

Full Length Research Paper

Expression and purification of human IL-2 protein from *Escherichia coli*

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Human interleukin 2 protein (IL-2) is an important cytokine found to be elevated in several types of cancer. A synthetic DNA sequence of the cDNA of mature IL-2 protein was cloned into the pRSET-B expression vector. The expressed IL-2 protein in *Escherichia coli* [BL21] was associated with the formation of insoluble inclusion bodies (IBs). The effect of different cultivation conditions (temperature, isopropyl- β -D-thiogalactoside concentration, and early harvest of cells) together with the incorporation of single or dual His-tag on the formation of IBs of the expressed protein was investigated. Yet, expression of soluble IL-2 was not achieved under any of the investigated conditions. A simple protocol for rapid and effective solubilization of these IBs was optimized. Using this protocol, together with subsequent purification using ion metal affinity chromatography, a purified His-IL2 protein was obtained in a yield of 5.1 mg/cell pellet of 1 L culture. In conclusion, the effect of different expression conditions on the solubility behavior of an expressed eukaryotic protein in *E. coli* was investigated using human IL-2 as a model protein. Moreover, the purified expressed protein could be used as a positive control in early diagnosis of tumors and in cancer research in Egypt.

Key words: Human interleukin-2, protein expression, inclusion bodies.

INTRODUCTION

Interleukins are proteins that play a vital role in intercellular communication among leukocytes. Such proteins are cytokines that are classified into different classes II-1 to II-38 (Akdis et al., 2016), in addition to IL-39 (Wang et al., 2016). Human interleukin-2 (IL-2) is a protein produced by human lymphocytes that have been stimulated by mitogens or antigens (Ju et al., 1987). IL-2 is first synthesized as a precursor polypeptide of 153 amino acids followed by cleavage of the first 20-amino

acids signal peptide resulting in the production of the mature secreted IL-2 protein (133 amino acids, 15 kDa) (Schoner et al., 1992). The mature protein is a potent cytokine that regulates innate lymphoid cells, acts as a B-cell growth factor, promotes antibody synthesis, and induces proliferation and differentiation of natural killer cells (NK) to enhance their cytolytic activities (Roediger et al., 2015). Additionally, it has been used in anti-tumor therapy (Sengupta et al., 2008; Janik et al., 1993), and in

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Table 1. Primers used in this study.

Primer	Sequence (5'-3')
IL2-F1	AGAAGAGGATCCggcacctacttcaagttctaca
IL2-R1	TCTTCTAAGCTTcaagtcagtggtgagatgatgct
IL2-R2	TCTTCTAAGCTTcaatggtgatggtgatggtgtcctgcagtcagtggtgagatgatgct

treatment of patients with disseminated cancers (Schoner et al., 1992). Several studies showed the increased expression levels of IL-2 in different types of cancer e.g. prostate cancer (Royuela et al., 2000), stomach and renal cancer (Lin et al., 1995), squamous cell carcinomas of the head and neck (Reichert et al., 1998), and neuroblastic tumors (Ridings et al., 1995). Therefore, the elevated expression levels of such cytokine could be used as a diagnostic marker for detection of actively proliferating tumors (Garcia-Tunon et al., 2004).

Recombinant protein expression in *Escherichia coli* is useful for different purposes. (i) The production of vaccines such as the expression of chicken anemia virus capsid protein VP1 (Lee et al., 2011). (ii) The production of protein that could be used in serodiagnosis of infections e.g. poliovirus (Uma et al., 2016), and *Brucella melitensis* (Cloekaert et al., 2001). (iii) Production of purified proteins that are sold by research companies to be used as positive control in different research experiments e.g. recombinant IL-2 in Western blot and ELISA (Gehman and Robb, 1984).

The use of *E. coli* as an expression system has many advantages e.g. rapid transformation process, high yield of expression, and the whole expression is less expensive in comparison with other hosts (Rosano and Ceccarelli, 2014). However, the higher expression rates of recombinant proteins in *E. coli* are often accompanied with the formation of insoluble aggregates of the target protein called inclusion bodies (IBs) (Singh et al., 2015). The aim of this study was to express the recombinant IL-2 protein in considerable amounts that could be used as a positive control for early diagnosis of tumors and in cancer research in Egypt. Another aim was to investigate the effect of different expression conditions on the solubility behavior of eukaryotic protein expression in *E. coli* using human IL-2 as a model protein.

MATERIALS AND METHODS

Bacterial strains and construction of recombinant plasmids harboring IL-2 gene

The *E. coli* strain Top10 (Thermo Fisher Scientific) was used for cloning, propagation and maintenance of the constructed plasmids; while the *E. coli* strain BL21 (DE3) [Invitrogen] was used for recombinant protein expression. Both strains were transformed using CaCl₂-heat shock method (El-Mowafy et al., 2013). Selection of positive transformants was performed on Luria-Bertani (LB) medium agar plates containing ampicillin (100 µg/ml). Positive

clones were further cultivated in LB medium containing ampicillin (100 µg/ml). Glycerol stocks of all strains were prepared and stored at -80°C till further use (El-Mowafy et al., 2013).

The cDNA of human IL-2 precursor (462 bases, 153 amino acids) (Devos et al., 1983) was synthesized and provided by Macrogen Inc (Korea) in a bacterial cloning plasmid (pIL2) with ampicillin as a selection marker.

The primers IL2-F1 and IL2-R1 (Table 1) were used for cloning of mature IL-2 (402 bp, 133 amino acids) from pIL2 into pRSET-B expression vector (Invitrogen). The amplified IL-2 gene and pRSET-B vector were digested with BamHI (NEB, UK) and HindIII (NEB, UK) followed by ligation with T4 DNA ligase enzyme (NEB) to form the plasmid pIL2-His. The cloned IL-2 gene was in frame with the nucleotides encoding the N-terminal 6 His-tag, which is already introduced by Invitrogen in the pRSET-B vector, resulting in the expression of His-IL2 protein. Similarly, another version of IL-2 was also cloned into pRSET-B vector as a BamHI/HindIII fragment from pIL2 using the primers IL2-F1 and IL2-R2 (Table 1) into pRSET-B expression vector to obtain the plasmid pIL2-His. The expressed IL-2 protein (His-IL2-His) from the latter plasmid was dual His-tagged at both terminals. This was achieved by introducing the nucleotides encoding the C-terminal 6 His-tag at the primer IL2-R2, in addition to the cloning of the BamHI/HindIII-digested polymerase chain reaction (PCR) fragment of IL-2 in frame with the nucleotides encoding the N-terminal 6 His-tag of the pRSET-B vector. Positive clones were confirmed by restriction digestion with BamHI and HindIII. All the cloning steps were performed using Phusion High-Fidelity DNA Polymerase (NEB, UK). All PCR reactions were performed in MultiGene™ Mini Personal Thermal Cycler (Labnet, Multigene gradient, Foster City, CA). The PCR products were electrophoresed on a 1.5% agarose gel and visualized by staining with ethidium bromide. The size of DNA fragments was confirmed using Hyperladder™ 100 bp (Bioline, England) and 1 kb DNA ladder (NEB, UK).

Expression of IL-2 in *E. coli* BL21 at different conditions

LB medium containing ampicillin (100 µg/ml) was inoculated from the glycerol stock of *E. coli* BL21 transformed with pRSET-B, pIL2-His or pIL2-His plasmids and incubated at 37°C with shaking (200 rpm). After overnight cultivation, subculturing into flasks containing 50 ml fresh medium supplemented with the antibiotic was performed so that the starting OD_{600nm} was 0.5 (Ma et al., 2006). The expression was induced with 0.5 mM isopropyl-β-D-thiogalactopyranoside (IPTG). Preliminary time course screening of expression was performed by harvesting 1.5 ml culture after 3, 4, and 5 h of induction followed by centrifugation at 878 × g for 5 min. The cell pellet was suspended in 30 µl 5x SDS gel loading buffer (0.25 M Tris-HCl, pH=6.8, 40% Glycerol, 8% SDS, 2.9 mM β-mercaptoethanol and 0.1% bromophenol blue) followed by heating at 95°C for 10 min (Laemmli UK, 1970). The resulting denatured protein samples were analyzed by 15% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), before staining with Coomassie Brilliant Blue (Laemmli UK, 1970). The size of protein bands was confirmed using chromatein prestained protein marker (Biosepes, China) or Roti®-Mark standard protein marker

(Roth, Germany).

For efficient lysis of the cell pellet after IPTG induction, sonication followed by enzymatic lysis was performed. Briefly, the whole cell pellet after IPTG induction was suspended in 3x volume lysis buffer (10 mM sodium phosphate buffer pH 7, 5 mM NaCl, 5 mM KCl, and protease inhibitor [Complete Ultra, mini, EDTA free, Roche]) (El-Mowafy et al., 2013). The bacterial suspension was subjected to sonication using a Soniprep (150 Sonicator TM-182; UK) in intervals of 30 s sonication and 30 s without sonication for a total of 5 min, while immersed in ice. To allow for enzymatic lysis, lysozyme solution (2 mg/ml) in 20 mM Tris-HCl (pH 8) was added to the sonicated cell suspension in a ratio of 3x volume of the initial cell pellet followed by immersion in ice for 30 min. Benzonase enzyme (EMD Millipore, USA) was added to the mixture to decrease the viscosity of the lysate by degradation of nucleic acids (Final concentration 25 U/ml). The soluble cell lysate (soluble protein extract) was obtained by centrifugation of the whole cell lysate at 11200xg for 10 min, at 4°C, from which an aliquot was denatured by heating in 5x SDS gel loading. Another aliquot was taken for the total protein extract (soluble and insoluble protein extract) before centrifugation, as a control, and was denatured by heating in 5x SDS gel loading (Laemmli UK, 1970).

The expression of the recombinant IL-2 protein in the total protein extract and the soluble cell lysate was investigated at different cultivation conditions by SDS-PAGE Coomassie staining. Such conditions included induction of expression at lower concentration of IPTG (0.25 mM), harvesting at earlier time of expression (3 h after IPTG induction), and expression of the protein at lower incubation temperature (35 or 30°C) (Sorensen and Mortensen, 2005a).

Recovery, purification and solubilization of IBs to obtain soluble purified IL-2 protein

The cell pellet from 100 ml culture was collected after IPTG induction and subjected to lysis by sonication and lysozyme enzyme before separation of the soluble protein extract as mentioned earlier. The protocol was carried out as described previously (Schoner et al., 1992) with few modifications. Briefly, the pellet (containing the disrupted cells, insoluble cellular proteins and IL-2 inclusions bodies) was suspended in 100 mM Tris-HCl (pH 8.5) containing triton 1% and urea 1 M. The mixture was left on ice for 10 min followed by centrifugation at 11200 xg for 10 min at 4°C. Washing of the pellet with triton and urea was repeated for additional 2 times, before washing with 100 mM Tris-HCl, pH 8.5. For solubilization of IBs, the pellet was resuspended in equal volume of 8 M guanidine HCl in 100 mM Tris-HCl (pH 8.5) and left in ice for 1 h with gentle shaking. The mixture was centrifuged at 11200 x g for 10 min at 4°C. The supernatant containing the solubilized IBs was subjected to Protino™ Ni-NTA agarose beads (Macherey-Nagel, Germany) to fish the His-tagged-IL2 protein according to the manufacturer's instructions. After elution of the Ni²⁺ bound His-tagged IL2 protein with imidazole (500 mM), excess imidazole concentration was removed by ultrafiltration with 100 mM Tris-HCl, pH 7 using Roti®-Spin MINI-3 centrifugal device (Roth, Germany). The purified IL-2 protein was stored at -80°C till further use (El-Mowafy et al., 2013).

Western blot

Western blot analysis of the expressed IL-2 proteins was performed using diluted (1:1000) 6-Histidine Epitope Tag Antibody [HRP] (Novus Biologicals, USA) as previously mentioned in El-Mowafy et al. (2013), except that the His-tagged protein bands were visualized by incubation of the membrane with tetramethylbenzidine (TMB) substrate solution (Sigma Aldrich, USA) at room temperature for

few minutes before capturing of photos (Shaaban et al., 2015).

Determination of protein content

The concentration of the purified IL-2 protein was measured using Bradford protein assay kit (Biospes, China) according to the manufacturer's instructions. The protein concentration was calculated from the standard curve of bovine serum albumin (positive control) after measurement of the absorbance at 595 nm using ELx808™ Absorbance Microplate Reader (Biotek Instruments Inc., Winooski, VT) (Shaaban et al., 2015). The buffer containing the purified IL-2 protein (100 mM Tris-HCl, pH 7) was used as blank (negative control).

RESULTS

PCR and cloning

The DNA sequence of mature IL-2 (402 bp) was successfully amplified from pl-IL2 plasmid (Figure 1A) and cloned into the pRSET-B vector, to allow for the bacterial expression of either the N-terminal His-tagged IL-2 (His-IL-2 protein from pl-His-IL2 plasmid) or the dually His-tagged IL-2 (His-IL2-His protein from pl-His-IL2-His plasmid). Cloning of both versions of IL-2 was confirmed by restriction digestion of the constructed plasmids (Figure 1B).

Recombinant expression of His-IL2 and His-IL2-His purified proteins

Bacterial expression of both versions of IL-2 protein was successfully detected at different time points following IPTG induction (Figure 2). Maximum production of the recombinant protein was observed after 4 h of induction (Figure 2). Therefore, further expression experiments were performed after 4 h of induction for both types of expressed IL-2 protein, unless otherwise specified.

Formation of IBs was detected in both versions of the expressed IL-2 by SDS-PAGE of the total protein extract and the soluble lysate of the transformed *E. coli* BL21 cells. The presence of the target protein band in the total protein extract, but not in the soluble lysate, confirmed the formation of insoluble IBs (lanes T and S in Figure 3A and B).

Purification and solubilization of IBs of His-IL2 and His-IL2-His proteins was successfully performed as indicated by SDS-PAGE Coomassie staining (Figure 3A and B) and Western blot detection using anti-histidine tag monoclonal antibody (Figure 3C).

Investigation of the effect of different expression conditions on the formation of insoluble IBs

Different expression conditions were used to avoid the formation of insoluble IBs. Decreasing the concentration

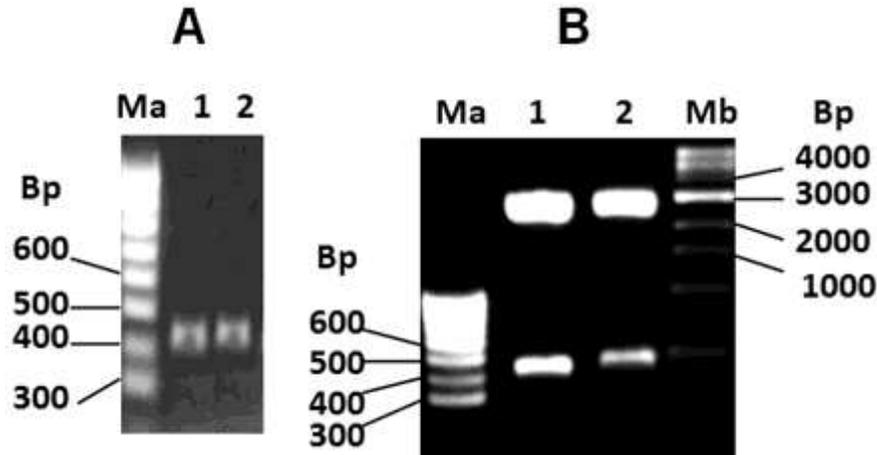


Figure 1. PCR amplification of IL-2 gene (A) and confirmation of successful cloning via restriction enzyme digestion (B). (A) PCR Amplification of mature IL-2 gene from the plasmid pI-IL2 using the primer pairs (IL2-F1, and IL2-R1) and (IL2-F1, and IL2-R2) as demonstrated in lane 1 (Expected size 427 bp) and lane 2 (Expected size 445 bp) respectively. Ma: Hyperladder 100 bp. (B) Enzymatic digestion of the plasmids pI-His-IL2 and pI-His-IL2-His via the enzymes BamHI and HindIII as shown in lane 1 (Expected fragments 2846 and 415 bp) and lane 2 (Expected fragments 2846 and 433 bp), respectively. Ma: Hyperladder 100 bp and Mb: 1 kb DNA ladder.

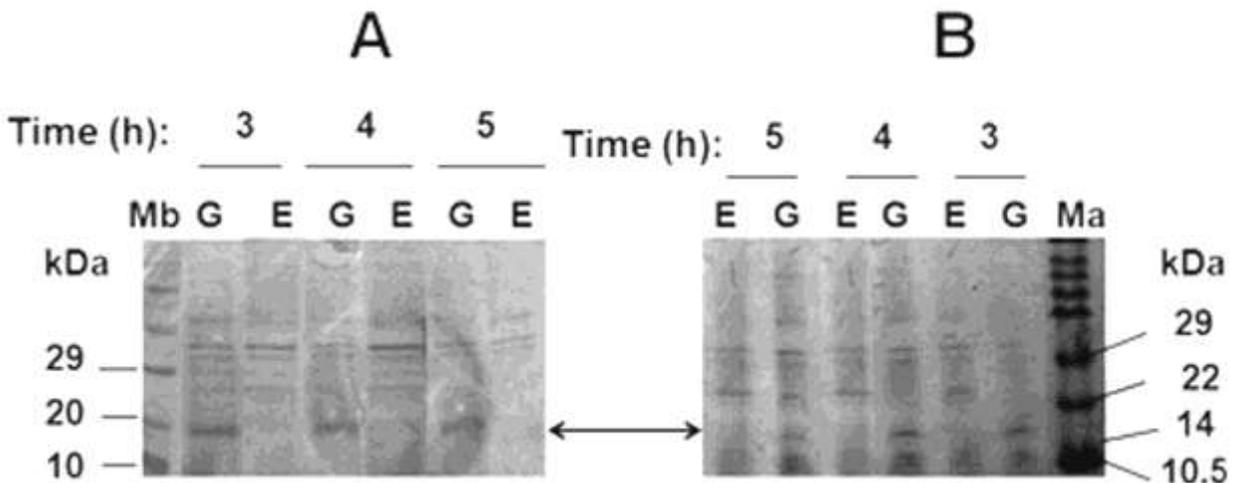


Figure 2. Time course screening of expression of His-IL2 protein (A) and His-IL2-His protein (B). SDS-PAGE analysis of total protein extract of *E. coli* BL21 cells transformed with empty pRSET-B vector (E), pI-His-IL2 (G in panel A), and pI-His-IL2-His (G in panel B) after 3, 4, and 5 h induction with IPTG (0.5 mM). Mb: Roti-Mark standard protein marker. Ma: Chromatein prestained protein marker. Expressed His-IL2 and His-IL2-His proteins are indicated by an arrow.

of IPTG to 0.25 mM (Figure 4A), earlier harvest of the induced cells (3 h of induction, Figure 4B), and decreasing the incubation temperature to 35 (Figure 4C) or 30°C (Figure 4D) did not help to avoid the formation of the IBs. This was indicated by the presence of the expressed His-IL-2 in the total protein extract, but not in the soluble lysate (lanes T and S in Figure 4).

Quantification of the yield of the purified recombinant IL-2 proteins

After purification of both versions of IL-2 protein via immobilized metal affinity chromatography of the solubilized inclusion bodies, the concentrations of purified proteins were determined. The yield of His-IL2 and His-

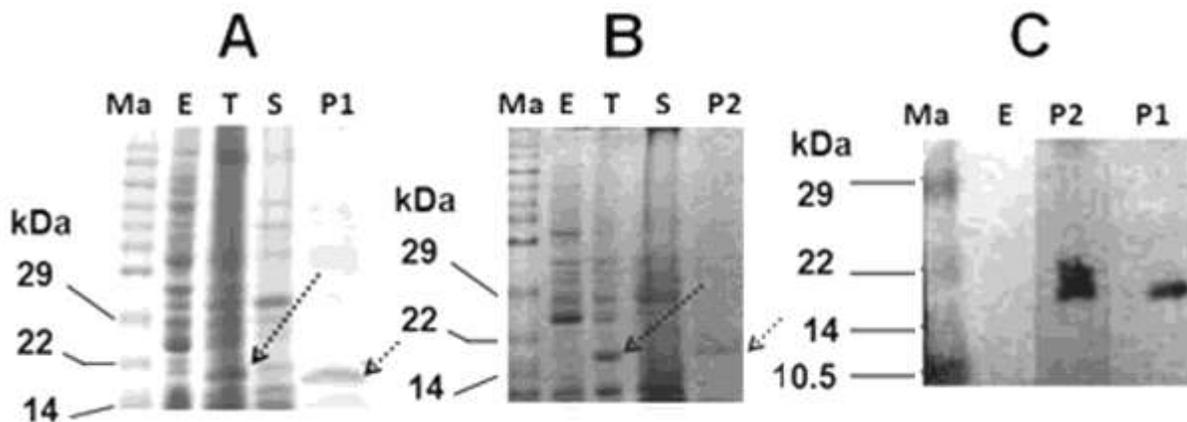


Figure 3. Coomassie staining of the purified His-IL2 and His-IL2-His proteins and their detection by Western Blot. SDS-PAGE analysis of total protein extract (T) or soluble lysate (S) of *E. coli* BL21 cells transformed with pI-His-IL2 (Panel A) or pI-His-IL2-His (Panel B) after 4 h induction with IPTG (0.5 mM). Panel C: Western blot detection of purified His-IL2 protein and His-IL2-His proteins. E: Total protein extract of BL21 cells transformed with empty pRSET-B vector, P1: Purified His-IL-2 protein, P2: Purified His-IL-2-His protein, Ma: Chromatin prestained protein marker. Expressed and purified His-IL2 and His-IL2-His proteins are indicated by arrows.

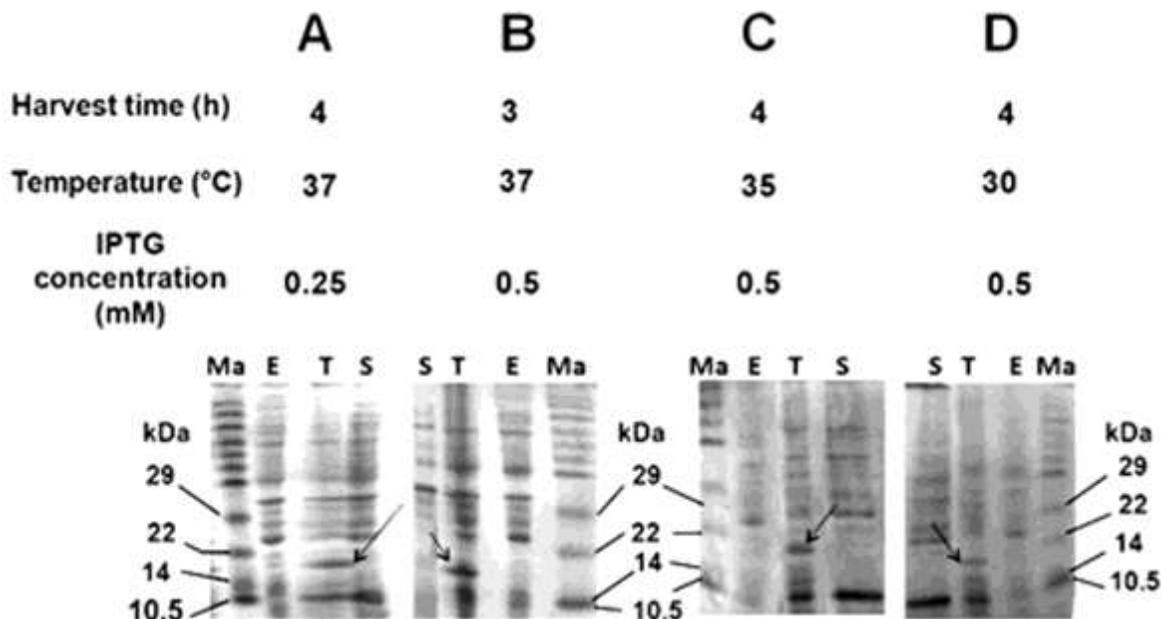


Figure 4. Coomassie staining of expressed His-IL2 protein under different cultivation conditions. SDS-PAGE analysis of total protein extract (T) or soluble lysate (S) of BL21 cells transformed with pI-His-IL2 after expression at different conditions. E: Total protein extract of BL21 cells transformed with empty pRSET-B vector, Ma: Chromatin prestained protein marker. Expressed His-IL2 protein is indicated by arrows.

IL-2-His purified proteins was 5.1 and 4.5 mg/cell pellet of 1 L culture, respectively.

DISCUSSION

Early studies on human IL-2 protein were restricted to the

protein purified from human T-cell leukemia cell line (Jurkat) (Robb et al., 1983; Stern et al., 1984). After recombinant protein technology, IL-2 was expressed after isolation of IL-2 mRNA from human leukemic T-cell line, followed by cloning of the complementary cDNA into a suitable vector. The cloned IL-2 was expressed in different hosts such as *E. coli* (Ju et al., 1987; Devos et

al., 1983; Rosenberg et al., 1984), insect cells (Smith et al., 1985), and monkey COS cells (Taniguchi et al., 1983). Most of the expressed IL-2 in *E. coli* was reported to be insoluble (forms IBs), however, the recombinant IL-2 was still retaining its biological activity even after extraction from SDS polyacrylamide gel (Devos et al., 1983).

In this study, the DNA sequence of the cloned IL-2 gene was derived from a synthesized DNA sequence rather than extraction of mRNA from human leukemic T-cell line and subsequent conversion into cDNA. An N-terminal His-tagged IL-2 (His-IL-2) protein was expressed in *E. coli* BL21 cells. Insoluble IBs of the recombinant His-IL-2 protein was formed, which was indicated by the absence of the target protein in the soluble lysate of the cells, despite its presence in the total protein extract (Figure 3A). A simple protocol was optimized for the solubilization of the insoluble aggregates of both versions of the expressed IL-2 protein (Figure 3). Such protocol depends on washing of the IBs with low concentrations of Tritone (1%) and urea (1 M) to get rid of cell wall and cell membrane components without solubilization of the IBs. The detergent guanidine hydrochloride (8 M) was used for solubilization of the IBs, followed by fishing of the His-tagged IL2 protein using Ni⁺² agarose beads to obtain the purified recombinant proteins (Figure 3) (Palmer and Wingfield 2004).

Different strategies were followed to prevent formation of IBs of the expressed recombinant His-IL2 protein. Incorporation of fusion tags was reported to increase the solubility of the expressed protein and subsequently could prevent formation of IBs (Costa et al., 2014; Sorensen and Mortensen, 2005b). Therefore, a dual His-tagged IL-2 protein (His-IL-2-His) was expressed to decrease the chance of IBs formation. However, the expression of this version of IL-2 protein (His-IL-2-His) was also associated with the formation of IBs (Figure 3B).

High expression rates of recombinant proteins in *E. coli* contribute greatly in IBs formation as described previously by Gatti-Lafranconi et al. (2011). Additionally, the use of high incubation temperature and high IPTG concentration often leads to a high expression rate and subsequent formation of IBs (Singh et al., 2015). Therefore, attempt was made to prevent the formation of IBs by decreasing the cultivation temperature to 35 or 30°C, and by decreasing the concentration of the expression-inducing agent to 0.25 mM. Additionally, the cells were harvested after 3 h of IPTG induction before they enter in the stage of high rate of protein expression. Nevertheless, none of these trials were useful to avoid the formation of IBs (Figure 4).

Conclusion

The impact of the different expression conditions of IL-2 protein on the formation of IBs was determined. Yet, IBs were still forming under all the investigated conditions.

This study provides an inexpensive protocol for the production of purified human IL-2 protein. The purified human IL-2 protein produced by this work could be used as a positive control for early diagnosis of tumors and in cancer research in Egypt. Additionally, the biological activity of the purified protein, after trying different refolding conditions, will be investigated in a future project.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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