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Cloning and Expression of a Phage Display Selected Single Chain Antibody for CD19

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Abstract

Introduction: CD19 is an important antigen in manners of immunotherapy and B cell development. Studies showed that presence of CD19 is essential for B cell differentiations in various stages of a B lymphocyte. In most B cell associated malignancies, CD19 is expressed in normal to high levels making it a strong marker for targeting malignant B cells. Single chain antibodies are a derivative of antibody which only composed of variable regions of the antibody joined to each other by a polypeptide linker. They have been used for various purposes such as diagnostics, therapy and these act like a targeting part in binding to other molecules. Production of this binding molecules in *E.coli* expression systems have been challenging because of inability of these hosts to correctly fold the recombinant protein. Therefore, expression and purification condition that improve the solubility of scFvs in this expression system may enable us to obtain a higher yield of functional scFvs for in vitro and in vivo application.

Materials and Methods: In this study, we used a pET expression system induced by IPTG in BL21 (DE3) strain to express our phage display derived anti-CD19 scFvs. For this purpose, we cloned and expressed three clones of scFvs selected by soluble panning of a human scFv library against our target (CD19 extracellular domain) in phage display. After selection of the positive colonies, bacterial crude extracts of each colony were prepared and their affinity was checked with ELISA against CD19.

Results: PZ7 was selected for cloning in pET28a vector, expression in BL21 and purification as it had highest affinity in crude extract ELISA. We observed the ~750 bp fragment of scFv after cloning in pET28a vector. Appropriate protein size was checked in SDS-PAGE before and after purification with NI-NTA. A protein band of ~27 kDa was confirmed in SDS-PAGE and western blotting. Furthermore, the sequence analysis showed that scFv PZ7 belonged to the human immunoglobulins family which our scFv library has derived from.

Conclusion: Successful implementation of the pET28a vector enabled efficient cloning and expression of a CD19-specific scFv antibody. The hybrid protein purification method employed demonstrates its potential for diverse protein types.

Keywords: CD19, Cloning, PZ7, pET28a, Phage display, scFv expression.

1. Introduction

CD19 is an important antigen in manners of immunotherapy and B cell development. Studies showed that presence of CD19 is essential for B cell differentiations in various stages of a B lymphocyte [1]. In most B cell associated malignancies, CD19 is expressed in normal to high levels, hence make it a strong marker for targeting malignant B cells[2].

Antibodies are a crucial part in our defense system with the ability of neutralizing objects likes viruses and bacteria and also helping other parts of the immune system to track foreign objects [3]. They act via specific binding to a target. Antibody binding

occurs through an antigen-binding site called paratope to a part in antigen called epitope. Antibodies are composed of constant and variable parts. Antigen binding property is related to the variable part of the antibody which acts like a lock and binds to a key region in the antigens [4]. Different binding molecules have been developed to target CD19 [5]. Zynlonta is a commercial antibody-drug conjugate that targets CD19 as the cell marker [6]. Nevertheless, due to large size of the antibodies and their expensive condition of expression in-vitro, their use in clinical studies has been limited. Improvements in genetic engineering have led to developments of various derivatives of antibodies for diagnosis and treatment of broad spectrum of disease such as cancers and infectious disease and also for drug delivery applications [7].

Single chain variable fragments (scFvs) are the most used derivatives of antibodies and composed only from variable parts of the heavy and light chains of the antibody joined by a polypeptide linker and have the capability of antigen binding. They can easily be engineered in-vitro for an increased affinity and specificity[3]. Different single chain antibodies have been developed for CD19 targeting. These antibody derivatives have some advantages over whole antibodies that makes them good candidate for cancer treatment and diagnosis. Better tumor penetration, easier development protocols and more convenient production methods are some of these advantages[8].

scFvs have an internal disulfide bonds which is important for their correct folding and function. They can be expressed in various expression systems like mammalian, yeast or prokaryotic expression systems. The latter has some advantages over the others. Rapid growth, less expensive production cost and high yield of expression are some reasons of priority of E.coli expression system over others [9–11]. Internal disulfide bonds of scFvs can't be formed in cytoplasm of E.coli and therefore most of the expressed protein precipitates in form of inclusion bodies as insoluble part. At this state, it is possible to refold these proteins to a soluble and functional form [12].

Recently, CD19 targeting CAR T cells have been employed to treat B cell malignancies. CAR T cell therapy is a form of adaptive therapy that uses either autologous or allogeneic tumor infiltrating lymphocytes for targeting cancerous cells. Identifying specific antigens to target tumor cells, is the key step to execute immunotherapy. This study aimed to select and characterize a specific scFv antibody to target CD19 for further development in CAR T cells.

A phage display derived scFv in pET28a expression vector was cloned and expressed in BL21. Purification using hybrid method was then performed and solubility and functionality of our scFv expressed in BL21 (DE3) was investigated.

2. Materials and Methods

Cloning of scFv into pET28a expression vector

After phage ELISA (data not shown), scFv sequence of the clone with highest affinity (named PZ7 afterwards) was amplified from phage display vector (pCOM3X) and cloned into pET28a using NcoI and HindIII restriction sites at 5' and 3' ends, respectively. Ligation product was then transformed into electro-competent BL21 (DE3) by electroporation and colonies were selected on kanamycin (50µg/ml) plate. Positive colonies were confirmed using colony PCR with T7 promoter and scFv reverse primer.

Expression of single chain antibodies in BL21 (DE3)

Among various positive colonies, one of them was selected to be cultured and incubated at 37˚C overnight with 220 rpm shake. Two percent v/v of cultured colony, PZ7, was used to inoculate 10ml LB medium and incubated at 37˚C on a shaker until the absorbance at 600 nm reached between 0.6 and 1. After cooling the culture down on ice, about 0.3 mM IPTG was used for induction of protein expression for 20 h at 16-18˚C with 160 rpm shaking. The culture was then centrifuged at 4˚C for 10 min at 4000 rpm. Pellets were dissolved in protein purification buffer (NaH2Po4 50mM, NaCl 500 mM, PMSF 1 mM) and incubated on ice for 1 h. Cells were then lysed by 3×1 min sonication on ice with 1 min intervals. Lysed cells were then centrifuged at 12000 rpm for 20 min at 4˚C. The supernatants from each colony was electrophoresed under denaturing condition on a 12.5% SDS PAGE to see if proteins were expressed at the correct size. It was also used for crude extract ELISA against CD19 ECD and then scFv was subjected to purification with a Ni-NTA affinity column.

ELISA

ELISA method was used to assess the affinity of crude extract of PZ7. In microtiter plates, 0.5 µg/ml of CD19 ECD (G&P Bioscience) was coated and incubated at 4˚C, overnight to block nonspecific regions, 300 µl/well of 3% skim milk in PBS was used at 37˚C for two hours. Then, wells were fully washed with PBS. Bacterial crude extract with 100 µl/well concentration was diluted at 1, 1/10, 1/100 and 1/1000 rates and was added and incubated at 4˚C, overnight. Three washing steps

with PBS were used for removing unbound proteins. 100 µl of HRP-conjugated anti-his tag antibody (1/5000 dilution in PBS) was added to each well and was incubated for 1h at 37˚C. PBS was used to wash the wells three times. 100 µl of TMB substrate was added to each well and incubated in dark at 37˚C for 15 to 30 min. 100 µl/well of 2M H2SO4 was added to each well and the absorbance was read at 450 nm.

scFv purification and SDS PAGE analysis

PZ7 in ELISA (named PZ7) was purified using Ni-NTA affinity column using hybrid purification method. Briefly, bacterial pellet was dissolved in appropriate volume (6ml to the pellet of 100 ml expression medium) of binding buffer (NaCl 500mM, H2PO4 20Mm) containing 6 M urea. It was incubated on ice for 1 h followed by 3×1 min sonication and centrifugation at 4˚C for 20 min. Supernatant were mixed with Ni-NTA resin and incubated 2 h at 4˚C with gentle shaking. Then, it was packed on a manually made colum. Then column was washed with 5 CV (Column volume) of binding buffer containing different concentration of imidazole (10, 20, 30, 40, 50 mM) and eluted with binding buffer with 250 and 500 mM imidazole and fractions were collected to evaluate protein concentration using Bradford assay. Purified protein was electrophoresed under denaturing condition on a 12% SDS PAGE.

Immunoblotting

Western blot was applied to detect his-tag in purified scFv colony Purified PZ7 was denatured under 12% SDS PAGE condition and then a nitrocellulose membrane, wet transfer system at 300mA for 2.5 hours. Three percent skim milk in PBS was used to block the membrane at RT for 1 hour with shaking followed by a washing with TBST. Membrane was then incubated overnight with HRP-conjugated anti-his tag antibody at a concentration of 1/7000 at 4°C. After three times washing with TBST, blots were developed by enhanced chemiluminescence (ECL) and a Western blot imaging system.

Alignment and sequence analysis of PZ7 single chain antibody

Plasmid containing PZ7 was sequenced by Pishgam co. and DNA sequence of PZ7 was aligned in NCBI BLAST tools to confirm the sequence. Then, it was translated to protein using EXPASY translation tools. Location of CDR regions of the antibody was determined using online IgG BLAST and IMGT database [13].

3. Results

Cloning of scFv fragments after phage display

PZ7 sequence amplified from phage vector using appropriate primers had a length of ~750 bp (Figure. 1 lane 1). After ligation into pET28a expression vector and transformation into BL21, one of the positive colonies with the appropriate length of ~900 bp (Figure. 2), amplified by T7 promoter and scFv-Rev primers, were selected for protein expression.

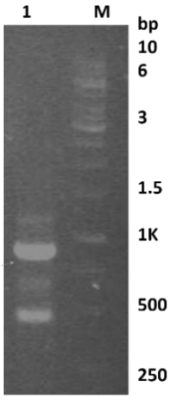

Figure. 1. Agarose gel electrophoresis of amplified PZ7. Lane 1: PCR product. Lane M: DNA marker 1kbp. The approximate 750 bp bp DNA fragments is the scFv size we expected

Protein expression and SDS-PAGE analysis

SDS-PAGE analysis of PZ7 clone showed the scFv size of ~33 kDa (Figure. 4). We expressed proteins of some other positive clones (Data not shown). Some of them had protein in correct size but some had lower molecular weight that possibly was due to immature stop codon or mutation in the PCRs.

ELISA

CD19 binding affinity of crude extracts of PZ7 with different concentration showed that PZ7 clone had concentration dependent binding affinity for CD19 ECD which indicate the specificity of CD19-PZ for each other. It was also shown after purification of PZ7 (Figure. 3).

Protein purification and immunoblotting

PZ7 protein was purified by Ni-NTA column (Figure. 4A) and confirmed by Western blotting using HRP-conjugated anti-his tag antibody (Figure. 4B). A band of around 33 kDa showed the correct size of the purified protein.

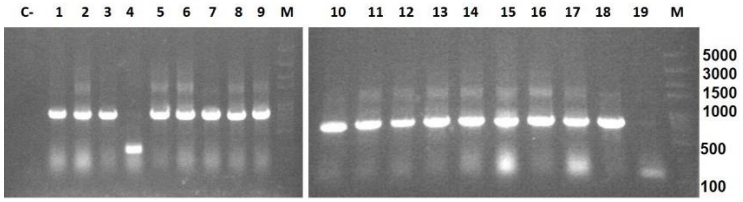

Figure. 2. Agarose gel electrophoresis of scFvs cloned in pET28a. PCR was carried out on single clones after cloning into pET28a. Lane c: negative control, lane 1 to 19: randomly selected clones from number 1 to 19. Lane M: DNA marker 100 bp. The approximate 900 bp DNA fragments confirms presence of PZ7 fragment as amplified by scFv. Some positive clones were selected for protein expression and further analysis

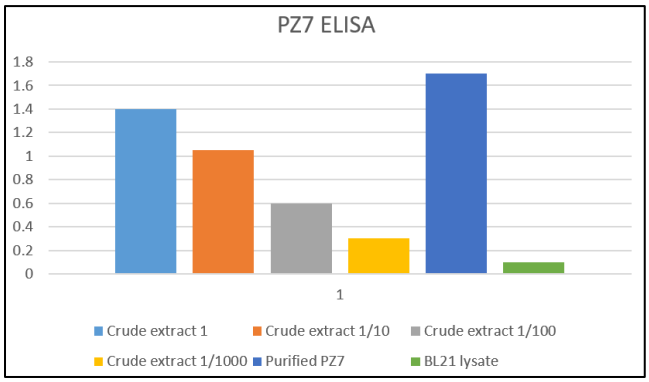

Figure 3. ELISA analysis of anti- CD19 scFv clone 7 (PZ7) expressed in BL21(DE3): soluble bacterial lysates with four different concentrations was used in ELISA. BL21 lysate was used as negative control and a His-tagged protein was used as a positive control. Purified PZ7 showed an absorbance of about 1.7 in ELISA

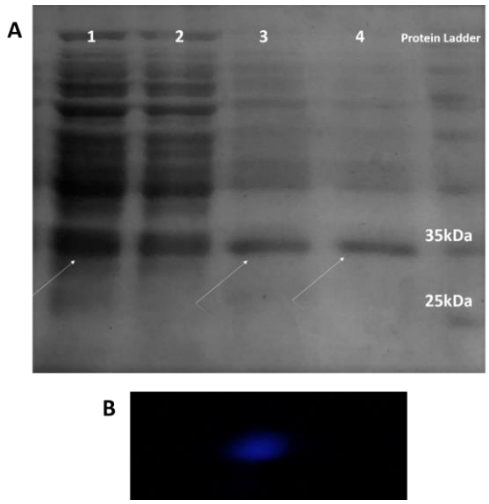

Figure 4. SDS-PAGE and western blot analysis of scFv PZ7 selected after second round of selection by phage display. A) Lane M:protein size marker. Lane 1: bacterial lysates of scFvs clone PZ7 induced by 0.3 mM IPTG in BL21. (scFv of ~33 kDa was shown by arrow). Lane 2: Ni-NTA affinity column flow through of the bacterial lysate (His-tagged protein was bound to the column). Lane 3: Fraction 1 of elution 250 mM of purified PZ7 by Ni-NTA affinity column. Lane 4: Fraction 2 of elution 250 mM of purified PZ7 by Ni-NTA affinity column. B) western blot analysis of PZ7 visualized by HRP conjugated anti-his tag antibody

Alignment and sequence analysis of PZ7 single chain antibody

Protein sequence of Heavy and light chain of PZ7 were submitted to IMGT/VQ server. BLAST analysis of heavy and light chain of PZ7 using NCBI BLAST tools showed the accuracy of sequencing result (Fig. 4). Complementary determining regions (CDRs) of heavy and light chain of scFv PZ7 protein sequence is summarized in table 1.

Table 1. Prediction of the hypervariable regions (CDR) of the scFv antibody domains using IMGT/VQ server

Region		Protein Sequence
Heavy chain	CDR1	GFNIKDTY
	CDR2	IDPANGNT
	CDR3	ARAGMENDYWYFDV
Light chain	CDR1	SSINSNY
	CDR2	RTS
	CDR3	QQGGSIPFT

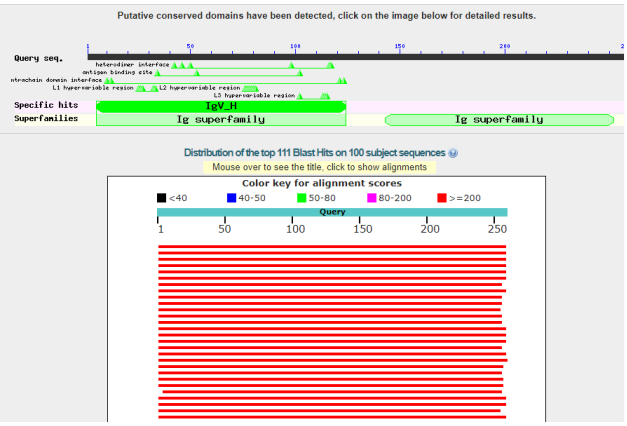

Figure. 5. Results of BLAST of scFv PZ7 in NCBI BLAST tools

4. Discussion

Bacteria are widely utilized in research for the expression of recombinant proteins, including antibodies. Expression vectors designed for bacterial systems enable high levels of protein production and offer control over the correct folding of the recombinant protein. However, the fast pace of protein production in bacteria often leads to the accumulation of proteins in insoluble aggregates known as inclusion bodies. This issue is particularly challenging during the expression of single-chain variable fragments (scFvs), where proper folding is crucial for their functionality. To address this problem, optimization of temperature conditions, IPTG concentration, and expression time becomes necessary to obtain recombinant proteins with the correct folding.

In our study, we employed the pET28a expression vector to produce our target protein and utilized a hybrid denaturation purification method to facilitate protein refolding. To enhance proper folding, we implemented lower concentrations of IPTG, reduced expression temperature, and extended the expression time, allowing for sufficient time for the protein to fold correctly. By expressing the scFv at a low temperature (16-18°C) for an extended period (18-20 hours) with a low concentration of IPTG (less than 0.5 mM) and moderate aeration conditions, we successfully obtained a soluble and functional scFv in BL21 (DE3).

Although the majority of the expressed protein accumulated in inclusion bodies, we implemented a hybrid purification method that enabled the isolation of functional scFv from the soluble fraction of the cell lysate. Furthermore, we examined the insoluble fraction to determine the extent of protein aggregation in the inclusion bodies. The hybrid denaturation method of purification, as demonstrated in our ELISA results, allowed us to obtain a functional scFv from the inclusion bodies, effectively recovering and refolding the protein.

In conclusion, our study highlights the feasibility of obtaining soluble and functional scFv in BL21 (DE3) through the optimization of temperature, IPTG concentration, and expression time. By implementing a hybrid denaturation purification method, we successfully isolated the scFv from the soluble fraction, overcoming the challenge of protein aggregation in inclusion bodies. These findings contribute to the development of strategies for the expression and purification of recombinant proteins, particularly scFvs, in bacterial systems, thereby expanding their potential applications in various research fields.

5. Conclusion

Recombinant protein expression is a process where obtaining a protein with the correct folding is of utmost importance. In this study, we demonstrated that our CD19-specific scFv possesses the capability of on-column refolding, indicating its ability to maintain its binding capacity. This finding suggests that the hybrid protein purification method employed in this research can be effectively utilized for a wide range of protein types.

In conclusion, this study demonstrates the successful implementation of the pET28a vector for cloning and expression of a single-chain variable fragment (scFv) antibody against CD19 in Escherichia coli BL21 (DE3). The utilization of the pET28a vector provided an efficient platform for gene cloning, offering tight control over gene expression and enabling the incorporation of a His-tag for facile purification. E. coli BL21 (DE3) exhibited remarkable compatibility as a host system, yielding high expression levels of the scFv antibody. The combination of the pET28a vector and E. coli BL21 (DE3) not only streamlined the

production process but also ensured scalability and cost-effectiveness.

Ethical Considerations

Compliance with ethical guidelines

This research is approved as a part of PhD dissertation program by medical school scientific committee of Tarbiat Modares University.

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Abbreviations

CD19: Cluster Differentiation 1, scFv: Single Chain Variable Fragment, PZ: CD19 binder scFv Payam Zandi (initials of the author).

Author's contributions

All authors equally contributed to preparing this article.

Conflict of interest

The Authors declare that there is no conflict of interest.

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