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# Pulsed Dilution Method for the Recovery of Aggregated Mouse TNF-α

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## Abstract

***Background:*** The expression of mouse tumor necrosis factor alpha (TNF-α) in *Escherichia coli* is a favorable way to get high yield of protein; however, the formation of cytoplasmic inclusion bodies, which is the consequence of insoluble accumulated proteins, is a major obstacle in this system. To overcome this obstacle, we used a pulsed dilution method to convert the product to its native conformation.
***Methods:*** Reducing agent and guanidine hydrochloride were used to solubilize inclusion bodies formed after TNF-(α) expression. Then, the refolding procedure was performed by pulsed dilution of the denatured protein into a refolding buffer. The properly-folded protein was purified by metal affinity chromatography.
***Results:*** SDS-PAGE showed a 19.9 kDa band related to the mature TNF-(α) protein. The protein was recognized by anti-mouse TNF-(α) on western blots. The final concentration of the purified recombinant TNF-(α) was 62.5 µg/mL.
***Conclusions:*** Our study demonstrates the efficiency of this method to produce a high yield of folded mature TNF- (α).

**Keywords:** *Escherichia coli*, Guanidine Hydrochloride, Inclusion Bodies, Mouse TNF-(α)

## Introduction

Currently, recombinant proteins are used in research, therapeutic, and industrial applications (1). Various eukaryotic and prokaryotic expression systems (2) are in use, and *Escherichia coli* (*E. coli*) being one popular prokaryotic host for the production of heterologous proteins. Advantages of this system include well-characterized genetics, ease of growth, cost-effectiveness, and high product yields. However, inclusion bodies (IBs), which are overexpressed protein aggregates, are considered a drawback (1-3). These IBs are misfolded, insoluble, biologically inactive protein (4). Normally, molecular chaperones such as hsp70 and hsp60 bind to newly-synthesized polypeptide chains and aid in their folding to native conformations (5, 6); however, they are often unable to prevent misfolding of excess recombinant molecules in expression systems (4). Hence, diverse methods have been employed to solubilize and refold IBs. The most common solubilization method is to use guanidine hydrochloride (GdnCl) as a chaotropic agent, with detergents and reducing agents (7). Afterward, the proteins can be renatured by procedures such as dilution, dialysis, chromatography, and solid phase matrix (8). Dilution with a suitable refolding buffer is effective and simple; however yields are generally low. Hence, chemical additives including amino acid derivatives, chaotropic agents, detergents, osmotic reagents such as glycerol and sucrose, and redox systems have been used to improve product yields (9). For each protein, it is critical to optimize conditions by choosing the proper additives (10). Tumor necrosis factor-α (TNF-α), a pluripotent cytokine produced by various cell types, plays an important role in immune response induction, inflammation, apoptosis, and cell proliferation. Dysregulation of this cytokine correlates with the severity of many diseases including insulin resistance, cancer, and

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autoimmune disorders (11). Furthermore, similarities between murine and human TNF-(α) allow the evaluation of the characteristics and clinical potential of this protein by murine TNF-(α) studies. Therefore, in this paper, we describe a pulsed-dilution method that includes GdnCl, EDTA, DTT, sucrose, glycerol, and glutathione as a redox system, to produce a soluble immature mouse TNF-(α).

## Materials and Methods

### *Cloning and expression of recombinant protein*

The cDNA was synthesized using oligo dT primers and reverse transcriptase (Parstous, Iran) from total mouse spleen RNA that was extracted with an RNA extraction kit (Parstous, Iran). The specific primers containing restriction sites were used to amplify the cDNA without the signal peptide. The sequences of these primers were: EcoRI-Forward; 5' GATCCGAATTCGCTCAGATCATCTTCTCA AAATTCGAG 3' and XhoI-Reverse; 5'GGTGCTCGAGCAGAGCAATGACTCCAAA GTAGACC 3'. The PCR products were purified from agarose gel (DNA gel extraction kit Parstous, Iran), cut with restriction enzymes, and ligated into pET-21b (+). After transformation of the vector into *E. coli* TOP10 cells, some isolated colonies were grown in ampicillin-LB medium for plasmid extraction (Parstous, Iran) and the accuracy of the sequence was confirmed by PCR and T7 sequencing primers (MACRO GEN Company). The selected plasmid was then transformed into *E. coli* BL21 (DE3) cells, which were cultured in 3 mL of LB medium containing 100 µg/mL ampicillin in logarithmic phase (OD 600 of 0.6). Isopropyl-beta-D-thiogalactopyranoside (IPTG) was then added in a concentration of 0.4 µg/mL to induce protein expression. After 18 hours of incubation at 37 °C, the cells were pelleted in a microfuge at 13400 (RCF) for 1 (min). SDS-polyacrylamide gel electrophoresis (SDS-PAGE) was performed on both the cell pellet and supernatant to detect protein expression.

### *Renaturation and refolding of inclusion bodies*

The collected pellet of bacteria was resuspended in 5 mL of solubilization buffer (50 mM Tris-HCl, pH 8.5, 100 mM KCl, 20mM DTT, 5 MGdnCl, and 1 mM EDTA), sonicated on ice for 5 (min), and incubated overnight at 4 °C and incubated at 37 °C for 2 h. After centrifugation for 10 (min) at 8000 (RCF), the supernatant containing the solubilized protein was added discontinuously to 300 mL refolding buffer (50 mM Tris-HCl pH 8.5, 0.4 M sucrose, 10% glycerol, 0.5% Triton X-100, 0.3 mM GSSG, 3 mM GSH). The solution was then incubated at 4 °C for 20 h and centrifuged for 10 (min) at 9000 (RCF) to separate the recombinant protein from the supernatant.

### *Metal affinity chromatography*

A 5 mL Ni-IDA column (Parstous, Mashhad, Iran) was first washed with 30 mL of distilled water and then equilibrated with 30 mL of start buffer [50 mM Tris-HCl pH 8.0, 0.5 M NaCl, and 5 mM imidazole]. The sample was mixed with 1.5 M NaCl and 5 mM imidazole and loaded onto the column. Afterward, 40 mL of start buffer and then 100 mL of wash buffer [50 mM Tris-HCl pH 8.0, 0.5 M NaCl] containing 5 mM imidazole and then with 200 mL of wash buffer containing 40 mM imidazole were passed through the column to remove unbound proteins. The attached proteins were eluted by adding elution buffer, [50 mM Tris-HCl pH 8.0, 0.5 M NaCl, and 500 mM imidazole]. Fractions were collected and dialyzed overnight in 150 mM phosphate-buffered saline (PBS) at 4 °C.

### *SDS-PAGE and Western* blotting

The dialyzed proteins and cell pellet were separated by 12.5% SDS-PAGE (Mini-Protean II Cell, Bio-Rad), transferred to polyvinylidene difluoride (PVDF) membrane, and incubated overnight at 4 °C with 2% bovine serum albumin (BSA) to block non-specific binding sites. Afterward, the membrane was incubated with biotin-conjugated anti-mouse TNF-(α) antibody (diluted 1:500 in 1% BSA) for 2 h at room temperature (RT) and then horseradish peroxidase-streptavidin (Bio-Rad, CA, USA). The protein band was visualized by chemiluminescence (Parstous, Mashhad, Iran).

### *Protein assay*

The protein concentration was measured using the bicinchoninic acid (BCA) method. Initially, the reagents and standards were prepared according to the kit procedure (Parstous, Iran) and the sample was diluted. Twenty-five µl of each

sample and 75 µl of working reagent were added into duplicate wells of the microtiter plate. The mixture was incubated at 60 °C for 60 (min). The optical densities (ODs) were measured at 562 nm and the sample concentrations were determined by comparing them to the standard curve.

## Results

After selection of transformed bacteria, the recombinant plasmid was extracted and assayed by PCR using T7 primers. The presence of a 709-bp band on the gel confirmed that the 469 bp TNF-(α) cDNA was ligated into to the 240-bp insertion site (Fig. 1). Moreover, the NCBI BLAST showed 100% homology between the sequenced fragment and the mouse TNF-(α) precursor.

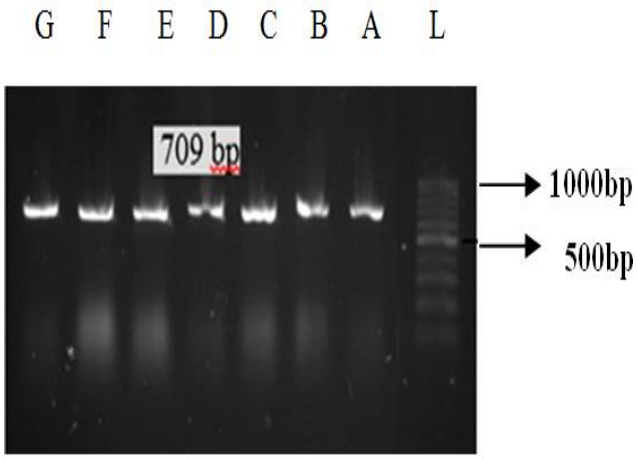


**Fig. 1.** Gel electrophoresis of the PCR product with T7 primers. Lane L: 100 bp ladder. Lanes A-G: confirmation of mouse TNF-a gene cloning into the recombinant plasmid.

Protein expression was evaluated by SDS-PAGE. A 19.9-kDa band was coincident with the recombinant TNF-(α). The band from the cell pellet was evidence for protein aggregation as (IBs) which are insoluble and cannot be soluble (Fig. 2).

The solubilized and refolded IBs were characterized by SDS-PAGE and western blotting. The SDS-PAGE showed a 19.9-kDa soluble protein that reacted with anti-mouse TNF-(α) antibody (Fig. 3). The final concentration of the refolded purified protein was 62.5 µg/mL.

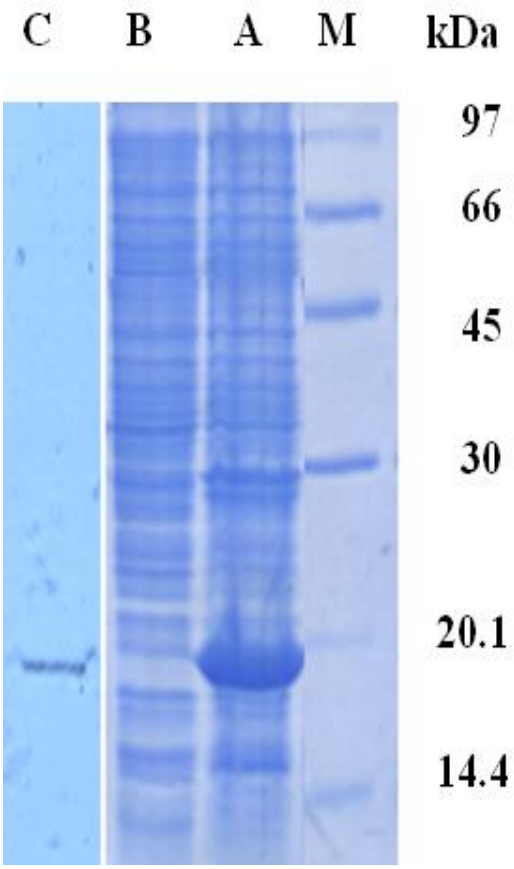


**Fig. 2.** SDS-PAGE of expressed proteins was performed by 12.5% acrylamide stained with Coomassie Blue. Lane M: protein marker. Lane A: pellet fraction of cell lysates. Lane B: supernatant fraction of cell lysate. Lane C: Purified refolded protein.

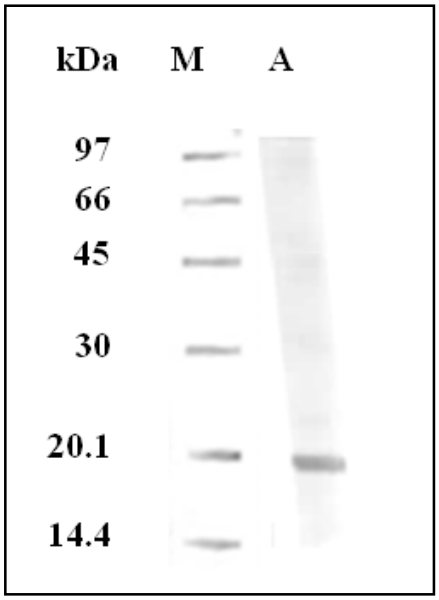


**Fig. 3.** Western blot analysis of purified recombinant TNF-(α). Lane M: protein marker. Lane A: Binding of Anti-Histag antibody to the 19.9 kDa band.

## Discussion

As discussed above, no universal method currently exists for solubilization and refolding of IBs that result from high-level expression of recombinant proteins (10). In this study, we assessed GdnCl, DTT, and EDTA for solubilization of immature mouse TNF-(α). Then the refolding buffer, supplemented with glutathione, sucrose, glycerol, triton X-100, and Tris was applied to improve the protein renaturation. For the first step, urea and GdnCl are two common denaturants that prevent non-covalent interactions, either by modification of solution properties or direct binding to protein (12). Here, GdnCl was preferred to urea due to its greater ability to solubilize sturdy IBs (13). The use of EDTA as a chelating agent and DTT as a reducing agent improve this process via prevention of cysteine residue oxidation, which is involved in disulfide bond formation (14). In the next step, high molar GdnCl (6 molar) was omitted because it can interfere with correct protein renaturation. Nevertheless, a low amount of this agent was added to the refolding buffer to prevent protein aggregation (15, 16). At this phase, folding enhancers such as glycerol and sucrose, chemical chaperones that bind with the folding intermediates, promote protein stabilization against denaturing stress and simplify protein collapse by reducing nonspecific hydrophilic interactions (17). The ability of glycerol, especially in combination with sucrose as an osmolyte, was defined by evaluating the efficacy of different osmolytes on aminoacylase and trehalose-6-phosphate hydrolase refolding (18, 19). Moreover, a direct correlation was observed between the refolding of lysozyme and glycerol concentration (up to 40%) (20).

To enhance correct disulfide bond formation, glutathione, which includes both reduced and oxidized forms of thiol agent, was used as a prevalent redox system (21). Also, we supplemented the buffer solution with triton X-100 and Tris, which have a synergistic effect on the recovery of native protein. These agents were used successfully by Ji-Cheng Pan and colleagues to refold arginine kinase (22). Apart from the chemical additives, a discontinuous dilution was used to increase the refolding yield and prevent protein aggregation, which is the result of intermolecular links of denatured protein at high concentrations (14). In conclusion, SDS-PAGE and western blotting showed that pulsed dilution is an efficient method to produce high yields of correctly-folded soluble immature mouse TNF-(α).

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