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EXTRACTION AND PURIFICATION OF RECOMBINANT SINGLE CHAIN ANTIBODY RECOGNIZING BLOOD TYPE A ANTIGEN

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ABSTRACT

In our previous study, we reported the expression of a recombinant single chain fragment variable (scFv) antibody that recognized blood type A antigen (antiA-scFv) in *E. coli*. When it was expressed as it is alone, antiA-scFv was produced as inclusion body. In contrast, SM/antiA-scFv was synthesized in soluble form when it was fused to small ubiquitin modifier (SUMO). Here, we present the extraction and purification of antiA-scFv in the inclusion body as well as in the soluble form and evaluate the antiA-scFv antibody activity. The results show that only fusion expression of soluble SM/antiA-scFv has biological activity of the antibody. SM/antiA-scFv was separated by fractional precipitation with 20% ammonium sulfate, and then washed with buffers to collect the pure antiA-scFv with SUMOprotease treatment. The purity of recombinant antibody was 89% and the yield of 64.9 mg/L of bacterial culture. The antibody has a polymer structure and could bind to purified antigen as well as agglutinate with red blood cell, but the specificity of the antibody was not good enough for the antigen and red blood cell of blood type A. This is the first report in Vietnam showing the extraction and purification of the recombinant single chain antibody recognizing antigen of ABO system using *E. coli* expression system. It can be considered as a reference for further studies to improve the specificity of recombinant antibody antiA-scFv to identify ABO-type blood antigens.

Keywords: *Escherichia coli*, antiA-scFv, blood type A, purification, single chain antibody.

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INTRODUCTION

The production of antibodies by hybridoma technology has been successfully applied in many areas of research, medical diagnostic and therapeutic applications such as in treatment of autoimmune diseases, infectious diseases and oncological diseases (Frenzel et al., 2013). However, in many cases, pure antigen is not available to induce immunity, especially with surface antigens or membrane protein antigens. These antigens are easy to lose their structures during purification process. Besides, hybridoma technology also has several limitations in cell-cell fusion mechanisms so that the fused hybrid cells (hybridomas) used in antibody production are unsustainable. Moreover, the production of monoclonal antibodies using hybridoma technology is very labourious and costly due to high-cost culture media for animal cells, strictly controlled cell culture conditions as well as storage conditions.

With the development of recombinant protein technology, single chain fragment variable (scFv) recombinant antibody, one of the most popular types of recombinant antibodies, is easily expressed in a functional form in *E. coli* (Ahmad et al., 2012; Spadiut et al., 2014). *E. coli* expression system is the most commonly used economical expression system because of its simple structure, well-known genetic background, high yield of target protein and its short generation time. Furthermore, scFv can also be genetically modified to enhance desirable properties such as affinity and specificity (Song et al., 2014).

However, the insoluble inclusion body formation of scFvs expressed in *E. coli* which often leads to low binding activity, unstable structure and toxic effect to host cells, is a significant obstacle. Another concern is the inability of bacteria to carry out eukaryotic post-translational modifications (PTMs) which is required by protein to fold and is therefore not suitable when glycosylation of antibody fragments or the fusion protein is required.

A variety of approaches to increase the expression and the proper folding as well as solubility of desired protein have been developed: (1) changing the vector, (2) changing the host strain, (3) adding of some chemicals during the induction, or (4) co-expression with other genes, (5) changing the gene sequences without changing the functional domain of protein.

Recombinant protein expressed intracellularly in the reduced environment of cytoplasm frequently forms in a insoluble inclusion bodies lacking biological activity (Wörn et al., 2000). Strategies to solubilize inclusion bodies under the presence of denaturing agents, followed by the refolding of the protein to regain function are not always successful. However, if a secretion vector is used, they can form in the periplasmic space which is advantageous in terms of protein folding and solubility. The antigen-binding fragment of an antibody was expressed as a fully functional and stable protein in *E. coli* in the oxidized periplasm that contributed to the correct formation of the intramolecular disulfide bonds and the hetero-association of the variable domains (Skerra & Plückthun, 1988). On the other hand, cysteine-free mutant antibody scFv lacking the conserved disulfide bonds could be expressed in a stable and functional form in the *E. coli* cytoplasm (Proba et al., 1998). Moreover, mutation of genes coding glutathione and thioredoxin reductase in host strains and co-expression of chaperones such as GroEL/ES, DnaK/J, DsbC, Skp, GroES/L, peptidyl prolyl-cis, transisomerase FkPa were applied to improve functional production of recombinant proteins (Bothmann & Plückthun, 2000; Friedrich et al., 2010).

MATERIALS AND METHODS

E. coli strains expressing recombinant protein antiA-scFv and protein SM/antiA-scFv generated from our previous study was used in this research (Dang et al., 2017).

The following reagents, chemicals, antibodies were also used in this study: ammonium persulfate (APS), N,N,N',N'-Tetramethyl-ethylenediamine (TEMED), glycerol, glycine, ethanol, methanol, SDS,

Tris, acrylamide, bis-acrylamide, coomassie, amonium sulfate (Merck, Germany); skimmilk (Difco, USA); ampiciline, 3,3′,5,5′-tetramethylbenzidine (TMB), ethylene glycol bis(succinimidyl succinate (EGS), ficin (Sigma, USA); Blood group A-BSA, B-BSA, BSA (Dextra, UK); red blood cells (National Institute of Hematology and Blood Transfusion, Vietnam); mouse monoclonal antibody against c-myc 1 mg/ml, peoxidase-labelled anti-mouse IgG **(**Sigma, USA).

Extraction of recombinant antiA-scFv from *E. coli*

After fermentation, the recombinant *E. coli* cells were harvested by centrifugation at 5,000 rpm for 10 min and resuspended in 20 mM Tris HCl, pH=8 to reach an optical density (OD_{600nm}) of 10. The cells were lysed by sonication on ice for 10 min at the frequency of 20 kHz. After sonication, the pellet was separated from the supernatant by centrifugation at 8,000 rpm in 10 min and subsequently resuspended in a equivalent volume in 20 mM Tris HCl, pH=8. Proteins in soluble and insoluble fractions were both examined by SDS-PAGE 12.6% (Laemmli 1970).

Denaturing purification of recombinant antiA-scFv

The inclusion bodies of recombinant antiA-scFv in 50 ml cell lysate were pelleted by centrifugation. The pelleted protein was solubilized in 15 ml of denaturing buffer, 6 M Guanidine-HCl. Residual insoluble matter was removed by centrifugation at 8,000 rpm for 10 min. The supernatant was collected and then loaded to the affinity chromatography column along with binding buffer (20 mM sodium phosphate; 0.5 M NaCl, 5 mM imidazol; 6 M GuHCl, pH=8). The non-binding proteins were washed with 10 column volume (CV) of binding buffer. The weakly bound proteins were washed with 10 CV of washing buffer (20 mM sodium phosphate; 0.5 M NaCl, 50 mM imidazol; 6 M GuHCl, pH=8). The bound proteins were eluted from the column in 2-ml fractions with elution buffer (20 mM sodium phosphate; 0.5 M NaCl; 400 mM imidazol; 6 M GuHCl; pH=8). The protein concentration in load, flow-through, wash and eluted fractions were determined by nanodrop. The refolding of eluted protein was performed using different buffer systems and its activity was checked.

Purification of soluble recombinant antiA-scFv

The antiA-scFv fused with SUMO (SM/antiAscFv) was expressed successfully in a soluble form (Dang et al., 2018) and the fusion protein was subsequently purified using Ni Sepharose affinity matrix to purify histidine-tagged protein. However, SM/antiA-scFv was stuck on the resin and was not eluted from the chromatography column even with 1 M imidazole. Thus, we had to change the purification strategy.

To purify SM/antiA-scFv by ammonium sulfate precipitation, 15% w/v $(NH_4)_2SO_4$ was added to the solution containing total soluble protein at 4^{o}C. After incubation at 4^{o}C for 30 min, the solution was centrifuged and both pellet and supernatant were collected. $(NH_4)_2SO_4$ was continuously added to the supernatant at the final concentration of 20% w/v to further precipitate protein containing SM/antiA-scFv. The precipitate was collected by centrifugation and washed with 20 mM Tris-HCl pH 8.

Cleavage of SUMO from SM/antiA-scFv by SUMO protease: The insoluble SM/antiA-scFv obtained after precipitation was cleaved with 0.025 U of SUMO protease at 30^{o}C for 3 hr (One enzyme unit will cut 100 µg substrate at the enzyme activity of 3,333 U/mg) in PBS pH 7.4 containing 2 mM DTT. After cleavage, the mixture was centrifuged at 8,000 rpm in 10 min. The supernatant was discarded and the pellet containing insoluble antiA-scFv was obtained.

To solubilize antiA-scFv pellet, insoluble antiA-scFv was washed with PBS pH 7.4 with 0.02% Tween-20 and 1% Triton X-100 and then solubilized in buffer containing 5% glycerol, 71.5 mM mercaptoethanol and 0.05% SDS. The solution was centrifuged at

8,000 rpm for 10 min to remove any remaining debris and collect the supernatant containing solubilized antiA-scFv. Then, antibody solution was loaded into a dialysis bag with a membrane molecular weight cut-off of 3 kDa and dialysed against PBS pH 7.4 with 5% glycerol. The concentration of soluble antiA-scFv was determined using a Nanodrop Spectro-photometer at 280 nm.

The purity of the product was evaluated by SDS-PAGE using Quantity One software (Biorad, UK). The bioactivity of recombinant antiA-scFv was assessed by ELISA using pure blood antigens and by the hemagglutination test using red blood cells.

Western blot analysis

Following SDS-PAGE, protein was transferred from gel onto PVDF blotting membrane at 15–20 V for 15 min using the Trans-blot Semi-dry system (Biorad, UK). Protein scFv was detected by Western blot using monoclonal antibody against C-myc (Dang et al., 2017). Briefly, membrane was incubated with 1,000-fold diluted primary antibody (antibody against C-myc) in 10 ml of 5% skimmed milk for 1 hr and then with 5,000-fold diluted secondary antibody (anti mouse IgG-peroxidase) in 10 ml 5% skimmed milk for another 1 hr. The detection was carried out by adding TMB substrate.

Enzyme-linked immunosorbent assay (ELISA)

100 µl each of antigen A/BSA, antigen B/BSA, and BSA (at concentration of 5 µg/ml in coating buffer) was added to each well of a flat bottom 96-well ELISA microtiter plate and incubated the plate overnight at 4^{o}C. After incubation, the solution was removed and the plated was washed with 200 µl wash buffer per well. Then 200 µl of blocking buffer was added to each well and the plate was incubated at room temperature (RT) for 30 min. The wells were washed 3 times with 200 µl wash buffer and 100 µl antiA-scFv (25 µg) was added to each well and incubated at RT for 60 min. The wells were washed 3 times with 200 µl wash buffer, and the conjugated secondary antibody (anti c-Myc antibody diluted 1000 times from stock 1 mg/ml) was added to each well and the plate was incubated at RT for 60 min. The solution was removed and the plate was washed 3 times. The 5000-fold diluted conjugated third antibody (anti-mouse IgG-peoxidase) was added to each well and the plate was incubated at RT for 60 min. The solution was removed and the plate was washed 3 times. The substrate solution was prepared by mixing acetate buffer, TMB and H_2O_2 and added to each well and incubated at RT within 5–30 min for colouring. The reaction was stopped by adding 100 µl of 2 M H_2SO_4 per well. The absorbance was measured at 450 nm.

Hemagglutination assay

A round-bottomed 96-well plate is preferred for this assay. To each well, 50 µl PBS pH 7.4 was added, then 50 µl of recombinant antiA-scFv solution at 0.5 mg/ml concentration was pipetted into the first column and serial two fold dilution of the recombinant protein was prepared. Then, 5 µl of 5% red blood cells was added to each well (type A: first row, type B: second row, type O: third row) and the plate was mixed gently. Negative control was PBS pH 7.4 without adding any type of blood cell. The plate was left at RT for 1 hr then the end=point of hemagglutination was visually determined. The antibody antiA-scFv being treated with 1 mM EGS at 25^{o}C for 30 min was also tested for its hemagglutination ability. Moreover, the hemagglutination test using ficin-treated red blood cells was also performed. For this, red blood cells type A (5%) was centrifuged at 4,000 rpm for 5 min and the supernatant was discarded. The red cells were washed 3 times with PBS pH 7,4 and then incubated with 0.1% ficin at 37^{o}C for 15-30 min. The mixture was centrifuged at 4,000 rpm for 5 min and the supernatant was discarded. The red cells were washed 3 times with PBS pH 7.4 and resuspended in the equivalent volume of PBS pH 7.4 to reach the prior concentration of 5%.

RESULTS

Purification and refolding of antiA-scFv

In the previous publication, we reported the result of production of antiA-scFv in *E. coli* using vector pET22b(+) as an expression vector (Dang et al., 2017). As the protein was expressed in the inclusion body form, the strategy for handling this protein including isolation of inclusion bodies, solubilization and refolding was necessary.

6M GuHCl was used to denature the insoluble antiA-scFv. The solubilised protein was then purified in denaturation condition using affinity chromatography (as protein was designed histidine-tagged). As shown in the chromatogram, the elution step at 400 mM imidazole produced one high peak. In the flow-through and wash steps, however, several minor peaks were observed which could be related to non-binding and non-specific binding proteins (Fig. 1a). Protein concentrations in each phase of chromatography as well as in the starting material (before loading to the column) were quantified by Nanodrop and the results were shown in Table 1. The elution fractions (E1-E7) contained the greatest amount of protein. Total amount of protein obtained in the elution step was 11.97 mg, equivalent to approximately 60% of the protein loaded on the column. The third elution fraction had the highest protein concentration of 2.4 mg/ml.

Table 1. Amount of protein in chromatography fractions

Phases of affinity chromatography		Protein concentration (mg/ml)	Volume (ml)	Total protein (mg)
Input sample before loading to column (TS)		1.34	15	20.10
Flow-through fraction (F)		0.22	16	3.52
Wash fraction		0.08	50	4.0
Elution fraction	E1	0.21	2	11.97
	E2	1.29	2	
	E3	2.40	2	
	E4	1.24	2	
	E5	0.51	2	
	E6	0.22	2	
	E7	0.11	2	

Based on SDS-PAGE analysis (Fig. 1b), the non-specifically bound proteins were removed during flow-through and wash fractions. Meanwhile, the target protein, antiA-svFv, bound efficiently to the resin and was collected only at the elution step with 400 nM imidazol. AntiA-scFv was the predominant protein fraction in the elution fractions 2, 3 and 4 (E2-4), consistent with the Nanodrop results. Thus, we concluded that the purification of antiA-scFv under denaturing condition was successful.

In order to regain biological fuction, after denaturing and purification, the refolding of antiA-scFv was performed by dialysing against buffer consisting of 50 mM Tris pH8, 8 mM KCl, 400 mM L-arginine, 2 mM GSH, 0.4 mM GSSG, 1mM EDTA to remove denaturing agents and allow the formation of the correct intramolecular associations. Refolded protein was incubated with EGS, an agent allowing proteins to be trimeric by chemical cross-linking. However, the refolded protein was still not active in hemagglutination test (data not shown), which means the recombinant antiA-scFv was produced without bioactivity.

Therefore, modifications in expression system aiming at enhancement of the soluble expression were considered. One of them was the use of SUMO fusion protein expression system.

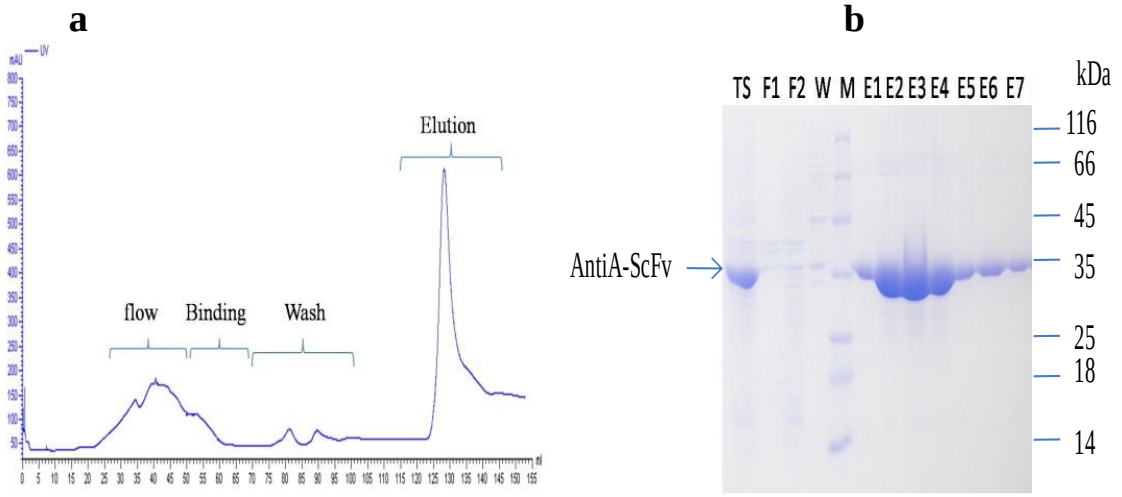


Figure 1. Affinity purification of recombinant antiA-scFv. (a). Chromatogram. Flow: the unbound proteins were removed when loading sample to the column; Binding: the unbound proteins were washed with binding buffer containing 5 mM imidazol; Wash: the unbound proteins were washed with wash buffer containing 50 mM imidazol; Elution: the bound proteins were eluted with elution buffer containing 400 mM imidazol. (b). SDS-PAGE gel analysis of affinity chromatography purification of recombinant antiA-scFv. Gel lanes were normalized to equivalent volume. TS. Total input protein (before loading to the column); F1-F2. Flow-through; W. Washing fractions; E1-E7. Elution fractions; M. Molecular Weight Marker

Purification of recombinant antiA-scFv fused with SUMO

The SUMO vector, as designed, has N-terminal polyhistidine (6xHis) tag (Dang et al., 2018) which facilitates purification of recombinant fusion protein with Ni-Sepharose resin. Therefore, total soluble fusion protein SM/antiA-scFv containing the 6xHis tag was purified through Ni-Sepharose affinity chromatography. The protein SM/antiA-scFv bound efficiently to the Ni^{2+} resin and was not washed off during loading and washing steps. However, very little amount of protein was obtained in elution step in comparison with the high amount of total protein loaded to the column. Purification of this fusion protein using ion-exchange column was also unsuccessful. The firm interation between sepharose-based resin and SM/antiA-scFv was only disruped when using denaturants (data not shown).

Thus, the purification of SM/antiA-scFv was conducted using precipitation with ammonium sulfate. The largest amount of SM/antiA-scFv was precipitated by 20% $(NH_4)_2SO_4$. In contrast, most of the proteins from *E. coli* and chaperone were precipitated at a higher concentration of ammonium sulfate (Fig. 2a). This result suggested the step for precipitation and removal of some undesired proteins from solution at 15% $(NH_4)_2SO_4$, followed by the increase of $(NH_4)_2SO_4$ to 20% to precipitate most of SM/antiA-scFv.

By centrifugation, precipitated SM/antiA-scFv was collected, washed and cleaved by SUMO protease. After cleaving the SUMO tag, anti-scFv was released from the fusion with SUMO, corresponding to a ~33 kDa band in SDS-PAGE gel. Protein antiA-scFv, in insoluble form, was easily separated from other constituents of the cleavage mixture by centrifugation and washed in buffer containing Tween 20 and Triton X100. In this wash step, some protein impurities were dissolved and separated from the antiA-scFv precipitate. The target protein

was solubilised in buffer containing 5% glycerol, 71.5 mM mercaptoethanol and 0.05% SDS and finally dialysed against PBS pH 7.4 with 5% glycerol (Fig. 2b). The obtained protein antiA-scFv after purification was tested for its bioactivity.

The final yields of purified antiA-scFv was approximately 64.9 mg/L of bacterial culture. This is relatively high compared to the productivity obtained by other studies at the same flask scale fermentation (Frenzel et al., 2013). For example, scFv was produced with a yield of 50 mg/L (Golchin et al., 2012) or 10.2 mg/L (Bu et al., 2013). In another research, only 0.5−1 mg scFv was recovered from 1 L of culture (Wu et al., 2007).

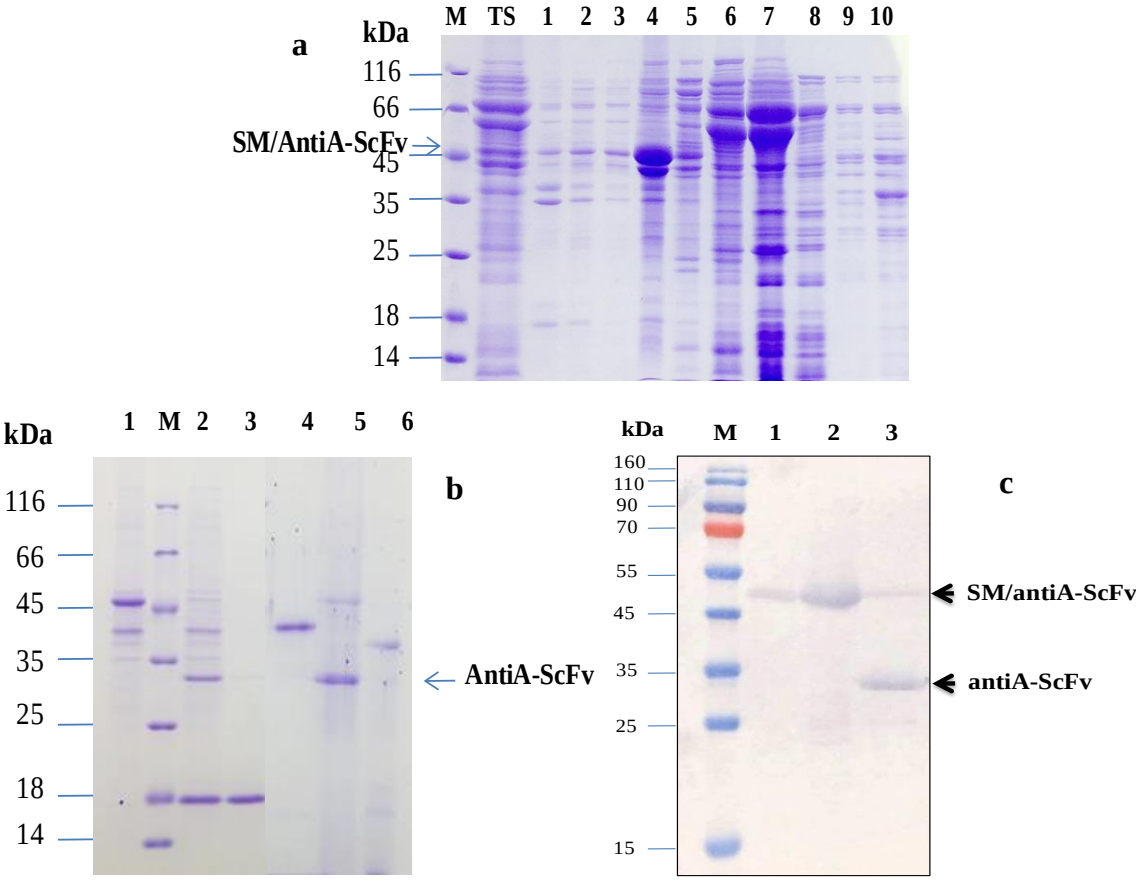


Figure 2. (a) Purification of SM/antiA-scFv by ammonium sulfate precipitation. TS. Total soluble protein SM/antiA-scFv; Lanes 1−10. Precipitation fractions at different $(NH_4)_2SO_4$ concentration: 5%, 10%, 15%, 20%, 25%, 30%, 35%, 40%, 45%, 50%; (b). SDS-PAGE analysis of SM/antiA-scFv cleaved by SUMO protease and purified antiA-scFv. Lane 1. SM/antiA-scFv, Lane 2. SM/antiA-scFv cleaved by SUMO protease, Lane 3. Soluble fraction after cleaving, Lane 4. Insoluble fraction seperated from cleavage mixture was washed in buffer containing Tween 20 and Triton X100, Lane 5. Insoluble fraction seperated from cleavage mixture (containing antiA-scFv) was solubilised in buffer containing 5% glycerol, 71.5 mM mercaptoethanol and 0.05% SDS, Lane 6. The remain insoluble fraction after the solubilization of antiA-scFv; (c). Western blot analysis of purified antiA-scFv. Lane 1. Total soluble protein SM/antiA-scFv, Lane 2. 20% ammonium sulfate precipitation fraction (containing SM/antiA-scFv), Lane 3. Purified antiA-scFv, M. Molecular Weight Marker (Fermentas)

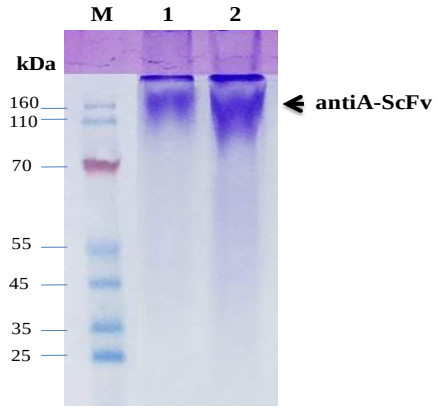


Figure 3. Nondenaturing PAGE analysis to demonstrate the polymezation of antiA-scFv. Lanes 1 and 2. 7 and 15 µg of purified antiA-scFv, respectively; M. protein marker (Fermentas)

Besides, nondenaturing PAGE analysis was used to visualize anti-scFv polymerization and the polymers were appeared as slow migrating bands on the gel forming a "ladder" of polymers with higher than 100 kDa in size (Fig. 3). From this result, we predicted that purified antiA-scFv was produced in a polymer-protein conjugate which could be applied directly to biological activity test.

Binding assay of recombinant antiA-scFv

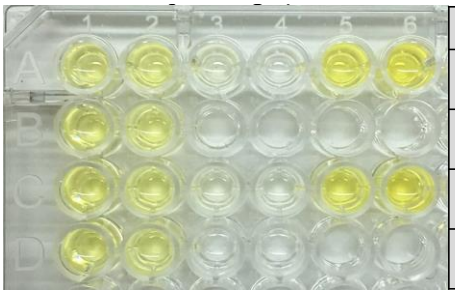
To analyse the biological activity of purified antiA-scFv, the specific binding activity of this recombinant protein was assessed by ELISA using pure antigens A/BSA, B/BSA and BSA. The higher signal of antiA-scFv bound to A/BSA and B/BSA antigen, at 0,403 and 0,338 respectively, was obtained comparing to BSA and negative control (wells without antigen). From this result, antiA-scFv bound to both A/BSA and B/BSA but showed 1.2-fold higher binding ability to A/BSA compared to B/BSA (Table 2).

Table 2. The binding activity of recombinant antiA-scFv was evaluated by ELISA.

Samples	A/BSA	B/BSA	BSA	Positive sample	Positive control-from company
TN1	0,396	0,353	0,036	0,663	0,838
TN2	0,389	0,353			
TN3	0,409	0,327	0,059	0,709	0,840
TN4	0,418	0,319			
TB	0,403 ± 0,013	0,338 ± 0,018	0,048 ± 0,016	0,686 ± 0,033	0,839 ± 0,001

Note: TN1- TN4. 4 replicates of each sample, 2 replicates for control. TB. average value calculated from all replicates for each sample, p-value < 0,01.

a. ELISA test result



b. Sample sites on ELISA dics

	1	2	3	4	5	6
A	A/BSA	B/BSA	BSA	ĐC(-) PBS	ĐC(+) Mẫu	ĐC(+) Hãng
B	A/BSA	B/BSA				
C	A/BSA	B/BSA	BSA	ĐC(-) PBS	ĐC(+) Mẫu	ĐC(+) Hãng
D	A/BSA	B/BSA				

Figure 4. The binding activity of recombinant antiA-scFv was evaluated by ELISA using pure antigens from red blood cells

In addition, the functional activity of the recombinant antiA-scFv was also assessed by a hemagglutination assay using type A, B and O human red blood cells. The results show that recombinant antiA-scFv showed hemagglutination of red blood cells at concentrations of or higher than 3.12 µg with type A, 6.25 µg with type B, and 25 µg with type O (Fig. 5a).

From the binding assays using pure antigen and red blood cells, recombinant antiA-scFv has low specificity in binding activity.

In other experiment, the incubation of antiA-scFv with EGS (an agent allowing protein to be trimeric by chemical cross-linking) increased its agglutination ability when hemagglutination of type A red blood cells starting from a concentration of 1.56 µg of antiA-scFv. When red blood cells was pre-treated with ficin, this activity was even increased further when hemagglutination started to happen from a concentration of 0.78 µg of antiA-scFv. While EGS is a bifunctional linker which facilitates tertiary structure of protein, ficin is known to enhance reactivity caused by antibodies against ABO blood group system. Therefore, the addition of EGS and the use of ficin pre-treated red cells will enhance the binding activity of recombinant antiA-scFv to the specific antigen on the surface of red blood cells in hemagglutination assay (Fig. 5b).

The key difference between A and B blood antigens is a singe sugar at the end of the antigen. To be specific, type A antigen has a terminal N-acetylgalatosamine whereas type B antigen has a terminal galactose. Since galactosamine is very similar to galactose, there is evidence that recombinant anti-A antibodies can elicit a cross-reaction with the B-specific terminal residue. Besides, the incomplete/incorrect formation of the 2 disulfide bridges structure could be responsible for the lack of specificity of recombinant anti A-scFv. Several approach could be considered to make *E. coli* more suitable for expression of disulfide-rich protein. These include introducing disulfide isomerase protein to enhance disulfide bond formation.

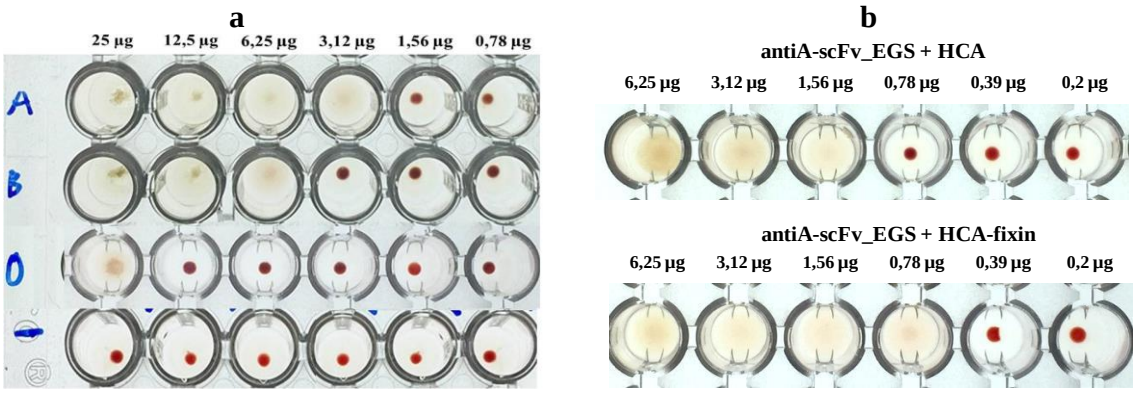


Figure 5. Hemagglutination assay of recombinant antiA-scFv. (a) Binding activity of recombinant antiA-scFv with antigens type A, B and O of red cells. (b) Binding activivy of recombinant antiA-scFv incubated with EGS with antigens type A, B and O of ficin pre-treated red cells

To the best of our knowledge, currently, no publication has reported the production of recombinant scFv of human antibody against antigens in the ABO-blood group but Rh-type blood system (Furuta et al., 1998).

CONCLUSION

Recombinant single chain antibody that recognized A-antigen (antiA-scFv) in ABO-blood system was expressed and purified with the purity of 89% and the yield of 64.9 mg/l

of culture. This recombinant antiA-scFv showed ability to hemagglutinate antigens of red blood cells but the binding specificity of its to A-antigen was limited.

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