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]

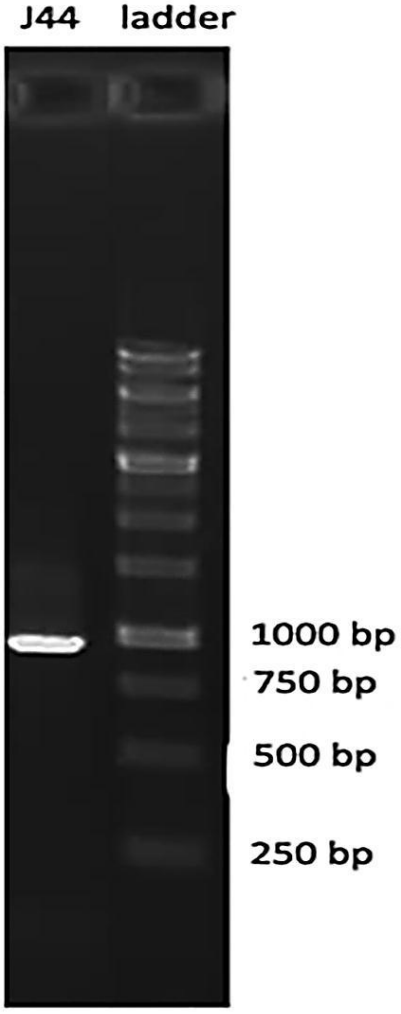

Figure 1. PCR product of J44 DNA coding sequence. PCR reaction on clone harboring phage dispaying J44 scFv using LMB3 (CAGGAAACAGCTATGAC) and pHEN (CTATGCGGCCCCATTCA) primers resulted in a band with the size of 930 bp on agarose gel 1%.

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.

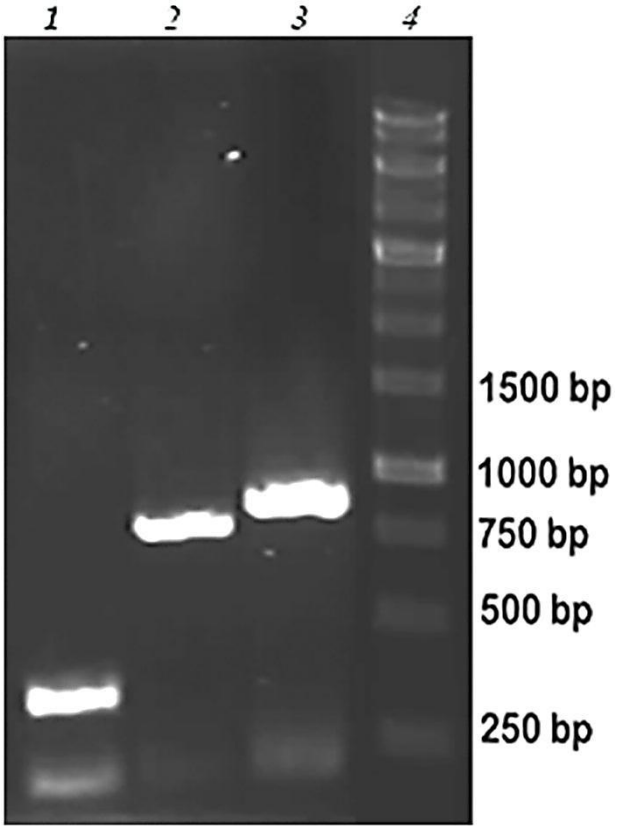

Figure 2. Single nucleotide mutagenesis on the J44 DNA coding sequence. Lane 1 is the PCR product performed on J44 coding sequence using F1 and R2 primers, lane 2 represents the PCR product using F2 and R1 primers conducted on J44 coding sequence, in lane 3 the PCR products from lane 1 and 2 were used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

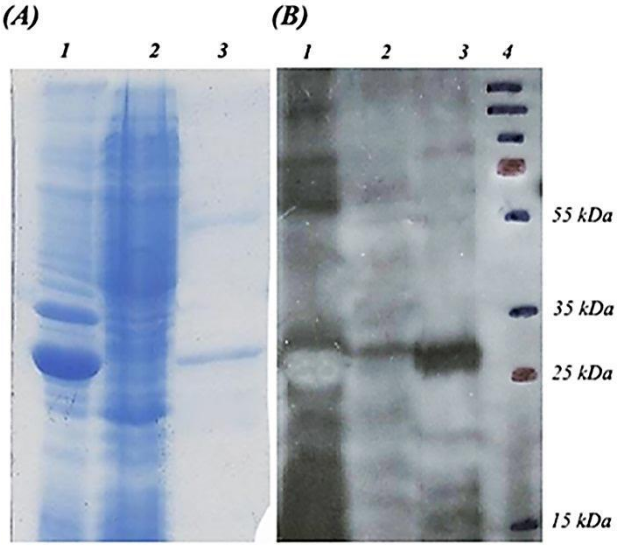

Figure 3. SDS-PAGE and western blot analyses of the produced J44 scFv antibody. The band around 28 kDa represents the produced J44 scFv antibody. In panel (A) lane 1 is the bacterial cell lysate, lane 2 represents the sample from soluble fraction and lane 3 is the sample related to affinity purified 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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