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MOLECULAR STUDIES ON THE CLONING AND EXPRESSION OF HUMAN BETA INTERFERON GENE IN *E. COLI*

MASTER OF SCIENCE

ZALİHE OMAY

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APPROVAL OF THE THESIS

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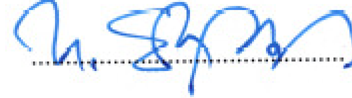
Member Assist. Prof. Dr. Yakup ERMURAT Bolu Abant Izzet Baysal University	

Prof. Dr. Ömer ÖZYURT

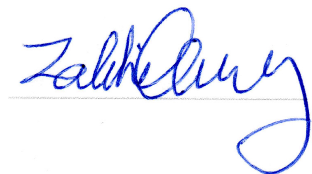
Director of Graduate School of Natural and Applied Sciences

This work is dedicated to my family

DECLARATION

I hereby declare that all information in this document has been obtained and presented in accordance with academic rules and ethical conduct. I also declare that, as required by these rules and conduct, I have fully cited and referenced all material and results that are not original to this work.

Zalihe OMAY



ABSTRACT

MOLECULAR STUDIES ON THE CLONING AND EXPRESSION OF HUMAN BETA INTERFERON GENE IN *E. COLI*
MSC THESIS
ZALİHE OMAY
BOLU ABANT IZZET BAYSAL UNIVERSITY GRADUATE SCHOOL OF NATURAL AND APPLIED SCIENCES
DEPARTMENT OF BIOLOGY
(SUPERVISOR: ASSOC. PROF. DR. MEHMET ÖZTÜRK)

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Human interferon beta (hIFNβ), produced by many cell types in mammals in response to viral and nonviral infections, acts as an immunoregulator and have a potential to become an important therapeutic drug for several diseases such as multiple sclerosis, asthma, hepatitis B and C, human papillomavirus and various cancers. Considering the clinical applications and convenience of the rIFNβ production due to its molecular size, generating a low-cost production system, which could provide as much as pure and high yield of this therapeutic protein, is very important. Prokaryotic secretory systems are mostly preferred for the production in order to secrete proteins into the periplasm or out of the cell membrane. Thus, secretoy system simplifies the downstream processing and reduces the protein degradation and cost. It is a quietly desirable and customisable system for secretion of IFNβ1b. In Turkey, licensed domestic production of recombinant proteins, imported at high prices, has not been achieved. There are some reports describing the construction of novel vectors expressing human rIFNβ1b in the periplasm of the *E. coli* cells. In this study, for production of rIFNβ1b protein in periplasmic space, codon optimisated rIFNβ1b gene according to *E. coli* codon table was provided by in silico bioinformatic tools. The obtained gene was cloned into pET22b expression vector which contains the pelB signal peptide. Then recombinant IFNβ1b peptide synthesis was performed in *E. coli*/BLR(DE)3 strain induced with 0.4 mM IPTG at 25°C and then isolated from the medium, periplasmic and cytoplasmic space of the host and they were pufied with the Ni-NTA affinity colon. The production of the rIFNβ1b, (~18 kDa) was determined by %12 SDS-PAGE and Western Blot analysis. Our results indicated that the intracellular soluble expression of the optimized IFNβ1b protein did not take place. Eventhough, this was not comfirmed by experimetally, we thought that because of the improper codon optimization, folded form of rIFNβ1b mRNA was produced and this structure prevented the translation of rIFNβ1b mRNA. Reconstruction of the prokaryotic secretion system and codon optimizations may improve the more convenient production and purification of rIFNβ1b protein.

KEYWORDS: Therapeutic protein, Interferon beta 1b, Expression, Purification, *E. coli*

ÖZET

İNSAN BETA İNTERFERON GENİNİN KLONLANMASI VE *E.COLİ*'DE EKSPRESYONU ÜZERİNE MOLEKÜLER ÇALIŞMALAR
YÜKSEK LİSANS TEZİ
ZALİHE OMAY
BOLU ABANT İZZET BAYSAL ÜNİVERSİTESİ
FEN BİLİMLERİ ENSTİTÜSÜ
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(TEZ DANIŞMANI: DOÇ. DR. MEHMET ÖZTÜRK)

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Viral ve viral olmayan enfeksiyonlara yanıt olarak memelilerde birçok hücre tipi tarafından üretilen İnsan interferon beta (hIFNβ), bir immünoregülatör görevi görür ve multipl skleroz, astım, hepatit B ve C, insan papilloma virüsü gibi çeşitli hastalıklar ve çeşitli kanserler için önemli bir terapötik ilaç olma potansiyeline sahiptir. Moleküler boyutu nedeniyle recombinant IFNβ1b (rIFNβ1b) üretiminin klinik uygulamaları ve elverişliliği göz önüne alındığında, bu terapötik proteinin saf ve yüksek verimini sağlayabilecek düşük maliyetli bir üretim sistemi oluşturmak çok önemlidir. Prokaryotik sekresyon sistemleri, protein üretiminde proteinleri periplazmaya veya hücre zarının dışına ekspres olması için çoğunlukla tercih edilir. Böylece, expresyon sistemi, sonraki işlemleri basitleştirir ve protein yıkımını ve maliyetini azaltır. IFNβ1b ekspresyonu için oldukça arzu edilen ve özelleştirilebilir bir sistemdir. Türkiye'de yüksek fiyatlarla ithal edilen lisanslı yerli rekombinant protein üretimi henüz gerçekleştirilememiştir. *E. coli* hücrelerinin periplazmasına insan rIFNβ1b proteinini ekspres eden yeni vektörlerin yapımını tarif eden bazı raporlar vardır. Bu çalışmada, insan rIFNβ1b proteinini periplazmik bölgede ekspresyonu için, *E. coli* kodon tablosuna göre optimize edilmiş rIFNβ1b geni, biyoinformatik araçlarla elde edildi. Elde edilen gen, pelB sinyal peptidini içeren pET22b ekspresyon vektörüne klonlandı. Daha rIFNβb peptit sentezi, 25°C'de 0.4 mM IPTG ile indüklenen *E. coli*/BLR(DE)3 suşunda gerçekleştirildi ve daha sonra konağın ortamından, periplazmik ve sitoplazmik bölgesinden izole edilip Ni-NTA affinite kolon ile pufiye edildi. Recombinant IFNβ1b, (~ 18 kDa), üretimi %12 SDS-PAGE ve Western Blot analizi ile belirlenmiştir. Sonuçlarımız, optimize edilmiş IFNβ1b proteininin hücre içi çözünür ekspresyonunun gerçekleşmediğini gösterdi. Bu durum deneysel olarak doğrulanamamasına rağmen, uygun olmayan kodon optimizasyonu nedeniyle rIFNβ1b mRNA'ların katlanmış formunun üretildiğini ve bu yapının rIFNβ1b mRNA'ların çevrilmesini engellediğini düşünülmektedir. Prokaryotik sekresyon sisteminin yeniden yapılandırılması ve kodon optimizasyonları, rIFNβ1b proteininin daha uygun üretimini ve saflaştırılmasını geliştirebilir.

ANAHTAR KELİMELER: Terapötik protein, İnterferon beta 1b, Ekspresyon, Saflaştırma, *E. coli*

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LIST OF ABBREVIATIONS AND SYMBOLS

FDA	: Food Drug Administration
FD&C Act	: Federal Food, Drug, and Cosmetic Act
DNA	: Deoxyribonucleic Acid
RNA	: Ribonucleic Acid
IFN	: Interferon
rhIFNβ1b	: Recombinant human interferon beta 1b
PEG	: Polyethylene Glycol
PSA	: Polysialic Acid
CHO	: Chinese Hamster Ovary
MS	: Multiple Sclerosis
IPTG	: Isopropyl-ß-D-thiogalactopyranoside
LB	: Luria bertani
OD_{600}	: Optical density at 600 nm
PAGE	: Polyacrylamide gel electrophoresis
PRR	: Pattern recognition receptors
PAMPS	: Pathogen associated molecular patterns
JAK-tyk	: Janus Associated Kinase family of tyrosine kinases
STAT	: Signal transducer and activator of transcription
ISRE	: Interferon-stimulated response element
ISG	: Interferon-stimulated genes
PVDF	: Polyvinylidene Fluoride

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1. INTRODUCTION

1.1 Therapeutic Proteins

Proteins are biomolecules or macromolecules composed of long amino acid chains called peptide. Proteins are the principal players of the cellular functions. Cells synthesize proper protein require for its function according to DNA coding information. Since proteins have diverse and dynamic role in cell biology, they take a part in the pharmaceutical industry as biopharmeceuticals or biotechnological drugs for therapeutic uses in preventing or curing disorders.

Biopharmaceuticals or biotechnologic drugs are protein/polipetide based on therapeutic agents obtained from living organisms (Nacak, 2012). The FDA has approved 100 proteins as therapeutics according to drug development process shown in Figure 1.1., Craik et al., (2013) and many others are ungoing clinical trials. Recombinant therapeutic proteins, known as biotechnological therapeutics, are approved as antibody-drug conjugates, biotechnology medicines, vaccines, cytokines, interferons and enzymes, (Leader et al., 2008). Biotechnological therapeutic proteins are used for various purposes, including prophylaxis, diagnosis, disease cure or management (Schmidt, 2004). Since the first-generation products are patent protected from their approved time for marketing in 1980s, Generic versions identical to conventional chemical drugs can be developed under engineered biotechnolgy area when patent expires. However, they cannot be manufactured exactly same compared to branded drugs, they must have the same:

- Dosage form
- Intended use
- Strength
- Safety
- Route of administration
- Quality
- Performance characteristics
- Active ingredients

The advantage of the generic drug production is being time saving. Instead of managing clinical trials to establish the safety and effectiveness, bio-equivalence studies and an Abbreviated New Drug Applications are preferred. Before approving a generic drug product, FDA carry out stringent assessment to make certain of the generic drug can be substituted for the branded drug. Although it is impossible to produce an exact copy of any biologic medicine, Biosimilars are highly identical to the original generics. Generic versions that show no clinically meaningful differences from branded drug called biosimilars or therapeutically equivalent.The Orange Book, The list of the *Approved Drug Products with Therapeutic Equivalence Evaluations,* identifies drug products approved on the basis of effectiveness and safety by the FDA under the Federal Food, Drug, and Cosmetic Act (the FD&C Act).

By the help of recombinant DNA technology these biotechnological proteins have proven themselves in the pharmaceutical industry (Regan and Jackson, 2003) as it allows protein modification and particular gene selection, they have low chance of immunologic rejection as well as they can produced efficiently and limitless quantitiy. Enhancing the number of biosimilars and biotechnologic drugs, increase the treatment options, accessibility and lowers the cost.

Therapeutic proteins have focus on interest in drug discovery and development since the first therapeutic protein recombinant human insulin expressed in *Escherichia coli* (*E. coli)* Goeddel et al. (1979), approved by the US-FDA in 1982. Recombinant protein insulin is the first commercially produced therapeutic protein for diabetes mellitus (Clark et al., 1982).

Therapeutic proteins are proven to be effective even as vaccines in treating many potentially fatal diseases like heart disorders, cancer and diabetes (Karacali et al., 2014; Akash et al., 2013a, 2013b; Ibrahim et al., 2013; Hermeling et al., 2004). Moreover therapeutic proteins have an advantage over other drugs since they are highly versatile, providing various pharmaceutical targets, specific mechanisms of action and show low toxicity levels. Despite these advantages, biotech proteins handle with the high molecular weight, short half-lives, instability, and immunogenicity and formulation limitations and effective delivery systems (Nacak, 2012 and Dipak et al., 2010).

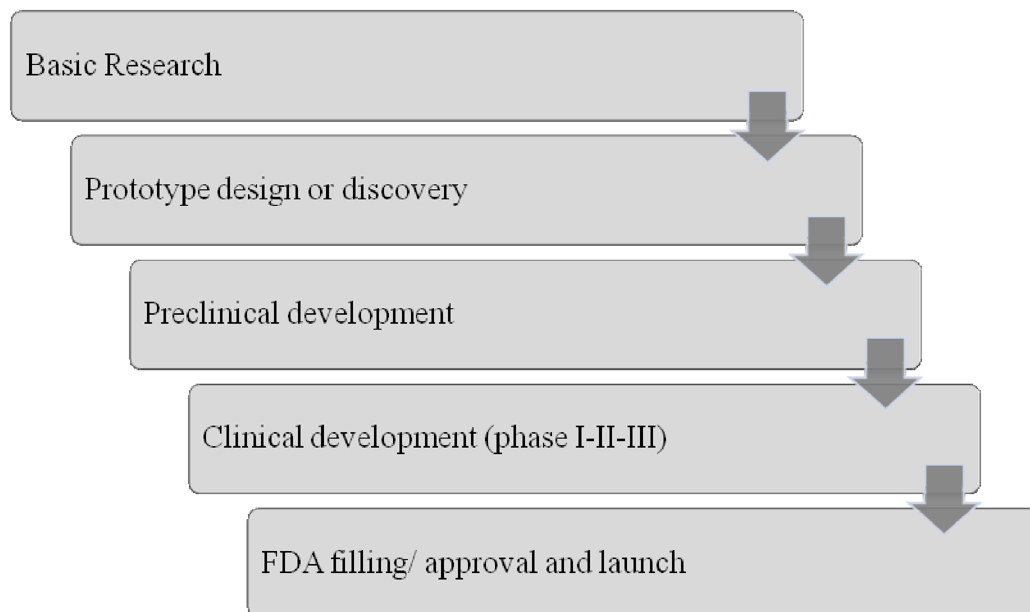


Figure 1.1. Drug development process

1.2 Classification of Therapeutic Proteins

Recently Leader and colleagues (2008) classified the therapeutic proteins in basis of their therapeutic application and function. They placed them in 4 groups, as summarized in Table 1.1.

Table 1.1. Classification of the therapeutic proteins (modified from Akash et al., 2015)

Functional and Applicational Classification of Therapeutic Proteins
Group I: Therapeutic proteins with enzymatic or regulatory activity • Ia: Replacing a protein that is a deficient or abnormal • Ib: Augmenting an existing pathway • Ic: Providing a novel function or activity
Group II: Therapeutic proteins with special targeting activity • IIa: Interfering with a molecule or organism • IIb: Delivering other compounds or protein
Group III: Therapeutic proteins as vacccines • IIIa: Protecting against a deleterious foreign agent • IIIb: Treating an autoimmune disease • IIIc: Treating cancer
Group IV: Therapeutic proteins as diagnostics • Therapeutic proteins used for diagnosis among group I, II, III

Group 1b includes the FDA approved proteins that are known to stimulate various immune and hematological responses such as erythropoietin for chronic anemia and interferon-alpha for the treatment of hepatitis C (Van et al., 2004; Corwin et al., 2002).

1.2.1 Production Systems of the Therapeutic Proteins

Usually, small molecular therapeutic drugs are uniformly produced and pure, while those biopharmaceuticals with a great number of amino acids are produced via living cells that cause adverse effects in patients and decrease the efficiency of drug (IFPMA, 2013). However shorter approval time of therapeutic proteins and obtaining far-reaching patent protection make proteins charming from a financial perspective compared with small molecular therapeutic drugs (Leader et al., 2008).

Recombinant DNA technology provides effective and limitless therapeutic protein production systems include bacteria, yeast, insect cells, mammalian cells, and transgenic animals and plants with advantages and disadvantages as shown in Table 1.2. Recently, in vitro system has been preferred for recombinant protein expression of therapeutic proteins (Ahmed et al., 2013; Martemyanov et al., 2001).

E. coli, *Saccharomyces cerevisiae* and *Pichia pastoris* are known to be best microbial organisms for expression and production of therapeutics. Microbial production system has more advantage than other system, can be optimized culture

conditions and being cheap. Moreover, it simplifies the purification steps that is essential for therapeutic usage (Gökbulut and Arslanoğlu, 2013; Kamionka, 2011; Wang et al., 2014; Ahmad et al., 2014)

Animal cell cultures include Chinese hamster ovary cells (CHO) which are another choice commonly used for the production of many therapeutics (Jayapal et al., 2007). However, required reach media conditions and low limited production in animal cell cultures makes it expensive process to produce biologically active protein. Further more, posttranslational modifications can carry out by using animal cell culture production and these modifications infect human immune system instead of treat.

By this way transgenic production system is drawn attention for cheaper production of various therapeutics using transgenic organisms. Transgenic systems are most effective than microbial productions however its high price. For instance, transgenic goats and sheep mammary glands are being used for the expression of the desired protein introduced their genome (Janne et al., 1992). Many transgenic plants have also been used to obtain a large volume of therapeutic proteins (Lis and Sharon, 1993).

After production of these therapeutics, they need to be purified from cell mixture (nonprotein contaminants) without affecting their biological activity using analytical method in order to identify a protein mixture and preparative methods for human usage. In order to produce large quantities for industrial usage includes the purification methods such as chromatography, precipitation, differential solubilization, extraction, and ultracentrifugation (Akash et al., 2015).

Following purification process proteins are concentrated by lyophilisation or ultrafiltration then formulated into products. All of these steps including quality-immunogenecity-efficiency tests are stricted by FDA preception. Traditionally cell culture methods takes 3 to 5 years to construct and cost 250-450 million dollars and of course need FDA approval and certification. Many researchers are developing progress for entire understanding of the basic formulation requirements and delivery of therapeutic proteins. Short half lifes, instability, drug permeability, pharmaco dynamic and formulation limitations of therapeutic proteins require effective delivery

systems. Several strategies have been developed to overcome this limitations and called second generation protein therapeutics, such as modifiying the formulation or protein structure. Nowadays, instead of the modification in the protein structure, polyethylene glycol (PEG) or polysialic acid (PSA) are covalently attached to therapeutic protein used to enhance its pharmalogical activity and decrease adverse effects. Also liposomes, polymeric microspheres, and polymeric nanoparticles, are used to help overcome drug formulation limitations (Banakar, 1997). For instance, in order to increase the plasma half-life of various therapeutic proteins genetically modified systems are generated, such as proteins fused with albumins (Yamashita and Hashida, 2013).

Oral, paternal, pulmonary and dermal delivery systems are used but therapeutics have sensitive structure effects rapidly by enzymatic reactions and show allergic reactions in vivo, Moreover, administration of therapeutic proteins by injection is optimum way that have minimum effect on their delicate structure and duration in patients. Recently, pulmonary delivery systems draw attention because of the rapid absorption with large surface area even more substantial formulation.

Table 1.2. Advantages and disadvantages of heterologous expression systems.

Expression systems	Advantages	Disadvantages
Prokaryotic Expression Systems	• Known genetics • Rapid growth rate • Cost efficient simple approach • Ability to secrete proteins into the periplasm resulting the simplify downstream processing	• Lack of post-translation modification • Low/moderate yield, • Poor biological activity of product • Presence of toxic material in the purified protein preparation
Eukaryotic Expression Systems	• Generate large quanitities • Grown in well-defined media • Can be easily adapted to fermentation allowing for large-scale, stable production • Presence of glycosylation machinery (for glycosylated proteins)	• Including hyper- or inappropriate-glycosylation of target proteins may causes immunogenicity • Have a slower growth rate than bacterial cells • Growing conditions often need to be optimized.
Insect Expression Systems	• Large-scale expression • Known insect cell biology • Obtaining recombinant proteins identical to their naturally occurring counterparts	• Expensive culture conditions • Significant toxicological and economic issues due to the use of viral transfection in this expression system

Table 1.3 (continued). Advantages and disadvantages of heterologous expression systems.

Expression systems	Advantages	Disadvantages
Plant Expression Systems	• Provide a cheap expression of recombinant proteins	• Expensive and time consuming
Cell Free Expression Systems	• Ability to assemble proteins with labelled or modified amino acids	• Expensive and very technically challenging to use.

1.3 Interferons

IFNs are the first cytokines that are produced by recombinant DNA techniques (Meager, 2002). Interferons are widely used therapeutic proteins in different brands for Hepatitis B, Chronic hepatitis C infection, melanoma, Kaposi's sarcoma, hairy-cell leukaemia, multiple sclerosis (MS), metastatic renal cell cancer and chronic granulomatous disease (Leader et al., 2008). Interferon therapies are classified into Group Ib with the growth factor therapies, according to Leader and collaques (2008). Even their precise pharmacological mechanism of action is unknown, they treat disease effectively as an antiviral, anti microbial and immunoregulator mediators.

1.4 Discovery of Interferons

In the field of virology, IFN which is derived from the English term 'interference' was incidentally discovered by Alick Isaacs and Jean Lindenmann while studying a heat inactivated influenza virus on the choriallantoic membrane of the chicken egg. These virologists demonstrated that the addition of influenza virus stimulated the production of interferon from infected cells. Interferons are soluble antiviral agents and have ability to regulate immune system by inhibiting the growth of live influenza virus on fresh membranes having ability to inhibition of viral replication in the infected cells (Isaac and Lindenmann, 1957; Chelbi-Alix and Wietzerbin, 2007)

Interferons are one of the members of cytokine family throughout chemokines, lymphokines, monokines, interleukins, tumor necrosis factor (Chelbi-

Alix and Wietzerbin, et al., 2007). Cytokines are secreted by host cells in response to pathogens and act as central regulatory mediators of immune response. In this way interferons have a potential to treat infections.

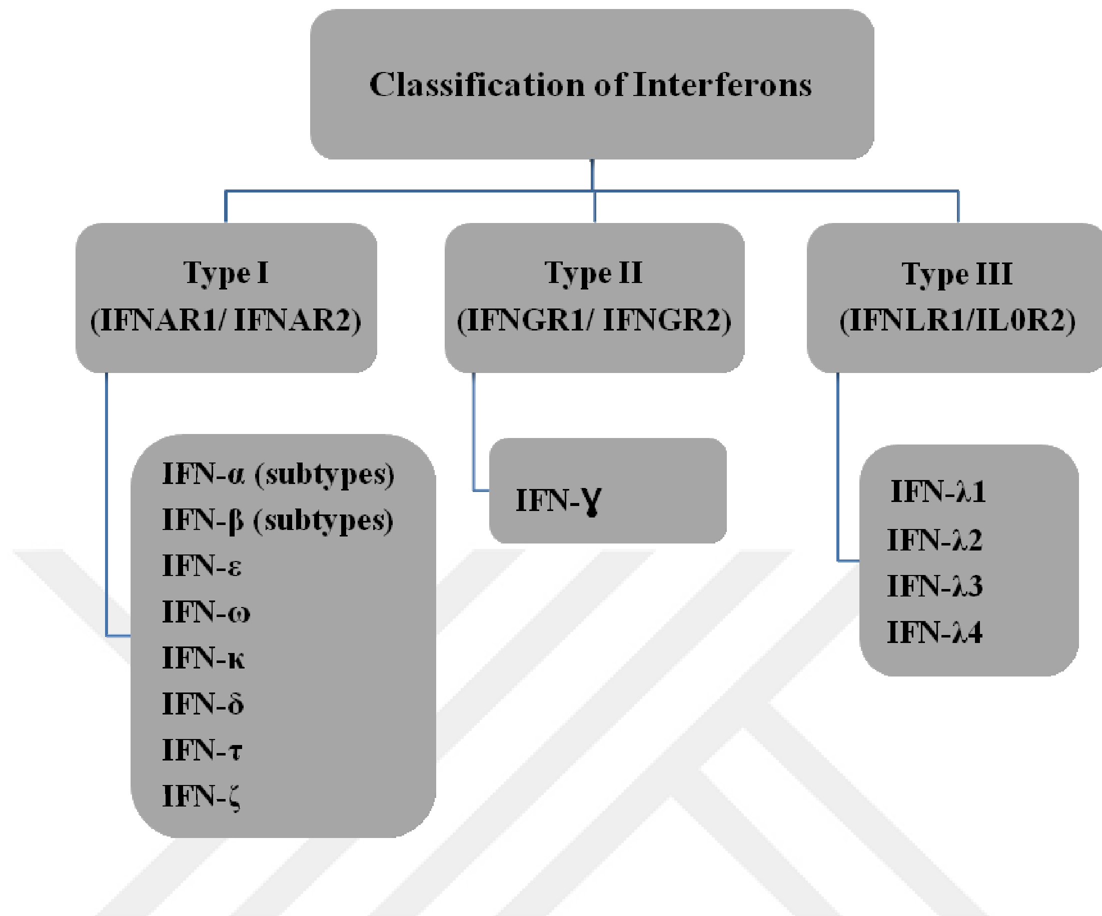
In 1957, the discovery of IFN serves as a cornerstone of immunology. After discovery, it was elucidated that interferon were produced in many animals, tissues, and cells. Next 20 years, for clinical application, interferons were partially purified and characterized from the medium where virus infected human white blood cells (Pestka, 2007). Interferon alpha (IFNα) and Interferon Beta (IFNβ) are the first successfully purified proteins by high performance liquid chromatography so that commercially available for chemical, biological, and immunological studies (Pestka, 2007).

1.5 Classification of Interferons and Interferon Receptors

Previously identified type I interferons have been divided into two classes on the basis of their induction in different cell types and lack of serological cross reactivity leukocyte (IFNα) and fibroblast (IFNβ) interferon (Stewart, 1979).

With the discovery of IFNɣ, Interferons were proceed to be classified in 3 types based on their cell origins called β, α, ɣ which originated from virus infected fibroblasts and leucocytes, activated T cells, respectively. Afterward, in 2003, discovering IFNλ and new distinct receptor caused rearrangement of interferon types (Wang et al, 2017). So, they classified into three subfamilies called type I, II, III based on their amino acid sequence, physical-biological properties and receptor specificity summarized in Table1.3. These subtypes, indicated by Greek letters, can be further divided into sub-categories. Generally, molecular weight of interferons range between 18kDa-100kDa with consist of 130-170 amino acids chains. Structurally, IFNs possess four-helical bundle topology and belong to hematopoietic growth factor family also known as helical cytokine family (Karpusas, et al. 1997; Pestka, et al. 2004).

Table 1.4. Classification of interferons and their receptors



1.5.1 Type I Interferons

This non glycosylated form of interferons comprised of 165-200 amino acids and lack of introns. This family contains 8 members shown in Table 1.3., and out of that IFNα and IFNβ are the most clinically important interferons and IFN delta (IFNδ), IFN tau (IFNτ) and IFN zeta (*IFNζ*) (known as limitin) are not reported in humans (Chevaliez and Pawlotsky, 2007; Pestka, 2004). Seventeen non allelic genes have been described in humans, gene encoding type I interferons located on chromosome 9 in humans (Pestka et al., 2004)

Type I IFN has pleiotropic effects in both the adaptive immune and innate responses; they are involved in cell differentiation and anti-tumor defenses besides antiviral effects. IFNAR1 and IFNAR2 are heterodimeric receptors for type 1 and Type I IFNs show high similarity and closeness of their structure such as IFN α and β has 30% sequence similarity and also, IFNα and IFNω share 60% homology

(Taniguchi 1980; Karpusas, 1997). Despite this closeness between each other they have differential physiological effects (Brierley and Fish, 2002).

1.5.1.1 Interferon Alfa

IFNα has 165 amino acid known as a leucocyte interferon as produced by infected leucocyte cells. Thirteen IFNα genes which produce thirteen distinct although structurally related IFN subtype proteins are reported (Rudick, 2011).

1.5.1.2 Interferon Beta

IFNβ is known as fibroblast interferon as produced by fibroblasts in response to virus or RNA stimulation and shows antiviral, antiproliferative and immune regulatory activity. It is also used as therapeutics for multiple sclerosis (MS) (Derynck et al, 1980). IFNβ has two subtypes, one of them is glycosylated protein IFNβ1a that differs from by two aminoacids residues from nonglycosylated IFNβ1b. Natural human IFNβ is composed of 166 amino acid residues and 9^{+} net charge. Recombinant Interferon Beta 1b (rIFNβ1b) is a non-glycosylated mutein (introduced by *in vitro* site-specific mutagenesis) of human protein having with 18.511 kDa molecular weight, produced by the cells of *E. coli*. It is biologically highly active since the cysteine 17 residue altered with serine to prevent the formation of undesired disulfide bonds (Meager, 2002). Recombinant hIFNβ is a drug of choice for the treatment of MS and several other diseases in humans (Chelbi-Alix and Wietzerbin, et al. 2007).

Recombinant Interferon β1b and Interferon β1a are known as effective therapeutic on relapsing MS disease. Commercially three formulations of Interferon β are currently available, IFNβ1b is made by Bayer Pharma (Betaferon®/Betaseron®), Novartis (Extavia), and Interferon β1a is made by Biogen (Avonex™) and Serono laboratories marketing interferon β1a under Rebif® brand (Bayas and Gold, 2003). Commercially available brand of the approved IFNα and IFNβ products are summarized in Table 1.4.

1.5.2 Type II Interferons

Type II interferons are single glycosylated proteins composed of 144 amino acids. They are produced by T lymphocytes of immune system, exhibit immune modulatory properties and support immune regulation and called immune IFNs. It has only 1 member IFNɣ, encoded by a gene that is located on chromosome-12 and IFNGR1 and IFNGR2 are type 2 receptors as shown in Table 1.3. Type II interferons have more immunomodulator effect as mediates cancel cell apoptosis however less antiviral than other type of interferons (Lee and Ashkar, 2018).

Both type I and type II IFNs play a critical role in host defense mechanism against intracellular bacteria, viruses and parasites (Casanova and Abel, 2004). However, Type II interferons have more immunomodulator effect as mediate cancer cell apoptosis however less antiviral than other type of interferons.

1.5.3 Type III Interferons

Unlike the type I and type II which are mostly expressed by cell receptors, type III is tissue specific and has only 1 member in response to protect mucosal entities and have antiviral effect by cooperation with IFN β as summarized in Tabe 1.3. Human express subtypes of λ (λ2, λ3) from chromosome-19 and IFNLR1 and IL0R2 are type III receptor.

According to Nature genetic, recently founded type III IFN which is expressed only small fraction of human population is called λ4 or IFNL4. Even it has only one type (IFNLR1) of type III receptors, it classified into type III as they have antiviral activity on type III receptor complex (Prokunina et al. 2013; Wang et al., 2017).

Table 1.5. Interferon beta and alfa products approved for clinical use (modified from Lipiåinen et al, 2015; Gary, 2018)

Product	Company/ Location	Production Host	Therapeutic Indication	Date Approved
PLEGRIDY (Peginterferob Beta-1a)	Biogen Idec Inc (UK)	CHO cells	Multiple sclerosis	2014 (EU&US)
REBIF (Interferon Beta-1a)	EMD Serono (UK)	CHO cells	Relapsing/remitting multiple sclerosis	2002(US) 1998(EU)
AVONEX (Interferon Beta-1a)	Biogen Idec Inc (UK)	CHO cells	Relapsing multiple sclerosis and Chronic hepatitis	1996(US) 1997(EU)
EXTAVIA (Interferon Beta-1b)	Novartis Europharm (UK), Novartis Pharmaceuticals (USA)	*E. coli*	Multiple sclerosis	1993(US) 1995(EU)
BETASERON (US) / BETAFERON (EU) (Interferon Beta-1b)	Bayer Pharma, Berlex Laboratories (USA), Chiron (USA)	*E. coli*	Multiple sclerosis	1993(US) 1995(EU)
PEG-INTRON/ SYLATRON (Peginterferon alfa-2b)	Merck Sharp & Dohme	*E. coli*	Chronic hepatitis C	2001(US) 2000(EU)
INTRON A/ ALFATRONOL (Interferon Alfa-2b)	Merck Sharp & Dohme	*E. coli*	Cancer, genital warts, hepatitis B and C and HPV	1986 (US) 2000 (EU)
PEGASYS (Peginterferon Alfa-2a)	Hoffman-La Roche (UK	*E. coli*	Hepatitis C	2002 (EU & US)

1.6 Clinical Applications and Adverse Effects of Interferons

The first clinical studies start using leukocyte IFN produced from white blood cells. However, major development in clinical applications was not undergone until pure recombinant IFNs became available (Strander, 1986). After the approval of IFN alfa which is the first cancer immunotherapy approved for treatment of hairy cell leukemia, in 1986, later approvals were trailed for Chronic Myelogenous Leukemia (CML), follicular non-hodgkin lymphoma (NHL), melanoma, and AIDS-related Kaposi's sarcoma. Studies showed that interferons found to be effective in some malignancies such as Hepatitis B and C and can be use for cancer vaccines development.

IFN gamma is a candidate for the treatment of non-viral infections, such as, parasitic diseases (Chagas disease, leishmaniasis). However, its clinical application has not been attempted yet (Murray et al., 1996).

The other type I interferon IFNβ subtypes, IFNβ1a and IFNβ1b subtypes, are first licensed by US FDA in 1993 for the treatment of Relapsing-Remitting Multiple Sclerosis (RRMS) (Rodriguez et al., 2010). In 1998, IFNβ1b was demonstrated the first available subtype by European Study Group with proven efficacy in the treatment of secondary progressive multiple sclerosis (SPMS) (Bayas and Gold, 2003).

In addition, interferons are used as an adjuvant or in drug combinations associated with radiotherapy and chemotherapy and to improve disease free or comprehensive survival in the high-risk melanoma treatment (Mocellin et al, 2010). Clinical studies with rhIFNβ generating by different pharmaceutical companies showing the data about the efficacy, safety, toxicity and biochemical studies are reported in the literature and several such studies are still ongoing (Walther and Hohlfeld, 1999; Choffllon, 2000; de Anders, et al., 2007).

The administration of IFNβ is associated with the variety of adverse effects. The most common side effects are injection-site reactions (pain, erythema, inflammation) and flu-like symptoms such as muscle aches, fever, chills, headache, and back pain. Serious allergic reactions, depression or suicidal thoughts, anxiety

insomnia, unusual changes in behavior or mood etc. are commonly seen in IFN treatments (Biogen Idec, 2007; Serono, 2007; Berlex Laboratories 2007).

1.7 Expression Systems Used to Produce rIFNβ

Previously, viral antigen induced human fibroblasts are used to obtain hIFNβ for clinical applications (Derynck et al., 1980). However, lack of safety and low yield are the serious obstacles of this method. For clinical use, high and pure yield of therapeutics are essential. By this way, the parameters that affect recombinant protein production such as, choice of expression system, growth conditions and purification procedures are still main matter, and there are residual attempts to develop profitable methods with the recombinant DNA technology.

Various heterologous expression systems such as mammalian, insect, yeast, and bacterial cells have been used for the production of rhIFNβ via recombinant DNA technology summarized in Table 1.5. (Madhavan and Sukumaran, 2016; Paz Maldonado, et al., 2007; Zago, et al., 2009; Rao, et al., 2009; Villela, et al., 2010; Moradian, et al., 2013)

There are two different active forms of recombinant hIFNβ used for MS; rhIFNβ1a and rhIFNβ1b. Recombinant hIFNβ1a is glycosylated form, has an identical aminoacid sequence with the naturally produced hIFNβ (Kagawa, et al., 1988; Antonetti et al., 2002). Recombinant IFNβ1b, non-glycosylated form, is produced by using prokaryotic expression systems (Mark, et al., 1984; Ghane et al, 2006)

The choice of host system from simple prokaryotics to high eukaryotics depends mainly on the size, structure, stability of the product as much as yield of production, acceptable cost and quality of final product. In the other word, the complexity of the expressed protein is evenly related with the complexity of host origin. Despite its cost and limited product yield, more than 50% of the therapeutic proteins are produced by using the mammalian cell lines; this expression system offers many advantages over other expression systems by obtaining identical to natural protein (Gellisen, 2005; Walsh, 2014).

Table 1.5. Vectors, hosts, strains and promoters used for preparation of the construct to express rhIFNβ1b.

rhIFNβ Production	Vector	Host
Insect cell line	• Baculovirus expression vector • Autographa californica nuclear polyhedrosis virus (AcNPV)	• *Spodoptera frugiperda* cells • *Trichoplusia ni* (Cabbage Loope) • Baculovirus-infected insect cells
Animal cell line	• pCI-neo expression vector	• Human embryonic kidney 293 (HEK 293) • Baby hamster kidney (BHK-21) • Chinese hamster ovary (CHO) • HeLa cells
Transgenic plant	• pIF-482	• *Nicotiana tabacum*
Yeast	• pPIC9IFN plasmid • pKlac1 vector	• *Pichia pastoris* • *Kluyveromyces lactis*
Prokaryotic	• pQE-30 • pKK223-3 • pGEMEX-1 • pET-23a(+) • pET-25b(+) • pT7 blue • pET-3a • pStaby plasmids • pET15b	• *Lactococcus lactis* • *E. coli* (BL21(DE3) and SE1 cells) • *Pseudomonas fluorescens*

1.8 *E. coli* as a Production Host

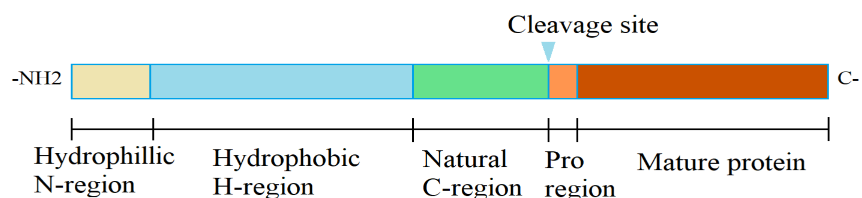
When setting up a process for production of a recombinant protein, the normal approach is to first try to express the protein of interest in *E. coli* which is a facultative gram-negative, rod shaped enteric eubacteria that has mutualistic life in intestines of mammals and commonly found in animal feces and on the edge of hot springs. For commercial purpose, cloning and expression of therapeutic proteins is preferred in *E. coli* expression systems over other systems (Gellisen, 2005; Peti and Page, 2007; Sivashanmugam, et al., 2009) due to its known genetics and rapid growth rate permitting high yield of production. *E. coli* known as a workhorse for the recombinant protein production (Baneyx, 1999; Studier, 2005; Bajaj, et al. 2015; Beladiya, et al., 2015; Iyengar, et al., 2015) that provides with the cost efficient and simple approach for expression of recombinant proteins and has ability to secrete proteins into the periplasm resulting the simplification of the downstream process as well rhIFNβ production by using *E. coli* expression system is widely reported (Allen, et al. 2015; Paz Maldonado, et al., 2007; Rao, et al., 2009; Villela, et al., 2010;

Fazeli, et al., 2013; Moradian, et al., 2013) and there are many US FDA approved therapeutic proteins produced by using *E. coli* expression systems (Walsh, 2014).

Gram negative organisms differ in membrane structure from gram positive organisms by having both an inner and outer membrane and have various secretion pathways. Studies show that overexpressed proteins accumulate in *E. coli* cytoplasm and periplasm or release into extracellular medium via another pathway (Cristo´bal et al., 1999; Choi and Lee, 2004; Ni and Chen, 2009) and simplfy downstream process (Shokri et al., 2003). Type I and type II pathways are commonly used for recombinant protein secretion through the six pathways for recombinant protein secretion in gram-negative prokaryotes (Cianciotto, 2005). Type I secretion is simplest pathway and has a single-step translocation of protein across both the outer and inner membranes (Binet et al., 1997). The type II secretion pathway is a two-step process targets proteins to the periplasmic space. The Tat and Sec are the two general pathways transporting proteins into the periplasm. The Sec pathway is universally conserved, essential and normally the main route of protein export.

The Tat system exports folded proteins into the periplasmic space whereas the Sec system is involved in the secretion of unfolded proteins across the cytoplasmic membrane schematisated in Figure1.3., Signal peptides consist of a hydrophobic core H-region, a neutral C-domain of about 6 residues and positively-charged 2-10 amino acid N-region, as pointed out in Figure 1.2., are required for all translocation pathways and allow to ease purification steps (Choi and Lee, 2004).

Standard Sec Signal Peptide:



Pel B Sec Signal peptide amino acid sequence:

Figure 1.2. Standard signal peptide

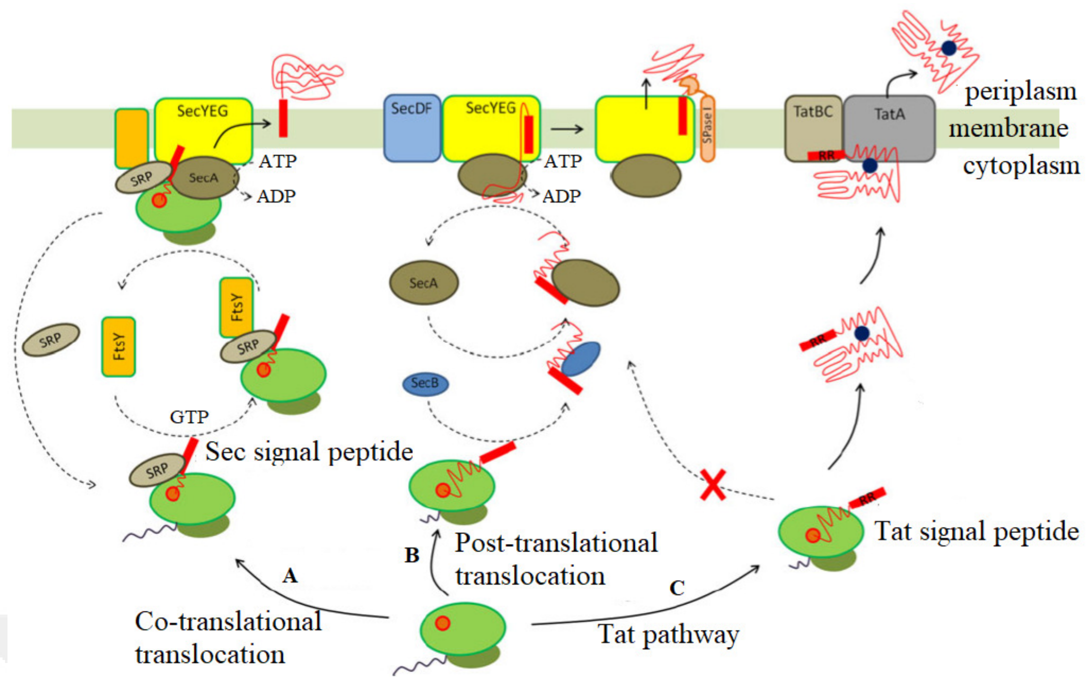


Figure 1.3. Schematic diagram of the signal peptide-based secretion pathway (modified from Low et al., 2013). The A and B represent the Sec A and Sec B pathways, and C represents the Tat pathway. A: Sec A pathway; membrane proteins and highly hydrophobic signal peptides follow the SRP route or co-translational translocation. Firstly forms a SRP-ribosome complex by binding the SRP, then FtsY receptor binds to the complex and guides it to the SecYEG translocon and the pre-protein enters the membrane through the SecYEG gate and being synthesized. B: Sec B pathway; less hydrophobic signal peptides and most periplasmic proteins follow the posttranslational translocation route. SecB signal binds to synthesized secreted protein and keeps it in an unfolded state. SecA binds to the signal peptide and guides it through the SecYEG translocon by hydrolyzing ATP. After the releasing of the pre-protein into the periplasm by SecDF assists, SPase I cleaves the signal peptide which releases the mature domain to the periplasm. C: Tat pathway; folded proteins that consist the Twin-arginine sequence follow the Tat route. After being synthesized, the pre-proteins fold rapidly into their native conformation and Tat protein is not recognized by the Sec components. TatBC recognizes the Tat protein and guides to the TatA translocon and results the mature domain release into the periplasm.

1.9 pET22b Expression Vector

The plasmid pET22b (Novagen) shown in Figure 1.4. and Figure 1.5. was used as a template to construct the expression vector that is able to produce target

rIFNβ1b in the *E. coli*/BLR(DE3) strain. pET22b expression vector, derivative of pBR322, contains a T7 promoter, lacI gene, N-terminal pelB (pectate lyase B, from Erwinia carotovora) signal sequence for potential periplasmic localization, and an optional C terminal poly-histidine tag for simplify the purification process. Cloning of the rIFNβ1b into pET22b between SacI and BamHI restriction sites results the new construct that encodes an N-terminal pelB sequence with the rIFNβ1b sequence following by a poly-histidine tag.

Periplasmic production reduces the contamination level whereas periplasmic secretion is not always possible, suitable for small scale procedure and periplasmic proteases may causes proteolysis (Jonasson et al., 2002).

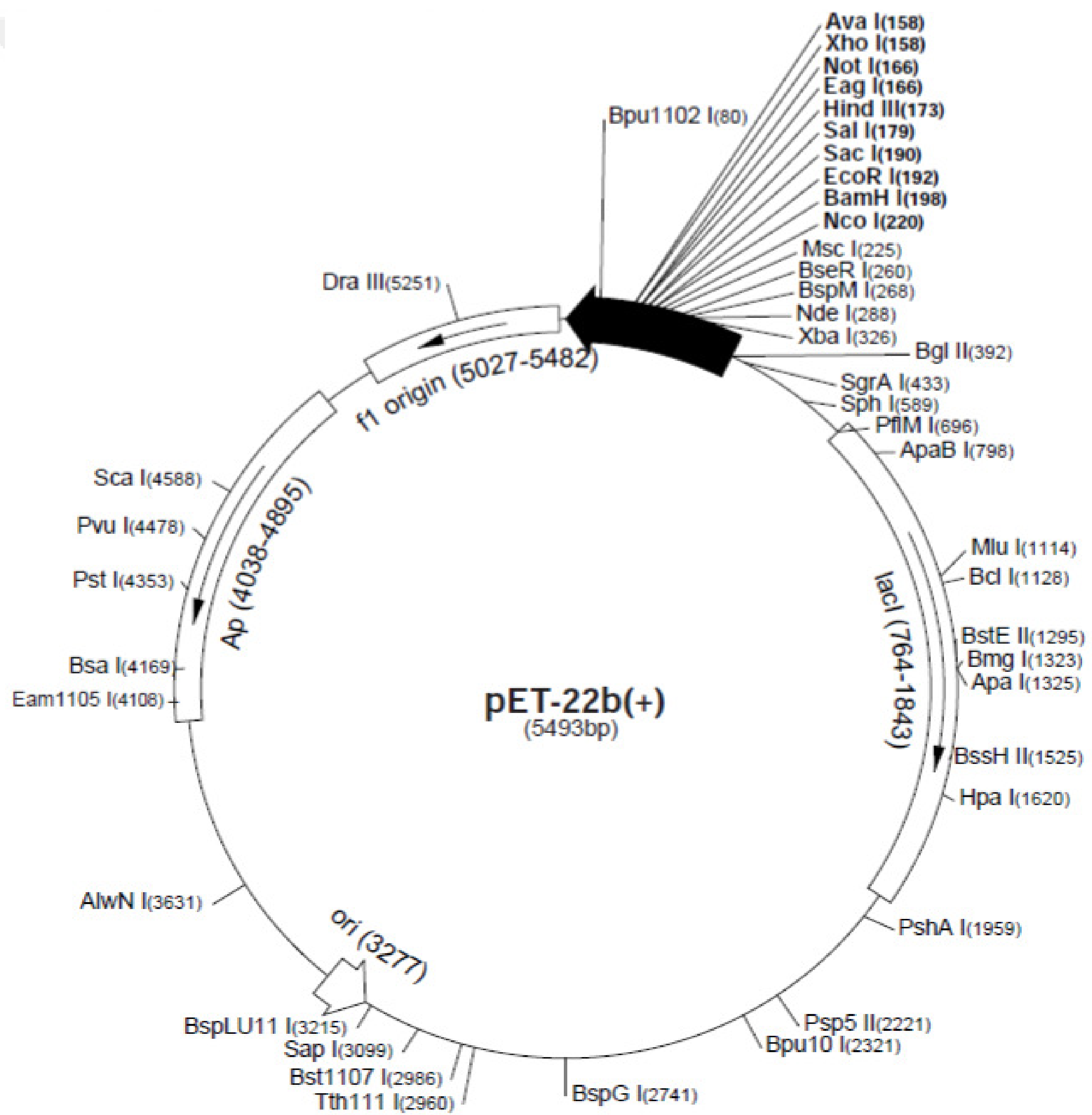


Figure 1.4. pET22b (+) expression plasmid vector map

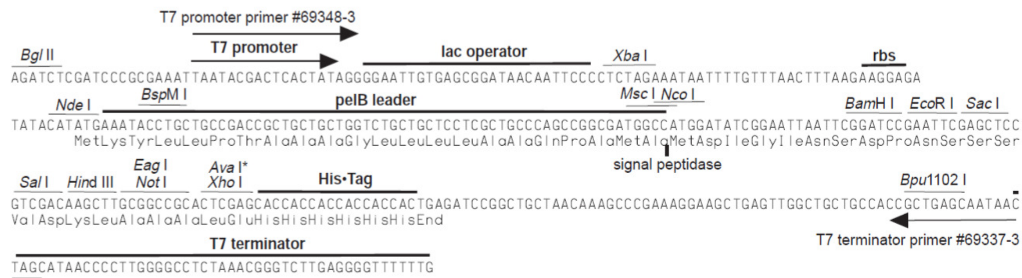


Figure 1.5. Cloning region of pET22b (+) expression plasmid vector

1.10 Induction of the Interferon Synthesis

Basicly IFN signaling is cell specific and stimulates with the presence of virus. Almost every cell produces interferons in response to stimulation by microbial products or chemical inducers. For instance, live or heat inactivated viruses, bacteria, protozoa, lipids and polysaccarides are known as microbial products (Isaacs & Lindenmann, 1957).

Production of IFNs requires presence of dsRNA virus, microbial products or chemical inducers. Pattern recognition receptors (PRRs) present on the target cells recognize the pathogen associated molecular patterns (PAMPs) that present in inducers and stimulates the IFN signaling pathways. Type I and type III interferons bind their distinct receptors and form heterodimeric complex which receptors cytoplasmic domains interact with jak-tyk Janus Associated Kinase (JAK) family of tyrosine kinases (Varedi, 2005) and start series of phosphorylation resulting activation of signal transducer and activator of transcription (STAT) proteins. Activated STAT proteins STAT1 and STAT2 accompany with IRF9 constitutes the ISGF-3 trimeric activator complex which translocates to the nucleus and bind to Interferon-Stimulated Response Element (ISRE) promoter and regulates the expression of Interferon-Stimulated Genes (ISG).

While type I and III interferons generate trimeric activator (Lau et al., 2000), dimeric activator complex (STAT) without IRF9 forms in type II class IFN and then

binds to GAS (IFN-gamma activated site) (Decker et al., 1991) and ISRE, resulting modulations of ISG and the released IFNs bind to the membrane receptors on target cells via autocrine and paracrine ways (Kang, et al., 2012; Rudick and Goelz, 2011; Borden, et al., 2007; Platanias, 2005).

Briefly, following secretion in response to a pathogen, interferons bind a homologous-heterologous receptor complex and induce transcription of genes such as those encoding chemokines and inflammatory cytokines. Even IFNs are structurally and genetically different and have interaction with different receptors; they activate by the same signal transduction pathways (Ernest et al., 2007). The JAK-STAT signaling pathway modulates by crosstalk between several proteins (Takaoka, 2000). This process was shown in Figure 1.6.

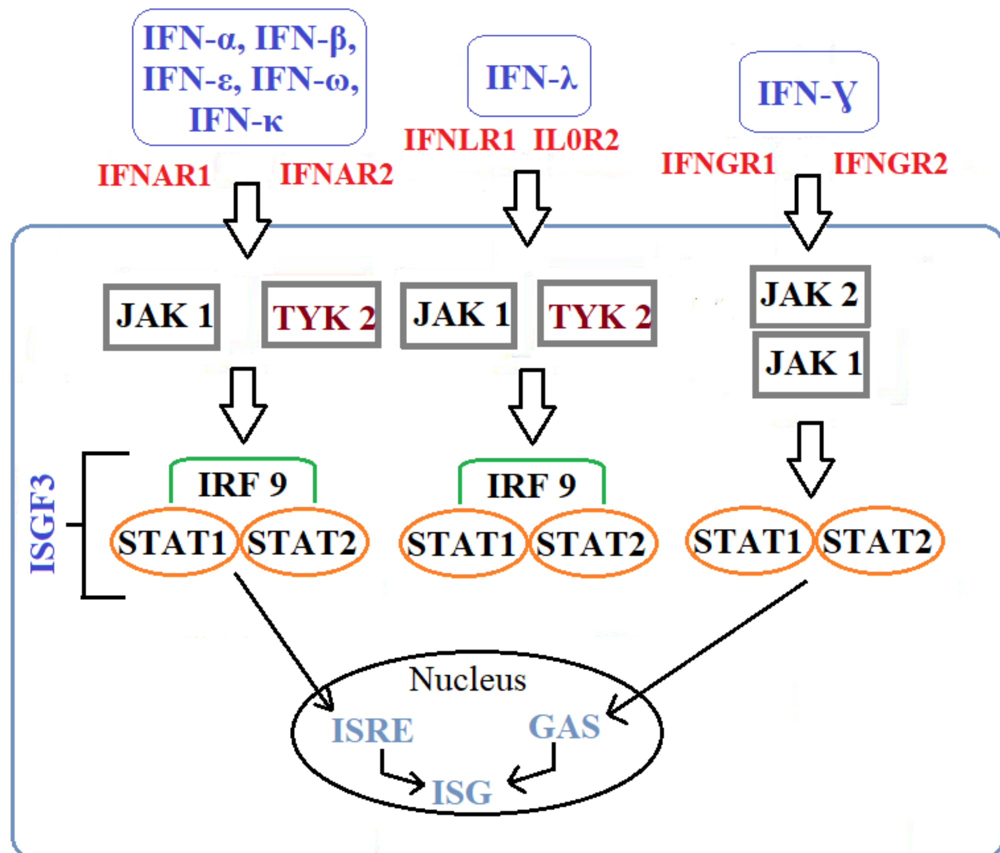


Figure 1.6. Antiviral response of interferons (modified from Dharampal et al., 2016)

2. AIM AND SCOPE OF THE STUDY

It seems that therapeutics is future drugs that will be choice for many diseases and more accessible besides traditional drugs. Even their cost and myriad production parameters, it was achieved by using biotechnology and yield to coming years as therapeutic era. It is possible to generate biosimilar drugs and approve them since current patented therapeutics are come up with expire date.

Technology development in health field needs serious knowledge and sales contract. Because of cumulative knowledge of therapeutics, developed countries have the rights of this drugs and this situation makes a financial load. Peptide drugs (biopharmaceuticals) usage in the diagnosis and treatment increases the intense use of modern biotechnology brought to our lives important innovations which is the importance of recombinant protein production imports. Proteins are needed to be purified from natural sources and expected yield is often not obtained. In addition, immunogenicity in products obtained from non-human organisms while creating problems, the risk of infection is not completely eliminated in human source products. So, under defined conditions, heterologous recombinant protein production by recombinant DNA technologies is the most appropriate alternative to overcome these obstacles.

In Turkey, pharmaceutical industry is gaining atraction in last 20 decades. Even therapeutic export isn't realisation yet, there is export of some drugs and start up companies are established such as Inovita, LifeSci, Koc University's Drug Research and Turgut İlaç that received approval from the Ministry of Health to develop generic products and being the first R&D laboratory with a research and development license. This study will enhance the awarenes about production of therapeutic proteins in Turkey.

This study is conducted to produce recombinant interferon beta 1b therapeutic protein since it is highly used for treatment or diagnosis in variaty of diseases, for instance using for MS, cancer, HIV and hepatitis C.

The purpose of this study is to construct an expression vector which allows effective and stable production of the cytoplasmic and periplasmic rhIFNβ1b, detection of the expression of these recombinant proteins by SDS- PAGE analysis and purification the cytoplasmic and periplasmic extracts by ÄKTAprime plus™ system.

To achieve these purposes, the synthetic IFNβ1b obtained by Oligomer Biotechnology Company (Ankara, Turkey) was cloned into pET22b expression vector. The new costruct containing rhIFNβ1b with pel B sequence and His-tag was transformed in to *E. coli*/ BLRDE3 strain. Expression carried out in *E. coli*/ BLRDE3 cells at 25°C and proteins were concentrated with TCA precipitation and Cryo-concentration methods. Then cytoplasmic and periplasmic extracts were purified and visualized on SDS PAGE then detected by Western Blot analysis.

3. MATERIALS AND METHODS

3.1 In Silico Analysis of rhIFNβ1b

The amino acid sequence of the hINF1b protein (ACCESSIONNC_ 000009, Gene ID: 3456 and NCBI Reference Sequence: NC_000009.12) shown in Figure 3.1 was obtained from NCBI (https://www.ncbi.nlm.nih.gov/). For an efficient expression of rhINFβ in *E. coli*, the codons of the hINFβ1b gene was optimized according to the optimal codon preference for *E. coli* with GC content adjustment (Öztürk and Ermurat, 2019). Cysteine 17 was also substituded for serine to prevent multimer formation and intramolecular disulfide bridges (Runkel et al., 1998).

In order to prepare pZOM construct, *Bam*HI and *Sac*I restriction sites were added to the 5' and 3' ends of the sythetic hINF1b gene, respectively (Figure 4.1). The restricition sites of designed gene were analyzed with Web Cutter (www.firstmarket.com/cutter/cut2.html) and aligments shown in Figure 4.3 were done by T-coffee programme (http://tcoffee.crg.cat/). Restriction analyses showed no restriction sites, interfering with cloning procedure and analysis of RNA structure of rhIFNβ1b, shown in Figure 4.2, was performed to find AU-rich elements (AREs) which determine the RNA stability with Freiburg RNA tools Expa RNA (rna.informatik.uni-freiburg.de/ExpaRNA) or the potential RNA instability motifs. Theoretically designed rhIFNβ1b gene was ordered from Oligomer Company.

Human Interferon beta 1b sequence:
>NC_000009.12:c21077942-21077104 Homo sapiens ch: 9, GRCh38.p13

```
ATG AGC TAC AAC TTG CTT GGA TTC CTA CAA AGA AGC AGC AAT
 M   S   Y   N   L   L   G   F   L   Q   R   S   S   N

TTT CAG TGT CAG AAG CTC CTG TGG CAA TTG AAT GGG AGG CTT
 F   Q   C   Q   K   L   L   W   Q   L   N   G   R   L

GAA TAC TGC CTC AAG GAC AGG ATG AAC TTT GAC ATC CCT GAG
 E   Y   C   L   K   D   R   M   N   F   D   I   P   E

GAG ATT AAG CAG CTG CAG CAG TTC CAG AAG GAG GAC GCC GCA
 E   I   K   Q   L   Q   Q   F   Q   K   E   D   A   A

TTG ACC ATC TAT GAG ATG CTC CAG AAC ATC TTT GCT ATT TTC

 L   T   I   Y   E   M   L   Q   N   I   F   A   I   F

AGA CAA GAT TCA TCT AGC ACT GGC TGG AAT GAG ACT ATT GTT

 R   Q   D   S   S   S   T   G   W   N   E   T   I   V

GAG AAC CTC CTG GCT AAT GTC TAT CAT CAG ATA AAC CAT CTG
 E   N   L   L   A   N   V   Y   H   Q   I   N   H   L

AAG ACA GTC CTG GAA GAA AAA CTG GAG AAA GAA GAT TTC ACC
 K   T   V   L   E   E   K   L   E   K   E   D   F   T

AGG GGA AAA CTC ATG AGC AGT CTG CAC CTG AAA AGA TAT TAT
 R   G   K   L   M   S   S   L   H   L   K   R   Y   Y

GGG AGG ATT CTG CAT TAC CTG AAG GCC AAG GAG TAC AGT CAC
 G   R   I   L   H   Y   L   K   A   K   E   Y   S   H

TGT GCC TGG ACC ATA GTC AGA GTG GAA ATC CTA AGG AAC TTT
 C   A   W   T   I   V   R   V   E   I   L   R   N   F

TAC TTC ATT AAC AGA CTT ACA GGT TAC CTC CGA AAC

 Y   F   I   N   R   L   T   G   Y   L   R   N
```

Figure 3.1. 166 Amino acid and 498 bp nucleotide residues of the hIFNβ1b (Gene ID: 3456) obtained from NCBI (https://www.ncbi.nlm.nih.gov/)

3.2 Preparation of the pZOM Expression Construct

3.2.1 Plasmid Vector Preparation

3.2.1.1 Bacterial Growth and Plasmid Isolation

The TOP10 containing pET22b(+) expression vector was streaked from glycerol freezer stocks to LB (Luria-Bertani) plate media supplemented with 100µg/ml ampicillin and incubated at 37°C for overnight. 5 ml of LB liquid culture supplemented with 100µg/ml ampicillin was inoculated with randomly selected single colony and incubated overnight at 175 rpm and 37°C. Cells were precipitated by centrifugation at 6000 rpm for 3 minutes and then pET22b(+) DNA was isolated from the cell pellet by Plasmid Miniprep Kit (Thermo Scientific, #K0503, Vilnius, Lithuania) as described in its protocol (Figure 4.4.).

3.2.1.2 Digestion of pET22b with *Sac*I and *Bam*HI-FD Restriction Enzymes

Previously isolated pET22b vector was first digested with *Sac*I (Fermentas, 10ul/ml) endonuclease for 1.5 hours at 37°C in reaction condition shown in Table 3.1. Then, largest DNA fragmnets were excised and purified from the 1% agarose gel (Sigma, USA) using the Thermo Scientific GeneJET Gel Extraction Kit (#K0691, EU) (Figure 4.6). Then, *Sac*I digested and purified pET22b vector was digested with *Bam*HI-FD (Thermo Scientific) at 37°C for 10 minutes in the conditon shown in Table 3.2. and whole double digested vector was loaded in 1% agarose gel (Sigma, USA) and extracted then purified by Thermo Scientific GeneJET Gel Extraction Kit (#K0691, Lithuania) as described in its protocol (Figure 4.7.).

Table 3.1. Digestion condition of pET22b vector with *Sac*I restriction enzyme

Reactant	Quantities
pET22b vector	40 µl
*Sac*I Enzyme	2 µl
FD Buffer (Thermo Scientific)	5 µl
ddH_2O	13 µl
Total volume	60 µl

Table 3.2. Digestion condition of predigested and purified pET22b vector with *Bam*HI-FD restriction enzyme.

Reactant	Quantities
Purified pET22b vector	50.0 µl
*Bam*HI-FD Enzyme	1.5 µl
FD Buffer (Thermo Scientific)	6.0 µl
ddH_2O	2.5 µl
Total volume	60.0 µl

3.2.2 Insert DNA Preparation

3.2.2.1 Preparation of the TOP10 and BLR(DE3) Competent Cells

Competent cells of TOP10 and BLR(DE3) were prepared according to competent cell protocol of Hanahan's method (Sambrook and Russel, 2018). TOP10 strain was used as the host strain for cloning of the interferon beta 1b gene, and BLR(DE3) strain was used as a host for the expression of this gene. They were streaked on LB medium from glycerol freezer stocks and exposed overnight incubation (14-16 hours) at 37°C to make single colonies. For starter liquid cultures, single colony of TOP10 and BLR(DE3) was selected from each plate and inoculated in 5 ml LB medium respectively then incubated nearly 16 hours at 175 rpm and 37°C. For the second liquid culture, 1:100 diluted starter liquid cultures were transferred into 50 ml LB broth separately and shake-incubated at 37°C for 2 hours at 125 rpm until the OD value of the cultures at 600nm reached 0.4 - 0.6. When they reached the desired OD value, cultures were allocated into 2 falcon tubes 20 ml volume. They were centrifuged at 4000 rpm and 4°C for 5 minutes. Each pellet was resuspended with 10 ml ice-cold $CaCl_2$ (100mM, pH: 7.0) and incubated on ice for 30 minutes. Then falcons were centrifuged again at 4000 rpm and 4°C for 10 minutes

and pellets were resuspended in 1 ml ice-cold 0.1 M $CaCl_2$ with 10% glycerol then transferred into 1.5 ml eppendorf tubes in100 ul volume and stored at -80°C.

3.2.2.2 Transformation of pUC57 Vector into *E. coli*/TOP10 Competent Cells

For insert preparation, lyophilized pUC57 vector containing synthetic rhIFNβ1b gene was dissolved in TE buffer (100ng/µl) and 2 µl from 1:10 diluted solution of pUC57 vector DNA was transferred into 50 µl of *E. coli*/TOP10 cloning strain and incubated on ice for 30 minutes. Then cells were heated at 42°C for 30 seconds and incubated on ice for 5 minutes. Afterward, 250 ul SOC media was added and placed in shaker at 175 rpm and 37°C for 1 hour. Cells were centrifuged 6000 rpm for 3 minutes and pellet was resuspended with pre-warmed 100 ul SOC media. Cells were spread on LB plate containing 100µg/ml ampicillin 60% and 40% ratio and incubated overnight at 37°C.

3.2.2.3 pUC57 DNA Isolation from Transformants

The *E. coli* TOP10 strain, that include pUC57 vector, were streaked on the LB medium supplemented 100µg/ml ampicillin. After the cells were grown enough at 37°C for 14-16 hours, a 3 ml LB liquid culture with ampicillin and randomly selected single colony from LB plate was shaked at 37°C overnight for the stock preparation and isolation purposes. The following day, one of the overnight cultures was centrifuged at 4000 rpm and 25°C for 4 minutes then resuspended in 1 ml LB broth. The pellet was collected by re-centrifuge at 6000 rpm for 3 minutes and stored in freezer at -20°C. Plasmid DNA isolation was carried out by Plasmid Miniprep Kit (Thermo Scientific, #K0503, Vilnius, Lithuania) as described in its protocol.

In order to confirm the presence of rIFNβ1b in pUC57 vector, that vector was digested with both *Eco*RI and *Hind*III endonucleases simultaneously and the 510 bp rIFNβ1b DNA was visualised on the 2% agarose gel (not shown).

3.2.2.4 Digestion of pUC57 Vector with *Sac*I and *Bam*HI-FD Enzymes

After isolation of pUC57 vector, firstly it was digested by *Sac*I at 37ºC for 2 hrs then *Bam*HI-FD was added and incubated 30 minutes more at 37°C as defined in Table 3.3. Double digested pUC57 vector was run in 1% agarose to visualize expected interferon beta 1b DNA fragment (Figure 4.8.).

Table 3.3. Digestion condition of pUC57 vector with *Sac*I and *Bam*HI-FD restriction enzymes

Reactant	Quantities
pUC57 vector	30 µl
FD Buffer	6 µl
*Sac*I enzyme	3 µl
*Bam*HI-FD	3 µl
ddH_2O	18 µl
Total volume	60 µl

3.2.2.5 Purification of rIFNβ1b protein

Whole digested pUC57 vector with *Sac*I/*Bam*HI was run on 1% sigma agarose gel and the 510 bp interferon beta 1b DNA fragment was purified by GeneJET Gel Extraction Kit (#K0691, Lithuania) as described in its protocol and controlled again by 1% agarose gel (Figure 4.8.).

3.3 Ligation of the pET22b vector with rIFNβ1b Gene

100 ng *Sac*I/*Bam*HI digested and purified pET22b vector was ligated with rhIFNβ1b gene in 1/3 ratio at 16°C for 16 hrs. The reaction was performed in 10 µl total volume shown in Table 3.4. The construct schematic map was shown in Figure 3.2. and Figure 3.3. The quantities of reaction were calculated with NEBioCalculator™ according to required mass formula as shown below:

Required mass insert (ng) = (bp insert/bp vector) X (Molar Ratio (3:1)) X (ng vector)

Table 3.4. Ligation conditions of pET22b vector with rIFNβ1b gene

Ligation reactants	Quantities
Vector	5 µl
Insert	3 µl
T4 ligase buffer (10X)	1 µl
T4 *ligase* Enzyme	1 µl
Total volume	10 µl

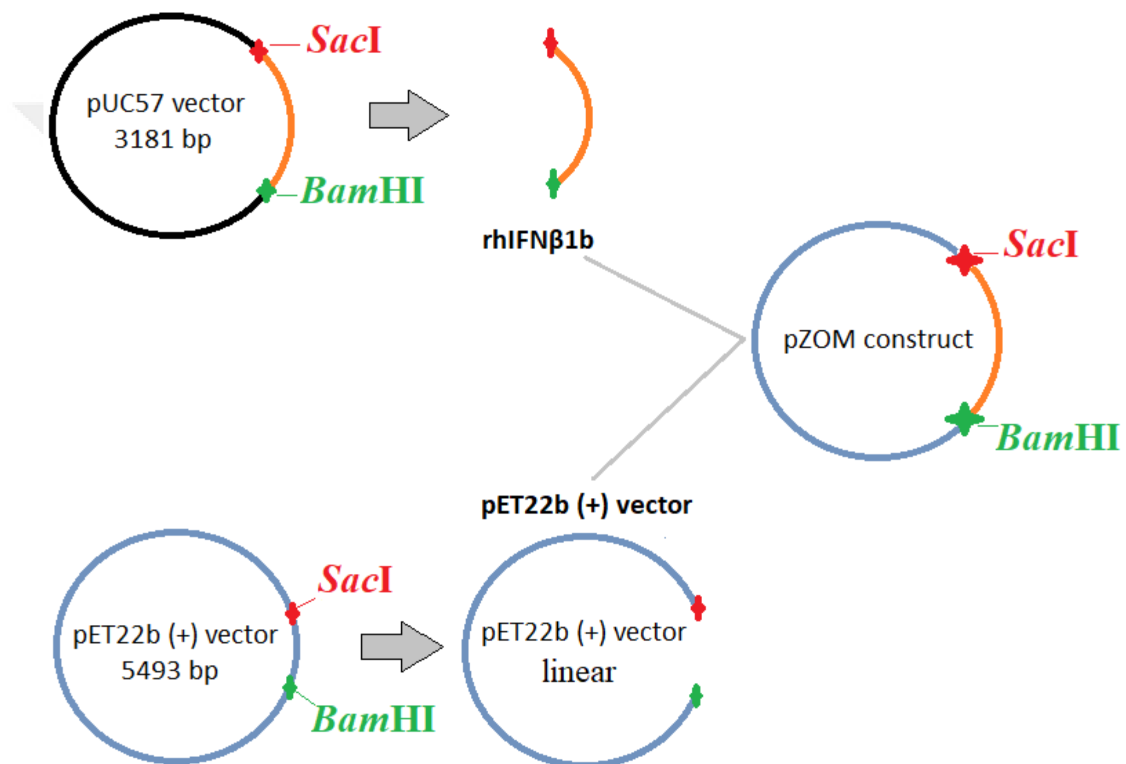


Figure 3.2. Schematic map of designed pZOM construct. The pET22b plasmid DNA isolated from TOP10 cells was digested by *Sac*I and *Bam*HI. In order to obtain the human IFNβ 1b for expression of the protein in *E. coli*, pUC57 was also digested with the same restriction enzymes and the 510 bp DNA fragment carrying rIFNβ1b gene was excised and purified from agarose gel. This DNA fragment was ligated into *Bam*HI/*Sac*I sites of pET22b expression vector, then transformed into *E. coli* BLR(DE3) host. The resulting construct was named as pZOM.

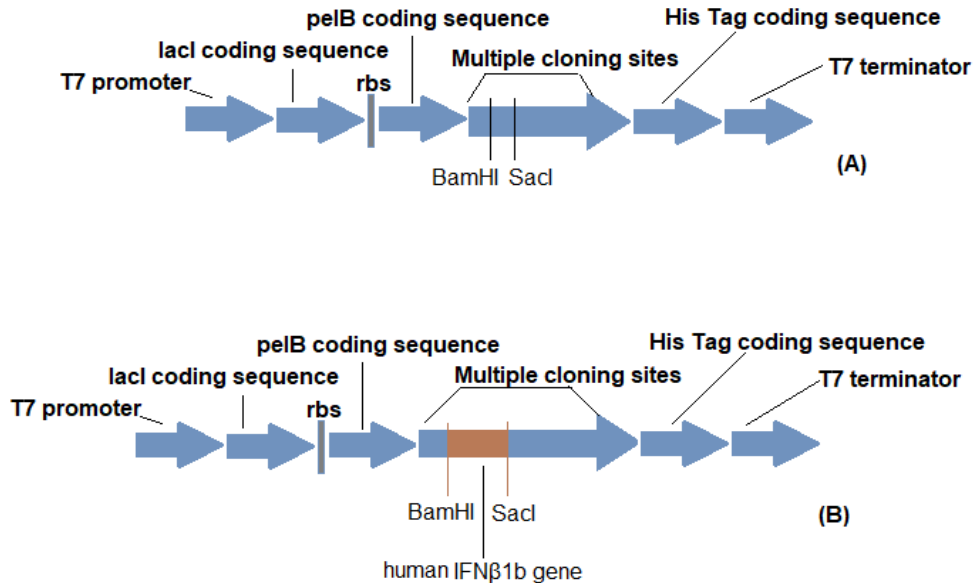


Figure 3.3. Schematic arrangement and composition of pET22b (+) and pZOM vector. (A) represents the pET22b **(+)** vector and (B) represents the pZOM vector

3.4 Transformation of the pZOM into *E. coli*/TOP10 Strains

TOP10 competent cells, kept in -80°C freezer, was thawed on ice for 10 minutes, then thet were incubated on ice with 3 µl ligation product for 30 minutes more. Following incubation, cells were heat shocked at 42°C for 30 seconds and finally incubated on ice 5 minutes. The transformants were mixed with 1 ml LB broth and shaked 175 rpm at 37°C for 1 hr. The pellet, obtained by centrifugation at 6000 rpm for 3 minutes, was resuspend in 100 µl LB broth and spread on the LB plate supplemented with ampicillin and then incubated 37°C for overnight. One of the randomly selected colonies was inoculated 3 ml LB broth supplemented with ampicillin and shaked at 37°C and 175 rpm for overnight. For the stock preparation and plasmid isolation, they were kept in -80°C freezer.

3.5 Plasmid DNA Isolation and Analysis of Construct

The cell pellets obtained from randomly selected transformants were isolated with Plasmid Miniprep Kit (Thermo Scientific, #K0503, Vilnius Lithuania) as described in its protocol (Table 3.5.) and checked on 1% agarose gel to show

whether rIFNβ1b gene was inserted into pET22b(+) vector or not, the DNAs, isolated from transformants which were digested with *Sac*I and *Bam*HI-FD enzymes with the same protocol previously used and desired DNA bands were visualized on 1% agarose gel (Figure 4.7).

Table 3.5. Control digestion condition of transformant with *Sac*I and *Bam*HI-FD restriction enzymes

Control digestion reactants	**Quantities**
Transformant DNA	2 µl
ddH_2O	1.9 µl
*Sac*I	0.3 µl
*Bam*HI-FD	0.3 µl
FD buffer	0.5 µl
Total volume	5 µl

3.6 Sequencing of the rIFNβ1b DNA in pZOM Construct

Sequencing of the rIFNβ1b in pZOM plasmid was done by Macrogen (Seoul, Korea) using universal T7 promoter (5′ TAATACGACTCACTATAG 3′) and T7 terminator (5′ GCTAGTTATTGCTCAGCGG 3′).

3.7 Stock Preparation

After confirmation of the rIFNβ1b gene orientation in pZOM plasmid, 3 ml LB broth medium with 100µg/ml ampicillin was inoculated and culture was incubated at 37°C and 175 rpm for overnight (nearly 14-16 hours) for storage purpose. Cell pellet was obtained by centrifugation at 5000 rpm for 4 minutes and resuspend in 4 ml of LB containing 30% glycerol and stored at -80°C in cryogenic tubes. Plasmid DNAs were isolated from these stocks and checked in 1% agarose gel.

3.8 Expression of rIFNβ1b and Preparation of Cell Extract

For recombinant rINFβ1b protein production, pZOM construct encoding rINFβ1b was transformed from *E. coli* TOP10 into the *E. coli*/BLR(DE3) host. At the same time, pET22b plasmid vector was also transformed from *E. coli* TOP10 into the *E. coli*/ BLR(DE3) host and this clone was used as a negative control. Glycerol stock of these cultures were prepared and checked in 1% agarose gel.

The glycerol stocks of *E. coli* harboring the pZOM plasmid and negative control pET22b(+) were used to inoculate 5 ml of LB broth supplemented with 100 μg/ml ampicillin and incubated overnight at 37°C and 175 rpm for nearly 14-16 hours. Overnight cultures were used for 2:100 (v/v) inoculations of 50 ml TB with 100 μg/ml ampicillin and 0.4 % glucose and incubated at 37°C and 175 rpm. IPTG was added to a final concentration of 0,4 mM when the OD_{600} value of cultures reached 0.4-0.6. When the OD_{600} values reached 1.5-2.0 at 25°C, the cells in stationary phase were harvested by centrifugation at 8000g and 4°C for 15 min.

3.8.1 Preparation of Media Fraction

In this step, the cell culture supernatant obtained at step 3.8 was concentrated with either Cryo-concentration developed by Ortíz and colleagues (2012) or TCA precipitation methods developed by Koontz (2014) as described 3.8.4 and 3.8.5 sections.

3.8.2 Preparation of Periplasmic Fraction

The pellets obtained from step 3.8 were resuspended with 1 ml hypertonic solution of freshly prepared TSE buffer containing 20% (w/v) sucrose, 30 mM Tris-Cl (pH:8.0) and 1 mM EDTA (pH:8.0) and incubated on ice for 30 minutes following centrifugation for 15 minutes at 5000g and 4°C. The supernatant was collected and cell pellets were resuspended in 1 ml hypotonic solution containing 5 mM ice-cold $MgSO_4$ and then incubated for 30 minutes on ice. The mixture was centrifuged and the supernatant of hypotonic solution was collected and combined with the supernatant from the hypertonic solution. Bradford method with BSA as a standard was used to regard the total protein content of the periplasmic fraction.

3.8.3 Preparation of Cytoplasmic Fraction

The pellets remained from periplasmic fraction were resuspend with 2 ml binding buffer pH:7.4 containing 20 mM Tris-HCl, 0.5 M NaCl. Lysozyme enzyme final concentration 1mg/ml was added and the mixture was incubated on ice for 30 minutes and then additional 10 minutes at 4°C. After the incubation, Tween20 (Polyethylene (20) sorbitan monolaurate), DNase and RNase were added in mixture final concentration of which are 1%, 5μg/ml and 5μg/ml respectively and incubated

at 4 °C for more 10 minutes. Then, insoluble debris was removed by centrifugation at 11000 rpm and 4°C for 20 minutes. The supernatant was collected by 0.45µm filter into a new eppendorf. The protein concentration of the cell extract was then measured by the Bradford method with Bovine Serum Albumin (BSA) as a standard. Proteins from fractioned samples were separated by 12% SDS-polyacrylamide gel with BIORAD mini protean Tetra cell (China).

3.8.4 Cryo-concentration Method

The remaining supernatant from centrifugation of the cell culture and periplasmic protein obtained with TSE-$MgSO_4$ buffer and the cytoplasmic protein obtained by binding buffer, containing 20 mM Tris HCl and 0.5 M NaCl, were frozen for 8-10 hours at -20 °C and then concentrated by centrifugation at 6000 rpm for 20 minutes at 4°C. The amount of protein in the concentrated samples was determined by Bradford assay and mixed with apropriate 5X SDS loading dye and incubated for 10 minutes in boiling water and loaded onto the 12% SDS gel.

3.8.5 TCA precipitation Method

To precipitate the cell culture supernatant protein and periplasmic supernatant protein, 100% TCA (10% of the total sample volume) was added, then supenatants were incubated for 30 minutes on ice and centrifugated at 10000g for 15 min at 4°C. The resulting pellets were washed with 500 µl acetone and then centrifugated at 1000 g for 15 minutes at 4°C and allowed to dry for 1 hour at room temperature. Pellets were dissolved in 100 µl of Tris HCl pH8.0 and protein was determined by Bradford method. An apropriate amount of 5x SDS loading dye was added and left for 10 minutes in boiling water and loaded onto the 12% SDS gel.

3.9 SDS-PAGE Electrophoresis

12 % of SDS PAGE electrophoresis was carried out and protein bands were developed by the Coomassie blue staining method. The Thermo Scientific™ PageRuler™ Prestained Protein ladder (#26616) was used as a standard (ranging from 10 kDa to 170 kDa, Lithuania). 5X Loading buffer (0.15 M Tris-base, 15% v/v

glycerol, 0.05% v/v bromophenol blue, 10% (v/v) β-mercapto ethanol) was added to samples and standards, prior to boiling them for 10 min. They were loaded onto the gel and ran for nearly 1 hour at 75 volts then 100 volts. After staining and destaining, the gels were visualized.

3.10 Partially Purification of the rIFNβ1b and SDS PAGE Analysis

In order to purify the desired protein, the samples of periplasmic and cytoplasmic extracts supposed to be containing 6xHis-tagged protein was purified by Ni-NTA (nickel-nitrilotriacetic acid) chromatography column which is based on the interaction between a transition Ni^{2+} ion immobilized on a matrix and the histidine site chains using the ÄKTAprime plus™.

The purified samples were then mixed with a 5X SDS loading dye and β-mercaptoethanol then incubated at 100°C for 10 minutes. The mixture was then loaded on a polyacrylamide gel (12%) and electrophoresed for nearly 1 hour at 75 volts and 3 hours at 100 volts according to Laemmli protocol (Laemmli, 1970). The gel was then stained with Coomasie Brilliant Blue G250 dye and shaked at room temperature for 30 minutes. The gel was destained by washing with destaining solution several times and then protein bands were visualized on gel.

3.11 Western Blotting

SDS-polyacrylamide gel electrophoresis was used to separate proteins using 12% gel. The gel was equilibrated in Blotting buffer for 15 minutes and protein bands were transferred to PVDF membrane for 1.50 hr at 20 V. Then the membrane was washed with TBST buffer without Tween 20 for 5 minutes and then blocked for 45 minutes in blocking buffer (5% skimmed milk and TBS-T) at room temperature. The gel was incubated in primary antibody rabbit polyclonal anti-IFNβ antibody (1:1000 dilution; Bioss) overnight at 4°C. After washing membrane for three times with TBS-T, second antibody HRP- antigoat rabbit IgG (1:4000 dilution, Proteintech Group) was added and incubated 1 hr in room temperature. Chemiluminescence detection kit Clarity Max™ Western ECL substrate (BIORAD) was used to visualize the protein bands. The protein bands were quantified using C-DiGit blot scanner (LI-COR Biosciences).

4. RESULTS AND DISCUSSIONS

4.1 RESULTS

In silico analysis of rhIFNβ1b gene was done by using bioinformatic tools. Codon optimization of hIFNβ1b according to *E.coli* codon table is shown in Figure 4.1. and optimized gene mRNA structure was checked as shown in Figure 4.2. Then, original sequence of hIFNβ1b and optimized IFNβ1b was aligned as seen in Figure 4.3. After in silico analysis, experimental analysis was done.

4.1.1 In silico Analysis of rhIFNβ1b Protein

Optimized human Interferon beta 1b sequence:

GGATCC *Bam*HI Site ATG AGC TAC AAC CTG CTG GGC TTT CTG CAG CGT AGC AGC AAC
M S Y N L L G F L Q R S S N
TTC CAG AGC CAA AAG CTG CTG TGG CAA CTG AAC GGT CGT CTG GAG
F Q S Q K L L W Q L N G R L E
TAC TGC CTG AAA GAC CGT ATG AAC TTC GAC ATC CCG GAG GAA ATT
Y C L K D R M N F D I P E E I
AAG CAG CTG CAG CAA TTT CAA AAA GAG GAC GCG GCG CTG ACC ATC
K Q L Q Q F Q K E D A A L T I
TAT GAA ATG CTG CAG AAC ATC TTC GCG ATT TTT CGT CAA GAC AGC
Y E M L Q N I F A I F R Q D S
AGC AGC ACC GGC TGG AAC GAA ACC ATC GTG GAA AAC CTG CTG GCG
S S T G W N E T I V E N L L A
AAC GTT TAC CAC CAG ATT AAC CAC CTG AAA ACC GTG CTG GAG GAA
N V Y H Q I N H L K T V L E E
AAG CTG GAG AAA GAG GAC TTT ACC CGT GGT AAG CTG ATG AGC AGC
K L E K E D F T R G K L M S S
CTG CAC CTG AAA CGT TAC TAT GGC CGT ATC CTG CAC TAC CTG AAG
L H L K R Y Y G R I L H Y L K
GCG AAA GAA TAT AGC CAC TGC GCG TGG ACC ATT GTT CGT GTG GAG
A K E Y S H C A W T I V R V E
ATT CTG CGT AAC TTC TAC TTT ATC AAC CGC CTG ACC GGC TAC CTG
I L R N F Y F I N R L T G Y L
CGT AAC GAGCTC *Sac*I Site
R N

Figure 4.1. Codon optimized 166 amino acid IFNβ1b sequence with *Bam*HI - FD and *Sac*I enzymes recongnition residues on 5' and 3' sites respectively.

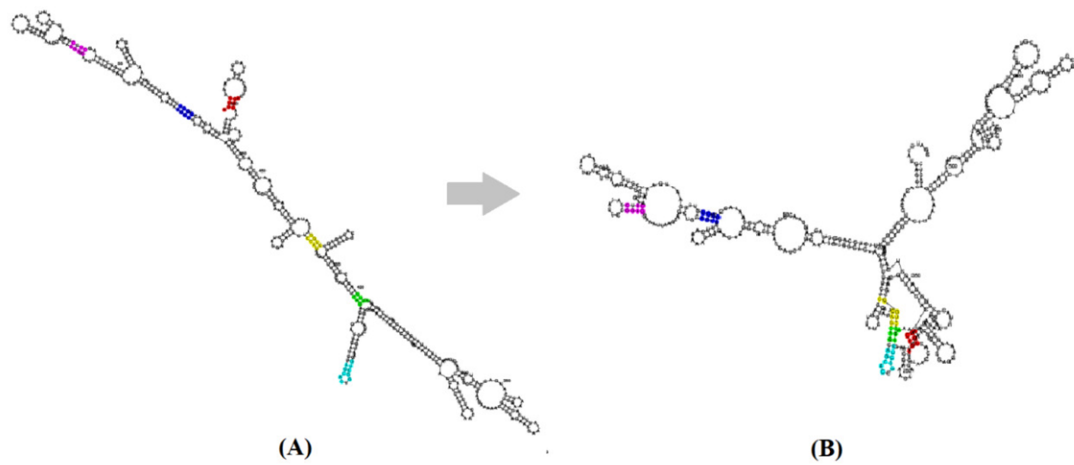


Figure 4.2. RNA structure of hIFNβ1b gene. (A) represents hIFNβ1b gene obtained from NCBI (NCBI Reference Sequence: NC_000009.12) and (B) represents optimized and restriction sites added rIFNβ1b gene.

```
MSA
The multiple sequence alignment result as produced by T-coffee.

T-COFFEE, Version_11.00.d625267
Cedric Notredame
SCORE=275
*
 BAD AVG GOOD
*
original     :  26
optimized    :  26
cons         :  27

original     AUGAGCUACAACUUGCUUGGAUUCCUACAAAGAAGCAGCAAUUUUCAGUGUCAGAAGCUCCUGUGGCAA
optimized    AUGAGCUACAACCUGCUGGGCUUUCUGCAGCGUAGCAGCAACUUCCAGAGCCAAAAGCUGCUGUGGCAA

cons         ************ **** ** ** ** **  * ******** ** *** * ** ***** *********

original     UUGAAUGGGAGGCUUGAAUACUGCCUCAAGGACAGGAUGAACUUUGACAUCCCUGAGGAGAUUAAGCAG
optimized    CUGAACGGUCGUCUGGAGUACUGCCUGAAAGACCGUAUGAACUUCGACAUCCCGGAGGAAAUUAAGCAG

cons          **** **  * ** ** ******** ** *** * ******** ******** ***** *********

original     CUGCAGCAGUUCCAGAAGGAGGACGCCGCAUUGACCAUCUAUGAGAUGCUCCAGAACAUCUUUGCUAUU
optimized    CUGCAGCAAUUUCAAAAAGAGGACGCGGCGCUGACCAUCUAUGAAAUGCUGCAGAACAUCUUCGCGAUU

cons         ******** ** ** ** ******** **  ************* ***** *********** ** ***

original     UUCAGACAAGAUUCAUCUAGCACUGGCUGGAAUGAGACUAUUGUUGAGAACCUCCUGGCUAAUGUCUAU
optimized    UUUCGUCAAGACAGCAGCAGCACCGGCUGGAACGAAACCAUCGUGGAAAACCUGCUGGCGAACGUUUAC

cons         **  * *****       ***** ******** ** ** ** ** ** ***** ***** ** ** **

original     CAUCAGAUAAACCAUCUGAAGACAGUCCUGGAAGAAAAACUGGAGAAAGAAGAUUUCACCAGGGGAAAA
optimized    CACCAGAUUAACCACCUGAAAACCGUGCUGGAGGAAAAGCUGGAGAAAGAGGACUUUACCCGUGGUAAG

cons         ** ***** ***** ***** ** ** ***** ***** *********** ** ** *** * ** **

original     CUCAUGAGCAGUCUGCACCUGAAAAGAUAUUAUGGGAGGAUUCUGCAUUACCUGAAGGCCAAGGAGUAC
optimized    CUGAUGAGCAGCCUGCACCUGAAACGUUACUAUGGCCGUAUCCUGCACUACCUGAAGGCGAAAGAAUAU

cons         ** ******** ************ * ** *****  * ** ***** *********** ** ** **

original     AGUCACUGUGCCUGGACCAUAGUCAGAGUGGAAAUCCUAAGGAACUUUUACUUCAUUAACAGACUUACA
optimized    AGCCACUGCGCGUGGACCAUUGUUCGUGUGGAGAUUCUGCGUAACUUCUACUUUAUCAACCGCCUGACC

cons         ** ***** ** ******** **  * ***** ** **  * ***** ***** ** *** * ** **

original     GGUUACCUCCGAAAC
optimized    GGCUACCUGCGUAAC

cons         ** ***** ** ***
```

Figure 4.3. Tcoffee alignment analysis of human originated and codon optimized rhIFNβ1b gene according to *E. coli* codon preference. NC_000009.12 is the original sequence of hIFNβ1b protein DNA and refers to oprimized hIFNβ1b protein DNA.

4.1.2 Experimental Analysis

4.1.3 Plasmid DNA Isolation of the pET22b Vector

The pET22b plasmid DNA was isolated by Plasmid Miniprep Kit (Thermo Scientific, #K0503, Vilnius Lithuania) as described in its protocol. The circular pET22b vector was loaded on a 1% agarose gel and stained with Ethidium bromide then visualized on UV-transilluminator as shown in Figure 4.4.

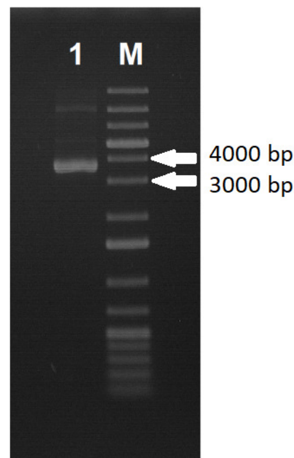


Figure 4.4. Isolation of the pET22b expression vector DNA. M represents the marker and line 1; circular pET22b vector DNA.

4.2 Digestion of pET22b with *Sac*I and *BamH*I-FD Restriction Enzymes

pET22b vector DNA (Figure 4.5.) was digested *Sac*I restriction enzyme then extracted from the 1% sigma agarose gel and run 1% agarose gel after purification (Figure 4.6 and Figure 4.7). Following the second digestion of pET22b vector DNA with *BamH*I-FD and purification of it was done for ligation and transformation process.

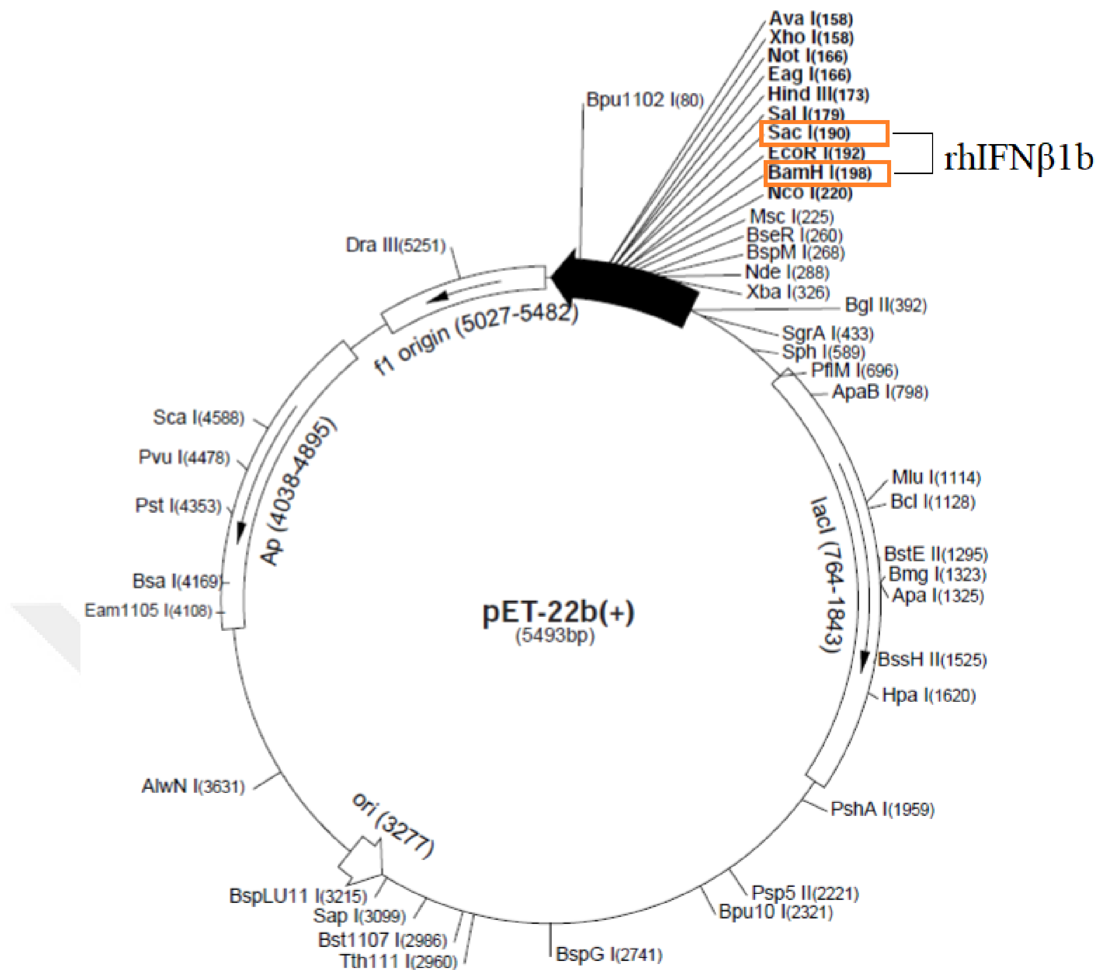


Figure 4.5. Map of the pET22b (+) expression vector DNA

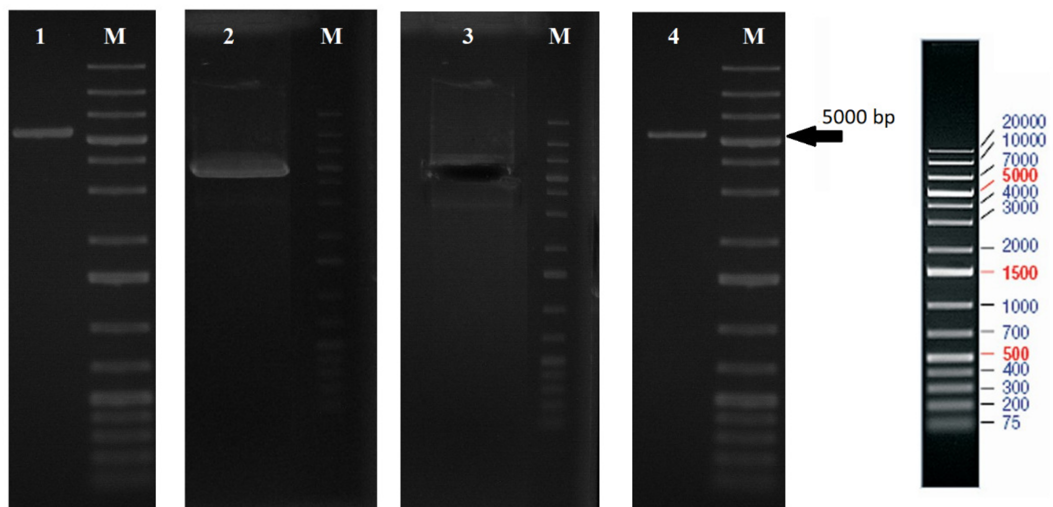


Figure 4.6. Digestion of pET22b expression vector DNA with *Sac*I restriction enzyme and purification of it. M represents the marker; line 1: *Sac*I digested pET22b DNA in a small volume; line 2: *Sac*I digested pET22b DNA in large volume; line 3: extracted *Sac*I digested pET22b DNA; line 4: purified *Sac*I digested pET22b DNA.

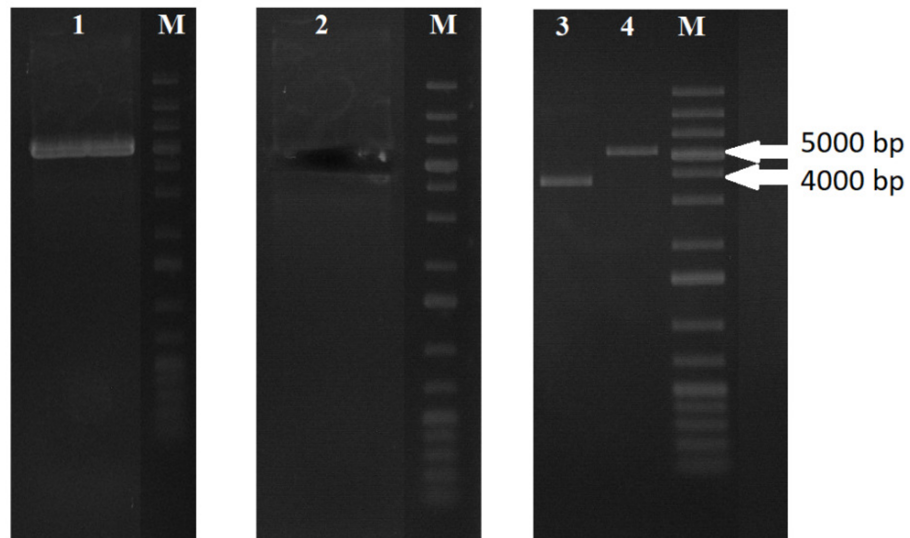


Figure 4.7. Digestion and purification of *Sac*I pre-digested pET22b vector DNA by *Bam*HI-FD restriction enzyme. M represents the marker; line 1: *Sac*I and *Bam*HI-FD digested pET22b DNA; line 2: extracted *Sac*I and *Bam*HI-FD digested pET 22b DNA; line 3: The circular pET22b plasmid DNA; line 4: gel purified pET22b vector DNA digested with *Sac*I/*Bam*HI.

4.3 Transformation of the pUC57 Construct Containing IFNβ1b Gene

Transformation and isolation of pUC57 DNA into TOP10 are shown in Figure 4.8. The isolated vector was digested with *Sac*I and *Bam*HI to extract the rhIFNβ1b gene and then purified to be ready for ligation with pre digested pET22b vector as shown below.

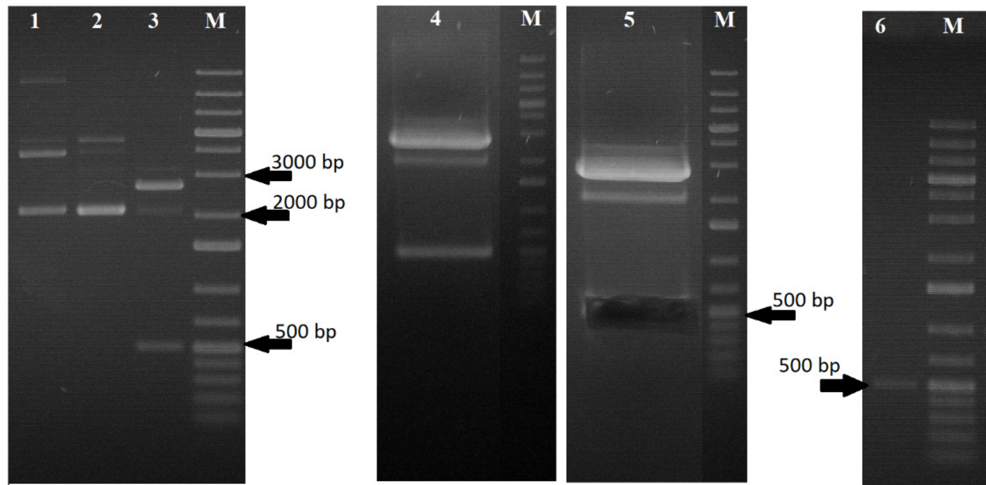


Figure 4.8. Transformation of pUC57 DNA into TOP10 and isolation of it. M represents DNA marker; line 1: pUC57 *Bsa*I free vector dissolved in TE buffer; line 2: pUC57 DNA isolated from the clone; line 3: double digested pUC57 DNA with *Sac*I and *Bam*HI-FD; line 4: entire double digested pUC57 DNA; line 5: large volume of *Sac*I/*Bam*HI digested pUC57 construct containing rIFNβ1b gene; line 6: agarose gel purified rIFNβ 1b gene**.**

4.4 Control of the IFNβ1b Gene Insertion

To control whether the rIFNβ1b gene was inserted into pET22b vector or not, isolated DNAs from transformant were digested with *Sac*I and *Bam*HI-FD enzymes and desired band visualized on 1% agarose gel as shown in Figure 4.9.

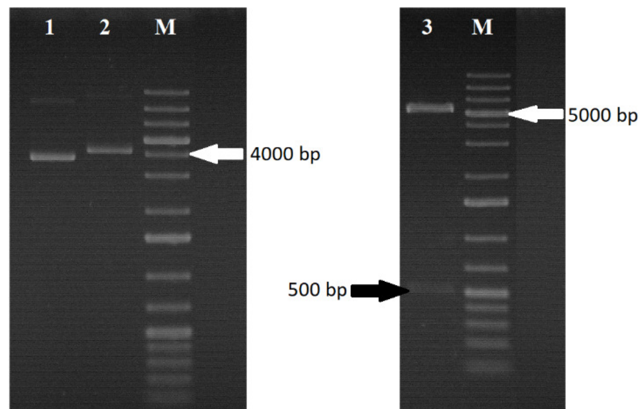


Figure 4.9. Control of the IFNβ1b gene insertion. M represents marker; line 1: uncut circular pET22b vector DNA; line 2: pZOM (pET22b vector DNA carrying rIFNβ1b gene); line 3: *Sac*I/*Bam*HI digested pUC57 construct containing rIFNβ1b gene.

4.5 Sequencing Results

Sequencing of the rIFNβ1b in pZOM vector, shown in Figure 4.10., was done by Macrogen (Seoul, Kore) using universal T7 promoter (forward) (5' TAATACGACTCACTATAG 3') and T7 terminator (reverse) 5' GCTAGTTATTGCTCAGCGG 3')

PelB Leader

*Bam*HI Restiriction Site

*Sac*I Restiriction Site

Histag Peptide

Figure 4.10. Sequencing of the rhIFNβ1b in pZOM vector.

4.6 SDS PAGE Analysis of Cell Extracts

4.6.1 SDS PAGE Analysis of Cytoplasmic Fraction

Pellets remained from periplasmic fraction was resuspend with 2 ml binding buffer pH:7.4 containing 20 mM Tris-HCl, 0.5 M NaCl and cell membrane was disturbed as described previous section 3.8.3 preparation of cytoplasmic fraction. The protein concentrations of the cell extracts were measured by the Bradford method then, proteins from fractioned samples were separated by 12% SDS-polyacrylamide gel with BIORAD mini protean Tetra cell as shown in Figure 4.11.

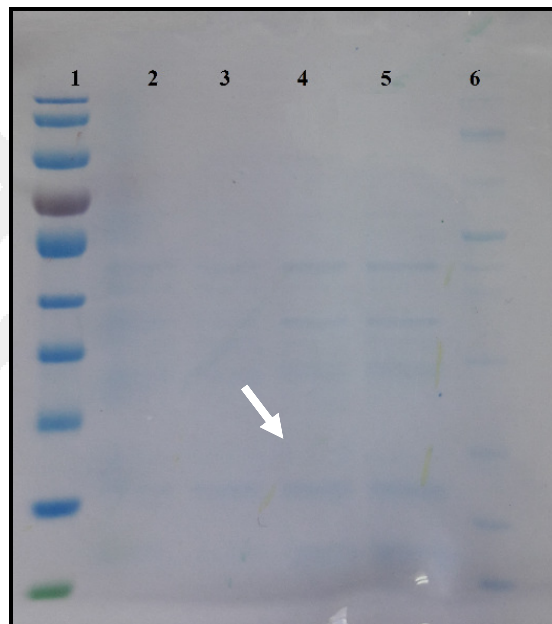


Figure 4.11. SDS PAGE analysis of samples from the cytoplasmic fractions. Line 1: prestained marker; line 2: sample from the cytoplasmic fraction in which BLR(DE)3/pET22b cells were grown; line 3: sample from the cytoplasmic fraction in which BLR(DE)3/pZOM cells were grown; line 4: sample from the cytoplasmic fraction IPTG ınduced BLR(DE)3/pET22b cells; line 5: sample from the cytoplasmic fraction IPTG ınduced BLR(DE)3/pZOM cells.

4.6.2 SDS PAGE Analysis of Periplasmic Fraction

After desired OD_{600} value was reached 1.5-2.0, cell cultures were centrifuged and cell culture media were separated from the cell pellet. TSE buffer and $MgSO_4$

buffer were added on cell pellet and centrifuged for 15 minutes at 5000g and 4°C. Then supernatant was removed from the pellet to obtain periplasmic protein. Cell lysis buffer was added to the pellet and centrifuged to obtain soluble cytoplasmic protein. Amount of protein concentrations of culture media, periplasmic and cytoplasmic fractions were determined with Bradford assay and mixed with 5X SDS loading dye the run in 12% SDS gel (Figure 4.12.).

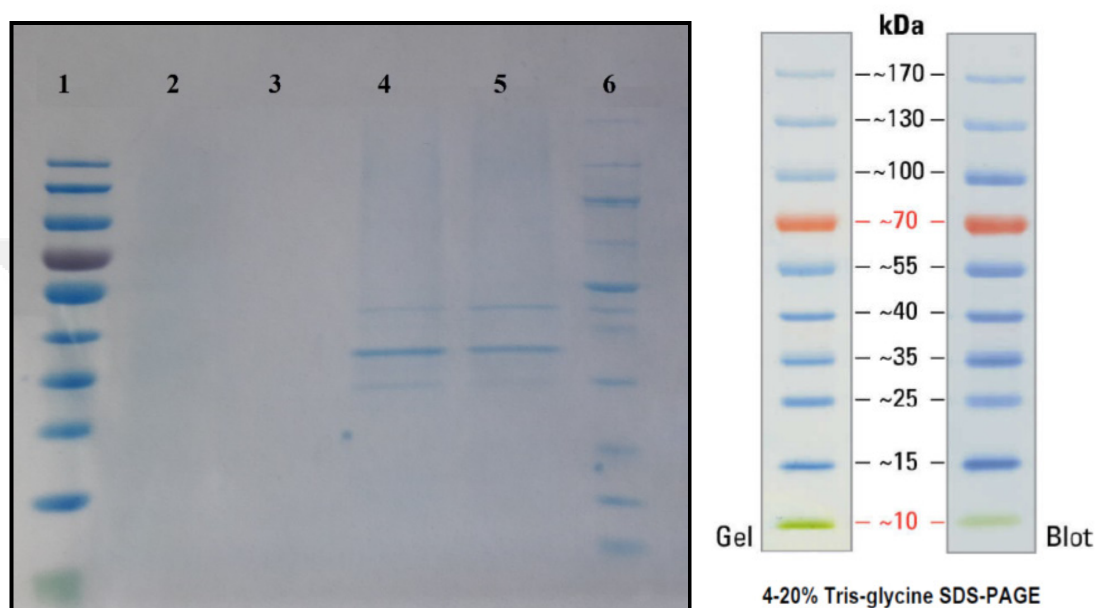


Figure 4.12. SDS PAGE analysis of periplasmic fractions. Line 1: prestained marker; line 2: sample from the periplasmic fraction in which BLR(DE)3/pET22b cells were grown; line 3: sample from the periplasmic fraction in which BLR(DE)3/pZOM cells were grown; line 4: sample from the periplasmic fraction IPTG induced BLR(DE)3/pET22b cells; line 5: sample from the periplasmic fraction IPTG ınduced BLR(DE)3/pZOM cells; line 6: unstained marker.

4.6.3 SDS PAGE Analysis of Culture Media

Cell culture medium supernatant was obtained by centrifugation at 8000g and 4°C for 15 minutes as described in section 3.8. Obtained samples were loaded on 12% SDS PAGE gel and separated as shown in Figure 4.13.

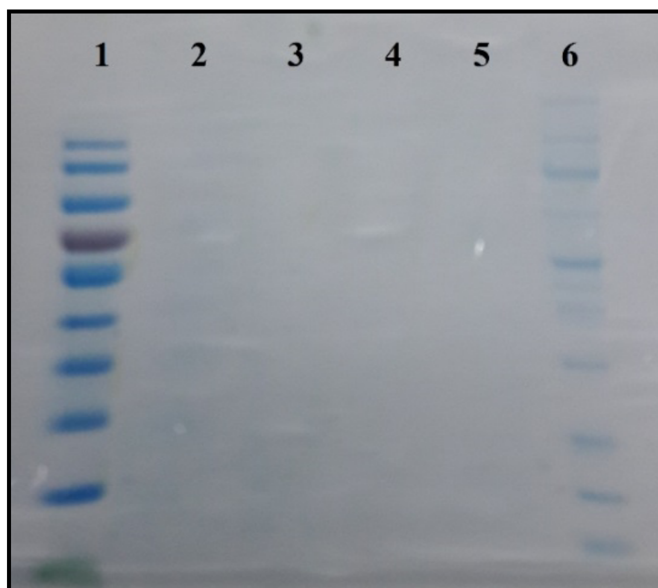


Figure 4.13. SDS PAGE analysis of samples from the culture media. Line 1: prestained marker; line 2: sample from the culture medium in which BLR(DE)3/pET22b cells were grown; line 3: sample from the culture medium in which BLR(DE)3/pZOM cells were grown; line 4: sample from the culture medium with IPTG and BLR(DE)3/pET22b; line 5: sample from the culture medium with IPTG and BLR(DE)3/pZOM; line 6: unstained marker.

4.6.4 SDS PAGE Analysis of TCA Precipitated Fractions

Cell culture media and periplasmic fractions in TSE-MgSO4 buffer, were precipitated using TCA method developed by Koontz (2014). Protein concentration was determined with Bradford assay and mixed apropriate 5X SDS loading dye then run in 12% SDS gel (Figure 4.14.).

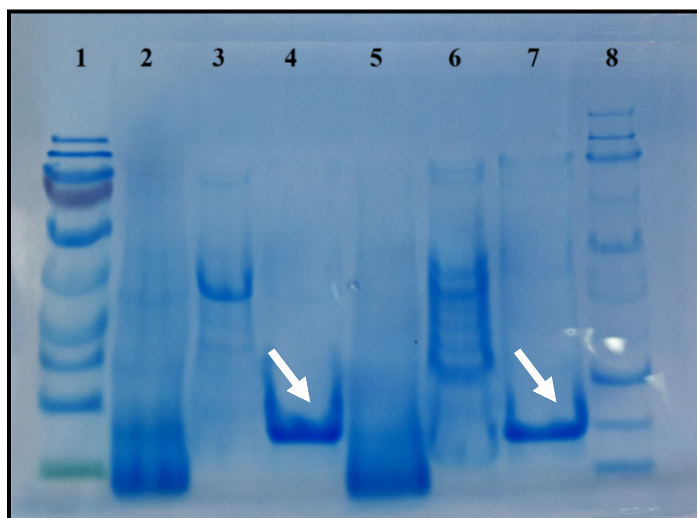


Figure 4.14. SDS PAGE analysis of TCA precipitated samples. Line 1: prestained marker; line 2: TCA precipitated culture medium sample in which BLR(DE)3/pZOM was grown; line 3: TCA precipitated periplasmic sample in which BLR(DE)3/pZOM was grown; line 4: TCA precipitated cytoplasmic sample in which BLR(DE)3/pZOM was grown; line 5: TCA precipitated culture medium sample in which BLR(DE)3/pET22b was grown; line 6: TCA precipitated periplasmic sample in which BLR(DE)3/pET22b was grown; line 7: TCA precipitated cytoplasmic sample in which BLR(DE)3/pET22b was grown; line 8: unstained marker

4.6.5 SDS PAGE Analysis of Cryo-Concentrated Fractions

Cell culture media, periplasmic and cytoplasmic fractions in TSE-MgSO4 buffer, obtained by centrifugation in step 4.7.2-4, were concentreted with cryo-concentration method developed by Ortíz et al. (2012). Both sample proteins and negative control were concentrated with cryo-concentration method, then protein concentrations were determined with Bradford assay and mixed apropriate 5X SDS loading dye then run in 12% SDS gel (Figure 4.15.).

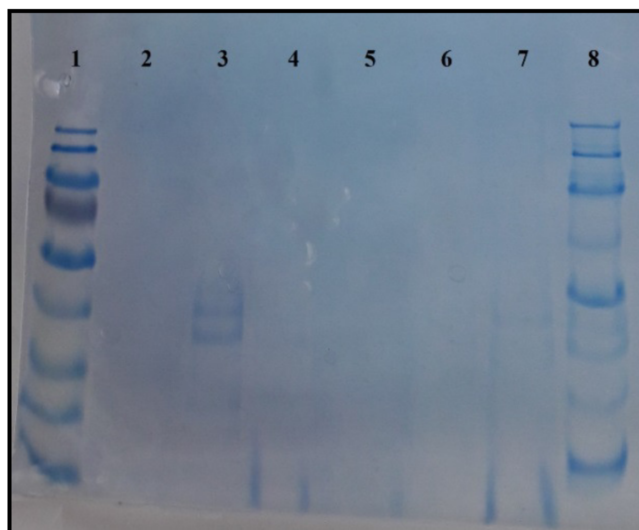


Figure 4.15. SDS PAGE analysis of cryo-concentrated samples. Line 1: prestained marker; line 2: cryo-concentrated culture medium sample in which BLR(DE)3/pZOM was grown; line 3: cryo concentrated periplasmic sample in which BLR(DE)3/pZOM was grown; line 4: cryo-concentrated cytoplasmic sample in which BLR(DE)3/pZOM was grown; line 5: cryo-concentrated culture medium sample in which BLR(DE)3/pET22b was grown; line 6: cryo-concentrated periplasmic sample in which BLR(DE)3/pET22b was grown; line 7: cryo-concentrated cytoplasmic sample in which BLR(DE)3/pET22b was grown; line 8: unstained marker.

4.6.6 SDS PAGE Analysis of the Partially Purified rIFNβ1b from Periplasm and Cytoplasm and Western Blot Analysis

After partially purification of periplasmic and cytoplasmic rIFNβ1b protein by ÄKTAprime plus™ system with Nikel Chealated column. According to peaks on purification graphic as shown in Figure 4.17, third tube may contain target purified protein was collected. Purified and unpurified protein samples from cytoplasmic/periplasmic samples and negative control were loaded and electrophoresed in 12% SDS gel. Then one of the gel was stained with staining solution (Figure 4.16) and the other gel was transferred in PVDF membrane for Western Blot Analysis (not shown). The SDS profile of rhIFNβ1b protein displayed a molecular band located between 10 and 15 kDa of molecular mass shown in Figure 4.16 which was not consistent with the theoretical molecular mass of rIFNβ1b proteins (18kDa).

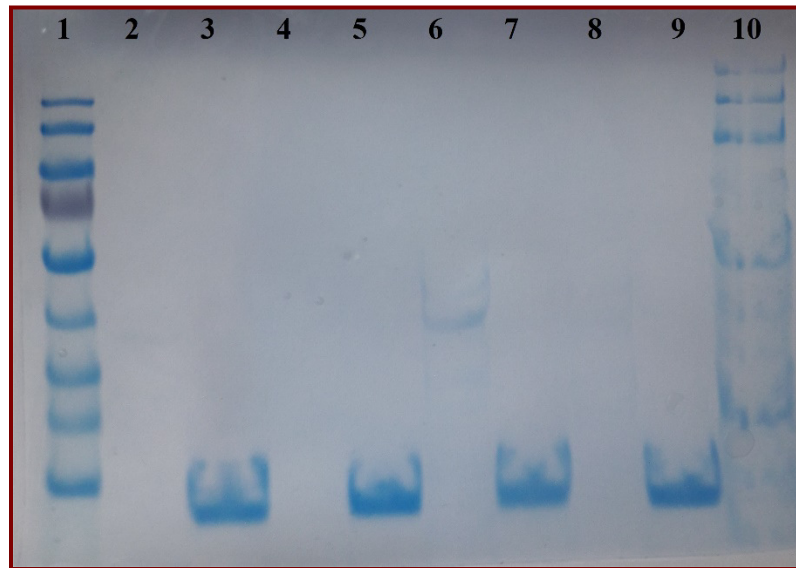


Figure 4.16. SDS PAGE analysis of the partially purified rIFNβ1b from periplasm and cytoplasm. Line 1: prestained marker; line 2: purified periplasmic fraction in which BLR(DE)3/pZOM was grown; line 3: purified cytoplasmic fraction in which BLR(DE)3/pZOM was grown; line 4: purified periplasmic fraction in which BLR(DE)3/pET22b was grown; line 5: purified cytoplasmic fraction in which BLR(DE)3/pET22b was grown; line 6: periplasmic fraction in which BLR(DE)3/pZOM was grown; line 7: cytoplasmic fraction in which BLR(DE)3/pZOM was grown; line 8: periplasmic fraction in which BLR(DE)3/pET22b was grown; line 9: cytoplasmic fraction in which BLR(DE)3/pET22b was grown; line 10: unstained marker.

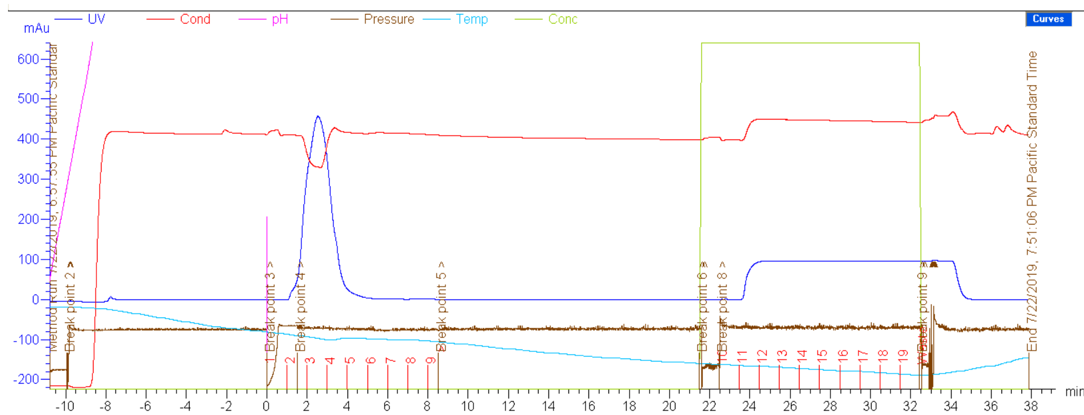


Figure 4.17. Purification graphic of rIFNβ1b protein. Peaks shows the third tube that may contain target protein.

5. DISCUSSION

Thanks to technological development and experience on proteins that can be used for production of the pharmaceuticals, these and similar therapeutic recombinant proteins are intended to be produced in large quantities and inexpensively using bacterial production systems to reduce costs. Production of heterologous recombinant proteins and use of these proteins is one of the important fields of study in Biotechnology.

E. coli expression system is commonly used for commercial purpose as it provides low cost, customisible process that increase protein activity and stability. We expected that, therapeutic protein production in prokaryotic cells will simplify the purification process by lowering the production cost and decrease the activity problem which occurs because of inclusion bodies in overexpressed proteins. Therefore, prokaryotic sec pathway was used for periplasmic secretion in order to obtain highly pure active protein while protecting *E. coli* from toxicity. Thus, it was thought that this method would be used for small or large-scale productions and it would certainly contribute to the development of new therapeutic indications for IFNs. Human IFNβ1b is previously produced by using various heterologous expression systems such as bacterial, yeast, insect and mammalian cells (Villela, et al., 2010; Moradian, et al., 2013; Ghane et al 2006; Zago, et al., 2009; Madhavan and Sukumaran, 2016; Skoko, et al 2003; Rao, et al., 2009; Paz Maldonado, et al., 2007)

Strains of TG1, XL1Blue, DH5α, M15, BL21(DE3) and JM109 of *E. coli* K12 were used for cloning and expression of the rIFN-β. The most expressions were belonging to *E. coli* BL21 (DE3) and Rosetta and the least to JM109. Plasmids pQE-30 pKK223-3 pGEMEX-1 and pET22b plasmids were used for the expression of synthetic IFNβ gene (Singh et al., 2012). Previosuly, pET25b, that involves pelB signal peptide and pET15b with a His-tag sequence that is binding N-terminal of the IFNβ1b gene was used to express soluble and insoluble forms of rIFNβ1b (Ghovvatti et al., 2018). According to previous reports, signal sequences can be highly useful and most important factors to promote high yield of recombinant heterologous

protein that are generally troublesome to express in *E. coli*. Several signal peptides were used in previous studies for the secretion of recombinant proteins in *E. coli*; such as PhoA, PelB, OmpA etc (Choi & Lee, 2004).

Previously reported that, the optimized process yielded 34% of rhIFN-β-1b with more than 99% purity (Rao et al.,2009) and high yield of IFNβ1b secretion fusion with PelB (Morowvat et al.,2014). Moreover, fusion with PelB signal peptide, facilitates the fully expression of IFNβ1b in the periplasmic space (Mobasher et al.,2016). According to Ghovvati, a therotical study shows the EXG1 signal peptide is one of the most suitable peptides for translocating IFN-beta 1b protein into the periplasmic space of *E. coli*. Signal.

In this study, 17th position cysteine amio acid of native hIFNβ1b gene was altered by serine and sacI and BamHI restiriction sites were added then subcloned into pET222b (+) vector then screened by using antibiotic-resistant markers (ampicillin) and sequenced. Desired protein was clonned into the MCS site of expression cell that consists a Histag signal peptide. Expression of protein was tried by using LB medium Terrific Broth (TB) medium at 25ºC by IPTG induction and to obtain more pure protein then purfication was done and the samples were examined by SDS and WB methods.

According to Warne and Thomes (1986), IFNβ is highly toxic protein for *E. coli* and expression of soluble active IFNβ is deleterious to host cell in early stages of cell growth thus decrease the production yield. However, expression of IFNβ1b in active form is simple and cost benefit as simplifies the downstream process by using pelB signal peptide for periplasmic localization. Previous studies also reported that the secondary structure of mRNA may inhibits the translational efficiency so, the future researches would be study of expression of the synthetic construct, employing codon substitution at the IFNβ1b gene and optimization of culture conditions. The expression of IFNβ1b in soluble form could not be determined in this study because of some unexpected problems were exists in these steps, so this study should be carried out under different conditions in future studies. Protein expression conditions can be optimized by using Taguchi's method or by Using Response Surface Methodology and the other down stream processes (SDS and WB) can be optimized advertently to obtain understandable results.

6. CONCLUSIONS AND RECOMMENDATIONS

The production of recombinant therapeutics is a major subject of biotechnology industry for the treatment of many diseases. Therefore, their therapeutic usage has improved the quantity and quality of the life for millions of patients. However, many questions remain unanswered in order to fully realize their potential. So, it is crucial to understand the mechanism. With the development of recombinant DNA technologies, many expression systems were used and they are still developing purposive.

In 2013, biotechnological drugs accounted for 22% of the pharmaceutical market. According to Evaluate Pharma 2014, biotechnological drug sales increased by 11% between 2006-2013. By 2020 the sales value is expected to be 30% according to steady accural. Production of rhIFNβ1b was studied in order to facilitate providing IFNβ1b drug as more cheaper for many patients around the world, especially from Turkey and in order to support the economic development in Turkey by exporting this protein.

Today, *E. coli* strains are preferred both in scientific studies and in commercial protein production due to their low cost and high purity of protein. Recombinant proteins are becoming increasingly important in the pharmaceutical industry because of the intended production in order to benefit from their healing effects as biopharmaceuticals. However, the secreted recombinant proteins used as drugs, need posttranslational modifications that affect drug efficacy, stability and biological activity. Recombinant proteins that do not have these modifications may have detrimental effects on the immune system. Therefore, in order to avoid unwanted side effects, the relationship between the precursor structures and cellular mechanisms of proteins needs to be well understood. As a result, it is crucial that the selection of expression systems should be selected carefully for the high quality recombinant protein production. Experimental results are influenced by various factors. Therefore, these parameters need to be optimized to obtain maximum efficiency with low cost are required in the studies. IFNs are glycoproteins in nature and are normally produced in almost every cell type in human and other mammalian

and provide defence mechanism in response to infections. Since interferon is specific to a particular species, it is necessary to obtain human cells again for use in the treatment of humans and expression of human interferon beta (hIFNβ1b) in prokaryotic cells is more useful process because of its low molecular weight and its non glycosylated form. The production of rIFNβ1b in *E. coli* is not naturally regulated and controlled by the host. For example, constitutive production of rIFNβ1b is not efficient for the host since it has negative effect on cellular growth. The use of either Lac and Tac promoter, provides the user to control and optimize the point of rIFNβ1b production. After sufficient cellular growth, the addition of IPTG converts the lac repressor (LacI) into its inactive form. Therefore, this allows the transcription of the target proteins.

In this study, the hIFNβ1b gene was optimized according to *E. coli* codon preference and cysteine at position 17 was altered by serine since it is required for a highly active and stable form of IFNβ. Since the vector pET22b has a pelB signal peptide, it was tought that PelB signal sequence would allow the protein to transport the periplasmic region of the host cell and to reduce purification cost. However, our SDS-PAGE and western blot analysis results showed that there was no expression of rIFNβ1b protein neither in the cytoplasm nor in periplasm. Probably because of the codon optimization the mRNA of rIFNβ1b might be higly folded and this higly folded form of mRNA prevented translation. This study may help to design new strategies for the production of IFNβ1b protein in *E. coli* cells.

In future, different codon optimization and expression parameters; pH, temperature, induction time and inducers, should be prefered to obtain understandable results. These conditions can be optimized by using Taguchi's method or by Using Response Surface Methodology. In addition, translation efficiency should be checked before downstream process and *E. coli* strains that have extra chaperons can be used as a host for expression of rIFNβ1b protein.

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APPENDICES

8. APPENDICES

Appendix A Tables

Table A1. Strains and their properties used in this study.

Strain	Genotype/relevant properties	Reference
BLR(DE3)	F^- *ompT gal dcm hsdS*$_B$($r_K^-m_B^-$) Δ*(srl-recA)306*: Tn*10amp resistance*	Novagen
TOP10	F- mcrA Δ(mrr-hsdRMS-mcrBC) φ80lacZΔM15 ΔlacX74 nupG recA1 araD139 Δ(ara-leu)7697 galE15 galK16 rpsL (Str^R) endA1 λ^-	Invitrogen

Table A2. Plasmids and their properties used in this study

Plasmid	Genotype /origin	Reference
pET22b(+)	PT7, Ampr, oripBR322, lacI, C-term. 6xHis N- Pelb signal	Novagen
pUC57-BsaI free	Pm13, Ampr, origin of puc19 lacz	Oligomer

Appendix B Bacterial Growth Media

✦ Luria Bertani (LB) medium

Contents	Amount for 1 liter
Tryptone	10 g
Yeast extract	5 g
NaCl	10 g

After dissolving contents in 900 ml upH_2O, pH is adjusted to 7.5 with NaOH/ HCl and upH_2O is added up to final volume 1 L and autoclaved at 121 ºC for 15 minutes.

✦ LB plate

Add 15% agar in 1L liquid LB medium and sterilized at 121 ºC for 15 minutes.

✦ Ampicillin (25 mg/ml)

250 mg Ampicillin Sodium Salt is dissolved in 9 ml upH_2O/ MilliQ water and volume adjusted up to 10 ml then sterilized by using a 0.22 µm filter and stored at -20 ºC.

✦ Lb plate with ampicillin

0.4 ml of ampicillin is added for each 100 ml of sterilized LB medium.

✦ Terrific Broth

TB contents	Amount	Final concentation
Yeast extract	24 g	24 g/L
Tryptone	20 g	20 g/L
Glycerol	4 ml	4 ml/ L
TB salt 10X	100 ml	1X
Final volume		**1 L**

Yeast extract, Tryptone and Glycerol are dissolved in ddH_2O until homogenized and adjust volume up to 1 L and sterilized by autoclave at 121 ºC

for 15 minutes separately from 10X TB salt. After cooling approximately 50 ℃ filter sterilized 100 ml TB salt is added in 900 ml TB content.

TB salt 10X

TB salt 10 X contents	**Amount**
KH2PO4	11,57 g
K2HPO4	62.7 g
Final volume	**0.5 L**

TB salt contents are dissolved in 400 ml upH_2O and up to volume 500 ml at room temperature pH 7.4, then sterilized through 0,22 µm filter.

Appendix C Buffers and solutions:

TE Buffer:

10 X TE Buffer	**Volume**
1 M Tris-HCl	100 ml
0.5 M EDTA pH 8.0	20 ml
Final volume	**Adjust final volume 1L with ddH_2O and autoclave.**

❖ **For competent cell preparation:**

- **0.1 M $CaCl_2$**

Dissolve 0.7351 g $CaCl_2$ (mw:147,02 g/mol) in 40 ml ddH_2O and adjust volume up to 50 ml then sterilize by 0.22 μm filter.

- **0.1 M $CaCl_2$ 10% Glycerol**

1 ml Glycerol is mixed with 9 ml 0.1 M $CaCl_2$ then sterilized by autoclave at 121 ºC for 15 minutes.

TAE Buffer:

10X TAE Buffer	**Amount**
Tris Base	48.4 g
Glacial Acetic Acid	11,42 ml
0.5 M EDTA pH 8.0	20.0 ml
Final volume	**Adjust volume up to 1 Lwith ddH_2O**

❖ **For cell extraction**

✦ **TSE Buffer:**

TSE Buffer contents	**Stock solution**	**Final concentration**
Tris HCl pH 8.0	1.0 M	30 mM
EDTA pH 8.0	0.5 M	1 mM
Sucrose		20 %
Final volume		**100 ml**

✦ **Binding Buffer**

Contents	**Amount**
20 mM Tris HCl	20 ml
0.5 M NaCl	100 ml
ddH_2O	580 ml
Final volume	**1 L**

✦ **5 mM MgSO4**

0,123 g MgSO4 (mw:246,48) is dissolved in 8 ml upH_2O then up to volume 10 ml an strerilized by autoclave.

✦ **30 mg/ml Lysozyme**

30 mg lysozyme is dissolved in 1 ml ddH_2O.

✦ **DNase 5mg/ml**

10 mg DNase is dissolved in 1 ml 0.15 M NaCl.

✦ **Bradford Reagent**

5 mg Bromophenol blue was dissolved into 4 ml methanol and adjust volume up to 5ml then mixed with 10 ml 85% H3PO4 and The final volume of the solution was adjusted to 50 ml with ddH_2O, then sterilized through the 22 µm filter and up to volume 100 ml. It was store at 4 ºC and prevented from sunlight.

✦ **Bovine Serum Albumin (1mg/ml)**

1 mg BSA was dissolved in 1 ml ddH_2O.

✦ **20 % Sucrose**

20 g sucrose was dissolved into 100 ml ddH_2O then sterilized by 22 µm filter.

❖ **For purification IFNβ1b protein**

✦ **Binding Buffer pH 7.4**

Contents	Amount
20 mM Tris HCl	20 ml
0.5 M NaCl	100 ml
30 mM Imidazole	300 ml
ddH_2O	580 ml
Final volume	**1 L**

✦ **Elution Buffer pH 7.4**

Contents	Amount
20 mM Tris HCl	10 ml
0.5 M NaCl	50 ml
0.5 M Imidazole	250 ml
ddH_2O	190 ml
Final volume	**500 ml**

❖ **Stock solutions:**

✦ **0.5 M EDTA pH 8.0**

18.62 g EDTA was dissolved 80 ml ddH_2O and adjusted pH 8.0 with NAOH and volume 100ml with ddH2O.

✦ **1 M Tris HCl pH 7.4**

6.1 g Trisma base (mw: 121,1 g/mol) was dissolved in 40 ml ddH_2O, adjusted pH 7.4 with HCl then up to volume 50 ml with ddH2O.

✦ **5 M NaCl**

58.44 g NaCl (mw: 58,44 g/mol) was dissolved in 100 ml ddH_2O then adjusted volume up to 200 ml and autoclave at 121 ºC 15 minutes.

✦ **1 M imidazole**

40,85 g Imidazole (mw: 68,08 g/mol) was dissolved in 500 ml ddH2O then adjust pH 7.4 with HCl, volume up to 600ml with ddH_2O. sterilize through 0.22 µm filter

✦ **1 M IPTG**

95,32 mg IPTG was dissolved in 4 ml ddH_2O and through the22 µm filter.

✦ **0.5 % Bromophenol Blue**

Mix 50 mg of bromophenol blue with 10 ml of ddH_2O and shake gently until dissolve.

✦ **10 % APS**

0.05 g APS was dissolved in 500 ul.

✦ **10 % SDS**

1g SDS was dissolved in 8 ml ddH_2O and adjusted volume up to 10 ml.

❖ **For SDS PAGE**

✦ **5X SDS loading dye**

Contents	**Amount**
Tris pH 6.8	1 ml
Glycerol	1 ml
10% SDS	400,0 µl
0.5% bromophenol blue	80.0 µl
Urea	2.4 g
B mercaptoethanol	5%
Final volume	**5 ml**

✦ **SDS Running buffer 10X**

Contents	Amount
Trisma base	7.5 g
Glycine	36.0 g
SDS	2.5 g
Final volüme	0.25 L

✦ **SDS staining solution**

Contents	Amount
Coomassie blue	1000 mg
Glacial acetic acid	100 ml
Methanol	400 ml
ddH_2O	500 ml
Final volume	**1 L**

✦ **SDS destaining solution**

Contents	Amount
Methanol	100 ml
Glacial acetic acid	100 ml
ddH_2O	800 ml
Final volume	**1 L**

❖ **For Western Blot analysis**

✦ **Blotting buffer (10x)**

Contents	Concentration
Trisma base	25 mM
Glycine	192 mM
ddH_2O	450 ml
Final volume	**500 ml**

✦ **Blotting Buffer (1x)**

100 ml from 10x Blotting buffer was add in 20% methanol and 0.1% SDS in 1L ddH_2O

✦ **TBST buffer (1x) (pH 7.4)**

Contents	Amount
Trisma base	3 g
NaCl	8 g
KCl	0.2 g
Tween 20	1 ml
Final volume	**1L**

All contents except Tween 20 are dissolved in 950 ml ddH_2O and pH adjusted with HCl to 7.4.

- **Blocking buffer (5% w/v)**

1.5 g skimmed milk is dissolved in 30 ml TBST (without Tween 20)

Appendix D Chemicals

Agar (Merck)

Agarose (Sigma)

Ammonium persulfate [APS] (Sigma)

Ampicillin (Sigma)

Bovine Serum Albumin (Sigma)

Bromophenol blue (Merck)

Calcium Chloride [minimum 93.0%, granular anhydrous] (Sigma)

Calcium Chloride Dehydrate [CaCl2] (Merck)

Coomassie Brilliant Blue G-250 (Fluka)

Destained Protein Ladder (Thermo Scientific)

dNTP Mix (Fermentas)

EDTA [Ethylenediaminetetraacetic acid] (Sigma)

EtBr [Ethidium Bromide] (Sigma)

Glacial Acetic acid (Carlo Erba)

Glycerol, (Sigma)

Glycine (Merck)

IPTG [isopropyl-beta-D-thiogalactopyranoside] (Thermo Scientific)

KCl (Thermo Scientific)

Methanol (Tekkim)

MgCI2 (Thermo Scientific)

Magnesium Sulfate hepta-hydrate (Sigma)

NaCl (Merck)

Page Ruller Prestain Protein Ladder (#26617) (Thermo Scientific)

Sodium Chloride [NaCl] (Merck)

Sodium Dodecyl Sulfate (Sigma)

Sodium Hydroxide [NaOH] (Merck)

Sucrose(saccharose) (Merck)

TEMED (Bio-Rad)

Trichloroacetic Acid (Merck)

Trizma Base (Sigma)

Tryptone (Lab-M)

Tween® 20 (Sigma)

Xylene Cyanol (Merck)

Yeast Extract Granulated (Merck)

1 kb Plus DNA Ladder (Fermentas)

6X Loading Dye (Fermentas)

IFN-β polyclonal rabbit antibody (cat no:0787R, Bioss)

HRP-goat anti rabbit IgG (cat no:00001-2, Proteintech Group)

Appendix E Equipment Used in This Study

-80 °C deepfreezes (Biolaps) and (Thermo scientific)

-20 °C deepfreeze (Arçelik)

34 °C and 37 °C Incubators (Nuve EN 500, Nuve FN 500)

34 °C and 37 °C shaker-incubator (Gerhardt)

+4 °C refrigerators (Arçelik)

Autoclave (Hirayana)

Centrifuge (Hettich Rotina 38R)

Platform centrifuge (Hettich Micro 120)

Electrophoresis system (Thermo Scientific)

ÄKTAprime plus™ Chromatography System

HisTrap™ HP (GE Healthcare)

Imaging system (UVP Photo Doc-It TM)

Micropipettes (Finnipipette, Rainin)

PCR (BIORAD T100 ™ THERMOCYCLER)

pH meter (HANNA HI 221)

Power supply (Thermo EC 250-90)

Shaker-heater (IKA RCT basic)

Spectrophotometer (HITACHI U-1900)

2UV™ Transilluminator

Vortex (Yellowline TTS2)

Water Purification System (Human Corporation)

Heidolph unimax 2010 platform shaker

SDS Miniprotean® Tetracell 4 gel system kit (BIORAD)

GeneJET Gel Extraction Kit (Thermo Scientific)

GeneJET Miniprep Kit (Thermo Scientific)

Western Blot System (BIORAD)

Mini Trans-Blot® Cell Module (cat no: 17038811, BIORAD)

Clarity Max™ Western ECL substrate (cat no: 1705061, BIORAD)

Appendix F Enzymes

*Bam*HI (Thermo Scientific) (ER0058)

*Sac*I (Fermentas) (ER1135)

Fast Digest Pack (Thermo Scientific)

Dnase (cat no: D5025) (Sigma)

Rnase (5mg/ml) (Sigma)

Lysozyme (Fluka)

T4 DNA Ligase (Fermentas)

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