrific broth contains:

Tryptone 12 g/l, yeast extract 24g/l, glycerol 8 g/l, $KH_2PO_4$ 2.2 g/l and $K_2HPO_4$ 9.4 g/l.

**Growth conditions**

Each colony of recombinant *E.coli* was incubated overnight in LB medium, then growth medium was centrifuged and pellets were inoculated in new and fresh medium of modified terrific broth.

**Expression of recombinant protein**

When growth culture reached to optical density [$OD_{600}$] of 0.6, then isopropylthio β galactoside [IPTG] was added to medium with a concentration of 1mM [1]. After 4 hours of incubation in 37°C samples were collected. Samples were centrifuged and supernatant was discarded. Then pellets were analyzed by sodium dodecyl sulfate polyacrilamide gel electrophoresis [SDS PAGE] under reducing conditions. SDS PAGE was prepared and then stained with coomassie Brilliant Blue R250 .

## RESULTS AND DISCUSSION

**Evaluation of gene optimization**

Analysis of IFN β 1b gene optimization for expression in *E.coli* was done through OptimumGene™ codon optimization tool and genscript rare codon analysis tool in Genscript site (http://www.genscript.com/cgi bin/tools/

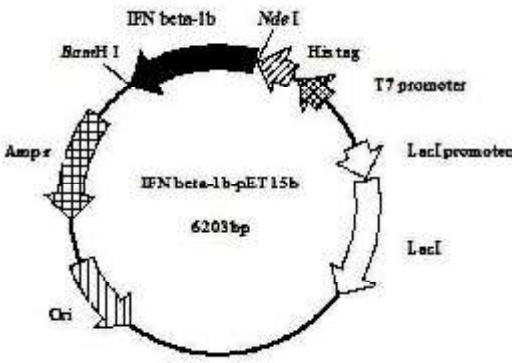


**Fig. 1.**

rare_codon_analysis) and high protein expression was predicted from this site and there were not any negative CIS elements and negative repeat elements in our sequence that can affect translation efficacy.

The RNA secondary structure and probability of hairpin or loop formation was studied by RNA secondary structure prediction software from http://www.genebee.msu.su/cgi bin/nph rna2.pl.

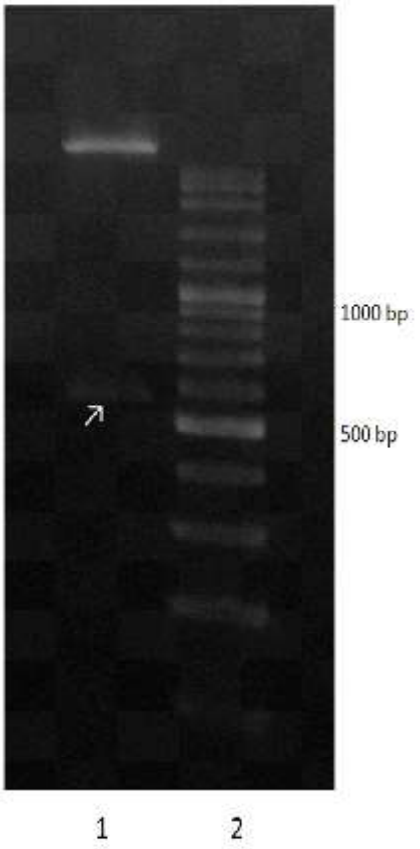


**Fig. 2.** Restriction analysis of constructed plasmid IFN-β-1b-pET15b with restriction enzymes *Nde*I and *Bam* HI. 1: Digested plasmid IFN-β-1b-pET15b. 2: DNA ladder (100 kb)

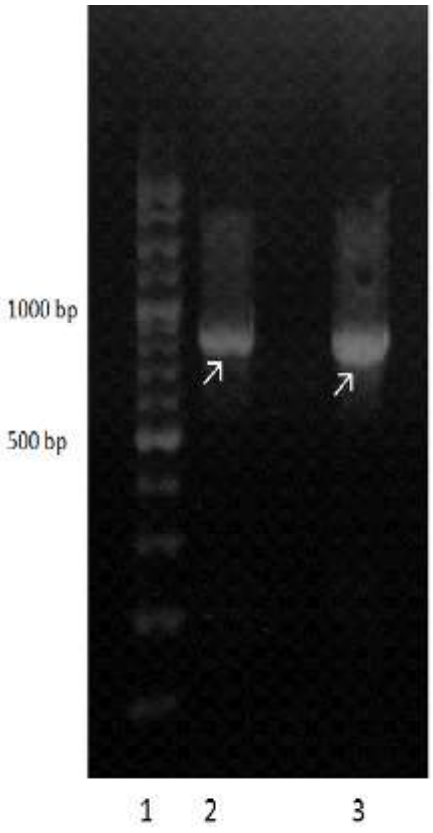


**Fig. 3.** PCR reaction for verification of IFN-β-1b gene cloning in pET15b with the standard T7 primers. 1: DNA ladder (100 kb). 2-3: samples from two different recombinant colonies

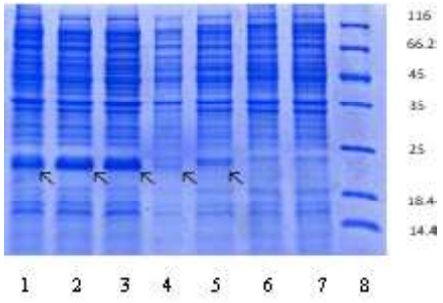


**Fig. 4.** 1-3: Single colonies of recombinant *E.coli* BL21 (DE3) in modified TB. 4-5: single colonies of recombinant DE3 in LB. 6-7 control *E.coli* BL21(DE3). 8: protein marker.

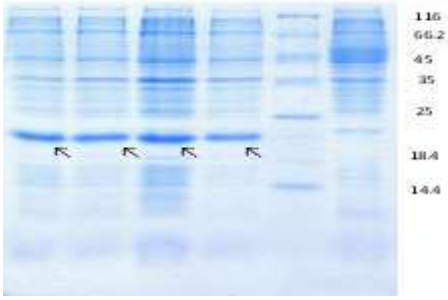


**Fig. 5.** 15% SDS-PAGE after 4 hours of induction in two step conditions with 1mM IPTG. 1-4: expression of recombinant IFN-b-pET15b in *E. coli* (DE3). 5: protein marker. 6: Negative control (*E. coli* (DE3))

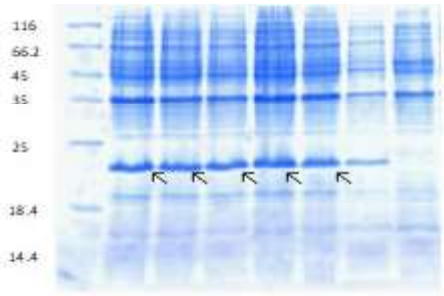


**Fig. 6.** 15% SDS-PAGE after 16 hours of induction with 1mM IPTG in two step production conditions. 1: protein marker, 2-7: different colonies of recombinant *E.coli* BL21(DE3). 8: control of *E.coli* BL21(DE3)

**Restriction analysis**

The recombinant plasmid IFN β 1b pET15b was digested with *Nde*I and *Bam*HI and was showed in agarose electerophoresis (Fig. 2).

**Verification of cloning**

After cloning of the synthetic IFN gene in pET15b, a PCR reaction for verification of cloning was done with the standard primers of T7 (Fig. 3).

**SDS PAGE analysis**

A comparison between two conditions of production was done. In one experiment, the expression was induced in a one step production, in LB medium after reaching the $OD_{600}$ of 0.6. In another condition the experiment was done in two steps, in step one a single colony was cultured to reach the $OD_{600}$ of 0.6, then the medium was centrifuged and pellets were transformed into new and fresh medium of modified terrific broth (second step) and induction was done by adding 1mM IPTG (Fig. 4).

After 4 hours and 16 hours of induction in two step production conditions, sampling was done and the pellets were prepared for SDS PAGE with 15% polyacrylamide analyzing under reducing conditions (Fig. 5 & Fig. 6).

Toward expression of heterologous proteins translated from foreign genes in prokaryotes like *E.coli* one problem is immediate degradation of mRNA due to differences in codon usage preference in eukaryotes and prokaryotes[10], for solving this problem the human interferon beta was optimized and codons were changed to get the best results in *E.coli* according to its codon usage preferences, and for prevention of inter and intra structure bond formation and aggregation, the cysteine in position 17 was changed to serine.

There are many expression systems for production of recombinant IFNβ such as CHO cell line[11] and human foreskin fibroblast [HFF][12]. In general due to toxicity and instability of IFN β, its expression is low[13-15]. IFN β is extremely toxic for *E.coli* cells, so its production in early stages of growth can decrease the rate and amount of production[16] in a study by Ghane *et al*., for overcoming this problem, they used from a fusion protein at the N terminal of IFN β gene[16]. The disadvantage of this method is the need of an additional step for separating of fusion protein. In another study by Gross *et al.* use of zinc ions had stabilizing effect on produced IFN β. There are many difficulties in purification and formulation of IFN β 1b because of its hydrophobicity and its tendency of forming aggregates[17]. Acidic productions such as carbonic acid, succinic acid and specially acetic and lactic acid as co products[2], are among the main problems reducing the yield of expressed recombinant proteins[14]. One approach for solving this problem is use of glycerol instead of glucose. Glycerol can block other metabolic pathways for getting energy form other carbon sources like glucose, as a result the concentration of inhibitory metabolisms like acetate will decrease[14]. In a study by Tan *et al.* they showed that using an anion exchange resin can enhance growth of *E. coli* and production of recombinant protein due to acetic acid removal from the culture medium[2]. For getting rid of by products that influence production efficacy, a two step process was designed, in step one a single colony was inoculated in 20 ml of LB medium for overnight, then the culture medium was centrifuged and the supernatant including acidic co products was discarded, then pellets were resuspended in new and fresh modified TB medium, after reaching the $OD_{600}$ of 0.6, expression was induced by adding IPTG. The other advantage of using modified TB medium is its buffered condition around pH of 7.2 which prevents decrease of pH due to co product formation[2]. Another problem is the toxicity of IFN produced, *E.coli* BL21 is a good host that tolerates this toxicity [18] .

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