Expression of recombinant human Interleukin ۲ (rhIL-۲) in *E. coli*

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Abstract

Background: interleukin-۲ (IL-۲) is a protein consisted of ۱۳۲ amino acids (MW = ۱۵,۳ kDa). Aldesleukin is the synthetic form of the protein which is used as an effective function for immuno system. Current study was aimed to investigate the expression of rhIL-۲ in *E. coli* BL۲۱ (DE۳) expression system in order to produce an active recombinant form of the protein. Methods: Firstly codon optimization was done for hIL-۲ gene. Then the gene was synthesized and inserted in pET-۲٤a vector by a cutting strategy included NdeI and BamHI-HF enzymes. In the next step, gene was run in agarose gel and purified. The constructed expression cassette was transformed into *E. coli* BL۲۱ (DE۳) cells through CaCl۲ heat shock method. Identification and confirmation of the transformed colonies were performed using screening PCR method. Synthesis of rhIL-۲ was induced by IPTG. The expression in induced strains was analyzed by SDS-PAGE and western blotting techniques**.** Results: Analysis of transformed *E. coli* strain with SDS-PAGE and western blotting techniques confirmed that gene was expressed in host cells. Molecular weight of the expressed protein was estimated to be ۷,٦ kDa. Conclusion: rhIL-۲ expression cassette for cloning and expression in *E. coli* was designed and the protein of interest was successfully induced and identified. *E. coli* BL ۲۱

(DE ۳) can be used as a suitable host for production of recombinant hIL-۲ and this technology has a potential to be localized.

Keywords: Interleukin ۲, *E. coli*, Gene expression, cloning

Introduction

Production of recombinant protein provides a suitable method for the commercializing the medical products. Another advantage of producing recombinant proteins is the more safety in comparison with sampling of fluids from individual (۱). IL-۲ is a member of a cytokine family, each member of which has a four alpha helix bundle (۲). IL-۲ has key roles in key functions of the immune system, tolerance and immunity, primarily via its direct effects on T cells. In the thymus, where T cells mature, it prevents autoimmune diseases by promoting the differentiation of certain immature T cells into regulatory T cells, which suppress other T cells that are otherwise primed to attack normal healthy cells in the body. IL-۲ also promotes the differentiation of T cells into effector T cells and into memory T cells when the initial T cell is also stimulated by an antigen, thus helping the body fight off infections(۳). Its expression and secretion is tightly regulated and functions as part of both transient positive and negative feedback loops in mounting immune responses and tamping them down. Through its role in the development of T cell immunologic memory, which depends upon the expansion of the number and function of antigen-selected T cell clones, it also has a key role in enduring cell-mediated immunity(٤). While the causes of itchiness are poorly understood, there is some evidence that IL-۲ may be involved in itchy psoriasis(٥). IL-۲ is manufactured using recombinant DNA technology and is marketed as a protein therapeutic called aldesleukin (branded as Proleukin) by Prometheus Laboratories, Inc. It has been approved by the Food and Drug Administration (FDA) and in several European

countries for the treatment of cancers (malignant melanoma, renal cell cancer) in large intermittent doses and has been extensively used in continuous doses(٦). IL-٢ is particularly important historically, as it is the first type I cytokine that was cloned, the first type I cytokine for which a receptor component was cloned, and was the first short-chain type I cytokine whose receptor structure was solved. Many general principles have been derived from studies of this cytokine, including its being the first cytokine demonstrated to act in a growth factor–like fashion through specific high-affinity receptors, analogous to the growth factors being studied by endocrinologists and biochemists(٧). Due to the high-throughput of recombinant DNA techniques, the commercial production of peptides and proteins in bacterial cells is performed this way (٨). One of the microorganisms which can be used for cloning and expression is a special strain of *E. coli*. This expression system has many advantages such as simple control of gene expression, protein efficiency (up to ٥٠٪ of total cell protein), having a cloning vector, and easy culture (٨, ٩). Our main goal was cloning and expression of recombinant human Interleukin ٢.

Materials and Methods

Design and synthesis of genes: recombinant human Interleukin ٢ is a non-glycosylated protein and the best expression system for this protein is *E. coli*. Thus, IL-٢ gene was optimized for this bacterium. This codon optimization was done since IL-٢ is a human protein, while the chosen host to produce the recombinant form of protein is *Escherichia coli* which was a prokaryotic cell. In essence, the codon usage pattern in *E. coli* is different from natural host that is human. Hence, in order to homogenize the codon usage pattern of the desired protein sequence and expression in the host and finally overexpression of gene, codon optimization and gene synthesize were performed by GenScript Corporation. IL-٢ sequence is shown below:

	IL-۲ gene (٤١١bp)
After optimization	CATATGCCGACCTCGTCCTCAACCAAGAAAACGCAACTGCAACTGGAACACCTGCTGCTGGATCTGCAAA TGATTCTGAACGGTATTAACAATTATAAAAACCCGAAACTGACCCGTATGCTGACGTTTAAATTCTACAT GCCGAAGAAAGCGACCGAACTGAAACATCTGCAGTGCCTGGAAGAAGAACTGAAACCGCTGGAAGAAGTC CTGAACCTGGCGCAAAGCAAAAATTTTCACCTGCGTCCGCGCGATCTGATTTCTAACATCAATGTGATTG TTCTGGAACTGAAAGGCAGTGAAACCACGTTCATGTGTGAATATGCGGACGAAACCGCCACGATTGTGGA ATTTCTGAACCGTTGGATTACCTTTTCGCAGTCTATTATTTCTACCCTGACCTGAGGATCC

NdeI restriction site	CATATG
BamHI restriction site	GGATCC
Stop codon	TGA

Then, this gene was sub-cloned into pUC۱۸ plasmid.

Cloning of human Interleukin ۲ gene in *E. coli* DH۵α: Cloning vector pUC۱۸ and IL-۲ gene were purchased from GenScript (USA). Initially, optimized IL-۲ gene that was sub-cloned into pUC۱۸. Cloning vector was transformed into *E. coli* DH۵α competent bacteria In order to replicate the plasmid. After the transformation, bacteria were cultured in LB Agar medium containing IPTG, X-Gal and ampicillin (۵۰ µg/ml final concentration), were incubated for ۱٦h in ۳۷°C temperature. Then, using white-blue screening of colonies, transformed bacteria that formed white single colonies were picked up by loop and cultured in

۱۰۰ml SOC medium and incubated in shaker incubator for ۱۲ h in ۳۷°C temperature. Next, bacterial sediment was prepared using centrifuge, and replicated plasmids were extracted by plasmid extraction kit (Qiagen, Netherlands). For plasmid confirmation, it was run in ۱٪ agarose gel electrophoresis. Extracted plasmid concentration was ۱۰۰ ng/µl.

Enzymatic digestion of pUC۱۸ cloning vector and pET-۲٤a expression vector: Firstly, this plasmid was digested with two restriction enzymes including NdeI and BamHI-HF (BioLabs (UK)), for separation of Interleukin ۲ gene from pUC۱۸ cloning vector. Then, it was run on ۱٪ agarose gel electrophoresis and purified with gel extraction kit (Qiagen, Netherlands) in order to extract the digested gene. Final concentration of obtained gene was ۱٤ ng/µl. pET-۲٤a was also digested by these two restriction enzymes and prepared for ligation.

Ligation of IL-۲ gene into pET۲٤-a expression vector: The equation below can be used to calculate the quantities of DNA necessary for a particular ratio:

$$\text{insert (ng)} = \frac{\text{vector (ng)} \times \text{size of insert (kb)}}{\text{size of vector (kb)}} \times \text{Molar ratio of (insert/vector)}$$

According to BioLab protocol, the best molar ratio is ۳:۱. ۳۰ µg of expression vector with ۱,۲۷ µg of IL-۲ gene and ٥ µl of T٤ DNA Ligase (BioLabs (UK)) are used for ligation.

Analysis of transformants by Colony screening: Colonies can be screened for inserts without plasmid preparation, by direct colony PCR using vector-specific primers. To verify ligation between the vector and insert, a ligation reaction can be analyzed directly by PCR using vector-specific primers was done using Colony screening method. For pET-۲٤a, appropriate primers for screening by colony PCR are T۷ Promoter Primer (TAATACGACTCACTATAGGG) and T۷ Terminator Primer (GCTAGTTATTGCTCAGCGG). According to pET manual from Novagen Company, a colony was picked up from an agar plate using a sterile toothpick. Colonies were Chosen that

were at least ۱ mm in diameter and we tried to get as many cells as possible. Then, each colony was transferred to a ۰٫۵-ml micro-tube containing ۵۰ µl of sterile water. Vortex was done to disperse the cells. Then the micro-tube was placed in boiling water or a heat block at ۹۹°C for ۵ min to lyse the cells and denature DNases. Next, it was centrifuged at ۱۲٬۰۰۰ × g for ۱ min to remove cell debris. ۱۰ µl of the supernatant were Transferred to a fresh ۰٫۵ ml micro-tube to do PCR with later and it was leaved on ice until use. Then a master mix was prepared for colony PCR by assembling the following components: ۱µl dNTP mix ۱۰mM, ۱µl upstream primer, ۱µl downstream primer with ۵pmol concentration, ۵µl ۱۰x buffer with $MgCl_2$, ۰٫۲۵ µl Taq DNA Polymerase, ۳۱٫۷۵ µl Nuclease Free Water were mixed. ٤۰ µl of the master mix were Added to each ۱۰ µl sample, were mixed gently and the samples were put in a thermal cycler.

Process in the thermal cycler for ۳۵ cycles, as follows:

- ۱ min at ۹٤°C
- ۱ min at the ۵۵°C
- ۲ min at ۷۲°C
- ٦ min final extension at ۷۲°C

Then after, ۱۰–۲۵ µl of the product of Colony PCR were loaded per lane on a ۱٪ agarose gel. A strong band was appeared that had a size corresponding to the total number of bases of the region between the two primers.

pET-۲٤a transformation into *E. coli* BL۲۱(DE۳) expression system and expression of rhIL-۲: pET-۲٤a expression vector and *E. coli* BL۲۱(DE۳) was purchased from Pasteur Institute (Iran). Expression vector carrying the desired gene, was transformed into *E. coli* BL۲۱ (DE۳). Then, it was cultured on LB Agar medium and incubated for ٦h in ۳۷°C. Next, single colonies were picked up from plate, each transferred to ۱۰۰ml LB Agar containing kanamycin (۳۰ µg/ml final concentration) medium and incubated in shaker incubator at ۳۷°C. Then, when $OD_{٦۰۰}$ reached ۲, IPTG (۱mM) was added and it was incubated again for ۵-٦ h.

Thus, protein expression was completed and IL-۲ protein was produced as inclusion bodies in bacterial cytoplasm. Finally, whole culture was centrifuged at ۱۰۰۰۰ x rpm.

Disruption of bacterial cell and cell lysis: After centrifugation, the sediment was weighed to obtain ۰,۲ g. Then, it was dissolved in DTT (٤۰۰ ml) and homogenized using SOAVI homogenizer (Parma, Italy) (٤ times with ۱۲۰۰ bar pressure). After the breakdown, the solution was centrifuged at ۱۱۰۰۰ rpm for ٥ minutes. The precipitate was weighed to obtain ۰,۱ g. Then the precipitate was dissolved in washing buffer (Triton (x۱۰۰) ۰,٥٪ $^{v}/_{v}$, Tris (٥۰mM) ،EDTA (٥mM), and DTT (۱mM)) and incubated for ٤۰ min at room temperature. Then, this solution was centrifuged at ۱٥۰۰۰ rpm for ۱٥ min. Then, precipitate was dissolved in ۱۰۰µl WFI (water for injection).

Analysis of recombinant protein expression: Gene expression was evaluated in the induced strain by SDS-PAGE and western blotting techniques. SDS-PAGE was done using ۱۰% poly-acrylamide gel and samples were stained by bromophenolblue. Finally western blotting was done using Primary polyclonal antibody against IL-۲ (Abcam corporation, USA)and IgG secondary antibody conjugated to HRP (Abcam corporation, USA). Western blotting analysis was performed using anti-IL-۲ polyclonal antibody as primary antibody, Goat anti-rabbit antibody (IgG-HRP) as secondary antibody and DAB, ٥۰X. Protein bands on SDS-PAGE were transferred to nitrocellulose membrane in order to identify the production of hrIL-۲ in *E. coli* BL۲۱ (DE۳). The first incubation of membrane was accomplished with bovine serum albumin (BSA) ۳٪ (w/v) in tris-buffered saline and PBS solutions for ۹۰ min to block unspecific bands on the membrane. The second incubation was done by anti-IL-۲ polyclonal antibody added at a dilution of ۱:٥۰۰ in PBS solution. Anti-IgG at a dilution of

۱:۱۰۰۰ in PBS solution was used in third incubation. Subsequently incubation was performed with diaminobenzidine solution (DAB) for ۲۰ min (۰,۵ mg/ml DAB, ۰,۱٪ H_2O_2).

Results

Cloning of IL-۲:

In order to confirm plasmid (pUC۱۸) replication in *E. coli* DH۵α, electrophoresis on ۱٪ agarose gel was done following the extraction process. Size of pUC۱۸ and IL-۲ gene are ۲٦۸٦ and ٤۰۵ bp respectively. NdeI site and BamHI restriction sites are located on nucleotide ۱۸۳ and ٤۲۹, respectively. Size of pUC۱۸ digested with NdeI and BamHI restriction enzymes is ۲٤٤۰bp. Therefore, size of pUC۱۸-IL-۲ is ۲۸٤۵bp. The result here (presence of ۲۸٤۵ bp band) indicated that pUC۱۸ plasmids is replicated successfully.

Enzymatic digestion of pUC۱۸ cloning vector and pET-۲٤a expression vector:

After digestion of the pUC۱۸ plasmid with two restriction enzymes (BamHI-HF and NdeI), electrophoresis was done on ۱٪ agarose gel, a ٤۱۱ bp band was detected which indicated that IL-۲ gene was sub-cloned into pUC۱۸ plasmid successfully (Figure ۱).

Colony PCR:

In order to confirm ligation of the gene into expression plasmid (pET-۲٤a), colony PCR was run. As it is shown (Figure ۲), lane ۲ contained IL-۲ sequence and the region located between two primers (٦۱۱ bp band).

SDS-PAGE:

After confirmation of IL-۲ ligation into pET-۲٤a, analysis of protein expression was done using SDS-PAGE technique. As it is shown here (Figure ۳), there was a ۱۵,۳ kDa band which indicated the expression of rhIL-۲ gene.

Western blotting:

Western blotting results also indicated the expression of rhIL-۲ by representing a ۱۵,۳ kDa band on nitrocellulose membrane (figure ٤).

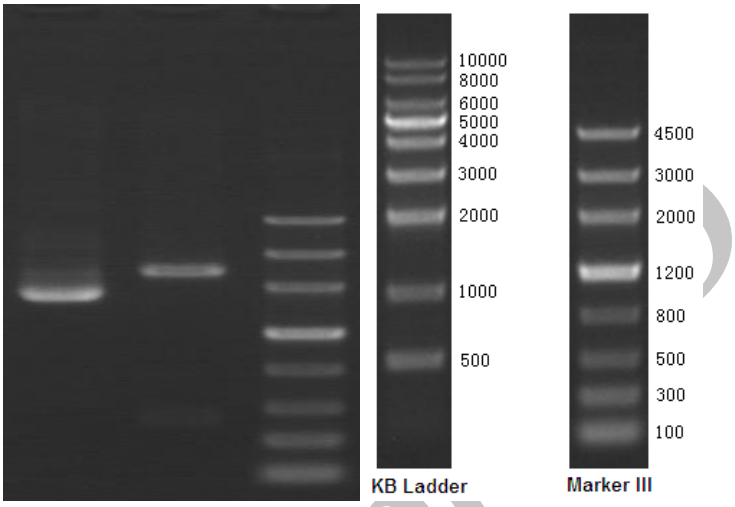

Figure ۱: Electrophoresis of the products of pUC۱۸ plasmid. Lane ۱ contained DNA ladder (Genscrip (USA)), lane ۲ contained ۲ bands representing pUC۱۸ and IL-۲.

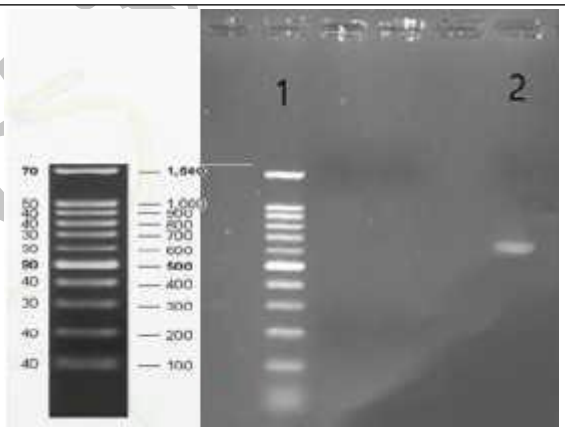

Figure ۲: Colony PCR of IL-۲ sequence integrated into pET-۲٤a. Lane ۱ contained DNA Ladder (Sinagene (Iran)) and lane ۲ contained IL-۲ sequence or the region located between two

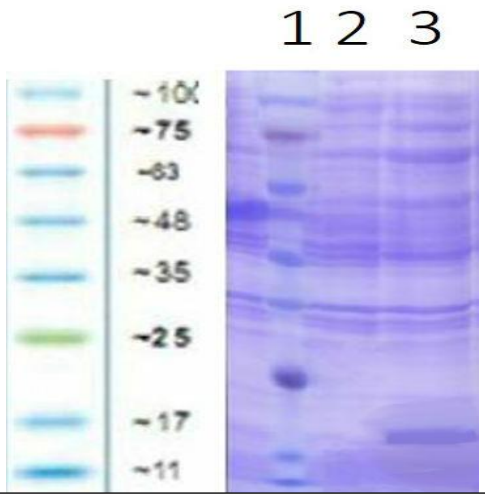

Figure ۳: SDS-PAGE for analysis the expression of IL-۲ protein in *E. coli* BL۲۱ (DE۳). Lane ۱(protein ladder) contained protein ladder (Sinagene (Iran)), lane ۳ contained a ۱۵,۳ kDa band, representing the expression of rhIL-۲ protein induced by IPTG. Lane ۲ representing the pattern of transformed BL۲۱ under-

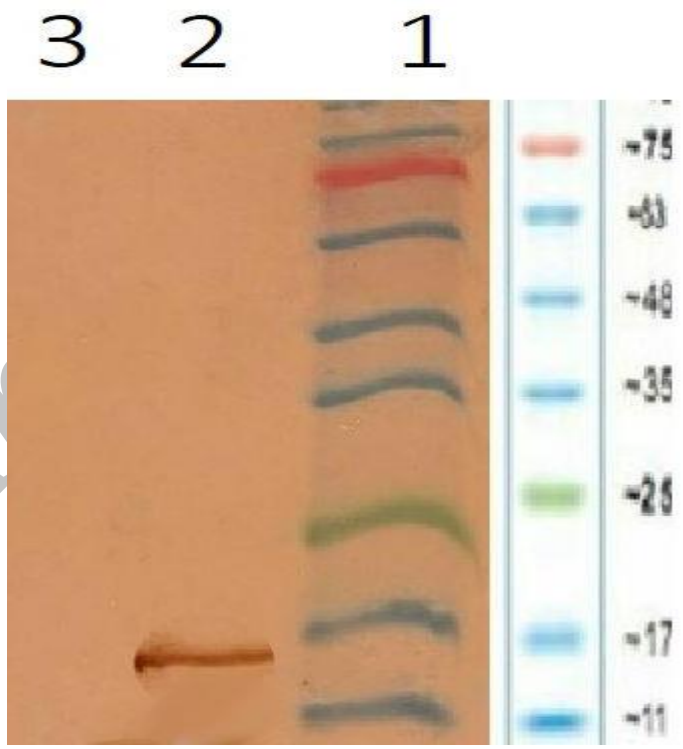

Figure٤: Analysis of the expression of rhIL-۲ using western blotting technique. Lane ۱ contained protein marker (Sinagene (Iran)), Lane ۲ contained rhIL-۲ protein and lane ۳ representing the pattern of transformed BL۲۱ under un-induction (without IPTG).

Discussion:

In this study, *Escherichia coli* BL۲۱(DE۳) was used as a cloning host to express human interleukin ۲. Human interleukin ۲ is an effective therapeutic protein for the treatment of cancers (malignant melanoma, renal cell cancer) in large intermittent doses and has been extensively used in continuous doses. So far, it has not been produced in Iran. So, the current study has made the first step for the localization of the technology of recombinant IL-۲ production in Iran. However, we investigated Cloning and expression of IL-۲ only in *E. coli*. A variety of Hosts are used for production of therapeutic proteins such as yeast and mammalian cells. Though, due to low expression levels and post-translational modifications of this hosts, *E. coli* is a more suitable expression host for many cases. Since IL-۲ is a non-glycosylated protein, *E. coli* can be a suitable host for large-scale production of it. in addition, *E. coli* is capable to produce high amounts of the recombinant protein. In other studies, different types of *E. coli* have been used, but *E. coli* BL۲۱(DE۳) was used for this study because this strain has been engineered for high level expression of protein for reasons stated above (۱۰). The prokaryotic_expression systems of IL-۲ such as *E. coli* W۳۱۱۰(۱۱), *E. coli* ۲۹٤(۱۲), *E. coli* HB۱۰۱/PLSD۱(۱۳) have been used but we selected *E. coli* BL۲۱ (DE۳) derivation of B۸۳٤ because it contains T۷ gene endogenously in its bacterial chromosome and produces T۷ RNA polymerase which in turn can increase the production of recombinant protein. Moreover, this strain possesses a deficiency in the lon protease and lack the ompT outer membrane protease. This deficiency prevents the recombinant protein from damage. Our priority to choose the host for protein expression was the high level production of the recombinant protein. The *E. coli* cytoplasm is regarded as the first choice in protein production owing to high efficiency (۱۱, ۱۴). However, inclusion body formation occurs as a

result of protein over-expression in cytoplasm (۱۵). Inclusion bodies can be an advantage for purification since ۱) they are easily isolated by centrifugation to yield highly concentrated and relatively pure protein, and ۲) inclusion body formation protects the protein from proteolytic attack. moreover, toxic proteins may not inhibit cell growth when present in inactive form as inclusion bodies (۱۶). Human IL-۲ has been expressed as inclusion bodies in *E. coli* cytoplasm. In this report, we investigated IL-۲ production in *E. coli* BL۲۱(DE۳) under control of T۷ promoter. To over-produce IL-۲, we used pET-۲٤a plasmid containing the powerful T۷ promoter. Given the importance of rhIL-۲, we decided to clone and express it in *Escherichia coli* BL۲۱(DE۳). Also, identification of the produced recombinant protein was specifically confirmed by SDS-PAGE and western blotting.

Conclusion:

In this study, the expression cassette for expression of human Interleukin-۲ in *Escherichia coli* was designed and the protein was identified successful. *E. coli* BL۲۱ (DE۳) can be used as a suitable host for the production of recombinant human Interleukin ۲ and this technology has the ability to be localized in Iran.

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