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Research Article

Production and Purification of a Novel Anti-TNF-α Single Chain Fragment Variable Antibody

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Abstract

Purpose: TNF-α is an inflammatory cytokine with a key role in initiation of inflammatory responses. Anti-TNF-α antibodies are being used in clinic for the purpose of diagnosis and treatment due to their high specificity. The objective of the current study was to express and purify an anti-TNF-α scFv antibody identified by phage display technology.
Methods: The DNA coding sequence of the identified scFv was cloned into pET28a vector and the corresponding protein was expressed as 6×His tagged using *E.coli BL21 (DE3) pLysS* expression system followed by affinity purification on Ni-Sepharose affinity column.
Results: The J44 scFv antibody was cloned into the expression vector and successfully expressed and purified. The purity of the scFv fraction was confirmed using SDS-PAGE analysis. Western blotting technique was used to detect expression of 6×His tagged protein.
Conclusion: In the current study an anti-TNF-α scFv antibody was successfully expressed in bacterial expression system and purified on affinity column. The purified protein can be used in different *in vitro* and *in vivo* experiments in order to elucidate its functionality.

Introduction

The emerge of recombinant protein technology has facilitated the production and purification of proteins. Over the past decades advances in the recombinant protein technology have given rise to the production of hundreds of protein based therapeutics applicable in clinics. In recombinant protein technology, attempts have been focused on setting up an efficient method for protein expression and purification to obtain a biologically active purified protein. Recombinant protein technology starts from cloning of gene, which includes generating the DNA fragment of interest from appropriate source followed by its insertion into a cloning vector, and finally introducing the recombinant DNA molecule into a host cell for protein production.

In many cases, the final goal of cloning is to express and purify the corresponding protein of the cloned DNA fragment. The first crucial step for successful recombinant protein production is the selection of appropriate expression platforms. Various factors such as quantity, purity, biological integrity and potential toxicity of the product are needed to be considered when choosing a suitable expression system.[1] Bacterial expression system, especially *E.coli,* is the first choice for laboratory scale production of recombinant proteins, although it suffers from producing proteins with poor solubility mostly due to lack of post-translational modification and formation of inclusion bodies.[2]

TNF-α is an important inflammatory cytokine, which was firstly identified by Carswell et al. in 1975, as an endotoxin-induced serum factor responsible for necrosis of the tumeric cells.[3] At the physiological levels, TNF-α is involved in maintaining homeostasis by regulating the body's circadian rhythm[4] as well as participating in immune responses,[5] embryonic development,[6] and sleep regulation.[7] Additionally, low levels of TNF-α stimulate fibroblast growth resulting in the remodeling and replacement of injured tissue. In spite of these important physiological roles, elevated amount of TNF-α is implicated in the pathogenesis of various human diseases, such as inflammatory diseases, atherosclerosis, osteoporosis, autoimmune disorders, allograft rejection, and cancer.[8] Because of the important role of TNF-α in pathogenesis of inflammatory diseases, much attention has being dedicated to find novel TNF-α inhibitors with the least side effects and expenses.

In the current investigation, we aimed to use recombinant protein technology in an effort to produce and purify an scFv antibody against TNF-α selected by phage display technology.

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Materials and Methods

Chemicals

Anti M13-HRP conjugated monoclonal antibody was prepared from Sino Biological Inc. (Beijing, P.R. China). Tryptone, yeast extract, Triton X-100, trypsin, potassium acetate, phenylmethylsulfonyl fluoride (PMSF), N,N,N',N'-tetramethylethylenediamine (TEMED), and urea were purchased from AppliChem (Darmstadt, Germany). Ni-Sepharose 4B was prepared from GE Healthcare Life Sciences (Sweden). Sodium azide (NaN3), β-mercaptoethanol, triethylamine (TEA), and methanol were from Merck (Darmstadt, Germany). Primers used in this work were ordered from FAZA Biotech (Tehran, Iran). Acrylamide, N,N'-methylene-bis-acrylamide, and PCR master kit were purchased from CinnaGen (Tehran, Iran). Agarose was from Invitrogen Ltd (Paisley, UK). Gel purification and plasmid mini extraction kits were obtained from Bioneer (South Korea). BM Chemiluminescence Western Blotting kit was purchased from roche Diagnostics GmbH (Mannheim, Germany). Mouse anti-His primary Antibody was prepared from GE Healthcare (Sweden). Goat anti-rabbit IgG-HRP secondary antibody was purchased from Santa Cruz Biotechnology (USA). All chemicals and reagents were of molecular biology grade. Ultra pure water (Milli-Q, Millipore Corporation, Bradford, MA, USA) was used for preparation of all solutions.

Cloning of scFv antibody DNA coding sequence

The expression of the selected scFv was performed using pET28a expression vector. To subclone the scFv coding gene in this vector, two sets of primers were used as indicated in Table 1. A pair of primers was designed for full-length amplification of scFv sequence and another pair of overlapping primers was used to mutate the amber stop codon (TAG) into tyrosine (TAT) in the DNA sequence of the selected scFv.

The designed primers were used to perform two colony PCR reactions on scFv sequence in order to clone scFv coding sequence into pET28a using the following steps. First in separate PCR reactions, F1 and R2, and F2 and R1 pairs of primers were used to perform two PCR reactions on an overnight bacterial sample inoculated by a single colony infected with the phagemid harboring the coding sequence for scFv of interest fused at the N-terminal of pIII minor coat protein. Then the PCR products from the first step were electrophoresed on 1 % agarose gel, extracted from the gel, and were used as the template in the next PCR reaction using F1 and R1 primers. The final product from the third PCR reaction was digested using NdeI and EcoRI restriction enzymes and subsequently was cloned into pET28a vector cut with the same restriction enzymes. The recombinant construct was transformed into *E coli* DH5α and plated on LB agar plates supplemented with kanamycin (50 mg/mL). The overnight grown colonies were used for recombinant construct extraction. Two PCR reactions using F1 and R1 primers as well as pET universal primers were carried out to confirm the appropriate insertion of the amplified coding sequence for the selected scFv into pET28a vector.

Table 1. The primers for performing mutating TAG amber stop into tyrosine

Primers	Primers for full length amplification	Overlapping primers
Forward	5’ CATGCATATGGCCGAGGTGCAGCT 3’ **(F1)**	5' GCTCGGACATATTACGCAGACTCCGTG 3' **(F2)**
Reverse	5’ GAGGAATTCTCACCGTTTGATTTCCACC 3’ **(R1)**	5' CACGGAGTCTGCGTAATATGTCCGAGC 3' **(R2)**

Expression and purification of anti-TNF-α scFv

The constructed scFv-pET28a vector was transformed into *E.coli BL21 (DE3) pLysS* for protein expression. A single colony of *E.coli BL21 (DE3) pLysS* containing the scFv-PET28a was used to inoculate 10 mL LB medium and cultured overnight. The overnight culture was diluted 1:50 in 1 LB medium and incubated at 37 °C while shaking. At OD of 0.6, IPTG with final concentration of 0.4 mM was added and the incubation temperature was set to 20 °C for 16 h. The overnight (O/N) culture was centrifuged at 3,000 *g* for 15 min and then the harvested bacterial pellet was resuspended in lysis buffer (Tris 50 mM pH 8, NaCl 150 mM, Triton 1%, lysozyme 0.1 mg/mL, DNAse 10 µg/mL, β-mercaptoethanol 0.1%, PMSF 1.4 mM). The suspension was sonicated five times on ice at 60% pulse for 30 s with pauses (30 s) to re-establish a low temperature. Bacterial debris was removed by centrifugation at 8,000 *g* for 20 min at 4 °C. The supernatant containing soluble fusion protein was subjected to the affinity chromatography column packed with Ni-Sepharose beads (GE Healthcare) that was pre-equilibrated with lysis buffer (without lysozyme) at 4 °C. After 1 h incubation, the column was washed with five column volumes pre-chilled wash-buffer (Tris 50 mM, NaCl 150 mM, β-Mercaptoethanol 0.1%) in order to remove non-specific proteins. To elute the bound protein, the elution buffer containing imidazole 500 mM, Na H_2PO_4 pH 7.4, and NaCl 500 mM was added on the beads and incubated for 30 min at room temperature with gentle shaking. Finally, the eluate containing 6×His tagged-scFv was recovered from the affinity column. SDS-PAGE analysis was used to visualize protein production and purification. The samples from *E.coli BL21 (DE3) pLysS* bacterial lysates induced for the production of scFv, the soluble fraction, and the purified scFv antibody were separated on 12% SDS–PAGE and transferred electrophoretically to a PVDF membrane in 48 mM Tris–HCl (pH 8.0), 39 mM glycine, 0.037% SDS, and 15% methanol. After overnight blocking with 5% BSA in TBST (10 mM Tris–HCl, 100 mM NaCl, pH 8.0, tween 20 0.05%) at 4 °C, the membrane was incubated with anti-6×His tag

monoclonal antibody for 1.5 h at room temperature. After three washes with TBST, the membrane was incubated with HRP-conjugated goat anti-mouse IgG for 1 h at room temperature. Following the five times washing the membrane using TBST, the bands were visualized using ECL western blotting detection reagent.

Results

Cloning of J44 scFv antibody coding sequence

During the phage display biopanning process against TNF-α using Tomlinson I and J libraries, phage displaying J44 scFv antibody was identified as the strongest TNF-α binder (data not shown). PCR reaction on the J44 scFv DNA coding sequence resulted in a band around 930 indicating that the selected scFv possesses both V_L and V_H (Figure 1). DNA sequencing of the isolated scFv revealed that the selected scFv contains an amber stop codon in its coding sequence, a common problem frequently reported when Tomlinson phage libraries were used for scFv selection against various targets.[9-12]

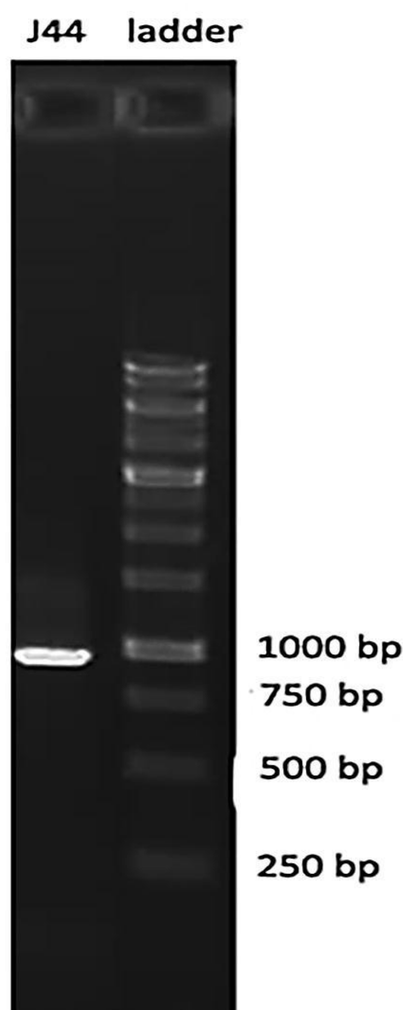

Figure 1. PCR product of J44 DNA coding sequence. PCR reaction on clone harboring phage dispaying J44 scFv using LMB3 (CAGGAAACAGCTATGAC) and pHEN (CTATGCGGCCCCATTCA) primers resulted in a band with the size of 930 bp on agarose gel 1%.

Although the amber codon allows the expression of scFv in suppressor bacterial strains like *E.coli TG1* and *ER2738*, the expression using *E.coli HB2151* (a nonsupressor strain) would be impossible. Therefore, in the case of J44 using overlapping primers the amber stop codon (TAG) was mutated into a codon encoding tyrosine (TAT) (Figure 2). The final PCR product was digested using restriction enzymes and cloned into pET28a vector cut with the same enzymes.

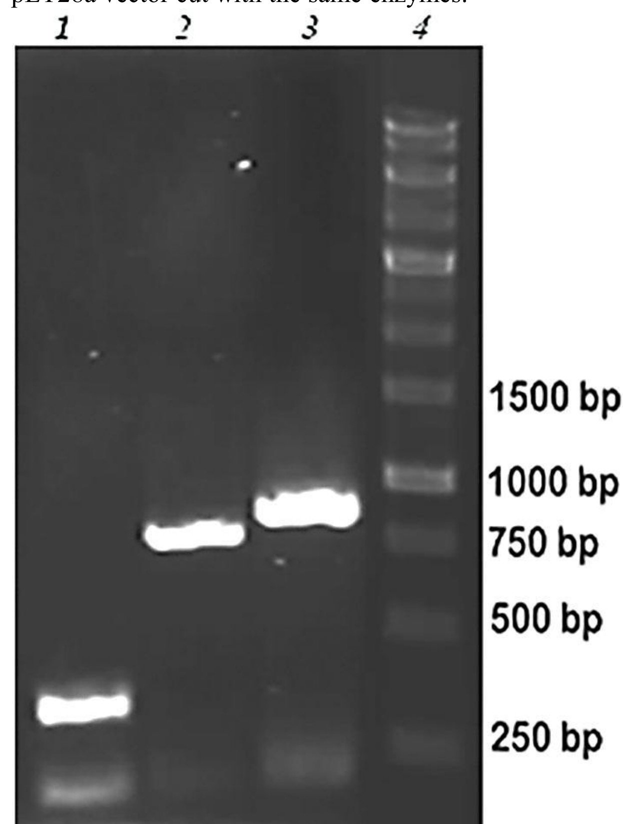

Figure 2. Single nucleotide mutagenesis on the J44 DNA coding sequence. Lane 1 is the PCR product performed on J44 coding sequence using F1 and R2 primers, lane 2 represents the PCR product using F2 and R1 primers conducted on J44 coding sequence, in lane 3 the PCR products from lane 1 and 2 were used as template for performing PCR reaction using F1 and R1 primers.

Expression and purification of J44 anti-TNF-α scFv antibody

The constructed vector was transformed into *BL21 (DE3) pLysS* for protein expression. Figure 3A shows SDS-PAGE analysis of the J44 scFv antibody expression and purification. The band around 28 kDa represents the purified scFv. Moreover, the presence of purified scFv antibody was also confirmed using western blotting technique. Figure 3B demonstrates the result of western blot in which the produced J44 antibody was detected in samples using anti-6×His tag antibody from bacterial cell lysate, soluble fraction, and the purified antibody.

Discussion

Antibodies are biological agents that are widely used as therapeutical agents due to their strong affinity and high specificity towards corresponding antigens. Until now,

several antibodies have been introduced to the market and are still being used for pathological conditions caused by excessive amounts of TNF-α, however, their use has been accompanied with serious side effects.[13-18] For example, immunogenic reactions are common side effects when infliximab is used in order to alleviate the symptoms regarding the elevated amount of TNF-α. Such hypersensitivity reactions are rarely observed in the case of other anti-TNF-α antibodies available in market such as adalimumab, golimumab, etanercept, certolizumab pegol[19] most likely due to the fact that they are fully humanized. Having said that, the humanized full antibodies also have some problems in their own right, such as issues related to their pharmacokinetics and high production costs, therefore, in the current study, we aimed to produce and purify an anti-TNF-α single chain fragment variable antibody isolated from a phage display library originated from non-immunnized human. These formats of antibodies are fully human and are small enough amenable to better pharmacokinetic properties. Sequencing of the coding region corresponding to J44 revealed the presence of an amber stop codon in a position at the CDR2 region. Such problem was also observed in similar studies using Tomlinson I and J libraries.[9-12] Therefore, the amber stop codon in the DNA sequence of J44 was mutated using sewing PCR by a pair of overlapping primers and the produced DNA fragment was cloned into pET28a vector. Using *BL21 (DE3) pLysS* bacterial expression system, J44 scFv antibody was produced as 6×His tagged protein and then was purified by applying Ni-Sepharose affinity column. Western blot immuno assay using anti-His antibody showed the successful expression of J44 scFv antibody (Figure 3).

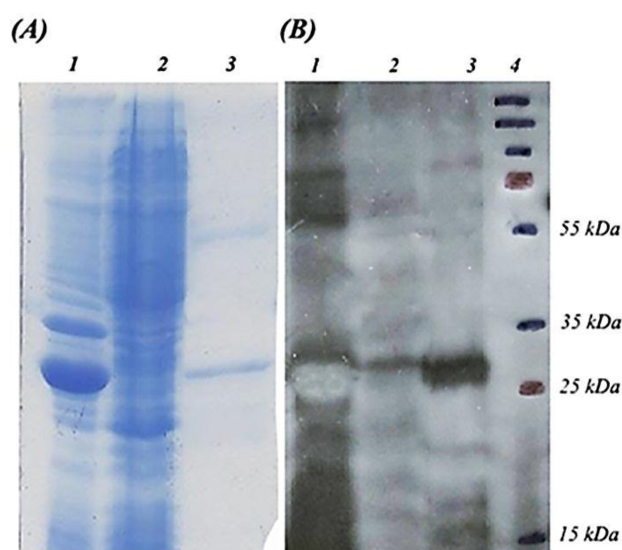

Figure 3. SDS-PAGE and western blot analyses of the produced J44 scFv antibody. The band around 28 kDa represents the produced J44 scFv antibody. In panel (A) lane 1 is the bacterial cell lysate, lane 2 represents the sample from soluble fraction and lane 3 is the sample related to affinity purified J44 scFv antibody. Panel (B) is the western blot analysis of the samples shown in panel A. Lanes 1, 2 , and 3 correspond to the lanes 1, 2, and 3 in panel A. Lane 4 is protein marker.

Production of single chain fragment variable antibodies in *E.coli* is a problematic issue as this format of antibody tends to form inclusion bodies in bacterial cytoplasm.[20-22] In several studies, the produced insoluble inclusion bodies were solublized using denaturing agents and subsequently were refolded by gradually decreasing the concentration of the denaturing agent.[23,24] Yang et al. successfully expressed and purified an anti-TNF-α scFv antibody in *E.coli Bl21 DE3* and evaluated its functionality using ELISA experiment and TNF-α cytotoxicity inhibition assay.[25] However, most of the expressed scFv was in the form of inclusion bodies found in insoluble fraction. Subsequently, they used harsh condition of protein unfoding (solublization) and refolding, which was a demanding work with the possibility of misfolding to occur during such a process.[26] In the current study, the identified anti-TNF-α scFv antibody coding sequence was inserted into pET28a expression vector and the corresponding protein was expressed in *E.coli BL21 (DE3) pLysS*. This bacterial expression system provides an efficient protein expression machinery for any gene under the control of T7 promoter. Furthermore, *E.coli BL21 (DE3) pLysS* contains a pLysS plasmid carrying T7 lysosyme encoding gene which lowers basal expression of target proteins under the control of T7 promoter.[27] As expected, in the present work the amount of J44 scFv antibody observed in bacterial pellet is prominent, however, it was possible to extract pure protein from the soluble fraction of the bacterial cell lysate in appreciable quantity using one-step on-column affinity purification. The presented expression method provides substantial amount of soluble scFv antibody for in-house laboratory uses and can be further refined in order to increase the percentages of the produced soluble form of the protein. More importantly, the purified anti-TNF-α scFv antibody in this work can readily be used in ELISA experiments to detect TNF-α and may show *in vitro* and *in vivo* anti-TNF-α activity.

Conclusion

In summary, the current study focused on expression and purification of an anti-TNF-α scFv antibody isolated previously through biopanning of human antibody libraries against TNF-α using phage display technology. The anti-TNF-α scFv antibody coding sequence was cloned into pET28a expression vector and the corresponding protein was produced in *E.coli BL21 (DE3) pLysS* expression system in research scale quantity. The functionality of the produced anti-TNF-α scFv antibody as well as its mode of interaction with TNF-α needs to be investigated in more details, which would provide an insight into the binding ability of the newly identified scFv antibody to TNF-α.

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Ethical Issues

Not applicable.

Conflict of Interest

The authors report no conflicts of interest.

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