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RESEARCH ARTICLE

PERIPLASMIC SECRETION, EXTRACTION AND PURIFICATION OF RECOMBINANT TAGLESS ANTI-TUMOR NECROSIS FACTOR (TNF) ALPHA SINGLE CHAIN VARIABLE FRAGMENT (SCFV) IN *E. COLI*

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ABSTRACT

Present day therapeutics against TNFα have some disadvantages in the form of cost, immunogenicity, etc., resulting in a need for an approach using small antigen binding antibody fragments called scFv (single chain variable fragment). In this study, construct with anti-TNFα scFv was developed and expressed in *E.coli* under optimal conditions of 30°C and 37 µM IPTG. Periplasmic secretion was achieved using 2% glycine and 1% Triton X-100in the culture medium. Addition of 0.5 M sorbitol ensured that the protein was expressed as a soluble form. Secreted protein was purified by immobilized metal affinity chromatography (IMAC) and confirmed by immunoblot.

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INTRODUCTION

E. coli is routinely used as an expression system for the expression of recombinant proteins including antibody fragments such as scFv (Wang *et al*., 2008). It is the most preferred system because it is cost effective, easily manipulated, fast growth cycles and easy large scale production. The disadvantage of as *E. coli* expression system include protein expression as inclusion bodies (Geng *et al*., 2008; Joosten *et al*., 2003; Liu *et al*., 2008; Mukai *et al*., 2006). In our work, we report that we overcome this disadvantage.

TNFα is a pleiotropic cytokine involved in a host of immuno regulatory functions, cell proliferation and differentiation. Monocytes and macrophages are the main producers of TNFα. It's unregulated response may result in patho-physiological conditions such as autoimmune disorders, which include rheumatic arthritis, psoriasis, refractory asthma, hidradenitis suppurativa, Crohn's disease, and ankylosing spondylitis (Feldmann *et al*., 2001). TNFα inhibitors (antibodies and fusion proteins) are used to treat such conditions associated with TNFα (Kavanaugh *et al*., 2009; Maini *et al*., 1999).

However, anti-TNFα therapeutics have been associated with side effects such as lymphomas, a lupus-like syndrome, congested heart failure, auto-antibodies induction, systemic side effects, injection site reactions, and demyelinating disease (Holt *et al*., 2008). These limitations can be minimized by using a genetically engineered scFv lacking Fc domain (Yokota *et al*., 1992).

scFv fragments consist of variable domains of the heavy and light chains linked by a flexible linker. They were described as small fragments capable of retaining the binding activity of the full IgG molecule, albeit in a monovalent fashion (Yokota *et al*., 1992; Bird *et al*., 1988). Liu *et al*. (2008; 2007) has reported expression of tetravalent mini-antibody against TNF (TNF-TeAb) and bivalent scFv against TNFα. A functionally active histidine-tagged scFv against TNFα had been expressed in *E. coli* using pET28a vector (Sushma *et al*., 2011).

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MATERIALS AND METHODS

Plasmid, *E. coli* strains, antigen and antibody, pET-20b(+) vector (Novagen-Cat. # 69739-3) used as the expression vector as it carries an N-terminal *pelB* signal sequence for periplasmic localization. *E. coli* strains, DH5α was used for cloning and maintaining of plasmids while BL21(DE3) was used as the expression host. Purified recombinant human TNFα was purchased from Sigma and mouse antihuman TNFα (MAK-hTNFα) from Strathmann Biotec AG. Pierce ECL Plus Western blotting substrate was also used. Restriction enzymes were purchased from New England Biolabs, USA. Gen Elute Gel extraction kit was purchased from Sigma. BIORAD ChemiDoc Imaging System was used to analyze immunoblots and PerkinElmer multiplate reader with EnSpire software was used for ELISA analyses.

Cloning of scFv in expression vector

Anti TNFα scFv was amplified from pET-28a/anti-TNFα scFv-His (Sushma *et al*., 2011) construct using primers 5'-GCCCATGGGACGCATTCATTCTGCAGCTGTC-3' (forward) and 5'-TGCTCGAGCTAGCCTCGAGGTTTTAT TTCCAGC-3' (reverse with a stop codon incorporated at the 3'end of the gene to avoid histidine tag) in an Eppendorf Master cycler ep gradient and cloned into pET-20b(+) using *NcoI* and *XhoI* restriction enzymes. The amplicon was gel purified using Sigma's Gen Elute Gel extraction kit and cloned into pET-20b(+) using *NcoI* and *Xhol* restriction sites. The construct was confirmed by restriction digestion analysis (*NcoI/XhoI*) and sequencing.

Expression and periplasmic extraction of scFv

The transformed bacterial clone BL21(DE3) selected from LB broth plates with ampicillin (100 µg/ml) was grown in LB broth medium with 100 µg/ml ampicillin at 37°C overnight in a rotatory shaking (300 rpm) incubator. The bacterial cells were pelleted by centrifugation and resuspended in Terrific broth (TB) containing ampicillin (100 µg/ml), IPTG (37 µM), 2% glycine and 1% Triton X-100 to an optical density (OD) at 600 nm of 4.0 for induction of protein expression (Yang *et al*., 1998). Sorbitol (1998) (0.5M) was also included in the expression medium for increased solubility of the recombinant protein (Sandee *et al*., 2005). The expression of the secreted (periplasmic) form of protein anti-TNFα scFv was induced at 30°C for 10 h in a rotatory shaking (300 rpm) incubator. After incubation, the spent media were collected, clarified by centrifugation, stored at 4°C and the cell pellet was subjected to shock treatment with 10mM EDTA (Humphreys *et al*., 1996) and 1mM PMSF in PBS (2% v/v of culture volume), pH 7.4 overnight at 4°C with gentle shaking (50rpm). The extract (periplasmic) was then centrifuged at 18000g, 4°C for 30minutes and the supernatant was stored at 4°C. The cell pellet was lysed with lysis buffer containing lysozyme and the cytoplasmic extract was separated from the pellet, which was resuspended in PBS containing 10% glycerol and 1mM PMSF. Buffer exchange (with PBS, pH 7.4) and sample concentration of the spent medium and the periplasmic extract was done using 10kDa centrifugation filters from Sartorius.

PAGE and immunoblot analyses

Protein concentration was estimated by Bradford's method (Bradford, 1976) and analyzed on 12% reduced sodium dodecyl sulphate (SDS)-poly acrylamide gel electrophoresis (PAGE) (Laemmli, 1970) stained by silver nitrate (Shevchenko, 1996). Spots of periplasmic extracts were made on NC membrane strip, incubated with TNFα, overnight at 4°C with constant but gentle mixing followed by the use of commercially available anti TNFα antibody (MAK-hTNFα) and HRP conjugated anti mouse antibody. Pierce ECL Plus Western blotting substrate was used for detection by chemiluminesence using BIORAD ChemiDoc Imaging System.

Purification of scFv

Purification of anti TNFα scFv from the periplasmic extract was done by IMAC using Novarose-IDA-Ni2+ at a flow rate of 1ml/min. 5mg of crude extract was loaded and elution was done by decreasing the pH. Flow through followed by elutions pH 6.0, 5.0 and 4.0 were collected as 2ml fractions. OD280 was measured and the chromatogram was prepared. The fractions were concentrated using 10kD cutoff centrifugal filters (Sartorius) and quantified by Bradford's method (Bradford, 1976). The fractions were then analyzed on SDS-PAGE (12% reduced gel) stained by silver nitrate. Peak fraction of scFv protein was obtained in pH 5.0 elution fraction.

Confirmation by ELISA

ELISA was done to confirm the scFv protein. The ELISA plate was coated with 50ng purified anti-TNFα scFv in 50µl (1µg/ml) of 0.1M carbonate buffer (pH 9.6) and incubated at 4°C overnight (Liu, 2008). A blank was set with only 0.1M carbonate buffer (pH 9.6) with no scFv being coated. The wells were then washed with 100µl of 1x PBS (pH 7.4) containing 0.05% Tween 20 and blocked with 5% skimmed milk in 1x PBS (pH 7.4) for 2 h at room temperature (RT). The wells were washed and TNFα was added at concentrations of 0.5ng, 5ng, 50ng and 500ng and incubated for 2 h at RT. The wells were washed and 10ng of commercial anti-TNFα antibody (MAK-hTNFα) was added and incubated at RT for 2 h. The wells were washed and HRP conjugated anti-mouse antibody at 1:20000 was added and incubated at RT for 2 h. Pierce ECL Plus Western blotting substrate was used for detection by chemiluminesence using PerkinElmer multiplate reader.

RESULTS

Generation of construct with anti-TNFα scFv

The scFv fragment was cloned into pET-20b(+) using *NcoI* and *XhoI* restriction enzymes. The construct developed was confirmed by restriction digestion with the release of ~770 bp fragment and sequencing.

Protein expression and confirmation

The protein was expressed and extracted from the periplasmic region. The protein extract was analyzed by SDS-PAGE (12% reduced) stained by silver nitrate (Figure 1).

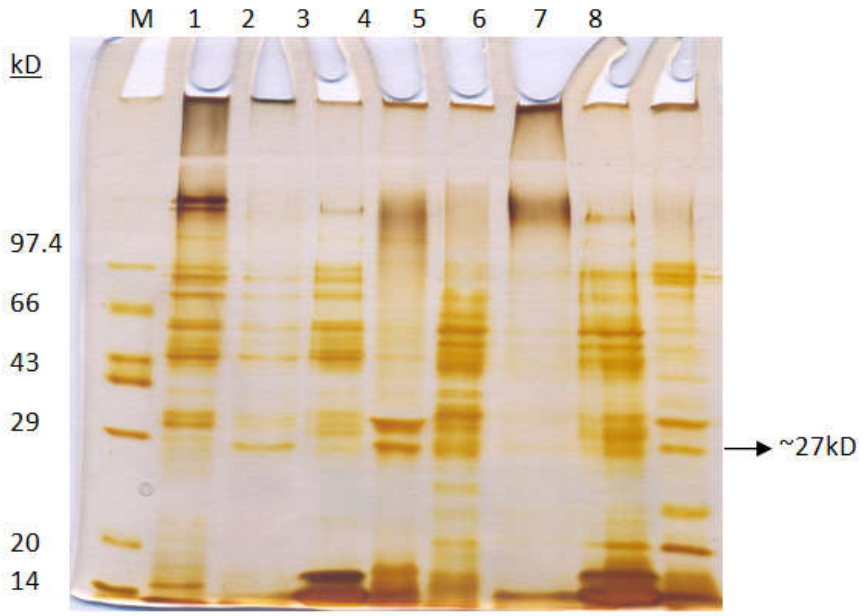

Figure 1. Silver stained SDS-PAGE (12%) gel analysis of extracts of construct induced (1-4) and vector induced (5-8) in the order: secreted, periplasmic, cytoplasmic and pellet fractions

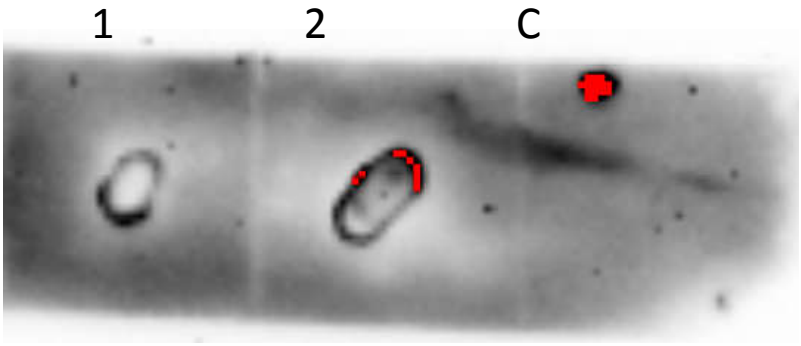

Figure 2. Detection of scFv by chemiluminesence using BIORAD ChemiDoc Imaging System. As compared to negative control (1) that of periplasmic extract of vector induced, presence of scFv protein in the periplasmic extract of construct induced (2) was confirmed. TNFα was used as positive control (C)

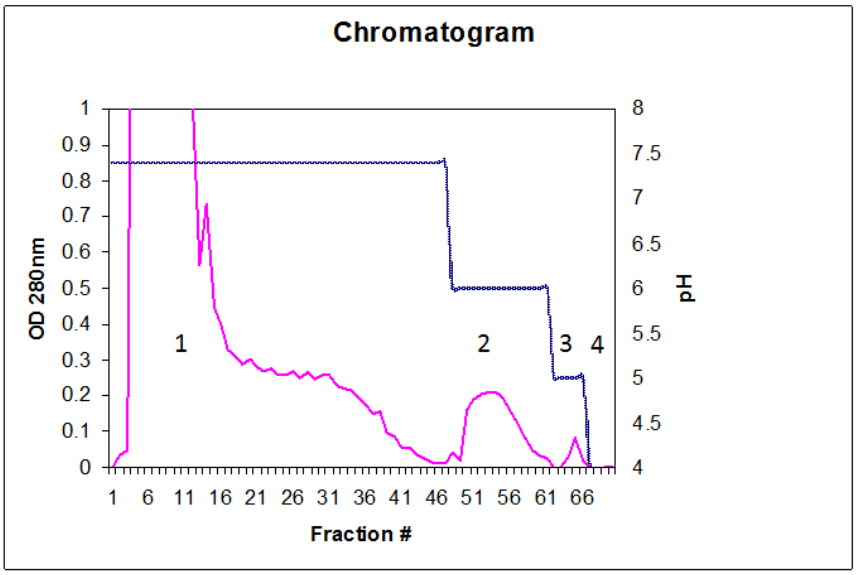

Figure 3a.

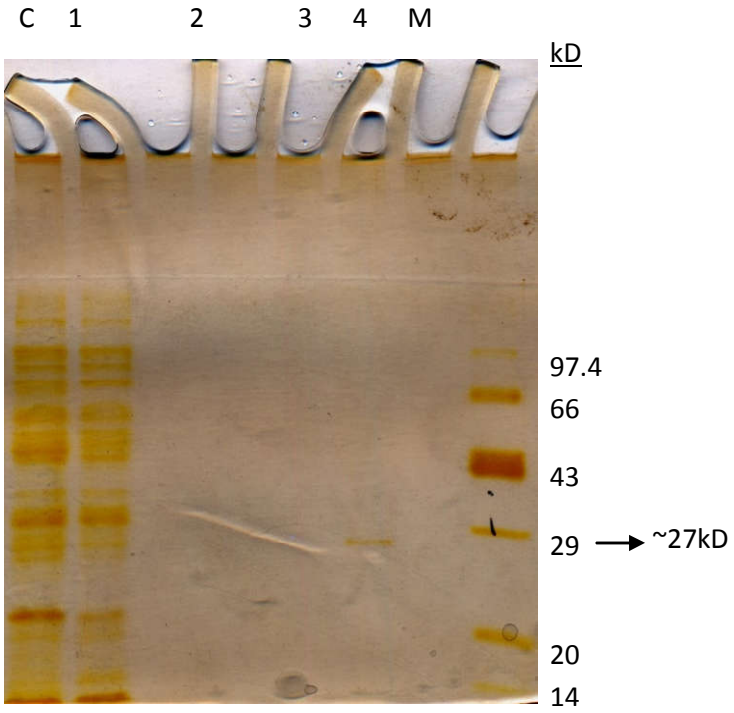

Figure 3b.

Figure 3. Purification and SDS PAGE analysis of soluble form of anti-TNFa-scFv from *E.coli* periplasmic extract. (a) Chromatogram shows elutions and protein profile (b) analyzed by silver stained SDS-PAGE (12% reduced gel) showing crude (C), flow-through (1), pH 6.0 (2), pH 5.0 (3) and pH 4.0 (4) elution fractions. The purified scFv protein ~27kDa was detected in pH 5.0 elution fraction (lane 3)

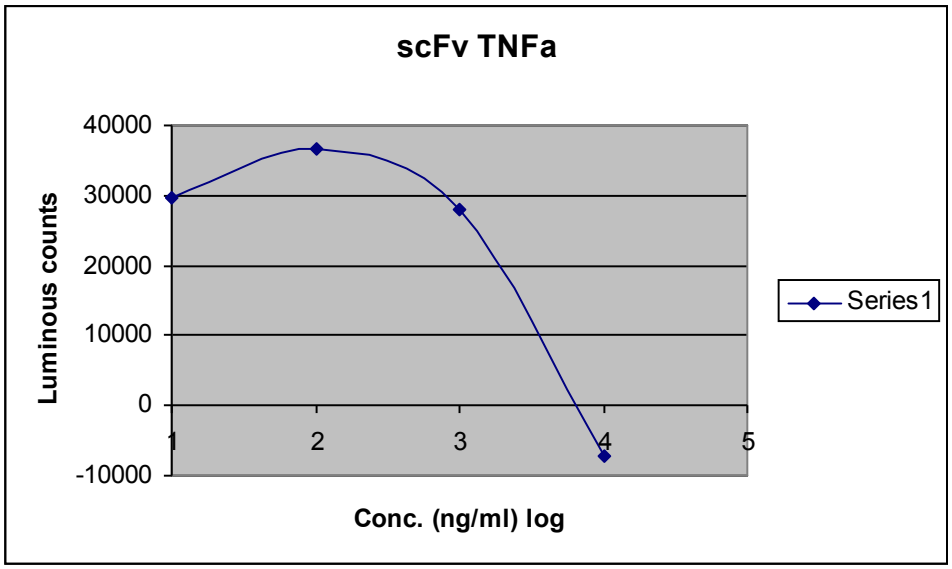

Figure 4. Graphical representation of ELISA showing the interaction between scFv and TNFα

Table 1. ELISA for scFv (50ng/50µl i.e. 1µg/ml) using TNFα and MAK-hTNFα. The blank value in terms of luminous counts was 117315. The luminous counts given are blank corrected

TNFα conc. (ng/50µl)	Representation of TNFα conc. (ng/ml)	Representation of TNFα conc. (ng/ml) log	Luminous counts
0.5	10	1	29575
5	100	2	36610
50	1000	3	28040
500	10000	4	-7345

~27kD band was observed in the periplasmic extract (lane 2) but not in that of periplasmic extract (lane 6) of vector induced sample indicating the expression of protein of interest. This was not observed in other extracts of construct induced as well. The presence of scFv protein in the periplasmic extract was confirmed by dot blot (Figure 2).

Purification of anti TNFα

OD280 of the fractions obtained from IMAC using Novarose-IDA-Ni(II) was measured and the chromatogram (Figure 3a) was prepared. The fractions were then analyzed on a reduced 12% reduced SDS-PAGE (Figure 3b) using silver nitrate. A pure protein band corresponding to ~27kD was observed in pH 5 elution fraction (lane 3, Figure 3b). The pure protein was confirmed by ELISA (Table 1) (Figure 4).

DISCUSSION

We were successful in cloning the gene of interest in pET-20b (+) expression vector and confirmed it by restriction analysis and sequencing. Our results indicate that we were able to express soluble scFv protein and that it was secreted into the periplasmic space. Unlike Yang *et al*. (1998), we did not get the recombinant protein in the spent medium. The addition of 0.5 M sorbitol and its effect on enhanced solubility of recombinant protein has already been proven (Sandee *et al.,* 2005). Previous reports on expression of scFv against TNFα indicate difficulties in expressing the protein in soluble form and have reported protein expression in either inclusion bodies (Geng *et al*., 2008; Liu *et al*., 2008; Chang *et al*., 2007) or partially soluble (40%) (Sushma *et al*., 2011). We have been able to overcome this problem by expressing tagless anti TNFα scFv completely in soluble form secreted in the periplasmic region, which was confirmed by dot blot. We were facing difficulties in detecting scFv by Western blot as we were unable to observe any detectable signal due to the indirect procedure used. So we took the dot blot detection along with ELISA to confirm scFv expression.

Based on the specific interaction of immobilized metal ion on the chromatographic matrix and the protein of interest, IMAC was used to purify the protein. The epoxy groups present in the matrix were coupled to iminodiacetic acid (IDA) to form metal IDA chelates for IMAC. Different metals like Cu(II), Ni(II), Zn(II) and Co(II) can be used for this as each of these metals show very distinct properties and binding under different pH and buffer conditions (Vancan *et al*., 2002; Porath *et al*., 1983; Prasanna *et al*., 2010; Todorova-Balvay *et al*., 2004). Of the 3 histidine amino acids, bioinformatics analysis showed the presence of 2 exposed histidines in the anti TNFα scFv protein. Accordingly, we tried Ni(II) as the ligand, so as to minimize retention of other host proteins and were successful in purifying scFv. We were able to purify the protein by IMAC using Novarose-IDA-Ni(II). The chromatogram of IMAC showed a peak at pH 6.0 elution but we were not able to detect any protein on SDS-PAGE. We assume that most of the proteins in pH 6.0 elution were less than 10000 kDa and hence were not retained by the 10 kDa centrifugal filters during buffer exchange and protein concentration steps. By densitometric scanning, it was estimated that 100ml of culture would yield 32 μg of protein of interest that corresponds to ~8% of periplasmic protein extract.

Conclusion

Our results indicate that we were able to optimize experiments for scFv secretion in the periplasmic region. We believe that it offers to be an attractive, cost effective expression system for the secretion of soluble scFv molecules. Further work on assessing biological activity of anti TNFα needs to be carried out to fully understand the efficacy of the therapeutic protein in *in vivo* models.

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