

## EFFICIENT REFOLDING OF THE RECOMBINANT HUMAN GLUCOCORTICOID-INDUCED TNF-RELATED RECEPTOR LIGAND PRODUCED IN *E. COLI* AS INCLUSION BODIES

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*În acest studiu a fost realizată renaturarea eficientă a GITRL-ului uman, producând sub forme insolubile în E. coli, folosind diferiți vectori recombinanți (pET20b-uGITRL, PETM52-uGITRL). Proteina insolubilă a fost dizolvată în soluție de clorhidrat de guanidină, după care renaturarea s-a realizat prin metoda de dizolvare rapidă în tampon redox, conținând glutatation redus și oxidat. Eficiența renaturării a fost de aproximativ 95%.*

*In this study it was realized an efficient refolding of the human GITRL produced in E. coli as inclusion bodies utilizing different recombinant vectors (pET20b-hGITRL, pETM52-hGITRL). The insoluble proteins were solubilized in guanidine hydrochloride and then were refolded by rapid dilution into a red-ox buffer composed of GSSG-GSH. Efficiency of the refolding was about 95%.*

**Keywords:** inclusion body, protein refolding, hGITRL, guanidine hydrochloride

### 1. Introduction

*E. coli* is one of the most widely used host for industrial and pharmaceutical protein production. One of the reasons seems to be that genetics of this organism are better characterized than those of any other microorganism [1]. Advantages of the *E. coli* expression system are: the inexpensive substrates, cultures can be grown fast, many foreign proteins expressed at high levels and a large number of cloning vectors are engineered to achieve the high-level synthesis of proteins and to facilitate the purification of recombinant gene products [1, 2].

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However, target proteins expressed at high levels in *E. coli* often accumulate within the cell as biologically inactive aggregates known as inclusion bodies (IBs) [2]. Although some recombinant proteins do occur in both the soluble and insoluble cell fractions, many others are only produced as IBs [3]. The proteins in IBs are usually not biologically active, so refolding of proteins is necessary to form their native conformations and to be biologically active [4]. Renaturation of proteins from inclusion bodies are usually performed by solubilization of the protein aggregate under high concentration of denaturants, along with a reducing agent such as  $\beta$ -mercaptoethanol, dithiothreitol or cysteine [5]. Solubilized proteins are then refolded by rapid dilution in refolding buffer. Removal of the denaturant is carried out by dialysis [6].

The formation of IBs can be advantageous. The resulted conglomerates provide high enrichment of the desired protein at an early stage of purification. Additionally, IB formation protects the recombinant protein against proteolysis from intracellular proteases [5, 6].

Our aim in this study is the realization of an efficient refolding of the human GITRL (hGITRL) produced in *E. coli* BL21 Star (DE3), as inclusion bodies.

The glucocorticoid-induced TNF-related receptor (GITR) and its ligand (GITRL) are members of the TNF receptor/ligand superfamily [7]. GITRL is a type II transmembrane protein with a short cytoplasmic region, a hydrophobic transmembrane domain and an extracellular domain [8]. GITRL attachment to GITR regulates diverse biological functions, including cell proliferation, differentiation and survival [7]. Human GITR (hGITR) is expressed by regulatory T cells [8], natural killer (NK) cells [9]. NK cells substantially contribute to cancer immunosurveillance and GITR triggering may impair NK cell effector function [9, 10]. GITRL is expressed on several human tumor cell lines; by this way tumor cells diminishes NK cell cytotoxicity, thus tumor cells survive [10].

In this study, we developed a high yield procedure for refolding of the hGITRL from inclusion body by rapid dilution method. The renaturation and purification of hGITRL from inclusion body would provide a way for further study the relation between GITR and GITRL.

## **2. Experimental**

### **Isolation and solubilization of IBs**

The coding sequence of the hGITRL was isolated from human brain cDNA and inserted in bacterial expression vectors (pET20b, pETM52). In the case of the pETM52-hGITRL construct, the multiple cloning site of pETM52 allows fusion of GITRL to the leaderless DsbA protein sequence, which promotes the formation the native structure of its fusion partners [11]. It was realized a

construction without fusion partner too (pET20b-hGITRL), because the fusion proteins often present nonspecific association, when the cleaving of the fusion partner is not successful.

The gene expression was realized in *E. coli* BL21 Star (DE3) cells [12, 13]. The bacterial pellets from 250 ml production culture was resuspended in 30 ml ultra pure water and stored at -20°C overnight. After thawing the cells, they were lysed by sonication, 3 cycles of 5 minutes at 70% amplitude was used, with 10 minutes pause between the cycles, the procedure was conducted on ice to prevent excess heat formation and denaturation of the proteins. The lysed cells were centrifuged at 18000 rpm for 30 min at 4°C. The supernatants were discarded and the IB pellets were washed three times with 30 ml of 10 mM Na-phosphate buffer pH 7.0, 0.1 M NaCl and 0,1%  $\beta$ -mercaptoethanol. This step helps separating the inclusion bodies from the rest of the cell proteins. The inclusion body was solubilized by 10 ml 6 M guanidine hydrochloride (GuHCl, Sigma) treatment, reduced with 50 mM Tris-HCl buffer pH 8.8 and 1 mM dithiothreitol (DTT, Serva) for overnight at room temperature.

#### Refolding of the solubilized hGITRL

The solubilized protein was refolded in a fast dilution red-ox system with oxidized and reduced glutathione (Sigma). The solubilized inclusion body was centrifuged at 14000 rpm for 10 min, filtered (0.22  $\mu$ m, Millipore) and stored at 4°C. In the case of the DsbA-hGITRL protein (obtained by transformation of pETM52-hGITRL vector in *E. coli* BL21 Star (DE3)) 2  $\mu$ l solubilized material (containing 6 M GuHCl) was diluted into 60 ml ice-cold refolding buffer (0.8 M Gu-HCl, 0.1 M Tris-HCl pH 8.0, 5 mM  $\text{CaCl}_2$ , 0.5 mM EDTA, 5 mM reduced glutathione (GSG) and 0.5 mM oxidized glutathione (GSSH)). In the case of the hGITRL protein (obtained by using the pET20b-hGITRL vector) the refolding buffer was supplemented with 1mM phenylmethylsulfonyl fluoride (PMSF, Sigma). In this case, the renaturation was realized without the refolding buffer containing 0.8 M GuHCl. The solution was stirred by magnetic stirring at 4°C for 8 h. The refolded protein was dialyzed against 10 mM Tris-HCl buffer pH 8.0 supplemented with 50 mM NaCl for 48 hours in 4 l of dialyzing buffer. Results of the refolding were verified by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE).

### 3. Results and discussion

Previous study reported that expression of human GITRL (hGITRL) in bacterial system resulted in formation of insoluble inclusion bodies [14]. In our study, majority of the hGITRL was found also to aggregate in inclusion bodies. Renaturation of the hGITRL from IBs involves four steps: isolation of IBs from *E.*

*coli* BL21 Star (DE3), solubilization of protein aggregates, refolding and purification of the solubilized hGITRL. Before starting the renaturation of the IBs, aggregates has to be solubilized by using a high concentration denaturants (guanidine hydrochloride or urea) [3]. In our study 6 M GuHCl was chosen. Refolding of solubilized inclusion bodies is initiated by the removal of the chaotropic reagents [5]. The most common method for renaturation of recombinant proteins is dilution of denatured proteins directly into the refolding buffer [6].

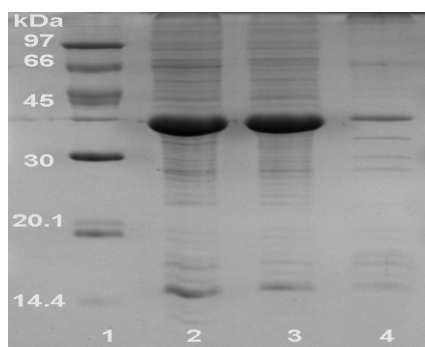


Fig. 1. SDS-PAGE of the hGITRL-DsbA refolding from inclusion body. Lane 1. protein molecular weight marker, Amersham; Lane 2. total refolded protein after dialyzing; Lane 3. soluble protein after dialyzing; Lane 4. insoluble protein after dialyzing.

If proteins contain disulfide bonds, the renaturation buffer needs to be supplemented with a red-ox system for formation of disulphide bonds [6]. Oxidation can be achieved using a mixture of reduced and oxidized reagents, such as reduced and oxidized glutathion, which are the most commonly used thiol reagents [3, 5, 6]. Other thiols, such as cysteine/cystine have also been utilized [5]. In this study the solubilized inclusion bodies were refolded by rapid dilution into a red-ox buffer composed of GSSG-GSH with or without 0.8 M GuHCl, and then were dialyzed against Tris-HCl buffer pH 8.0.

Fig. 1. represents the visualization of soluble and insoluble proteins by SDS-PAGE. Band intensities were quantified by scanning densitometry using the GeneSnap software. The percentage of the soluble protein was calculated by the comparing densitometry values of the soluble proteins with total refolded proteins. In the case of fusion protein DsbA-hGITRL, efficiency of the refolding was about 95%.

In the case of the hGITRL obtained by using pET20b vector, the majority of the expressed proteins were found in inclusion bodies too. Fig. 2A. shows that refolding efficiency of hGITRL without fusion partner is about 95% when the

refolding buffer contained 0.8 M GuHCl. The efficiency of the refolding decreased to 10 % without containing GuHCl (Fig. 2B).

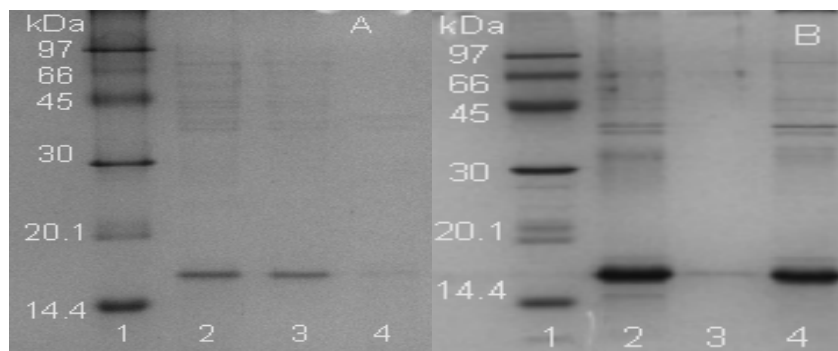


Fig. 2. SDS-PAGE of the hGITRL renaturation from inclusion body. A. refolding of the hGITRL, when the refolding buffer contains 0.8 M GuHCl; B. refolding of the hGITRL without GuHCl in the refolding buffer.

Lane 1. protein molecular weight marker, Amersham; Lane 2. total refolded protein after dialyzing; Lane 3. soluble protein after dialyzing; Lane 4. insoluble protein after dialyzing.

#### 4. Conclusions

In this study it was realized the refolding of the hGITRL obtained as inclusion bodies. The insoluble proteins were solubilized in Gu-HCl, and then were refolded by dilution of the solubilized proteins into a red-ox buffer composed of GSSG-GSH in the presence of the GuHCl. In the case of hGITRL without fusion partner, the refolding was most efficient when the refolding buffer contained 0.8 M GuHCl then during refolding without GuHCl. Although the fusion protein and the protein without the fusion partner were produced as inclusion bodies, the efficiency of the refolding for both proteins was about 95%. In conclusion the renaturation method used in our study led to high recovery of the proteins. Our aim for the future is to test the hGITRL activity and the interaction of recombinant hGITRL and hGITR expressed on the surface of different human T cell lines. In final an antibody will be prepared which can be used for tumor detection.

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