Selection of Aflatoxin B1 Mimic Epitope Peptides by Phage Display

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Abstract: Aflatoxin B1 (AFB1) is a natural pollutant with strong toxicity and carcinogenicity. It not only causes huge economic losses, but also poses a serious threat to human, livestock and poultry health. To acquire the mimic epitope peptide of aflatoxin B1 and establish a non-toxic detection system of aflatoxin, an anti-afb13c7 monoclonal antibody was used as the target molecule, and the mimic epitopes of AFB1 were screened from the phage random 7 peptide library. A total of 39 phage clones were selected for verification. 36 of them could specifically bind to the antibody, and 30 of them could be inhibited by AFB1, whose DNA was extracted and sequenced. The results showed that 30 positive phage particles were actually 17 phage clones. The common sequence was histidine (H) - proline (P) - tryptophan (W), abbreviated as xxxxhpw, xxxhpwx, xxhpwxx, xhpwxxx (X was any amino acid). The linear range, detection limit and half inhibitory concentration (IC50) of the 17 positive phage particles were similar. The linear range was 1-2000 pg / ml.

1 INTRODUCTION

Aflatoxin B1 (AFB1) is a secondary metabolite produced by Aspergillus flavus, Aspergillus parasiticus and Aspergillus wasabi, which has been proved to be carcinogenic, teratogenic and mutagenic (Riikka, 2017, Rushing, 2018). AFB1 is easy to pollute wheat, rice and other agricultural products, which poses a serious threat to human, livestock and poultry health (Anja, 2016, Zhao, 2015). The method of monitoring and detecting AFB1 is important to prevent its harm, which has great research significance.

Domestic and foreign scholars have done a lot of research on the detection methods of AFB1.

Since the immunoassay for AFB1 have to use the toxin both in free and conjugated forms, it may pose a toxicity risk to kit manufacturers and users, so it is an urgent problem to find the substitute of AFB1 standard (Liu, 2016). The epitope peptide of AFB1 McAb was successfully screened by phage display technology in this study.

2 MATERIALS AND METHODS

2.1 Reagents and Instruments

The anti-AFB1 monoclonal antibody was made in Laboratory of Jiangxi Normal University of science and technology. Aflatoxin B1 was purchased from Solarbio. IPTG and X-gal were purchased from Golden clone Biotechnology Co., Ltd. Random phage 7 peptide library and E.coli ER2738 were purchased from NEW ENGLAND BioLabs. Agar Powder was purchased from Chembase. Polyethylene glycol. PEG-8000 was purchased from Shanghai Shenggong biology Co., Ltd. Mulitiskan MK3 microplate reader (Thermo, USA). Centrifuge5804r freezing centrifuge (Eppendorf, USA). Washing machine (Thermo, USA). Pipette gun (Thermo, USA). Polystyrene 96-well microtiter plates (Costar).

2.2 Panning and Identification of Positive Mimetic Peptides

The specific operation process is as follows (Wang, 2018, Shi, 2019)

The solid phase carrier (Polystyrene 96-well microtiter plates) was coated with monoclonal antibody and incubated with phage library for 60

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minutes. The unconjugated phage was washed out, and the specific binding phage was washed down to amplify the phage. The above steps were repeated for 3 times and the phage titer was determined. The final product was confirmed by positive clone sequencing, and the binding of the selected peptide to the monoclonal antibody was detected by ELISA. The specific steps are as follows.

A. The anti-AFB1 monoclonal antibody was dissolved in the PBS of 0.1M pH 7.4 (100, 75, 50μg/mL), and the 100μL/pore 4°C package was spent overnight.

B. Discard the liquid in the hole, wash it 3 times with TBST (including 0.1% Tween20), put the board upside down on a clean tissue and pat it hard to remove the residual solution. Add 3% BSA-PBS 300μL/hole 4°C to seal for 2 hours.

C. Wash it 3 times with TBST (including 0.1%-0.05% Tween20), add the phage peptide bank (Ph.D.-7 library) 100μL per hole (dillute it with TBST at 1:10, containing phage about 2×1011pfu), and shake gently at 16-22°C. It should be 1h.

D. Discard the liquid in the hole, wash it 10 times with TBST, and wash away the unbined bacteriophages.

E. Add the lotion (Gly-HCL pH2.2) 100μL per hole, shake it gently at 16-22°C for 8 minutes, suck out the lotion, and add 15μL neutralizing buffer.

F. Except for 10μL for titer determination after neutralization, the rest are added to 20mL inoculation with the LB culture medium of E.coli ER2738, which is in the pre- logarithmic growth stage for amplification culture.

G. After 4.5 hours of oscillation culture of 37°C, 4°C 10000rpm centrifugal for 10 minutes, 1/6 volume of PEG/NaCl is added to the supernatant, and 4°C precipitates overnight.

H.4°C centrifugal 15min (10000rpm), remove the upper clearing, then use 1 mL TBST suspended phages, and add 1/6 volume of PEG / NaCl ice to incubate 60 minutes. 4°C centrifugal 15min (10000rpm), de-clearing, precipitation with 200μL TBST suspension and 10μL to measure titer.

A. Inoculated with E.coli ER2738 monobacterium colony in 5-10mL LB medium, rocker cultured to logarithmic mid-term (OD600 at about 0.5).

B. When cells grow, melt the upper agar and divide it into 3mL equal parts in the sterilization test tube, and dilute one tube for each phage. Store at 45°C for later use.

C.37°C warm up the LB/IPTG/Xgal plate, and take one tablet for each phage dilution.

D. The bacteriophages to be tested are diluted with a 10x series of LB medium.

E. When the bacterial culture reaches the middle logarithmic, it is divided into 200μL equal parts in the trace centrifugal tube, and each phage dilutes one tube.

F. Add 10μL bacteriophages with different dilution degrees to each tube, quickly oscillate and mix well, and warm up 16-22oC for 1-5 min. Add infected cells to the upper agar culture tube pre-temperatured at 45°C, mix one tube quickly at a time, and immediately pour them on the LB/IPTG/Xgal plate pre-temperatured at 37°C. Tilt the plate appropriately to spread the upper agar evenly.

G. After the plate is cooled for 5 minutes, put it upside down at 37°C for the night. Check the plate and count the number of spots on the plate with ~102 phage plaques. Then multiply this number by dilution factor to obtain the empty spot formation unit (pfu) titer per 10μL phage. DNA of positive phage clones was extracted and sequenced

A. Inoculate overnight cultures of E.coli ER2738 at 1: 100 dilution on LB medium and 1 mL into culture tubes. One tube per clone to be identified.

B. Using a sterilized toothpick or tip, pick a blue plaque into the 1 mL culture tube above. Note: The plaque should be selected from a total of less than 100 plaque to ensure that each plaque selected contains only one DNA sequence.

C. 37 °C shaker culture 4.5-5 h.

D. The cultures were transferred into microcentrifuge tubes and centrifuged at 4C 30 sec (10000 rpm). The supernatant is transferred into a fresh tube and centrifuged again. 80% supernatant was transferred to fresh centrifuge tube with pipette. This was the amplified phage reservoir, stored at 4 °C or diluted with sterile glycerin 1: 1 and stored at -20 °C.

E. coli ER2738 was inoculated in 20 mL LB medium and cultured at 37 °C to prologarithmic stage. Alternatively, the overnight culture of E.coli ER2738 was diluted in a 1: 100 ratio in 20 mL LB medium.

F. Adding 5L phage reservoir to each tube of Ecoli ER 2738 culture medium, aerated at 37 °C for 4.5 h.

G. The above cultures were transferred into centrifuge tubes and centrifuged at 10,000 rpm for 10 min. The supernatant is moved into a fresh centrifuge tube and centrifuged again.

H. Take 80% supernatant in fresh centrifuge tube, add 1 / 6 volume of PEG / NaCl. Precipitation at 4 °C overnight.

I.4℃ 10000rpm 15 min Centrifugally precipitate and discard the supernatant.

J. Precipitate was resuspended in 1 mL TBS. The suspension was transferred to a microcentrifuge tube and centrifuged at 4 °C for 5 min (10000 rpm) to remove the residual cells.

K. supernatant was transferred into a fresh microcentrifuge tube and added 1 / 6 volume of PEG / NaCl to precipitate. Ice action 15-60 min. Centrifuge 10 min (10000 rpm) at 4 °C, discard the supernatant, and centrifuge briefly to remove the residual supernatants. The precipitate was resuspended in 50 L TBS and the titer of phage was determined. 4 °C storage.

A. Monoclonal antibody against AFB1 was dissolved in 0.1 M pH 7.4PBS (10 μg / mL) and coated overnight with 100 μL / well 4 °C.

B. PBST washed plate three times, 3% skim milk 300μL / hole 4 °C closed 2h.

C. Addition of 1 μg / mL of AFB1 (soluble in 20% methanol PBS) 50 μL and PBST diluted 50 μL. (Dilution ratio was twice the maximum dilution for identification of specifically bound positive phage clones with absorbance values of 1.0-1.5), 37 °C 1 h.

D. PBST washed 6 times and added horseradish peroxidase labeled anti-M13 monoclonal antibody (100 μL / well); 37 °C acts for 1h. Wash 6 times. Benzidine (OPD) color, 2M H2SO4 terminated reaction, OD value was measured. The original peptide library coated as positive control, McAb coated with original peptide as negative control, monoclonal antibody coated with PBST as blank control. According to the ratio of OD value (S) of test sample to OD (N) of negative control, the S / N was more than 2.1. The phage which can be blocked by AFB1 molecule is the mimotope of AFB1.

500μl of the phage-containing supernatant was transferred to a fresh microfuge tube and added with 200μl PEG/NaCl. The mix was let stand at room temperature for 10 minutes, then it was centrifuged for 10 minutes, supernatant was discarded. Pellet was suspended thoroughly in 100μl Iodide Buffer and added with 250μl ethanol. The resuspended solution was incubated for 10 minutes at room temperature and spun for 10 minutes, supernatant was discarded, the pellet was washed in 70% ethanol and dried briefly under vacuum. The pellet was suspended in 30μl TE buffer. Take 5ul of the above solution for nucleic acid electrophoresis, and send the rest to the Shanghai Shenggong biology Co., Ltd for sequencing.

2.3 Establishment of Competitive ELISA Standard Curve with AFB1 Mimic Epitope

Dilute AFB1 standard stock solution into a series of concentration standard solutions. According to the competitive steps under the optimized conditions, the luminescence value was determined through experiments, and the standard curve was established (the logarithm of the toxin standard solution concentration was the abscissa and the binding rate was the ordinate). Specific steps are as follows

A. After amplification and purification of positive phage particles, the optimal antibody coating concentration and phage concentration were determined by matrix titration.

B. anti-AFB1 monoclonal antibody soluble in 0.1 M pH7.4PBS, 100 μ L / pore 4°C was coated overnight. The original peptide library was coated as positive control, the monoclonal antibody coated with primitive peptide library as negative control and the mAb coated with PBST as blank control.

C. PBST washed plate three times, 3% skim milk 300μL / hole 4°C closed 2h.

D. Adding concentrations of 200, 100, 50, 25, 12.5, 6.25, 4, 2, 1, 0.5, 0.25, 0.125, 0.10, 0.05, 0 ng / mL AFB1 standard (dissolved in 20% methanol PBS) 50 μ L and positive phage 50 μL, shake and mix, keep moisture at 37°C for 1 hour.

E. PBST washed 6 times, added horseradish peroxidase labeled anti-M13 monoclonal antibody (100 μL / pore), 37°C for 1 h.

F. PBST washed 6 times. The reaction was terminated by 2M H2SO4. The optical density (OD value) at 450 nm was determined. The binding rate (%) = B / B0 × 100% (B0 is the OD value without AFB1 and B is the value of OD with AFB1).

Drawing Competition Inhibition Curve.

3 RESULTS

3.1 Analysis of DNA Sequence and Polypeptide Core Series

After DNA sequencing, the amino acid sequence was translated by the DNAMAN software. The 30 phage particles are actually 17 phage clones., No. A1-17 The common sequence of aflatoxin mimic epitope peptide is histidine (H) - proline (P) - tryptophan (W), abbreviated as xxxhpwx, xhpwxxx, xxxxhpw, xxhpwxx. X is any amino acid. The DNA and amino acid sequences are shown in Table 1.

Table. 1 The inserted DNA sequences of positive phages

Phage	Number	DNA sequence	Petide sequence
A1	1	AGATACAAAGTAGGAACCAGC	SMF**HPW**S
A2	2	CCACGCACCGTAGGAACCAGA	GAW**HP**W**S**
A3	1	ATAGTAGGAACCTCAACCACC	Y**HPW**SW
A4	1	GTCGTAAACACCGTAGGCACC	QHLW**HP**W
A5	6	ACCGGACACAAAGTAGGAACC	WPVF**HP**W
A6	1	CTCCAAAAAGTAGGAACCAGC	EVF**HPW**S
A7	2	ATAGGCCGAACCGTAGGAACC	YPAW**HP**W
A8	1	CTAAAAGGCACCGTAGGCACC	DFPW**HP**W
A9	2	CTAGTAGGCACCCACATAAGC	D**HPW**VYS
A10	3	CGCGTAGGCACCCCAGGATAA	A**HPW**GPI
A11	2	AGCCAAACCGTAGGAACCAGA	SVW**HPW**S
A12	2	ATAGTAGGAACCAGACACTGC	Y**HPW**SVT
A13	1	AAACACGTAGGCACCTAAACC	FV**HPW**IW
A14	2	TTATGCGTAGGAACCAAAACC	NT**HPW**FW
A15	1	CAATTACAAATAGTAGGAACC	VNVY**HP**W
A16	1	TGAAACGTAGGCACCGTAACC	TL**HPW**HW
A17	1	CACGTCGGAATAGTAGGCACC	VQPY**HP**W

3.2 Establishment of Standard Curve of Non-toxic ELISA with AFB1 Mimic Epitope

The standard curve of ELISA was established by coating 96 well plate with anti AFB1 monoclonal antibody (3c7) and incubating with phage particles a1-a17 with mimic epitope of AFB1. The results showed that the standard curve of ELISA based on these phage particles showed a good linear relationship. The linear range, detection limit and half inhibitory concentration (IC50) were basically similar, and the linear range was 1-2000 pg / ml (as shown in Fig1).

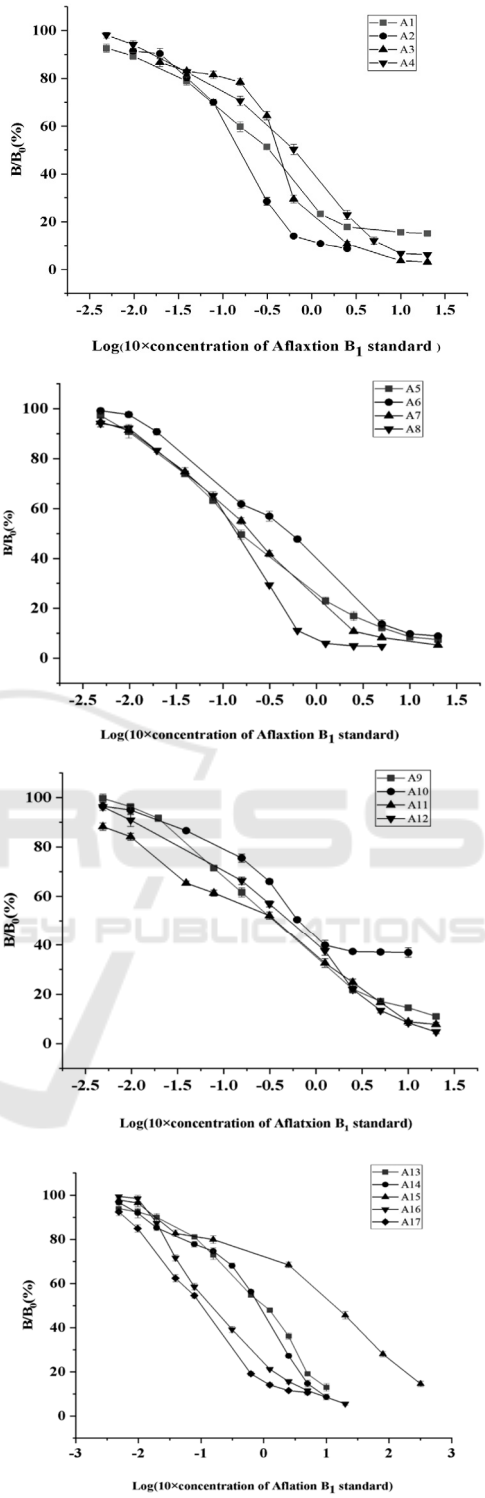

Figure 1 The standard curves of competitive ELISA of A1-A17

4 CONCLUSION

Appropriate concentration of target protein is beneficial to the screening of mimic peptides. Reducing target protein concentration may improve the specificity of washing. In order to screen antigen mimic epitopes with high affinity, the detergent concentration of the first round of elutriation was 0.1%, and that of the second round of elutriation was 0.5%. The number of ligands that have affinity with the target protein at the beginning of panning is very small. If the detergent concentration is too high, the ligands with weak binding force may be lost. In the second round, due to the amplification of ligands, the concentration of detergent can be increased, and high affinity ligands are more easily elutriated.

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