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BIOTECHNOLOGY METHODS

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Expression of V_H-linker-V_L orientation-dependent single-chain Fv antibody fragment derived from hybridoma 2E6 against aflatoxin B_1 in *Escherichia coli*

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Abstract Aflatoxin B_1 (AFB_1) is a toxic secondary metabolic product, which threatens human and animal health. Antibody is a key factor for immunoassay against toxic stuff like AFB_1, and single-chain Fv antibody fragment (scFv) has become a popular format of genetically engineered antibody. In this study, four hybridoma cell lines against AFB_1 were obtained, and then *scFvs 2E6* derived from hybridoma cell line 2E6 were constructed in different V_H/V_L orientations. Subsequently, *scFvs 2E6* were expressed in *E. coli* BL21(DE3) mainly in the form of inclusion body. SDS-PAGE, Western blot and ELISA were employed to characterize *scFvs 2E6*. The results revealed that the yield of inclusion body of *scFvs 2E6* in either V_H/V_L orientation was similar; however, only the scFv in V_H-linker-V_L orientation showed anti-AFB_1 bioactivity after refolding. The present study underscores the importance of choosing optimal V_H/V_L orientation for scFv construction, and scFv may be favorable for immunoassays in food industry.

Keywords Aflatoxin B_1 · scFv · Biotechnology · Hybridoma · ELISA · Food safety

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Introduction

Mycotoxins are toxic secondary metabolites of fungi mainly belonging to the genera: *Aspergillus*, *Penicillium*, *Fusarium*, and *Alternaria* [18, 21, 23, 28]. Many foods can be contaminated by mycotoxins during any phase of the production and transformation of food products [39]. Food frequently contaminated with mycotoxins includes corn, wheat, barley, rice, oats, nuts, milk, cheese, peanuts, and cottonseed [37].

Therefore, there is a significant demand for routine screening of mycotoxins in human food and animal feed. So far, methods for mycotoxins detection mainly include thin layer chromatography, liquid chromatography, and immunological methods, such as enzyme-linked immunosorbent assay (ELISA), membrane-based immunoassay, fluorescent polarization, and so on [23]. The superiority of immunological methods leads to a demand for large amounts of high-quality antibodies.

Recently, rapid developments in biotechnology have brought new methods to obtain specific antibodies. And among them, single-chain Fv antibody fragment (scFv) has gained substantial interests. scFv consists of the

variable regions of the heavy (V_H) and light (V_L) chains of an immunoglobulin, and they are linked by a flexible linker [1, 2]. There are several advantages of employing scFv as antibody. First, it retains the specific affinity to the antigen, although this is usually lower than that of the original antibody; second, scFv can be produced in large quantities using bacterial expression systems at low cost; and third, it is easy to manipulate for different applications. For example, the fusion between scFv and marker molecules becomes simple to operate [32, 33, 35]. However, scFv also harbors some problems, such as instability, aggregation, and a tendency to dimerize, which are the major obstacles to scFv's commercialization. Even so, there is little doubt that their strengths, as tools in immunoassays, also should not be underestimated [3].

Aflatoxin B_1 (AFB_1), the most toxic mycotoxin mainly produced by fungi of *Aspergillus flavus*, *A. parasiticus* [6, 24] and *A. nomius* [10], is a potent carcinogen, teratogen, and mutagen [27, 28]. It is listed in Group I carcinogen by the International Agency for Research on Cancer (IARC) [12].

In the present study, four bioactive hybridoma cell lines against AFB_1 were obtained through cell fusion after immunization of BALB/c mice with aflatoxin B_1-cationized bovine serum albumin (AFB_1-cBSA), and then $2E6V_H$ and $2E6V_L$ genes were successfully amplified from the cDNA of hybridoma cell line 2E6. Finally, the construction and expression of anti-AFB_1 *scFvs 2E6* (scFv $2E6V_L$-linker-$2E6V_H$ and scFv $2E6V_H$-linker-$2E6V_L$) in both V_H/V_L orientations were described. In addition, immunological characterization of each scFv was performed using Western blot and ELISA. As far as we know, although there were some publications about anti-AFB_1 scFv [7, 16, 17, 22, 36], most of these scFv genes were derived from phage display. In addition, there are few reports describing the effect of the orientation of V_H/V_L on scFv. Our research revealed that the yields of *scFvs 2E6* in both orientations were similar while the bioactivity of *scFvs 2E6* was V_H-linker-V_L orientation dependent. The results would benefit the field of genetically engineered antibody design, which, in turn, contributes to the production of high quality antibodies for immunoassay against toxic substance in food industry.

Materials and methods

Materials

Freund's adjuvant, hypoxanthine, aminopterin, thymidine (HAT), Bradford reagent, bovine serum albumin (BSA), and ovalbumin (OVA) were purchased from Sigma (MO, USA). Polyvinylidene fluoride (PVDF) membrane, unstained marker, and Precision Plus protein standards were purchased from Bio-Rad (GA, USA). Vector pET-3d and *Escherichia coli* (*E. coli*) BL21(DE3) used for expression were purchased from Novagen (MA, USA). Vector pMD 19-T was purchased from Takara (Kyoto, Japan). AFB_1-cBSA (*nAFB*$_1$: *ncBSA* = 5.2:1) and AFB_1-cOVA (*nAFB*$_1$: *ncOVA* = 3.2:1) were prepared in our laboratory [39]. All other reagents were of analytical grade.

Production and characterization of monoclonal antibodies

AFB_1-cBSA was used for the immunization of BALB/c mice. After three immunizations (50 μg per mouse per time), the mice with the highest serum sensitivity as detected by an indirect competitive ELISA (icELISA) previously described [39] were boosted intravenously with 25 μg of AFB_1-cBSA. And 72 h later, spleen cells were collected and fused with myeloma line Sp2/0 using polyethylene glycol 4000 [13, 14]. Hybridoma cells were grown in 96-well plates and screened on day 14 for reactivity against AFB_1 by icELISA. Positive wells were cloned from a single cell in 96-well plates. Hybridoma clones (or hybridoma cell lines) were further characterized for specific reactivity against AFB_1 with comparison to AFB_2, AFG_1, AFG_2, and AFM_1 by icELISA, and 50 % inhibitory concentration (IC_{50}) was defined as the sensitivity of antibody. The specific reactivity of each antibody from different hybridoma cell lines was measured by the index cross-reactivity ratio (%), which was calculated as the ratio of (the sensitivity of each antibody against AFB_1/the sensitivity of each antibody against analyte) × 100.

Cloning of V_H/V_L gene and construction of pET-3d *scFvs 2E6*

Total RNA was isolated from hybridoma cell lines 2C10 and 2E6, and then used as template for cDNA synthesis, respectively. Primers (Table 1) were designed to amplify V_H/V_L gene according to Zhou et al [38]. PCR products were analyzed on 1 % agarose gel and then cloned into pMD 19-T vectors for sequencing.

scFvs 2E6 in different V_H/V_L orientations were assembled by splicing-overlap-extension polymerase chain reaction (SOE-PCR) with a $(Gly_4Ser)_3$ linker. *Nco*I and *Bam*HI restriction sites were added onto the resultant *scFvs 2E6* through modified primers, and then the *scFvs 2E6*-encoding genes were ligated into vector pET-3d (Fig. 1). The constructs were confirmed by sequencing.

Expression of *scFvs 2E6*

Escherichia coli BL21(DE3) was transformed with the expression vector pET-3d *scFvs 2E6*. Each kind of transformant was selected by growing on Luria–Bertani (LB) agar

Table 1 Primers for V_H and V_L gene amplification

Primer	Oligonucleotide sequence
Lambda V_L forward	CTTGGGCTGACCTAGGACAGT
Lambda V_L reverse	CAGGCTGTTGTGACTCAGGAA
Kappa V_L forward 1	CCGTTTYAKYTCCARCTT
Kappa V_L forward 2	GGATACAGTTGGTGCAGCATC
Kappa V_L reverse 1	GATRTTKTGATRACSCA
Kappa V_L reverse2	GAY ATTGTGMTRACYCARTCT
Kappa V_L reverse 3	GAY ATCCAGMTGACWCAGWCT
Kappa V_L reverse 4	CAAATTGTTCTCACCCAGTCT
V_H forward 1	TGCAGAGACAGTGACCAGAGT
V_H forward 2	TGAGGAGACTGTGAGAGTGGT
V_H forward 3	TGAGGAGACGGTGACYGWGGT
V_H reverse 1	GATGTGAAGCTTCAGGAGTC
V_H reverse 2	CAGGTGCAGCTGAAGSAGTC
V_H reverse 3	CAGGTTACTCTGAAAGAGTC
V_H reverse 4	GAGGTCCAGCTGCARCARTC
V_H reverse 5	GAGGTGAAGCTGGTGGARTC

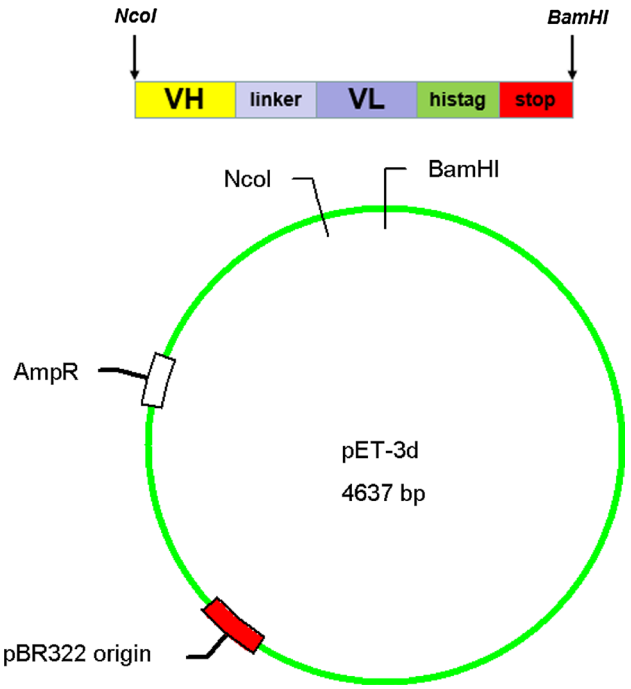

Fig. 1 Schematic diagram of the pET-3d scFv2E6V_H-linker-2E6V_L expression vector. Genes and feature were not drawn to scale

plates supplemented with 50 µg/mL carbenicillin. Single colony of each scFv was cultured overnight in 5 mL LB medium supplemented with 0.5 % glucose and 50 µg/mL carbenicillin (LBC) at 30 °C. This overnight culture was employed to inoculate a 50 mL batch fresh LBC medium, which was subsequently used to inoculate 500 mL LBC for expression when the OD_{600nm} reached approximately 0.8. Expression of the *scFvs 2E6* was induced with 0.4 mM isopropyl β-D-1-thiogalactopyranoside (IPTG). After 2.5 h incubation at 37 °C, 1 mL of each *E. coli* BL21(DE3) culture was collected for SDS-PAGE and Western blot analysis. As a negative control, *E. coli* BL21(DE3) transformant containing only pET-3d was processed in the same way.

The rest of the cultures were harvested by centrifugation at 3,000*g* for 10 min at 4 °C. The supernatant was decanted and the pellet was washed with Tris-buffered saline (TBS) for three times before it was frozen at −80 °C. To lyse cells, pellets were resuspended in 40 mL suspension buffer (20 mM Tris, 5 mM EDTA, pH 8.0) with 100 µg/mL lysozyme and 12.5 U/mL DNase I (Roche, IN, USA). After being shaken at 4 °C for 1 h, the cell pellets were disrupted by sonication and incubated for another 1 h at 4 °C with 0.025 g sodium deoxycholate and 570 µL of a 70 % Tergitol solution (Sigma, CA, USA). The insoluble material was collected by centrifugation at 16,000*g* for 30 min at 4 °C, and resuspended in B-PER Bacterial Protein Extraction Reagent (Thermo Scientific, IL, USA). To obtain inclusion body, the resuspension was followed by centrifugation again. Subsequently, the inclusion body was washed twice with washing buffer (20 mM Tris, 1 mM EDTA, 1.5 M Urea, 0.5 M NaCl, 1 % Triton X-100, pH 8.0) and then twice with TBS to remove EDTA. The inclusion body was resuspended in 30 mL of dissolving buffer (50 mM Tris, 8 M urea, 0.5 M NaCl, 5 mM 2-mercaptoethanol (β-ME), 10 mM imidazole, pH 8.0) and stirred overnight at 4 °C to solubilize the pellet. Finally, the sample was centrifuged at 10,000*g* for 20 min at 4 °C to remove any undissolved pellet. 1 mL of each scFv in the solubilized inclusion body was purified by Ni^+-NTA column under denaturation condition, and the concentration of the inclusion body was determined through Bradford assay with BSA as a standard.

SDS-PAGE and Western blot analysis of the *scFvs 2E6*

Samples were separated on 4–20 % Tris/Glycine gels (Invitrogen, NY, USA) and were electrophoretically transferred to PVDF membrane using 10 mM CAPS buffer and 10 % methanol. The transfer was first confirmed by SYPRO ruby protein blot stain (Thermo Scientific, IL, USA), and then the membrane was blocked with 3 % BSA in TBS containing 0.05 % Tween-20(TBST) for 1 h at room temperature. To probe for *scFvs 2E6*, primary antibody Goat Anti-Mouse Kappa-BIOT (Southern Biotech, AL, USA) was used at a 1:4,000 dilution in TBST, followed by streptavidin–HRP conjugates (Southern Biotech, AL, USA) at a 1:10,000 dilution. Each antibody solution was in contact with the membrane for 1 h at room temperature. After that, the membrane was washed five times (3 min each time) with TBST, followed by color development using 1-Step Ultra TMB-Blotting solution (Thermo Scientific, IL, USA).

Table 2 The sensitivity and cross-reactivity of hybridoma cell lines for AFB_1 and its analogs

Cell line	Sensitivity (ng/mL) and cross-reactivity ratio (%)				
	AFB_1	AFB_2	AFG_1	AFG_2	AFM_1
2E6	85.76	1,087.55 (7.89 %)	3,839.65 (2.23 %)	–	–
2C10	9.54	–	47.56 (20.06 %)	901.08 (1.06 %)	–
4D11	117.92	1,061.66 (11.11 %)	611.64 (19.28 %)	1,722.68 (6.85 %)	3,230.03 (3.65 %)
2H1	203.00	–	5,854.22 (3.47 %)	–	–

– Not detected

Renaturation of *scFvs 2E6* on nickel-chelating fast flow column

The renaturation of *scFvs 2E6* in different V_H/V_L orientations were performed according to Guo et al. [8] with modifications, respectively. A 1.5 mL nickel-chelating fast flow column was washed with 10 column volume of water and then equilibrated with 10 mL binding buffer [50 mM Tris (pH 8.0), 8 M urea, 0.5 M NaCl, 5 mM β-ME, and 20 mM imidazole]. After that, 1.5 mL of denatured scFv was added to the column, and it was washed with 10 mL binding buffer. The bound protein was refolded by applying refolding buffer [50 Mm Tris (pH 8.0), 0.5 M NaCl, and 20 mM imidazole] with a step-gradient of 8–0.5 M (8, 6, 4, 3, 2, 1, 0.5) urea at a flow rate of 0.5 mL/min in an equal volume of 10 mL. Oxidizing conditions were introduced with 1 mM glutathione disulfide and 1 mM glutathione. The refolded protein was washed with buffer [50 mM Tris (pH 8.0), 0.25 M NaCl, 0.25 M urea, and 120 mM imidazole], and then eluted by refolding buffer containing 0.25 M urea, 0.25 M NaCl, 0.05 % Tween-20, and 0.25 M imidazole.

ELISA characterization of *scFvs 2E6*

To determine the sensitivity of *scFvs 2E6*, 96-well plates (Nunc MaxiSorp®) were filled with 100 ng of AFB_1-BSA (Sigma, MA, USA) and incubated at 4 °C for 12 h. The wells were washed three times with TBST and decanted. The wells were blocked with 5 % skimmed milk in TBST for 2 h at 37 °C. After the wells were washed, 100 μL of scFv diluted in TBST as well as 10 μL of AFB_1 standard solution (100–0.5 and 0 μg/mL) were added to each well and incubated at 37 °C for 1 h. After washing, 100 μL of Goat Anti-Mouse Kappa-BIOT was added to each well and incubated at 37 °C for 1 h. After another washing, the wells were filled with 100 μL of streptavidin–HRP conjugates and incubated at 37 °C for 1 h. Then the wells were washed and 100 μL of 1-Step Ultra TMB ELISA substrate was added. The reaction was stopped by adding 50 μL of 2 M sulfuric acid 15 min later. Absorbance was measured at 450 nm using microplate auto-reader (Bio-Tec instruments, VT, USA).

The absorbance in wells with zero analyte concentration (B_0) was taken as 100 % scFv binding level. The scFv binding rate (%) for each concentration of AFB_1 (B) ($n = 3$) was calculated as $B/B_0 \times 100$. The non-specific binding was subtracted from the average of B_0 and average of B, respectively.

The specificity of *scFvs 2E6* was also determined by method mentioned in the above part "Production and characterization of monoclonal antibodies".

Results and discussion

Production and characterization of monoclonal antibodies

Nine hybridoma cell lines were obtained by using hybridoma technique. According to the icELISA, four hybridoma cell lines (2E6, 2C10, 4D11, and 2H1) were found to produce antibody against AFB_1. The sensitivity of each antibody was shown in Table 2, and the results indicated that the sensitivity of 2C10 was the best (9.54 ng/mL), followed by 2E6 (85.76 ng/mL), 4D11 (117.92 ng/mL), and 2H1 (203 ng/mL), respectively. Meanwhile, AFB_2, AFG_1, AFG_2, and AFM_1 were employed to investigate the specificity of each cell line. As shown in Table 2, each cell line showed cross-reactivity toward AFG_1, and among these four cell lines, 2C10 and 4D11 possessed a cross-reactivity ratio of about 20 %, which was a little higher than that of 2E6 and 2H1. Meanwhile, 2E6 and 4D11 recognized AFB_2 with cross-reactivity of 7.89 and 11.11 %, respectively. Besides, each cell line possessed weak cross-reactivity for AFG_2 and AFM_1. Therefore, monoclonal antibodies from all the four hybridoma cell lines showed good specificity. Two hybridoma cell lines (2C10 and 2E6) were selected to construct scFv based on their relative higher sensitivity against AFB_1 than other hybridoma cell lines.

Construction, expression renaturation, and purification of the *scFvs 2E6*

After the acquisition of cDNA from each hybridoma cell line, only reasonable V_H and V_L genes were obtained from cell line 2E6 by applying the primers, which were confirmed through the website NCBI BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi). Besides, the NCBI BLAST results suggested that the V_L gene was of Kappa type.

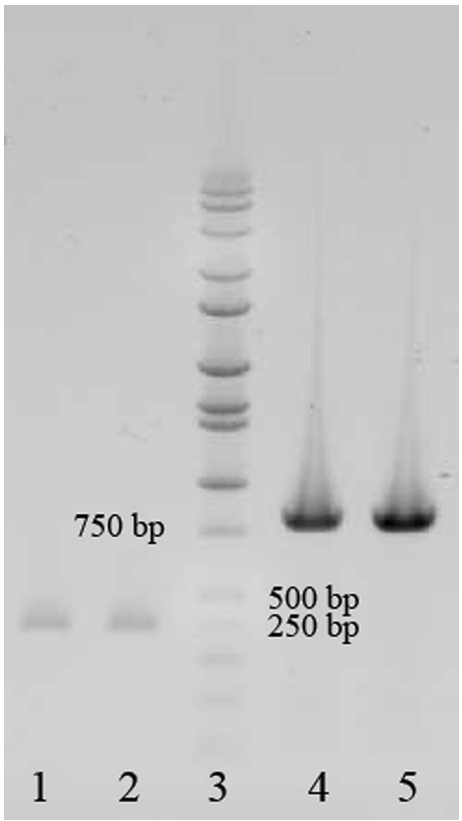

Fig. 2 Agarose gel of V_H/V_L gene from cell line 2E6 and *scFvs 2E6* genes. *Lane 1* $2E6V_H$ gene, *lane 2* $2E6V_L$ gene, *lane 3* DL2000 Plus DNA Marker, *lane 4* scFv $2E6V_L$-linker-$2E6V_H$ gene, *lane 5* scFv $2E6V_H$-linker-$2E6V_L$ gene

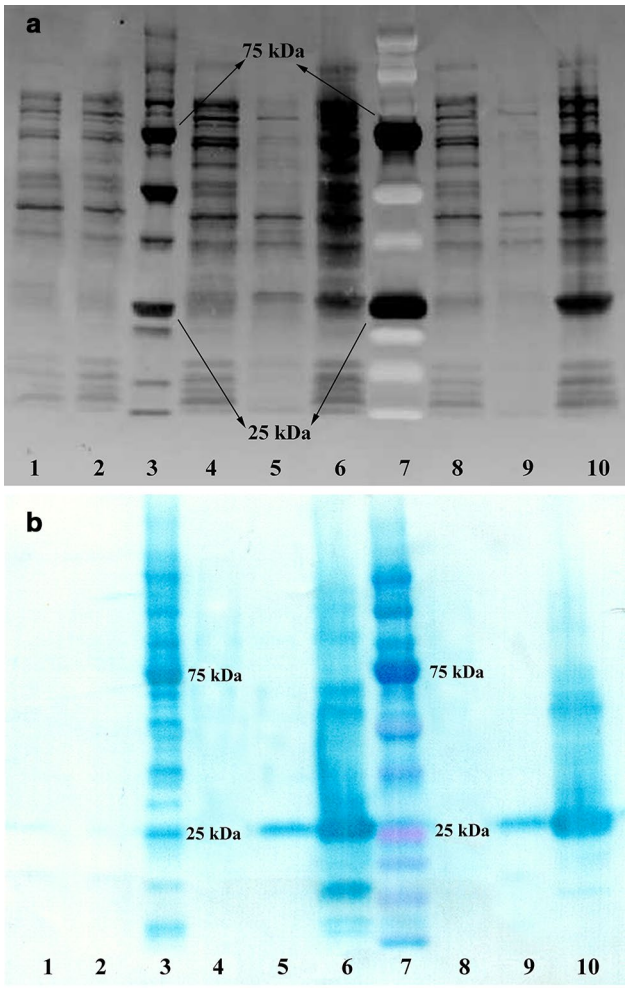

Fig. 3 SYPRO ruby protein blot stain and Western blot analysis of scFv expression. **a** SYPRO ruby protein blot stain analysis of scFv expression. Total protein from *E. coli* BL21(DE3) containing plasmid pET-3d before induction (*lane 1*) and after induction (*lane 2*), *lane 3* unstained marker, *lane 4* total protein from *E. coli* BL21(DE3) containing plasmid pET-3d scFv $2E6V_L$-linker-$2E6V_H$ before induction; soluble (*lane 5*) and insoluble (*lane 6*) protein from an equal amount of *E. coli* BL21(DE3) containing plasmid pET-3d scFv $2E6V_L$-linker-$2E6V_H$ after induction, *lane 7* precision plus protein standard, *lane 8* total protein from *E. coli* BL21(DE3) containing plasmid pET-3d scFv $2E6V_H$-linker-$2E6V_L$ before induction; soluble (*lane 9*) and insoluble (*lane 10*) protein from an equal amount of *E. coli* BL21(DE3) containing plasmid pET-3d scFv $2E6V_H$-linker-$2E6V_L$ after induction. Not equal amounts of samples were loaded in each *lane*. **b** Western blot analysis of scFv expression. The samples were loaded as in **a**

The reason why reasonable V_H and V_L genes could not be obtained from cell line 2C10 might lie in that limited primers had been tried or V_H/V_L-encoding gene was mutated in vivo. Therefore, V_H and V_L genes from cell line 2E6 were applied in the subsequent research.

As shown in Fig. 2, the $2E6V_H$ gene was about 351 bp while the $2E6V_L$ gene was about 348 bp. $2E6V_H$, $2E6V_L$, and the linker were connected by SOE-PCR to generate *scFvs 2E6* genes with the size of about 750 bp (Fig. 2). The expression plasmids for pET-3d *scFvs 2E6* were constructed as described. In the resultant construct, the hexahistidine sequence and a stop codon were fused to the C terminus of the *scFvs 2E6* genes. Sequencing analysis was consistent exactly with the targeted gene sequences.

To confirm the transfer of protein samples to PVDF membrane and the expression of *scFvs 2E6,* SYPRO ruby protein blot was first performed. As shown in Fig. 3a, the *scFvs 2E6* were successfully expressed, and they were mainly in the form of inclusion body. The molecular weight of recombinant *scFvs 2E6* was found to be ~27 kDa, which agreed with the predicted size of the scFv according to the amino acid sequence. On the other hand, no scFv product was observed in transformants containing only pET-3d. When Goat Anti-Mouse Kappa-BIOT antibody was employed in Western blot, the *scFvs 2E6* showed strips (monomer, ~27 kDa; dimer, ~54 kDa), in addition, some soluble proteins were found to be of similar size with *scFvs 2E6* (Fig. 3b). However, bioactivity of soluble proteins to AFB_1 determined by indirect ELISA was not obviously

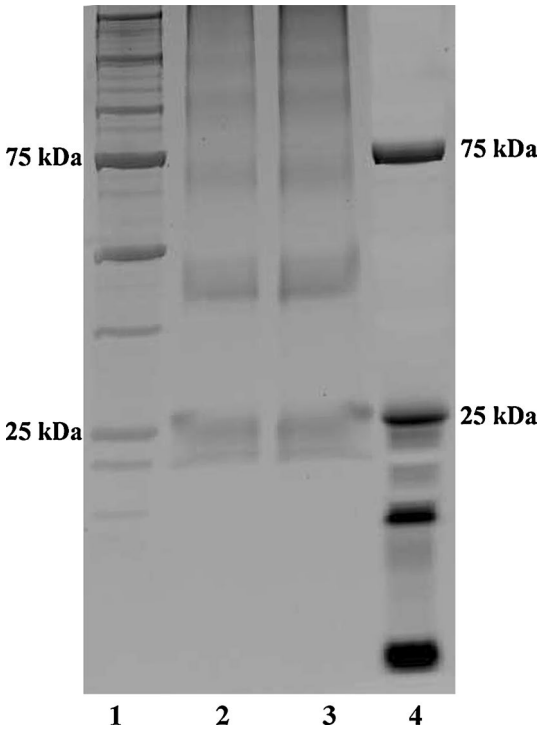

Fig. 4 SDS-PAGE analysis of refolded *scFvs 2E6*. *Lane 1* unstained marker, *lane2* refolded scFv 2E6V_L-linker-2E6V_H, *lane3* refolded scFv 2E6V_H-linker-2E6V_L, *lane 4* precision plus protein standard

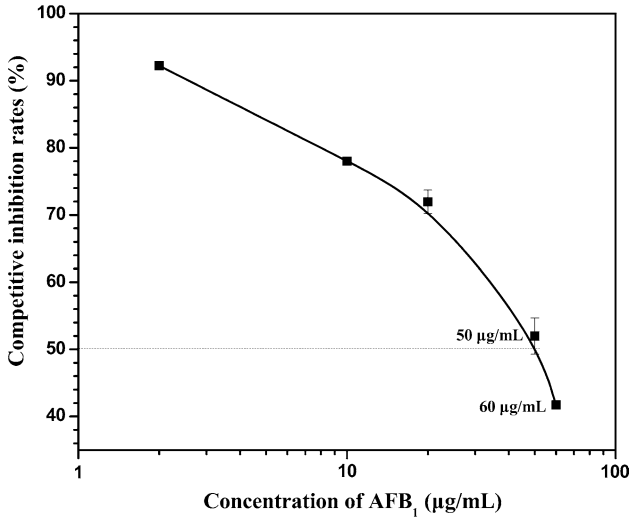

Fig. 5 The competitive inhibition curve of scFv 2E6$V_H$$V_H$-linker-2E6$V_L$ against AFB_1. Each point represented the mean ± SD of three replicates

observed, and the reason might be that not enough soluble (correctly folded) proteins were obtained from 500 mL of *E. coli* BL21 (DE3) culture.

After cell lysis and washing, inclusion body solution was obtained. And 1 mL inclusion body of each scFv was purified through Ni^+-NTA column. According to the Bradford assay results, there was no obvious difference in the total yield of inclusion body in either V_H/V_L orientation. The *scFvs 2E6* in the form of inclusion body were then loaded onto a nickel-chelating fast flow column under denaturing conditions and refolded by washing with a step-gradient Urea. As is well known, protein refolding is one of the fundamental problems in genetic engineering, and researchers described a variety of methods for refolding. However, due to different characterization of each protein, there is no universal method for refolding. In this study, the *scFvs 2E6* were subcloned into pET-3d and expressed as a hexa-histidine tail fusion protein, which made it feasible to purify the recombinant protein with ease, and contributed to the renaturation by nickel-chelating fast flow column. In addition to the method of on-column refolding, other previously published methods, such as stepwise dialysis [20, 31] and dilution method [34], were also tried. Fortunately, correctly refolded *scFvs 2E6* were obtained with each method. However, it should be noted that the reason why the on-column refolding method was chosen in this study was that the aggregation caused by intermolecular interactions was minimal when the refolding molecules were isolated by binding to the Ni^+-NTA via His-tag [5].

scFvs 2E6 were subjected to SDS-PAGE analysis after refolding, and the band of ~27 kDa was detected (Fig. 4). Some additional bands were also observed, which might represent dimers (~55 kDa) and multimers of the scFvs.

The determination of *scFvs 2E6* sensitivity

The refolded *scFvs 2E6* in dimers or multimers might still be capable of binding with the antigen. Therefore, the refolded scFv was directly used for bioactivity investigation in ELISA. First, an indirect immunoassay check board titration was carried out [4] for immunoreagents optimization. The result suggested that scFv 2E6V_L-linker-2E6V_H showed almost no affinity toward antigen AFB_1 while scFv 2E6V_H-linker-2E6V_L exhibited higher affinity, and the optimal working concentrations of coating antigen and scFv 2E6V_H-linker-2E6V_L were 2 and 0.1 mg/mL, respectively. Second, the competitive inhibition curve of scFv 2E6V_H-linker-2E6V_L was obtained by icELISA, which indicated that to achieve 50 % inhibition of binding (IC_{50}), the concentration of AFB_1 was required to be about 50 μg/mL (Fig. 5). The results also suggested that cross-reactivity reactions were not observed when analogs (AFB2, AFG1, AFG2, and AFM1) were at a concentration of 50 μg/mL.

scFv can be constructed in different V_H/V_L orientations. The V_H-linker-V_L construct typically exhibits euclidean distances between linker termini of 18–24 Å, whereas the V_L-linker-V_H orientation generally exhibits distances that are 5–10 Å longer for the same Fv [11]. Thus, different

orientation of a domain may distort the native Fv conformation [29]. Luo et al. [15] showed that successful secretion of recombinant scFv in *Pichia pastoris* was V_L-linker-V_H orientation dependent, but there have been substantial publications about scFv in the orientation of V_H-linker-V_L [19, 25, 26]. Besides, some studies reported that the orientation of V_H and V_L genes can have an impact on the yield of scFv [9, 30]. However, in this study, the difference in the yield of *scFvs 2E6* in either V_H/V_L orientation was not obvious. Therefore, the impact of V_H/V_L orientation on the bioactivity and the yield of scFv seemed to be uncertain.

Accordingly, the sensitivity of the scFv 2E6V_H-linker-2E6V_L obtained was relatively low with comparison to the monoclonal antibody, and the reasons for it might be that scFv was monovalent, or that it was incompletely refolded, or that it lacked post-translational modifications, which indicated that the scFv was not in a natural state. Undoubtedly, this would limit its application in immunoassays, and how the V_H/V_L orientation determines the characteristics of scFv still remains a question. Consequently, in order to extend the application of scFv in immunoassay, future efforts will be focused on the detailed analysis of these problems.

Conclusion

In conclusion, we described the production of a panel of monoclonal antibodies against AFB_1, and we successfully cloned the V_H/V_L gene from hybridoma cell line 2E6 in this study. Subsequently, *scFvs 2E6* in different V_H/V_L orientation were constructed and expressed in *E. coli* BL21(DE3). Afterward, Western blot and ELISA were performed, and the results demonstrated that *scFvs 2E6* could be refolded from inclusion body and its bioactivity was V_H-linker-V_L orientation dependent. Therefore, this study provides some guideposts for molecular designs of scFv aimed at improving its specificity and affinity, which would benefit the immunoassay of toxic substances in food industry.

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