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Short communication

Recovery of active anti TNF-α ScFv through matrix-assisted refolding of bacterial inclusion bodies using CIM monolithic support

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ABSTRACT

Anti TNF-α molecules are important as therapeutic agents for many of the autoimmune diseases in chronic stage. Here we report the expression and purification of a recombinant single chain variable fragment (ScFv) specific to TNF-α from inclusion bodies. In contrast to the conventional on column refolding using the soft gel supports, an efficient methodology using monolithic matrix has been employed. Nickel (II) coupled to convective interaction media (CIM) support was utilized for this purpose with 6 M guanidine hydrochloride (GuHCl) as the chaotropic agent. The protein purified after solubilization and refolding proved to be biologically active with an IC_{50} value of 15 μg. To the best of our knowledge, this is the first report showing the application of methacrylate based chromatographic supports for matrix-assisted refolding and purification of *Escherichia coli* inclusion bodies. The results are promising to elaborate the methodology further to exploit the potential positive features of monoliths in protein refolding science.

1. Introduction

Many recombinant proteins produced using different expression systems have been employed in the therapeutic field and some are in the pre-clinical trial or clinical trial [1]. Among the expression systems, mammalian cell culture systems are the most preferred ones for the therapeutic protein production. Other systems include, insect cells, yeast, bacteria and plant cells are also been reported [2]. The bacterial expression systems, used mainly for the expression of small proteins are characterized by the simplicity, cost effectiveness and less technical difficulties despite the disadvantages like lack of post translational modifications and the formation of protein aggregates [3]. In this aspect, ScFvs, which consist of only the heavy and light chain variable regions of an antibody, can be very well expressed in *Escherichia coli* expression system.

Further to the post translational modification issues, the formation of inclusion bodies have been a major problem in *E. coli* expression system [3]. It is attributed that, high level expression of recombinant protein often results in protein aggregation, leads to the formation of inclusion bodies *in vivo* [4]. Several ScFv proteins had been expressed in *E. coli* in the past [5] and many of them were shown to be forming inclusion bodies. Even though, the formation of inclusion bodies is not desired, the proteins present in inclusion bodies have some advantages like, less contamination from native proteins and protection from endogenous proteolytic degradation [6]. Refolding of inclusion bodies can be attained by a number of methods including direct dilution, dialysis, diafilteration, chromatographic methods (size exclusion, and hydrophobic based), matrix-assisted refolding, and chemical-assisted refolding [7]. Conventional methods of dialysis or extreme dilution are effective but slow and yield large volumes of diluted protein. Recovery of the active and properly folded protein from this method is of great concern.

In matrix-assisted refolding, the solubilized and unfolded protein is attached to a solid support prior to changing from denaturing to native buffer conditions [8]. This approach avoids the unwanted intermolecular interaction between aggregation-prone folding intermediates. Binding of the solubilized protein to the matrix requires the formation of a stable protein–matrix complex, withstanding the presence of chaotropic agents [9]. However, after changing to native buffer conditions, the detachment of the refolded target protein from the matrix should be easily accomplished. Due to the selective binding, matrix-assisted refolding can combine the renaturation of the target protein along with its separation from host cell protein contaminants [10]. As far as we know, the convective interaction media CIM supports have not been used in the matrix-assisted refolding and purification of inclusion bodies.

In this study, a recombinant ScFv specific to TNF-α was expressed in *E. coli*. To refold and purify more than 60% of the ScFv detected in the inclusion bodies, a novel and efficient methodology

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using monolithic matrix combined with metal affinity has been developed. CIM monoliths were previously used in our study to purify the soluble form of anti TNF-α ScFv [11] and in this report we explored the possibility of using CIM-IDA-Ni (II) disks for concurrent refolding and purification of insoluble anti TNF-α ScFv.

2. Materials and methods

Expression plasmid pET28a was purchased from Novagen. *E. coli* strains DH5α and BL21 (DE3) were used as a host system for cloning and protein expression, respectively. Human breast cancer cell line – MCF-7 – were cultured in DMEM medium containing 10% (v/v) FBS (fetal bovine serum) at 37 °C in a 5% CO_2 incubator. Recombinant human TNF-α, MTT reagent, cyclohexamide, Murine anti His-tag antibody and molecular biology regents were purchased from Sigma. Rabbit monoclonal antibody against human TNF-α was purchased from Epitomics, U.S.A. Restriction endonucleases were from New England Biolabs, U.S.A and CIM supports were from BIA separation, Slovenia. Protein purification was carried out using AKTA prime FPLC chromatographic system from Amersham Biosciences.

2.1. Cloning of ScFv in expression vector

The generation of an expression construct for anti TNF-α ScFv has been reported previously [11]. In short, the DNA fragment encoding anti TNF-α ScFv cDNA was cloned in pET28a and used in the protein expression studies.

2.2. Protein expression and SDS-PAGE analysis

The protein expression and SDS PAGE analysis was performed as described elsewhere [11]. In short, Fresh competent *E. coli* BL21 (DE3) strain was transformed with the construct pET28a-ScFv. A 100 ml culture was raised with a 1 ml culture of a single colony inoculum grown for 12 h with appropriate antibiotics. Protein expression was induced by 0.2 mM isopropyl-β-D-thiogalactopyranoside at 0.6 OD and left for 6 h at 37 °C. The cells were harvested by centrifugation at 14,000 rpm for 10 min and resuspended in lysis buffer (20 mM PBS, 1 M NaCl, 2 mM PMSF). After the cells were sonicated, and soluble and insoluble fractions were separated by centrifugation. Protein concentration was estimated by Bradford's method with bovine serum albumin as a standard. Equal concentrations of protein were analyzed by SDS PAGE using Bio-Rad mini protein system (Bio-Rad Laboratories). The resolved protein samples were visualized by staining with Coomassie brilliant blue.

2.3. Solubilization of anti TNF-α ScFv expressed in inclusion bodies

The inclusion body obtained from 100 ml culture was washed thrice with 0.5 M GuHCl containing lysis buffer and centrifuged at 14,000 rpm for 10 min at 4 °C to pellet down washed inclusion bodies. For solubilization of inclusion bodies, the pellet was resuspended in two different solubilization buffers containing 8 M urea and 6 M GuHCl. These two preparations were kept for magnetic stirring at 4 °C for 12 h and at 37 °C for 3 h to check the efficiency of the methods. The solubilized protein was analyzed by SDS-PAGE.

2.4. On-column refolding and purification of inclusion bodies

For this, CIM-IDA-Ni (II) column was equilibrated with equilibration buffer (20 mM phosphate buffer containing 6 M GuHCl, 0.5 M NaCl, 1 mM DTT and 5 mM Imidazole – pH 7.4.). After equilibration, the solubilized inclusion body was loaded onto the column and unbound proteins were washed extensively with equilibration buffer and a series of buffers containing decreasing concentration (4 M, 2 M, 1 M and 0.5 M) of GuHCl. For this, the buffers with different GuHCl concentrations were passed through the column for 10 min at a flow rate of 0.5 ml/min and 10 fractions of 0.5 ml volume were collected. The bound proteins were eluted by elution buffer (20 mM phosphate buffer containing 0.5 M imidazole, 0.5 M NaCl, 5% glycerol, 500 μM reduced glutathione (GSH), pH 7.4) The fractions were concentrated and the protein profile was analyzed by SDS-PAGE. The presence of anti TNF-α in the purified fractions was confirmed by Western blot analysis using anti His-tag antibody.

2.5. MCF-7 cytotoxicity assay with rhTNF-α

Refolded and purified anti TNF-α ScFv was assayed for its activity by MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, a yellow tetrazole), assay using the principle of TNF-α induced apoptosis in MCF-7 cells. To perform this experiment, MCF-7 cells were seeded onto a 96-well plate at a density of 10,000 cells/well and incubated at 37 °C with 5% CO_2 for 24 h. After 24 h, the cells were treated with different concentrations of TNF-α (0, 2.5, 5, 10, 20, 40 ng/ml) in the presence of 1 μg/ml of cyclohexamide in order to determine the IC_{50} value of TNF-α to induce apoptosis in MCF-7 cells. In the next set of experiments, cells were treated with IC_{50} concentration of TNF-α with increasing concentration of refolded and purified ScFv (1.25–50 μg/ml). Commercial rabbit anti TNF-α monoclonal antibody was used as positive control and a nonspecific ScFv (anti HCG ScFv) was used as negative control. After 36 h, the cells were incubated with 20 μl MTT (5 mg/ml) for 4 h. The purple crystals formed were solubilized using DMSO and the absorbance was measured at 595 nm with a micro plate reader (Floustar Optima, Germany). All samples in this experiment were run in triplicate. The average of the absorbance values was plotted against the ScFv concentration. The IC_{50} value of refolded and purified anti TNF-α ScFv was calculated.

3. Results and discussion

Soluble expression and purification of a ScFv against human TNF-α has been reported previously [11]. Since the expression showed nearly 60% of the protein in the inclusion bodies (Fig. 1), refolding experiments were carried out to solubilize the inclusion bodies. The results obtained with two different chaotropic agents, using the conditions specified in Section 2 showed that 12 h stirring with 6 M GuHCl containing buffer at 4 °C gave maximum solubilization among all the tested parameters (Fig. 2, Lane 8).

Soft gel support matrices are the conventional solid supports for the on-column refolding of denatured proteins, which has a number of undesirable features like, time consuming, laborious and have technical drawbacks. These problems can be addressed with solid monolithic supports which form a continuous stationary phases that are cast as a homogenous column in a single piece with agglomeration-type or fibrous microstructures [12]. For refolding, 2 mg of the solubilized inclusion bodies was loaded onto CIM-IDA-Ni (II) column pre-equilibrated with the equilibration buffer. While washing the column with equilibration buffer containing decreasing concentrations of GuHCl, most of the unbound or sticky proteins were removed. This gradual drop in GuHCl concentration facilitated refolding of unfolded proteins on column. With the increasing concentrations of imidazole used, 0.5 M concentration eluted the His tagged proteins from the column (Fig. 3A). SDS-PAGE analysis of the eluted protein fractions (65%) showed that protein was purified to homogeneity (Fig. 3B and Table 1). The use of monolithic columns in the effective purification of many recombinant proteins and proteins from its natural sources had been reported [13].

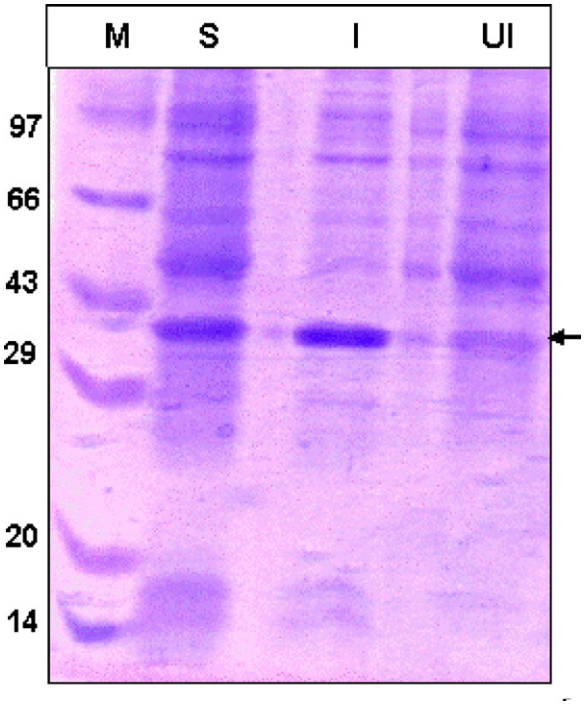

Fig. 1. Expression of anti TNF-α ScFv in *E. coli*, BL-21(DE3). Anti TNF-α ScFv expression analyzed by SDS PAGE and coomassie staining. M – protein molecular weight marker, S – soluble fraction, I – inclusion bodies, UI – un-induced control. The arrow indicates the 33 kDa anti TNF-α ScFv.

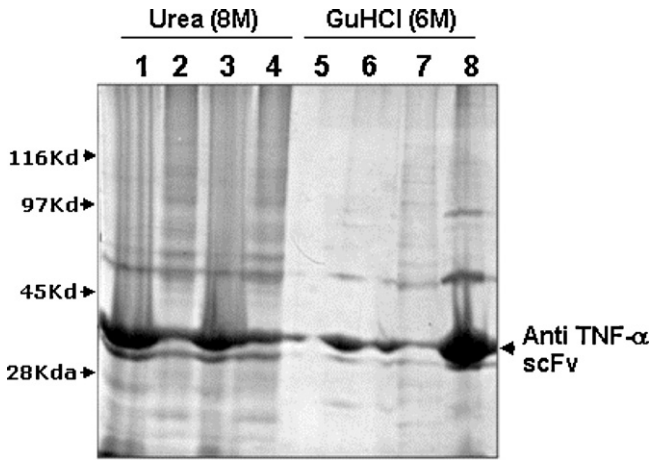

Fig. 2. Comparison of solubilization efficiency using 8 M urea and 6 M GuHCl at different time points. Inclusion bodies were solubilized using two different chaotropic agents, 8 M urea and 6 M in two sets, 3 h at 37 °C and 12 h at 4 °C. Lane 1, 2, 5, 6 and 3, 4, 7, 8 – insoluble and soluble fractions solubilized with 8 M urea and 6 M GuHCl for 3 h and 12 h, respectively.

Table 1
Purification table for inclusion bodies.

Chromatographic column	Load (mg)	Flow through (mg)	0.5 M imidazole (mg)	ScFv recovery (%)
CIM-IDA-Ni (II) 0.34 ml disk	2	0.45	1.29	64.5

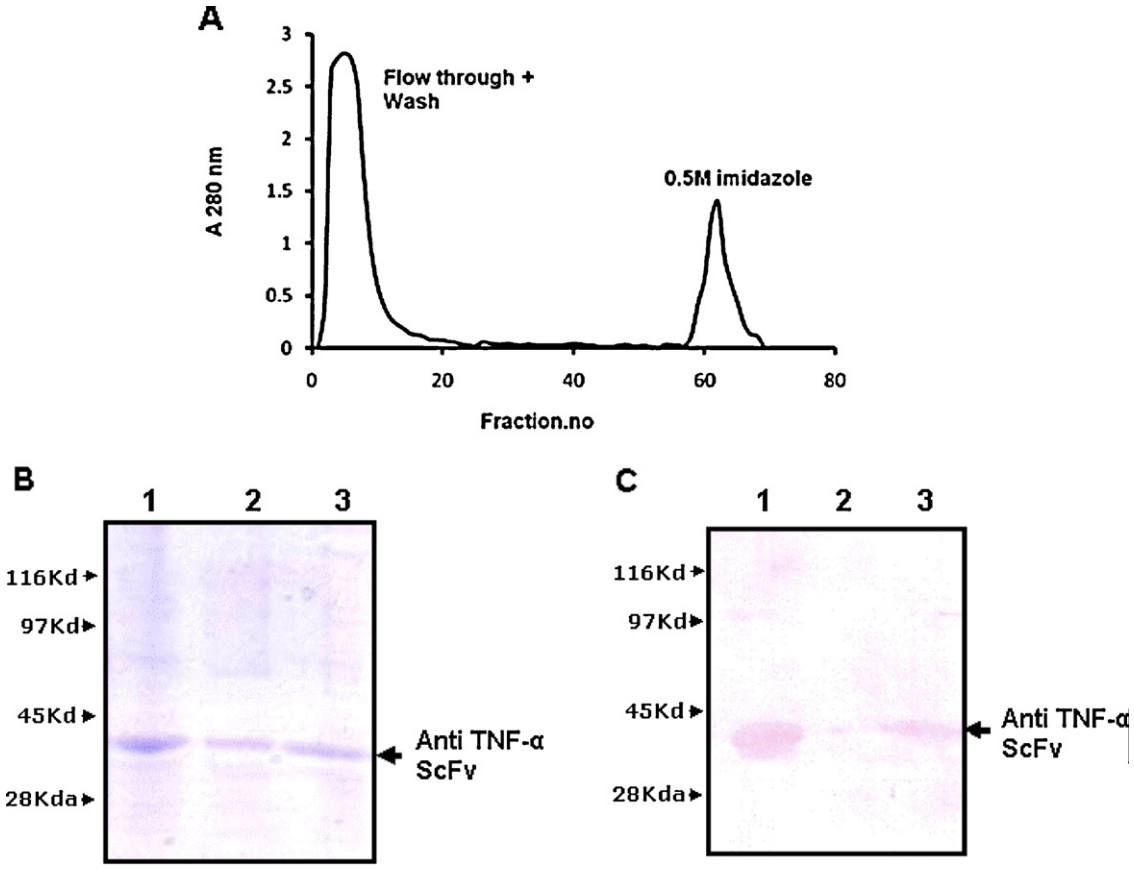

Fig. 3. Purification of solubilized inclusion bodies. Chromatogram shows the ScFv elution peak with 0.5 M imidazole (a). The protein profile of the peak was analyzed in SDS-PAGE (b) and the presence of ScFv was confirmed by Western blot analysis (c), Lane 1 – solubilized inclusion bodies as load, Lane 2 – flow through, Lane 3 – 0.5 M imidazole elution.

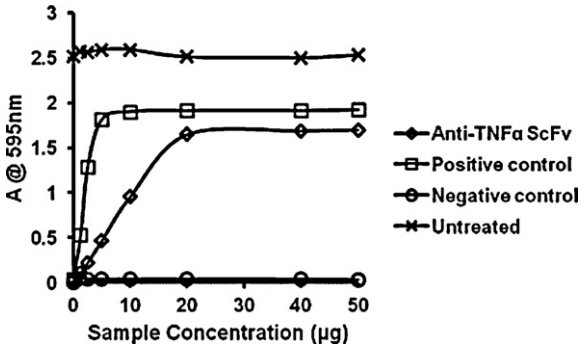

Fig. 4. Inhibition of TNF-α induced cytotoxicity by refolded anti TNF-α ScFv in MCF-7 cells. The anti TNF-α ScFv protected MCF-7 cells from rhTNF-α induced cytotoxicity. The experiment was done as per the methods given in Section 2.5. Commercially available anti TNF-α antibody was used as a positive control along with a nonspecific ScFv (anti HCG ScFv) as the negative control. Control cells were without any treatment.

Peterka et al. reported the isolation of histidine containing proteins to high purity using CIM-IMAC with a single chromatographic step [14]. The evenly distributed ligands on CIM discs compared to soft gels facilitate the bound proteins to be well distributed on the matrix, preventing protein aggregation. Considering the features, purity, yield and purification speed, the monolithic supports are found to be superior to the conventional columns [15]. Western blot analysis with anti His-tag antibody confirmed the presence of anti TNF-α ScFv in the purified fraction (Fig. 3C).

When compared to refolding in-solution, matrix-assisted refolding yields higher percentage of active protein. This was proved in a previous study in which, 5 tested proteins showed better refolding by matrix-assisted strategy than refolding in-solution [5]. There are several reports on the matrix-assisted refolding of ScFv using soft gel matrices. When a His-tagged ScFv developed against isoferritin (IP10 ScFv) was refolded by soft gel based Ni-chelating chromatography, a refolding yield of 45% and an overall bioactive protein yield of 40 mg/l were obtained [16]. In another report, 77% refolding yield was observed for an anti TNF-α ScFv with Ni-NTA system [17]. In the present experiments, CIM monoliths were giving reasonably good refolding yield (65%) (Table 1) within 50 min, where as the Ni-NTA assisted refolding needed around 6 h for the chromatographic run [17] to complete. Easy and straight forward scale up with CIM-IMAC has been reported in the previous work [11].

Refolded ScFv protected MCF-7 cell lines from TNF-α induced apoptosis in dose-dependent manner. The optical density at 595 nm increased when the ScFv concentration increased up to 20 μg/ml, then the curve gets saturated. In this assay, the minimum amount of refolded ScFv required to get 50% inhibition of cytotoxicity (IC_{50}) was 15 μg (Fig. 4), whereas, the same percentage of cytotoxic inhibition was achieved with 8 μg of soluble ScFv and 5 μg of positive control [11]. A nonspecific ScFv (anti HCG ScFv) did not show any protective effects. These data suggests that the refolded and purified ScFv is in its active form. The protein aggregation observed in the initial experiments was reduced to a significant level after adding glycerol and GSH in the elution buffer. The sample was centrifuged to remove the aggregates and the supernatant was taken for the assay. In this step, the purified ScFv concentration in the sample would have been reduced leading to the decline in activity of refolded ScFv compared to the soluble fraction.

4. Conclusions

The preliminary studies to assess the possibility for the exploitation of CIM support in matrix-assisted refolding and purification of inclusion bodies was done. CIM supports were found to be a suitable matrix in the refolding experiments with the positive features like fewer steps in the experimental procedure, completion of the entire protocol in less than an hour and easy scale up. Our results shows that monolithic columns could be more efficient compared to soft gel matrices in matrix-assisted refolding and purification of inclusion bodies.

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