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Screening and protein structure analysis of anti-aflatoxin B1 single-chain antibody by phage display: Postprint

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Abstract

Aflatoxin B1 (AFB1) is a highly toxic and widely contaminating mycotoxin, and establishing efficient, accurate, and rapid detection methods for AFB1 is of great significance. Utilizing a phagemid-assisted phage display system to construct a single-chain variable fragment (scFv) library and applying a "panning-elution" strategy is one of the commonly used methods for screening high-affinity ligands. Simultaneously combining computer-aided approaches such as homology modeling and molecular docking to analyze the binding sites and key amino acids between antibody and antigen provides a foundation for antibody engineering at the genetic level. In this study, the heavy chain variable region and light chain variable region were amplified from the spleen cells of AFB1-BSA immunized mice, the assembled scFv fragment was inserted into the phagemid pCANTAB5e to construct a phage display scFv library, and using different concentrations of AFB1-OVA as antigen, an anti-AFB1 scFv with relatively good affinity was screened from the library, with an affinity constant of 8$\times10^{5}$ L/mol; according to homology modeling and molecular docking, it was found that when binding to the antigen AFB1, Tyr33, Ser52, and Tyr102 in the scFv play key roles, binding to AFB1 via π-π conjugated bonds, hydrogen bonds, and van der Waals forces, respectively.

Full Text

Preamble

Screening of Anti-Aflatoxin B1 Single-Chain Antibody Based on Phage Display Technology and Analysis of Its Protein Structure[1]

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Abstract

Aflatoxin B1 (AFB1) is a highly toxic and widely contaminating mycotoxin, making it crucial to establish efficient, accurate, and rapid detection methods for AFB1. The phagemid-helper phage display system is commonly used to construct single-chain variable fragment (scFv) libraries, and the "panning-elution" strategy is an effective approach for screening high-affinity ligands. Combined with computer-aided methods such as homology modeling and molecular docking, the binding sites and key amino acids between antibody and antigen can be analyzed to provide a basis for antibody modification at the genetic level. In this study, we amplified the heavy chain variable region (VH) and light chain variable region (VL) from spleen cells of AFB1-BSA-immunized mice. The assembled scFv fragment was inserted into the phagemid pCANTAB5e to construct a phage display single-chain antibody library. Using different concentrations of AFB1-OVA as coating antigen, an anti-AFB1 scFv with good affinity was screened from the library, with an affinity constant of 8×10^{5} L/mol. Homology modeling and molecular docking revealed that Tyr33, Ser52, and Tyr102 in the scFv play key roles in binding to AFB1 through π-π conjugated bonds, hydrogen bonds, and van der Waals forces, respectively.

Keywords: Aflatoxin B1; single-chain antibody; phagemid-helper phage display; homology modeling; molecular docking

1. Materials and Methods

Six-week-old female Balb/c mice (Hubei Experimental Animal Research Center); *E. coli* TG1 and pCANTAB5e vector (Beijing Baokewei Shian Biotechnology Co., Ltd.); helper phage M13KO7 and HRP-labeled anti-M13 antibody (GE Healthcare, USA); Easy Taq polymerase, DNA markers, restriction enzymes SfiI, NotI, NdeI, and XhoI (New England Biolabs, USA); TRIzol® Reagent RNA extraction kit (Thermo Fisher Scientific, USA), QIAGEN Plasmid Mini Kit, QIAquick Gel Extraction Kit (QIAGEN, USA); AFB1 standard (Sigma-Aldrich, USA); RevertAid™ cDNA synthesis kit (Roche, USA); other reagents were of analytical grade from domestic sources.

Equipment included a T100™ Thermal Cycler PCR instrument and gel imaging system (Bio-Rad, USA), and a Biorad 5810R refrigerated high-speed centrifuge (Eppendorf, Germany).

1.2.1 Animal Immunization

For primary immunization, AFB1-BSA was emulsified with an equal volume of Freund' s complete adjuvant and injected subcutaneously at multiple points on the back of mice. After a 30-day interval, the same dose of immunogen (50 g per mouse) was emulsified with an equal volume of Freund' s incomplete adjuvant for booster immunizations. On day 10 after each immunization, tail blood was collected and antiserum titers were measured according to reference [16].

1.2.2 scFv Amplification and Assembly

The mouse with the highest antiserum titer was selected, and spleen RNA was extracted using TRIzol® Reagent. The spleen was ground into powder with liquid nitrogen, mixed with 1 mL TRIzol reagent, and incubated at room temperature for 30 min. After complete dissolution, 300 L chloroform was added, mixed thoroughly, and incubated for 10 min, followed by centrifugation at 14,000 rpm for 15 min. The supernatant (500 L) was transferred to a sterile centrifuge tube, mixed with 800 L isopropanol, incubated for 10 min, and centrifuged at 14,000 rpm for 15 min. The pellet was washed twice with 70% ethanol, and finally dissolved in 50 L DEPC-treated sterile water. RNA concentration was measured and quality was assessed by agarose gel electrophoresis.

Using mRNA as template, cDNA was synthesized by reverse transcription. With 1 L cDNA as template, specific primers designed according to Shen Beifen [17] were used to amplify VH and VL genes. Equal amounts of VH and VL gene fragments were used as mutual templates for the first round of fusion PCR to randomly assemble them into scFv genes. PCR conditions were as described in reference [17]. After completion, VH5' primers containing SfiI restriction sites (recognition sequence GGCCCAGCCGGCC) and VL3' primers containing NotI restriction sites (recognition sequence GCGGCCGC) were added to the system for a second PCR amplification.

1.2.3 Phage Antibody Library Construction, Identification and Panning

The scFv gene and pCANTAB5e plasmid were double-digested with restriction enzymes SfiI and NotI, ligated with T4 DNA ligase, and transformed into competent *E. coli* TG1 cells. A 100 L aliquot of the culture was plated on 2×YT solid medium containing 100 g/mL ampicillin. Library capacity was estimated based on colony counts, and random clones were selected for sequencing identification.

Panning and amplification of positive phages were performed according to reference [18]. A 96-well microtiter plate was coated with 100 L of 20 g/mL AFB1-OVA at 4 °C overnight. The coating solution was discarded the next day, and the plate was washed three times with PBST. After blocking with 2% skim milk for 2 h, the plate was washed three times with PBST. Then 100 L of phage antibody library was added and incubated at 37 °C for 2 h, followed by ten PBST washes. The plate was blotted dry, and 200 L Gly-HCl buffer was added for elution with gentle shaking at 37 °C for 6 min. Immediately, 25 L Tris-HCl buffer (pH 8.9) was added to neutralize the eluate. The eluate was collected to infect *E. coli* TG1 (OD_{600}nm = 0.4). A portion was used to determine titer, while the remaining phages were precipitated with PEG/NaCl solution and amplified for the next round of panning. The "panning-enrichment" process was repeated four times, with AFB1-OVA coating concentrations of 20 g/mL, 20 g/mL, 10 g/mL, and 10 g/mL for each round, respectively.

1.2.4 scFv Phage Clone Identification and Sequence Analysis

From the selection plates of round 5, 46 single clones were randomly picked and tested by phage ELISA: AFB1-OVA (diluted in coating buffer) was coated overnight at 4 °C, blocked with 3% skim milk for 2 h, and washed three times with PBST. After blotting dry, phage monoclonal supernatants were added. Following three PBST washes, HRP-labeled anti-M13 secondary antibody was added. After 1 h incubation, TMB substrate was added for color development, and absorbance at 450 nm was measured.

According to reference [19], the OD_{450}nm value of test samples was defined as P, and that of negative controls as N. Clones with $P/N \geq 2.1$ were considered positive, while $P/N < 1.5$ were considered negative.

Plasmids from positive phage clones were extracted, and DNA sequences were determined using universal primers S1 and S6 for pCANTAB5e.

1.2.5 Positive scFv Prokaryotic Expression and Purification

PCR primers containing NdeI and XhoI restriction sites and a His-Tag were designed to amplify the positive scFv. The product was ligated with pET-30a plasmid to construct the pET-30a-scFv recombinant plasmid. After verification, the plasmid was transformed into competent *E. coli* BL21 (DE3) for expression. When the culture OD_{600}nm reached 0.6–1.0, IPTG was added to a final concentration of 1 mM, and induction was carried out at 18 °C for 16 h. Cells were harvested and lysed by ultrasonication. After centrifugation, the pellet was resuspended in PBS buffer and sonicated on ice (2 s on, 2 s off, 100 W power, 30 min). The supernatant was purified using Ni-NTA affinity chromatography, and protein expression was analyzed by SDS-PAGE.

1.2.6 scFv Characterization

The physicochemical properties of the anti-AFB1 scFv, including theoretical molecular weight, isoelectric point, thermal stability index, extinction coefficient, and average hydrophilicity, were predicted online using the Protparam tool on the ExPASy website (https://web.expasy.org/cgi-bin/protparam/protparam).

Various methods exist for measuring antibody affinity constants, and different methods may yield different values. Based on scFv titer determination and following the method of Beatty [20] for measuring antibody affinity constants, we calculated the affinity constant of our single-chain antibody. The principle is as follows: with constant enzyme-labeled antigen concentration, antibodies are serially diluted. Assuming 100% binding at very high antibody concentrations, the reciprocal of the antibody concentration at which binding reaches 50% is defined as the affinity. The antibody affinity constant calculation formula

1.2.7 Homology Modeling and Molecular Docking

The amino acid sequence of scFv was submitted to the SWISS-MODEL server (http://swissmodel.expasy.org/). Through sequence alignment, templates with high similarity to the target protein and known structures were identified. The Align Structures (MODELER) command in DS software was used for structural alignment of templates. The Align Multiple Sequences command was used to align the deduced scFv amino acid sequence with the aligned templates. The Build Homology Models command was then used for homology modeling.

The model with the smallest PDF Total Energy value was selected as the initial model. The Model Antibody Loops command was used to optimize the antibody variable regions to obtain the final model. The Ramachandran Plot command was used to generate Ramachandran plots for final model evaluation.

The 3D structure of AFB1 was downloaded from PubChem (http://pubchem.ncbi.nlm.nih.gov). The Dock Ligands command was used to perform molecular docking of scFv with AFB1. The Receptor-Ligand Hydrogen Bonds and Receptor-Ligand Bumps commands were used to predict intermolecular forces. The Draw Ligand Interaction Diagram command was used to generate 2D interaction diagrams. The binding interactions and key binding sites between scFv and AFB1 were analyzed.

2. Results

2.1 VH and VL Gene Amplification and scFv Assembly

After five immunizations, the mouse serum titer reached 2×10^{6}. Total RNA was extracted from spleen cells and used as template for reverse transcription. VH and VL gene fragments were specifically amplified, yielding single specific bands for each. After recovering the VH and VL gene fragments, directional amplification of VH-linker-VL was performed to construct the single-chain antibody gene, which was approximately 750 bp in size. The results are shown in Figure 1 [Figure 1: see original paper], and the fragment size matched the theoretical value.

[Figure 1: see original paper] Figure 1. Amplification of variable regions VH and VL genes and scFv construction

2.2 Anti-AFB1 Phage Display Antibody Library Construction and Panning

After phagemid transformation into *E. coli*, plate counting estimated the phage scFv antibody library capacity at approximately 5×10^{6} cfu/mL. After helper phage superinfection, the phage display scFv library capacity reached 2×10^{13} pfu/mL.

The library capacity obtained in this study was 2–3 orders of magnitude lower than reported in the literature [15,16]. Library capacity is an important indica-

tor for evaluating library quality and a critical factor for successful selection of positive clones.

After five rounds of panning, the input and eluted phage titers were determined for each round. As shown in Table 1 , the captured phages in round 5 were enriched 6.96-fold compared to round 1. Ninety-two single colonies were randomly picked from the plates after round 5, and phage ELISA was used to measure the binding activity of culture supernatants to antigen. Clones with P/N values (OD of positive wells/OD of negative wells) ≥ 2.1 were considered positive. A total of 13 positive clones were obtained. Plasmid extraction and sequencing revealed that all 13 positive clones had the same DNA sequence. The translated amino acid sequence is shown in Figure 2 [Figure 2: see original paper], with the black underlined portion representing the VH-VL linker. This positive sequence was designated as scFv.

Table 1. Enrichment of phage display anti-AFB1 scFv by immunoaffinity panning

AFB1-OVA concentration: 20, 20, 10, 10, 5 g/mL
Phage input: $2.1\times10^{13}, 1.3\times10^{13}, 1.5\times10^{13}, 2.5\times10^{13}, 1.8\times10^{13}$ cfu/mL
Phage captured: $5\times10^{2}, 9\times10^{2}, 1.2\times10^{3}, 1.6\times10^{3}, 3\times10^{3}$ cfu/mL

[Figure 2: see original paper] Figure 2. Amino acid sequence of anti-AFB1 scFv (underlined portion indicates the linker sequence)

2.3 Anti-AFB1 scFv Recombinant Expression and Property Analysis

After constructing the recombinant expression vector pET-30a-scFv, it was transformed into *E. coli* BL21 (DE3) for induced expression. SDS-PAGE was used to analyze the expression of purified recombinant scFv protein. As shown in Figure 3 [Figure 3: see original paper], the expressed scFv had a molecular weight of approximately 28 kDa and was soluble.

[Figure 3: see original paper] Figure 3. SDS-PAGE analysis of purified scFv-A/B. M: Protein marker; Lane 1: Purified scFv.

The physicochemical properties of anti-AFB1 scFv were predicted using the Protparam tool. Results showed a theoretical molecular weight of 25.8 kDa, theoretical isoelectric point of 8.56, and grand average of hydropathicity (GRAVY) of -0.366 (negative value indicates hydrophilic nature). The instability index was 54.93, indicating suboptimal stability, which is one of the main limitations for scFv applications [13]. Through affinity measurement and calculation using the formula, the scFv affinity constant was determined to be 8×10^{5} L/mol.

2.4 scFv Bioinformatics Analysis

2.4.1 Homology Modeling and Molecular Docking of scFv Through homology modeling and antibody loop optimization, a 3D structural model of

scFv was obtained, as shown in Figure 4A [Figure 4: see original paper]. The heavy chain region is indicated in blue, and the light chain region in red. The heavy and light chains together form a pocket region that is predicted to enable high-affinity binding.

[Figure 4: see original paper] Figure 4. 3D model of anti-AFB1 scFv (A) and its molecular docking model with AFB1 (B).

Ramachandran plot analysis showed that the frequency of amino acids with unreasonable spatial orientation in the scFv 3D structure model was 13/226 (5.7%), which falls within the excellent model range ($ $10%), indicating a high-quality Ramachandran plot and thus a reasonable 3D model. Most of the amino acids with unreasonable spatial orientation were located in the linker peptide connecting heavy and light chains, and their orientation has minimal impact on molecular docking results.

Based on the constructed 3D structure and downloaded small molecule model, the spatial conformation of the binding complex was simulated. The binding sites of scFv with AFB1 were identified as Tyr33, Ser52, and Tyr102 on the heavy chain. Tyr33 binds through π-π conjugated bonds, Ser52 through hydrogen bonds, and Tyr102 through π-π conjugated bonds and van der Waals forces, as shown in Figure 4B and Figure 5 [Figure 5: see original paper].

[Figure 5: see original paper] Figure 5. 2D molecular docking diagram of scFv-AFB1.

3. Discussion

Phage antibody libraries are a commonly used technology for antibody preparation. In this study, we immunized mice, amplified antibody variable region genes, and constructed an anti-AFB1 single-chain antibody gene library with a capacity of 10^6 magnitude. Library capacity is an important indicator for evaluating library quality and a critical condition for successful selection of positive clones.

The library capacity obtained in this study was 2–3 orders of magnitude lower than reported in the literature [15,16]. This may be due to the use of $CaCl_2$ chemical transformation; subsequent studies could attempt electroporation to reduce loss of single-chain antibody genes during library construction and improve transformation efficiency [21]. Additionally, using the Cre-LoxP *in vivo* recombination system to construct phage antibody libraries is an effective method to increase library capacity. Sun et al. [22] introduced the Cre-LoxP homologous recombination system into the primary library, enabling free recombination between different antibody genes, doubling library capacity and diversity, and obtaining a phage antibody library of 10^{11} magnitude.

The advantage of single-chain antibodies lies in their smaller molecular weight, only one-quarter that of conventional antibodies, with better solubility and tissue penetration. In this study, a single-chain antibody scFv with relatively high

affinity was obtained after five rounds of screening from the phage display antibody library, with an affinity constant of 8×10^{5} L/mol, which is slightly lower than reported in references [23,24]. This may be because connecting VH and VL through a short peptide often results in lower affinity than the original antibody [25]. *In vitro* antibody affinity maturation is a highly efficient method for improving antibody affinity [26]. Common strategies for *in vitro* affinity maturation include: (1) Error-prone PCR to introduce point mutations [27]; (2) Recombination of CDR regions that play major roles in antigen binding, particularly CDR3 region shuffling, which can greatly improve antibody affinity [28]. Randomization of CDR3 amino acid sequences also increases synthetic antibody library diversity and capacity, with CDR3-modified libraries reaching capacities above 10^9 [29]; (3) Chain shuffling technology—Sun et al. [30] constructed a heavy chain-shuffled library by replacing the light chain variable region and screened a human antibody against rabies virus glycoprotein with affinity reaching 10^9 magnitude; (4) DNA shuffling—researchers [31] combined error-prone PCR and DNA shuffling to randomly mutate heavy and light chain variable regions of an anti-hepatocellular carcinoma scFv, obtaining an antibody that maintained original specificity while affinity increased more than 2-fold.

Meanwhile, library source, construction method, and antibody type all influence the binding mode and capacity between antibody and AFB1 to some extent (Table 2) [32-35]. Using homology modeling to predict scFv tertiary structure and docking it with the AFB1 model, the most stable docking conformation was obtained after optimization. Based on the 3D and 2D docking diagrams, key amino acids in scFv during antigen AFB1 binding and the antigen-antibody binding modes can be clearly identified. In future studies, site-directed saturation mutagenesis can be used for *in vitro* affinity optimization of this scFv. By introducing site-directed mutations at key amino acids to obtain mutants substituted with each of the other 19 natural amino acids, a site-directed saturation mutagenesis library of anti-AFB1 single-chain antibodies can be constructed. Simultaneously, randomization of CDR3 region amino acid sequences can be performed to screen for scFv with improved affinity, providing strong support for the development of bioinformatics in the field of anti-AFB1 antibody screening.

Table 2. Comparison of binding modes of different antibodies to AFB1

Antibody Type	Library Source	Binding Mode to AFB1
Nb-G8 nanobody	Phage display nanobody immune library	Thr32, Ile33, Phe49, Typ54, Tyr106, and Val112 residues may directly participate in epitope formation

Antibody Type	Library Source	Binding Mode to AFB1
scFv-H4	Tomlinson I+J library	AFB1 is primarily enveloped in a hydrophobic cavity formed by Ala91, Pro95, Phe98 in light chain and Lys96, Thr97, His95 in heavy chain, with hydrogen bonds forming between the difuran ring and Asp
Monoclonal antibody	Hybridoma cell line 1C11	Ser49 and Phe103 in heavy chain bind AFB1 through hydrogen bonds and hydrophobic interactions
scFv	Anti-AFB1 monoclonal antibody hybridoma cell line	In tertiary structure, the linker pulls VH and VL regions close to each other, forming a typical groove structure as the antigen-binding region
scFv	Phagemid-helper phage display system constructed single-chain antibody library	Tyr33, Ser52, and Tyr102 play key roles in binding to AFB1 through π-π conjugated bonds, hydrogen bonds, and van der Waals forces, respectively

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