**Short Communication**

# Bacterial overexpression of the human interleukin-2 in insoluble form via the pET Trx fusion system

**Sohrab Boozarpour[1], Majid Sadeghizadeh[1]*, Mohammad Ali Shokrgozar[2], Saman Hosseinkhani[3], Seyed Abbas Shojaosadati[4], Sara Gharavi[5], Ghasem Ahangari[6], Bijan Ranjbar[7]***

*[1]Department of Genetics, Faculty of Biological Sciences, Tarbiat Modares University, P.O. Box 14115-154, Tehran, I.R. Iran [2]National Cell Bank of Iran, Pasteur Institute, P.O. Box 1316943551, Tehran, I.R. Iran [3]Department of Biochemistry, Faculty of Biological Sciences, Tarbiat Modares University, P.O. Box 14115-154,Tehran, I.R. Iran [4]Biotechnology Group of Chemical Engineering, Faculty of Engineering, Tarbiat Modares University, P.O. Box 14115-143, Tehran, I.R. Iran [5]Department of Biology, Alzahra University, P.O. Box 1993891176, Tehran, I.R. Iran [6]Department of Medical Genetics, National Institute of Genetic Engineering and Biotechnology, P.O. Box 14155-6343, Tehran, I.R. Iran [7]Department of Biophysics, Faculty of Biological Sciences, Tarbiat Modares University, P.O. Box 14115-154, Tehran, I.R. Iran*

***Abstract***
Selection of a system for successful recombinant protein production is important. The aim of this study was to produce high levels of human interleukin-2 (hIL-2) in soluble form. To this end, the pET32a vector in *Escherichia coli* BL21 (DE3) was used as an expression system, since it was previously used for the production of mouse IL-2 in soluble form. The results indicated that contrary to expectations, the expressed protein was in the form of inclusion bodies and perhaps amino acid differences between human and mouse IL-2 should be determinant. The hIL-2 protein is a small peptide, therefore its recovery as a biologically functional protein by the process of refolding may be feasible and could lead to high yields at the industrial scale.
***Keywords*:** Human IL-2; Soluble protein; Inclusion body; Overexpression; *E. coli*

Human interleukin-2 (IL-2) is a protein which plays a central role in immunological responses (Minami *et al*., 1993). Therefore, soon after its discovery, extensive studies were carried out regarding the cloning (Minami *et al*., 1993), gene structure (Holbrook *et al*., 1984), high level expression (Williams *et al*., 1988) and crystallography of the protein (Bazan, 1992). The genetic locus for the human IL-2 is located on the long arm of chromosome 4 (Holbrook *et al*., 1984), which encodes a human IL-2 precursor, with a molecular weight of approximately 15 kDa (Kashima *et al*., 1985) , consisting of 153 amino acids. The 20 amino acids at the N-terminal of hIL-2 comprise a signal peptide that is excluded from the mature IL-2 (Cerretti *et al*., 1986). Post-translational modifications include glycosylation of Thr3 (Robb *et al*., 1981) and the formation of disulfide bridges between cysteines located on amino acid residues 58 and 105 (Gomez *et al*., 1998). Human IL-2 glycosylation does not affect its biological activity *in vitro* and is probably effective only in increasing the half life and solubility of the protein in biological systems (Sarah and Kathleen, 2004). This protein is postulated to play a role in various cancers especially renal cell cancer (RCC), melanoma as well as AIDS (Sereti *et al*., 2002). Human IL-2 has been cloned in several microorganisms as host cells (Hyung *et al*., 2005), among which in this work, *E. coli* BL21 carrying the pET32a vector was considered for the overexpression of hIL-2 in sol-

**Correspondence to:* **Majid Sadeghizadeh** and **Bijan Ranjbar,** Ph.D.
*Tel: +98 21 82884717; Fax: +98 21 82884484*
*E-mail: sadeghma@modares.ac.ir and ranjbarb@modares.ac.ir*

uble form (Robert *et al*., 1995).

In this study, peripheral blood mononuclear cells (PBMCs) were isolated from a healthy donor by means of Ficoll (Baharafshan Co., Iran). The cells were stimulated with 10 µg/ml of phytohemagglutinin (PHA) (Sigma, Chemical Co., USA) for 24 h at 37°C. Total RNA (3 µg) was used in the reverse transcription reaction mixture containing oligo-dT or specific primers, following the standard protocol (first strand cDNA synthesis, Fermentas, Lithuania). Two gene-specific primers (ABA1 and ABA2) were designed using the sequence of mature hIL-2 available in the GenBank database to amplify hIL-2 cDNA lacking the signal peptide (accession number: BC070338); *Nco*I and *Bpu*1102I restriction enzyme sites were introduced into the ABA1 (upstream) and ABA2 (downstream) primers, respectively. The designed primer sequences were as follows:

ABA1: 5′CGAGTCCATGGACCTACTTCAAGTTCTACA 3′

ABA2: 5′CAAGCTCAGCCTAGTCAGTGTTGAGATGATGC 3′

The underlined nucleotide sequences represent the *Nco*I and *Bpu*1102I restriction enzyme recognition sites, respectively.

A standard Polymerase Chain Reaction (PCR) protocol using the synthesized cDNA, gene-specific primers (ABA1 and ABA2), 10x PCR buffer, dNTP (20 mM) , $MgCl_2$ (50 mM) and *Taq* DNA polymerase (5 U/µl) (Cinnagen, Iran) was employed. Altogether, 35 cycles involves denaturation for 35 s at 94ºC , annealing for 35 s at 62ºC , primer extension for 40 s at 72ºC followed by 72°C for 30 min were performed using the thermal cycler (Bioneer, South Korea). The PCR products were then analyzed by agarose gel electrophoresis and ligated into the pTZ57R/T vector.

In order to subclone the fragment, the products and the pET32a expression vector (Novagen, USA) were double-digested with *Nco*I and *Bpu*1102I restriction enzymes (Fermentas, Lithuania). The digested products were then purified with a gel purification kit (Bioneer, South Korea), following which ligation was carried out and recombinant colonies obtained by screening. One of the choose hIL-2 recombinant colony after DNA purification and agarose gel analysis was sequenced (MWG BIOTECH, Germany); the obtained nucleotide sequence was aligned with the sequence of hIL-2 (accession number: BC070338) available in the GenBank.

cDNA was synthesized by means of the oligo-dT, gene specific primer and M-MuLV (Fermentas, Lithuania) as reverse transcriptase, then used for Reverse Transcription-PCR, from which a 421 bp fragment was obtained (Fig. 1). The fragment was subcloned into the pET32a expression vector; colony selection was performed by means of single and double digestion with *Nco*I and *Bpu*1102I restriction enzymes. Fragments of the correct size (410 and 5900 bp in length) are depicted in Figure 2. Cloning was confirmed by aligning the nucleotides with the sequence of hIL-2 from GenBank, which showed complete similarity. *E. coli* BL21 (DE3) was transformed with the recombinant plasmid. For protein expression, the standard protocol (pET System Manual, Novagen) was used; however, 2xYT was replaced with LB and grown for 1, 3, 5 and 7 h at 30ºC. The expressed protein was extracted and analyzed by 12.5% (w/v) SDS-PAGE. Western blot hybridization was performed using the mouse anti-IL2 monoclonal antibody (Abcam, UK) as the primary antibody and conjugate goat-anti-mouse HRP as the secondary antibody (Sigma, USA), according to the standard protocol (Sambrook and Russel, 2001).

As indicated in Figure 3, the results obtained by 12.5% (w/v) SDS-PAGE show the expected IL-2 protein band obtained from the bacterial pellet corresponds to the 35 kDa marker band; whereas this band

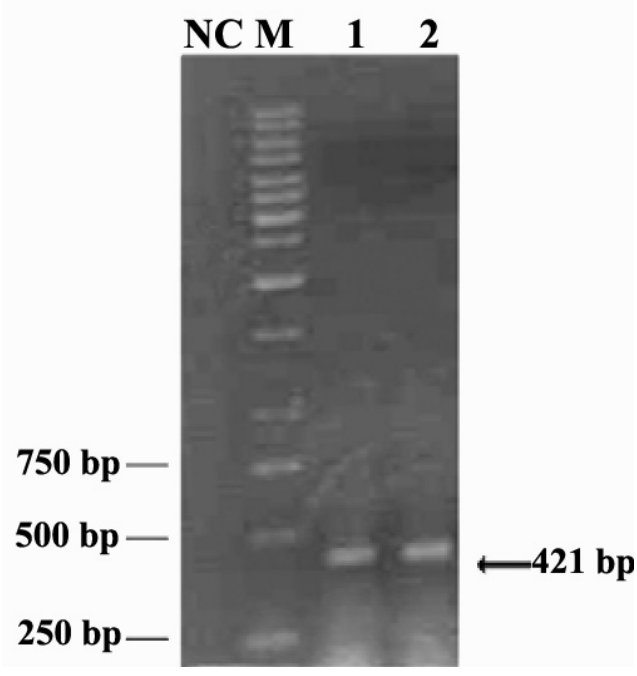


**Figure 1.** RT-PCR of human interleukin-2. NC: negative control (no DNA); M: Marker (Fermentas, GeneRuler 1kb DNA Ladder); 1: RT-PCR products using oligo-dT; 2: RT-PCR products using specific primers.

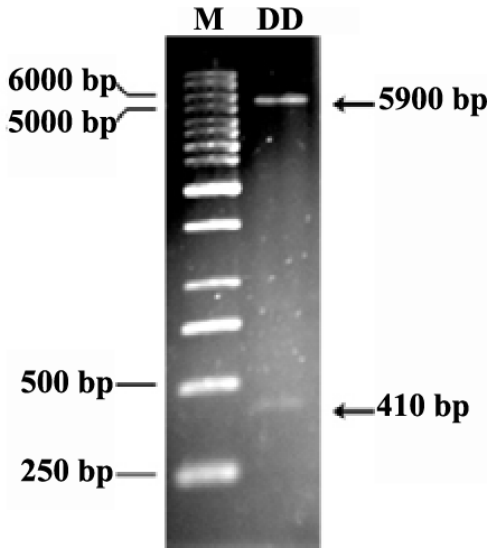


**Figure 2.** Confirmation of constructs by double digestion with *Nco*I and *Bpu*1102I restriction enzymes. M: marker (Fermentas); DD: construct double digested with *Nco*I and *Bpu*1102I restriction enzymes.

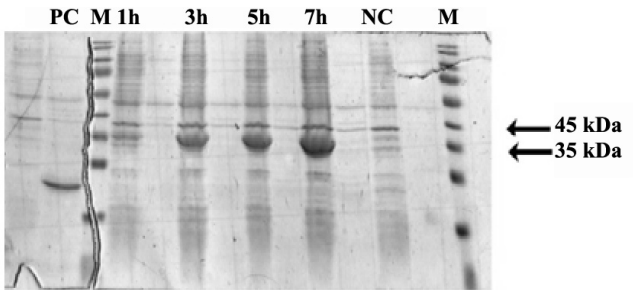


**Figure 3.** Protein expression analysis of the cloned IL-2 gene at 1-7 h using 12.5% (w/v) SDS-PAGE. M: marker;NC: negative control (*E.coli* BL21 (DE3) with uninduced pET32a plasmid), where both lanes are negative control, with left lane representing the pellet and right lane representing the supernatant; PC: positive control (*E.coli* BL21 (DE3) with pET32a plasmid induced by IPTG 1mM), where both lanes are positive control, with left lane representing the pellet and the right lane representing the supernatant; 1,3,5,7 h: induction times. Left lane for each induction time is the pellet and right lane for each induction time is the supernatant of the expressed IL-2 protein.

is not visible in the sample derived from the supernatant. Also, in the supernatant obtained from *E. coli* BL21 (DE3) containing the pET32a plasmid without the IL-2 insert, which was induced by IPTG 1mM, the expected 20.4 kDa band was observed (peptides include the trxA, S and His tags). In the negative control (*E. coli* BL21 (DE3) with the uninduced pET32a plasmid, 35 and 20.4 kDa bands were not seen reflecting the controlled conditions in the expression experiments. After 7 h, hIL-2 obtained from the bacterial pellet was overexpressed (Fig. 3). In order to prove the identity of the expressed protein, a specific antibody was used in the Western blot. The specific reactivity of the used antibody with the 35 kDa IL-2 protein in the pellet but not in the supernatant is shown in Figure 4.

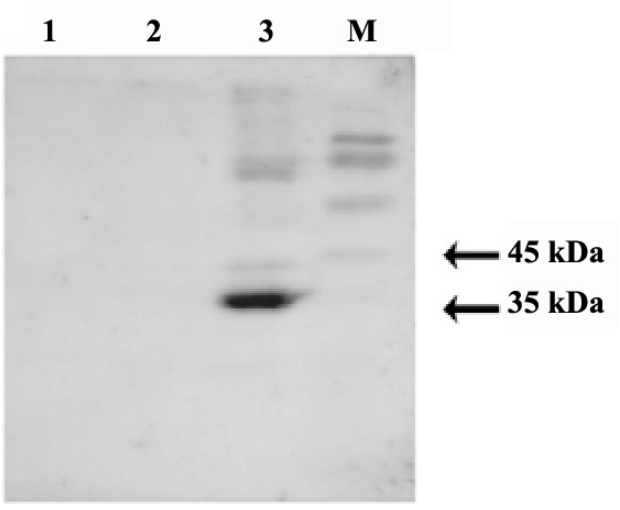


**Figure 4.** Western blot confirmation of the expressed human IL-2 protein. 1: negative control; 2: bacterial supernatant of the expressed hIL-2; 3: bacterial pellet of the expressed hIL-2; M: protein marker (Fermantas).

Production of recombinant proteins as inclusion bodies at the industrial scale has several advantages. Endogenous proteinases may degrade the soluble recombinant proteins and these proteins could have toxic effects on the host cell (Idicula-Thomas and Balaji, 2007). Furthermore, the overexpression of proteins in the inclusion body form makes their isolation and purification easy (Clark, 2001). Human IL-2 with 133 amino acids has many pharmaceutical properties of significant value (Sarah and Kathleen, 2004).

This study reports the cloning and expression of human IL-2 in a bacterial system in order to evaluate the overexpression and the production of soluble hIL-2 at the industrial scale. IL-2 has been previously cloned in several recombinant expression systems (Hyung *et al*., 2005), and has shown high levels of expression in *E. coli* BL21, when compared with other systems (Williams *et al*., 1988). On the other hand, Novy *et al*. (1995) have shown that if mouse IL-2 is cloned into the *Nco*I and *Bpu*1102I sites of the pET-32a vector and then transferred into *E. coli* BL21 (DE3), most of the IL-2 is produced in soluble form, which may be related to the trxA tag and deletion of

```
Human APTSSST--------------KKTQLQLEHLLLDLQMILNGINNYKNPKLTRMLTFKFYM 46
Mouse APTSSSTSSSTAEAQQQQQQQQQQQQHLEQLLMDLQELLSRMENYRNLKLPRMLTFKFYL 60
      *******               :: * :**:**:*** :*. ::**:* **.********:

Human PKKATELKHLQCLEEELKPLEEVLNLAQSKNFHLRPRD-LISNINVIVLELKGSETTFMC105
Mouse PKQATELKDLQCLEDELGPLRHVLDLTQSKSFQLEDAENFISNIRVTVVKLKGSDNTFEC120
      **:*****.*****:** **..**:*:***.*:*.  : :****.* *::****:.** *

Human EYADETATIVEFLNRWITFCQSIISTLT-133
Mouse QFDDESATVVDFLRRWIAFCQSIISTSPQ149
      :: **:**:*:**.***:******** .
```

**Figure 5.** Alignment of amino acid sequences of human and mouse interleukin-2.

the additional amino acids coded by the multiple cloning site in the vector. Therefore, in this study, pET32a and *E. coli* BL21 (DE3) were chosen and the same restriction enzymes were used for the cloning procedure to obtain high levels of expression for hIL-2 in the soluble form. Precursor human interleukin-2 has a signal peptide which is not recognized by *E.coli*. The primers designed in this study were targeted to amplify hIL-2 gene without the signal peptide. A soluble product was expected; however, SDS-PAGE and Western blotting showed (Fig. 3 and 4) that IL-2 does not appear as a soluble form even after 7 h, and maximum expression is observed in the insoluble form.

Some properties of the expressed protein, such as disulfide bonds (Lilie *et al*., 1998; Makrides, 1996), post-translational modifications (Zhang *et al*., 1998), hydrophobicity, net charge and secondary structure are determining factors in the formation of inclusion bodies (Chiti *et al*., 2003). Other factors which determine the formation of inclusion bodies are related to the environment of the expressed protein, such as pH, temperature (Yon, 2002). Protein concentration and kinetics of protein translation in the context of rare codons are also deciding factors (Komar *et al*., 1999).

In certain polypeptides, the compositions of N- and C-terminal residues are found to be more critical in determining the intrinsic propensity for inclusion bodies as compared to the rest of the protein (Idicula-Thomas and Balaji, 2007). As previous studies indicate, the presence of three Cysteine residues that bind incorrectly, does not affect the solubility of the hIL-2 protein (Arakawa *et al*., 1986). However, as shown in this study, the IL-2 protein is produced in the insoluble form, contradicting previous studies carried out with the mouse IL-2, which shows high solubility in the supernatant under the same conditions, and even the same restriction sites. Presumably, the expression of protein in the inclusion body could be related to differences in the amino acid sequences between the human and the mouse (Cerretti *et al*., 1986). Following the investigation of amino acid sequences of human and mouse IL-2, a 12 amino acid glutamine motif was found in the N-terminal of the mouse IL-2 not represented in the human IL-2, which is probably pivotal in determining the polarity and solubility of mouse IL-2 (Fig. 5). The other postulation is that environmental factors are critical in differences observed between this and Novy *et al.* (1995) study. Since hIL-2 is a small peptide, correct refolding of biologically active protein is readily feasible and hence can ensure overall protein production levels, higher than its soluble form.

## Acknowledgments

This work was supported by the Center of Excellence for Biotechnology and the Research Council of Tarbiat Modares University (Grant number: TMU 88-8-74). The opinions or assertions contained within are private views of the authors and should not be construed as official or necessarily reflecting the views of the Research Council of Tarbiat Modares University.

## References

Arakawa T, Boone T, Davis JM, Kenney WC (1986). Structure of unfolded and refolded recombinant derived [Ala 125] interleukin-2. *Biochemistry* 25: 8274-8277.

Bazan JF (1992). Unraveling the structure of IL-2. *Science* 257: 410-413.

Cerretti DP, McKereghan K, Larsen A, Cantrell MA, Anderson D, Gillis S, Cosman D, Baker PE (1986). Cloning, sequence, and expression of bovine interleukin 2. *Proc Natl Acad Sci USA*. 83: 3223-3227.

Chiti F, Stefani M, Taddei N, Ramponi G and Dobson CM (2003). Rationalization of the effects of mutations on peptide and protein aggregation rates. *Nature* 424: 805-808.

Clark EDB (2001). Protein refolding for industrial purposes. *Curr*

*Opin Biotechnol*. 12: 202-207.

Gomez J,Gonzalez A, Martinez-A C, Rebollo A (1998). IL-2-induced cellular events. *Crit Rev Immunol*. 18: 185-220.

Holbrook NJ, Smith KA, Fornace AJ Jr, Comeau CM, Wiskocil RL, Crabtree GR (1984). T-cell growth factor: complete nucleotide sequence and organization of the gene in normal and malignant cells. *Proc Natl Acad Sci USA*. 81: 1634-1638.

Hyung JC, Hwa SS, Hye JL, Hye SC, Nimish ND, Minh QP, William EB (2005). Comparative production of human interleukin-2 fused with green fluorescent protein in several recombinant expression systems. *Biochem Engin J*. 24: 225-233.

Idicula-Thomas S, Balaji PV (2007). Protein aggregation: A perspective from amyloid and inclusion-body formation. *Curr Sci*. 92: 758-767.

Kashima N, Nishi-Takaoka C, Fujita T, Taki S, Yamada G, Hamuro J, Taniguchi T (1985). Unique structure of murine interleukin-2 as deduced from cloned cDNAs. *Nature* 313: 402-404.

Komar AA, Lesnik T and Reiss C (1999). Synonymous codon substitutions affect ribosome traffic and protein folding during *in vitro* translation. *FEBS Lett*. 462: 387-391.

Lilie H, Schwarz E and Rudolph R (1998). Advances in refolding of proteins produced in *E. coli*. *Curr Opin Biotechnol*. 9: 497-501.

Makrides SC (1996). Strategies for achieving high-level expression of genes in *E. coli*. *Microbiol Rev*. 60: 512-538.

Minami Y, Kono T, Miyazaki T, Taniguchi T (1993). The IL-2 receptor complex: Its structure, function, and target genes. *Annu Rev Immunol*. 11: 245-268.

Robb RJ, Munck A, Smith KA (1981). T cell growth factor receptors. Quantitation, specificity, and biological relevance. *J Exp Med*. 154: 1455-1474.

Robert N, James B, Keith Y, Robert M (1995). Novagen, Inc. pET TRX Fusion System for Increased Solubility of Proteins Expressed in *E. coli*.

Sambrook J and Russel DW (2001). 'Molecular cloning. A laboratory manual', Cold Spring Harbor Laboratory Press.

Sarah LG, Kathleen DL (2004). Overview of interleukin-2 function, production and clinical applications. *Cytokine* 28: 109-123.

Sereti I, Martinez-Wilson H, Metcalf JA, Baseler MW, Hallahan CW, Hahn B, Hengel RL, Davey RL, Kovacs JA, Lane HC (2002). Long-term effects of intermittent interleukin 2 therapy in patients with HIV infection: characterization of a novel subset of CD4(C)/CD25(C). *T cells Blood*. 100: 2159e67.

Williams DP, Regier D, Akiyoshi D, Genbauffe F, Murphy JR (1988). Design, synthesis and expression of a human interleukin-2 gene incorporating the codon usage bias found in highly expressed Escherichia coli genes. *Nucleic Acids Res*. 16: 10453-10467.

Yon JM (2002). Protein folding in the post-genomic era. *J Cell Mol Med*. 6: 307-327.

Zhang Y, Olsen DR, Nguyen KB, Olson PS, Rhodes ET and Mascarenhas D (1998). Expression of eukaryotic proteins in soluble form in *E. coli*. *Protein Exp Purif*. 12: 159-165.