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Production and Hosting by Knowledge E

Research Article

Optimization of Cloning Conditions for High-level Production of Recombinant Mouse Interleukin-2 in *Escherichia coli*

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Abstract

Backgrounds and objectives: Many proteins have been expressed so far in bacterial host. Due to its simple culture conditions, having a short life cycle, and easily genetic manipulation, *E.coli* have been regarded as a preferable host to produce recombinant proteins, but protein cloning in bacterial host have many challenges. Therefore, we aimed to review some of these problems by an experience from mice IL-2 recombinant.
Methods: cDNA synthesis was performed after RNA extraction of mouse splenocytes. PCR product purification carried out after IL-2 coding sequence amplification and was ligated into the pET-21b (+) vector and transformed into the competent BL21 *E.coli*. Expression and purification of recombinant mouse IL-2 were done using IPTG inducer and metal affinity chromatography respectively.
Results: DNA sequencing confirmed the accuracy of the insertion process. A 23 kDa exogenous protein was observed on the SDS-PAGE. Specificity and concentration of produced mouse recombinant IL-2 protein were confirmed by western blotting and BCA methods.
Conclusion: Recombinant IL-2 was produced in BL21 and pET-21b (+) expression system at 24°C in the soluble form.

Keywords: Recombinant protein; pET-21b (+); IL-2 protein; Cloning

1. Introduction

Despite the development of various type of simple and sophisticated various methods of protein expression and purification during the past decade, protein solubility is still a major obstacle in the cloning process. Due to the increased cloning projects and information obtained from previous studies showed reduction (ranging between 13% and 23%) of the heterologous proteins expression in *E.coli* and expression of some kinases,

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phosphatases, membrane-associated proteins and many other enzymes in insoluble form, new attempt guided towards substitution of modified expression methods that can improve the solubility of the expressed protein.(7, 8). Because protein activity demands to fold into precise three-dimensional structures (9) and functional protein should be refolded by time-consuming from inclusion bodies (10), some irregular results make the process arduous (11). Therefore, straight soluble production would be highly desirable.

Moreover, several factors should be considered to expression desired protein in the soluble form. These factors including a choice of suitable *E.coli* expression strain host (12), diminish the temperature (13), selection of different promoters or induction situations (14), using molecular chaperones and folding modifier in the same condition of expression (15) and uses the compatible vector to the expression host. The latest can improve expression and yield of the favorite protein consequently. A vector can assert solubility and/or fold in one of three ways: First, facilitate binding to a protein that is highly soluble in itself, e.g. glutathione-S-transferase (GST), thioredoxin (Trx), N utilization substance A (NusA). Second Providing conditions for better linkage to the enzyme responsible for breaking disulfide bonds formation (e.g. thioredoxin, DsbA, DsbC) (16), and finally simplification the translocation of protein into the peri-plasmic space by forming signal sequence (17).

Mouse IL-2 (also known as TCGF) is a ~17 kDa factor produced mainly by a specific subset of T cells. The important role of T cells in the immunity system consisting of cell cycle progression, clonal expansion, and proliferation of activated T cells as well as NK cells, LAK cells, monocytes and B cells growth factor secretion. Simplify the soluble expression of this protein can make the process easy for further studies on the utility of this product particularly in the development of diagnostic methods such as ELISA and promote proliferation of mouse T lymphocytes in culture. Therefore, the aim of this study was to clone the mouse IL-2 for optimization of eukaryotic proteins cloning condition.

2. Material and Methods

2.1. RNA isolation and RT- PCR

Total RNA was isolated from mouse splenocytes using RNA extraction kit (Pars Tous, Iran, Cat no. A101231). The cDNA was constructed by using the reverse transcription reaction mixture containing oligo-dT primers and prepared Master Mix following the standard protocol (Pars Tous, Iran, Cat no. A101161).

The amplification of coding region of IL2 was done using two IL-2 gene-specific primers designed base on the sequence of mature mouse IL-2 available in the Unipart database. These primers contained *EcoRI* and *XhoI* restriction enzyme sites without the signal peptide. The designed primers sequences were: *EcoR1*–Forward 5'GATCC-GAATTCGGCACCCACTTCAAGCTCCACT3' and *XhoI*–Reverse 5'TGGTGCTCGAGTTG-AGGGCTTGTTG AGATGATGC3'.

A standard Polymerase Chain Reaction (PCR) protocol was conducted as follows: DNA template (10 ng) Gene-specific primers (10 pmol/µl), 10x PCR buffer, dNTP (10 mM), MgCl2 (25 mM) and KlenTaq DNA polymerase (5 U/µl) (Pars Tous, Iran, Cat no. C101121). The

PCR amplification conditions was initial denaturation at 94°C for 3 min, 28 cycles of denaturation at 94°C for 45s, primer annealing at 58°C for 30s and DNA extension at 72°C for 30s. Finally, the reaction was carried out at 72°C for 3 min.

2.2. Cloning and expression

A 447 bp PCR fragment from IL2 gene was assessed by agarose gel electrophoresis. The PCR product was extracted from agarose gel using DNA extraction kit (Pars Tous, Iran, Cat no. A101221), and after digestion with *EcoRI* and *XhoI*, than digested product was ligated into pET-21b (+) vector with DNA ligase according manufacture instructions (Thermo Fisher Scientific, USA).

The recombinant plasmid transformed into competent TOP10 *E.coli* cells as previously describe (18).. Inserted colonies were screened for ampicillin resistance. After ensuring the accuracy of the sequence of inserted plasmid (Macro gene, Germany), transformation into the competent *E.coli* BL21 cells was performed and induced by 0.2mM Isopropyl β- Dthiogalactoside (IPTG) and incubated for 18 hours at 24°C . The bacteria were harvested by centrifugation at 13400 rpm for 1 min. After re-suspension in the lysis buffer (Potassium Phosphate buffers (50 mM, pH 7.8), Glycerol 10%, Triton x-100 0.5%, Nacl 200 mM) and sonication, protein expression was confirmed by loading supernatant and cell pellet on the SDS-PAGE. Expressed recombinant protein was employed to a (Ni-IDA) chromatography column in order to purify histidine-tagged proteins according to the manufacturer's instructions (Pars Tous, Iran, Cat no. A101271), in briefly, 10ml bacteria lysate was passed through chromatography column. Then, column was washed with wash buffer I (Potassium Phosphate buffers (50 mM, pH 7.8), Nacl 200 mM) and wash buffer II (wash I, 20 mM Imidazole) respectively. Finally, protein was eluted with elution buffer (wash I, 250 mM Imidazole). The purified protein was dialyzed in PBS buffer at 4°C for 24 hours.

2.3. Western blot analysis

After running the expressed purified recombinant mouse IL-2 protein on the %12.5 electrophoresis gel, they were blotted to polyvinylidene difluoride (PVDF) membrane and blocked with BSA 2% overnight at 4°C. After washing, lanes were incubated with 1:500 diluted biotinylated anti-mouse IL-2 (Ebioscience, Vienna, Austria) for 2 hours at room temperature. Then the membranes were incubated with 1:20000 diluted horseradish peroxidase-streptavidin (HRP) conjugate (Bio-Rad, CA, USA) 1 hour at RT. In the last step, the specific reaction was detected by Chemiluminescent substrate (Pars Tous, Iran, Cat no. B111420).

2.4. Protein concentration quantification

Bicinchoninic Acid (BCA) protein assay method was used in order to quantify the concentration of recombinant protein. All samples and reagents were prepared according manufacture instructions (Pars Tous, Iran, Cat no. A101251) and tested in a duplicate way.

The optical density of samples was detected at 562 nm and final concentration obtained by comparing the result with the standard curve.

3. Results

3.1. Amplification, cloning and sequence of cDNA coding mouse IL-2

As reported in Unipart database the length of mouse IL-2 cDNA is 510 bp (accession number: PO4351). After amplification of mouse IL-2, we were able to obtain a fragment about 447 bp (Figure 1). The fragment was inserted into the pET-21b (+) expression vector; one clone was confirmed using PCR with T7 primers. The PCR product showed a fragment of approximately 687 bp (Figure 2). Finally, aligning the nucleotide sequences with the sequence of mouse IL-2 deposited from GenBank revealed complete homology (Figure 3).

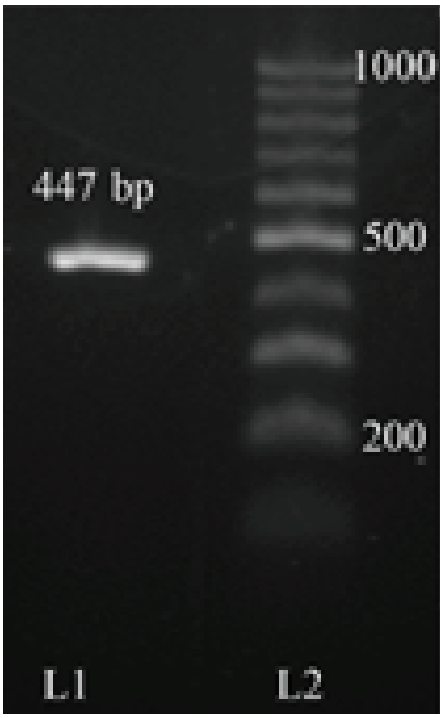

Figure 1: Agarose gel electrophoresis of PCR product after amplification of the mouse IL-2 coding region using the specific primers. Lane 1: PCR product of 447 bp mouse IL-2. Lane 2: DNA marker (100 bp, Fermentas).

3.2. Expression and purification

The recombinant plasmid was transformed into the expression host *E.coli* strain BL21. After IPTG induction for 18h, 23 kDa recombinant mouse IL-2 was expressed on SDS–PAGE electrophoresis gel in the soluble form (Figure 4). For the purification of the protein,

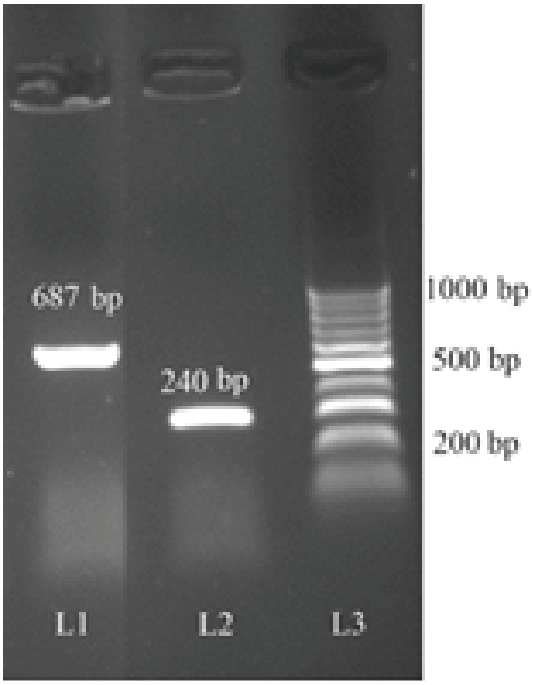

Figure 2: Colony-PCR screening with T7 universal primers. Lane 1: 687 bp recombinant plasmid , Lane 2: plasmid without insert, Lane 3: DNA marker (100 bp, Fermentas).

Score	Expect	Identities	Gaps	Strand
209 bits(113)	2e-51	113/113(100%)	0/113(0%)	Plus/Minus

Features: interleukin-2 precursor

```
Query  336       AGGGCTCTGACAACACATTTGAGTGCCAATTCGATGATGAGTCAGCAACTGTGGTGGACT  395
                  ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  37007753  AGGGCTCTGACAACACATTTGAGTGCCAATTCGATGATGAGTCAGCAACTGTGGTGGACT  37007694

Query  396       TTCTGAGGAGATGGATAGCCTTCTGTCAAAGCATCATCTCAACAAGCCCTCAA  448
                  ||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  37007693  TTCTGAGGAGATGGATAGCCTTCTGTCAAAGCATCATCTCAACAAGCCCTCAA  37007641
```

Score	Expect	Identities	Gaps	Strand
278 bits(150)	4e-72	150/150(100%)	0/150(0%)	Plus/Minus

Features: interleukin-2 precursor

```
Query  189       AGGCCACAGAATTGAAAGATCTTCAGTGCCTAGAAGATGAACTTGGACCTCTGCGGCATG  248
                  ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  37009676  AGGCCACAGAATTGAAAGATCTTCAGTGCCTAGAAGATGAACTTGGACCTCTGCGGCATG  37009617

Query  249       TTCTGGATTTGACTCAAAGCAAAAGCTTTCAATTGGAAGATGCTGAGAATTTCATCAGCA  308
                  ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  37009616  TTCTGGATTTGACTCAAAGCAAAAGCTTTCAATTGGAAGATGCTGAGAATTTCATCAGCA  37009557

Query  309       ATATCAGAGTAACTGTTGTAAAACTAAAGG  338
                  ||||||||||||||||||||||||||||||
Sbjct  37009556  ATATCAGAGTAACTGTTGTAAAACTAAAGG  37009527
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Figure 3: Aligning the nucleotide sequences with the sequence of mouse IL-2 from GenBank.

the cells were sonicated on ice and employed to affinity chromatography (Figure 5) as well as purified using dialyzes.

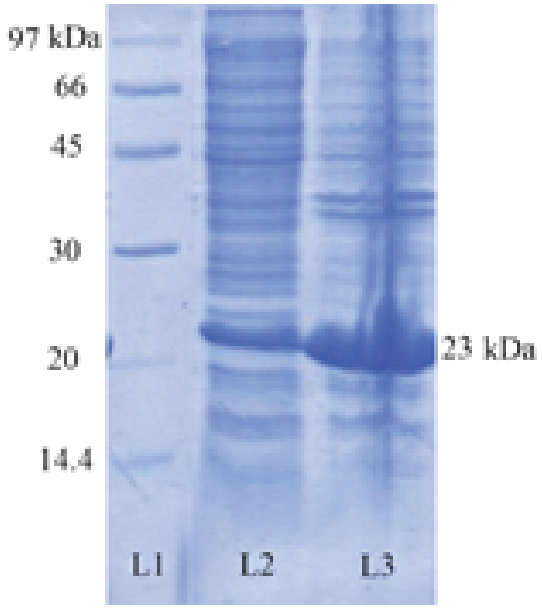

Figure 4: SDS-PAGE for analyzing of Mouse IL-2 expression with Coomassie blue staining. Lane 1: The Protein size marker. Lane 2: Expressed IL-2 protein in the supernatant. Lane 3: Expressed IL-2 protein (23 kDa) in the precipitate.

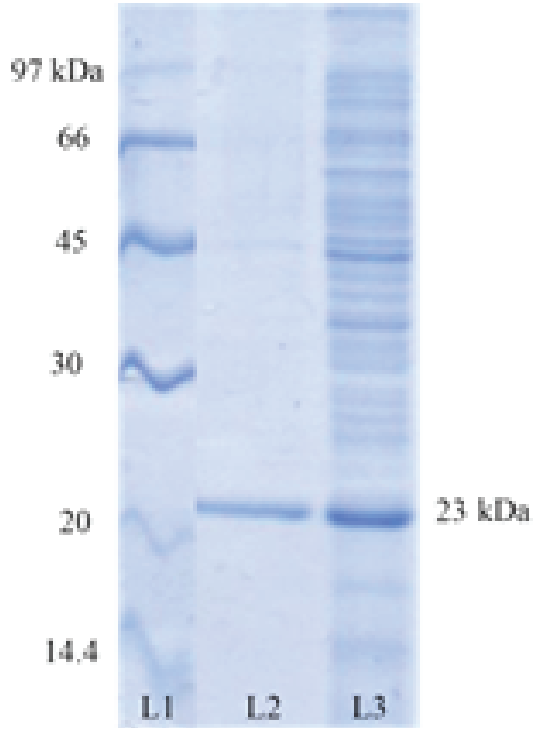

Figure 5: SDS-PAGE for analyzing the purity of mouse IL-2 protein after Nickel affinity chromatography. Lane1: The Protein size marker. Lane 2: Purified protein in 23 kDa. Lane 3: expressed recombinant mouse Il-2 befor purification.

3.3. Immunoblotting

Immunoblotting of the mouse IL-2 protein with 1:500 diluted biotinylated anti-mouse IL-2 was done and results showed that our protein was expressed as a 23kDa fragment (Figure 6). Also, we noticed that IL-2 folded as a 46 kDa dimer protein.

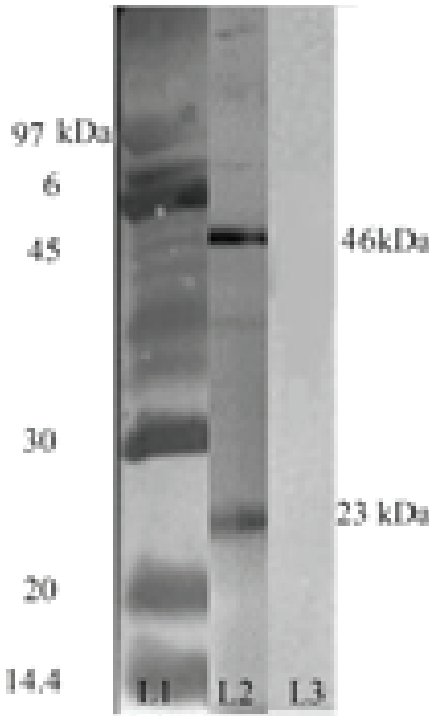

Figure 6: Western blotting of purified recombinant mouse IL-2. Lane 1: Low molecular weight protein size marker. Lane 2: Purified recombinant protein. Lane 3: Negative control BL21.

4. Discussion

It is ideal overcoming the problem of non-soluble expression of various proteins and simplification the process of production of recombinant proteins. Checking the solubility of desired protein with available software would be useful before cloning the target gene into the particular vector. Despite obtained information based on available software that showed the low percentage of soluble expression of mouse IL-2, in the current study, we have produced recombinant mouse IL-2 in the soluble form using pET-21b (+) vector in BL21 (DE3) *E. coli* strain.

One of the most important steps in the process of cloning is choosing the best vector can provide our interests in the process of cloning. Vector selection depends on the promoter with which the gene of interest is to be cloned and different fusion 'tags' which influence solubilization of recombinant protein in *E.coli* host (19). In this study, we used pET-21b (+) vector and the reason for choosing mentioned vector is eliminating the fusion tag removal step because of the existence of a smaller tag, a 6-Histidine tag (His-tag) in this vector, in comparison with other pET vectors such as pET-32a (+) which is previously used in mouse IL-2 cloning process (20). Since pET-32a (+) contain a long fusion tag

which adds 109-aa, this large size can interfere with the activity of recombinant protein if the tag is not cleaved properly. In the other hand, appropriate enzyme sites must be designed into the vector in order to precise cleavage and complete removal extra amino acid fused with recombinant protein. In addition due to the interference of long tags such as thioredoxin with the structure and the function of desired recombinant protein, therefore it must be removed after the expression and purification process (20, 21).

In the recent study, we observed the higher molecular weight of produced recombinant protein than we expected. This experience also has been implied by Yan et al. The reason for this phenomenon is related to various protein conformation and formation of a disulfide bond between protein monomers resulting in formation protein in dimer form. Moreover, the existence of His-tag at the end of a protein can have an effect on the conformational structure of target protein resulting in changing the mobility of protein on the SDS-PAGE gel electrophoresis (22).

In our experiment, we have expressed recombinant mouse IL-2 in the soluble form by optimizing temperature in 24˚C. According to the previous study the optimum temperature for induction of recombinant proteins is 37˚C (23). However, inclusion body formation is facilitated in 37˚C. In addition, the temperature can be an effective factor in the expression rate of recombinant protein production as well as appropriate folding of the recombinant protein (24). Optimization the time incubation and decreasing temperature to lower (15-20˚C) can affect the growth of bacteria, rate of protein production and reduction of aggregation of intended protein (25, 26).

Using IPTG to induce lac repressor-regulated promoters can influence the cell mass as well as yield of desired protein (27). Protein expression can be regulated by optimizing the concentration of IPTG. Some proteins need to be induced slowly with lower IPTG concentrations while others, higher amounts of IPTG is desired (28). Since establishing optimum concentration will gain empirically by varying the IPTG concentration between 0.01 and 5 mm, we set up 0.2 mM concentration of IPTG achieved by examining different concentration of IPTG.

5. Conclusion

We facilitated cloning and expression of mouse recombinant IL-2 using by vector pET-21b (+) in the soluble form. This cytokine was expressed at 24˚C in large scale without any refolding and it is suitable for commercial aims. In order to uses of this cytokine for clinical use, it is better that it cloned in eukaryotic hosts such as pichia pastoris.

Acknowledgment

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Author Contributions

AA performed experiments and prepared the draft of the paper. MH collaborated on experiments and manuscript preparation. KM developed the main idea, and performed the statistical analysis. MJM assessed the obtained data and edit the manuscript critically. SEBK designed the work, and read the manuscript. MS developed the main idea, read and edit the manuscript critically. All authors have read and approved the manuscript.

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