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RESEARCH PAPER

Cyclic peptide: a safe and effective alternative to synthetic aflatoxin B_1-competitive antigens

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Abstract

Aflatoxin B_1 (AFB_1) is one of the major mycotoxins, which naturally occurs in food and agricultural products. In this study, a cyclic peptide (CVPSKPGLC) mimicking AFB_1 was used to develop a biotinylated peptide–based immunoassay (bp-ELISA) for AFB_1 determination. This cyclic peptide was isolated from a commercially available phage-displayed random 7-mer cyclic peptide library, and then synthesized chemically. Instead of phage particles, the peptide was biotinylated and used to detect AFB_1 by bp-ELISA, with an IC_{50} of 0.92 ng/mL, which was approximately 60-fold better than that of phage ELISA. Good recoveries (83–102%) were obtained in spiked rice and corn samples, which were further validated by high-performance liquid chromatography-fluorescence detector. As better sensitivities (0.92–1.21 ng/mL) were obtained by bp-ELISA even using selected anti-AFB_1 antibodies prepared previously in laboratory, this cyclic peptide is suitable as a substitute for synthetic competitive AFB_1 antigens in food contamination monitoring.

Keywords Aflatoxin B_1 · Phage display · Cyclic peptide · Immunoassay

Introduction

Aflatoxins (AFTs), mainly produced by *Aspergillus flavus* and *Aspergillus parasiticus* in food and agri-products, were first discovered in the 1960s in the UK during an outbreak of "turkey X" disease [1]. Aflatoxin B_1 (AFB_1) is considered to be the most toxic aflatoxin, and is classified as a group I carcinogenic substance by the International Agency for Research on Cancer in 1993 [2]. Owing to its toxicological and carcinogenic effects on humans, maximum allowable limits of AFB_1 in food and agri-products have been established by national and international institutions. The limit of total aflatoxins (aflatoxin B_1, B_2, G_1, and G_2) or AFB_1 alone has been set in the range of 2.0–20 μg/kg [3, 4]. Importantly, AFB_1 is stable and unresponsive to heat and alkali treatment, and hence, difficult to neutralize after contamination [5]. Therefore, it is highly desirable to develop a safe, sensitive, and economically inexpensive assay to detect AFB_1 in foodstuff.

When compared with analytical methods such as thin-layer chromatography, high-performance liquid chromatography (HPLC), and liquid chromatography-tandem mass spectrometry [6–8], antibody-based immunoassays are more suitable methods for AFB_1 detection owing to their simplicity, high-throughput detection, and comparable sensitivity and specificity [9]. However, AFB_1 is a low molecular weight (MW) chemical (MW < 1500 Da) [10] that is mainly detected in a competitive manner. Accordingly, competing antigens are synthesized and optimum performance is obtained by using batches of trials [11]. Typically, a heterologous system, which

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differs with regard to the hapten or carrier protein between the immunogen and competitive antigen, is always employed to produce more sensitive assays [12, 13]. However, this system requires a considerable amount of time to synthesize competing antigens and to achieve their conjugation with carrier proteins, which is elaborate and inevitably necessitates handling of the toxin [14]. In contrast, phage-displayed technology is an attractive and powerful tool applied in peptide identification used in mycotoxin immunoassays [15–18] because peptides displayed on phage particles can mimic competitive antigens that bind to the antibody by recombinant DNA technology [19]. However, compared with phage particles, the peptides alone, acting as mimotopes of AFB_1, possess some incomparable advantages as follows: (1) peptides are small in molecular weight and induce weak immunogenicity [20]; (2) peptides are flexible in synthesis and chemical conjugation, either be synthesized chemically or expressed by fusion with signal proteins [21]; (3) they reduce biological risk (phage-infecting *Escherichia coli*) [22]; (4) they are not affected by phage production (more than eight amino acids may not support phage production in pVIII system [23]); and (5) peptide-based biosensors always present high sensitivity, selectivity, and long-term stability [24].

The synthetic peptides have been proven to be suitable for application in immunoassay industry [25]. In a previous study, a biotinylated 12-mer peptide "GMVQTIF-GGGSK" was synthesized and used as a competing antigen to develop an ELISA for ochratoxin A with a IC_{50} of 0.024 ng/mL [26]. Synthetic peptides derived from phages have also been produced to develop microarray-based immunoassays for fumonisin B_1 [27] and non-competitive two-site immunoassays for molinate and clomazone [28]. Besides, mimotope peptides can be fused and expressed with maltose-binding proteins [29] and core streptavidin in *E. coli* ER2738 [22]. However, the fusion protein may be expressed in inclusion bodies and need a series of complicated steps to be refolded [22].

Owing to fewer three-dimensional conformations, constrained peptides (with disulfide bonds between two cysteine residues) always present enhanced binding toward target molecules and more resistance to hydrolysis unconstrained (linear) peptide [30]. Peptide cyclization is an easy way to create constrained structures of short peptide sequences [31], and the constrained ring size and structures of the cyclic peptides presented the differences on the favorable binding affinities toward target molecules [32]. In the present study, anti-AFB_1 peptides displayed on phage particles were isolated from a disulfide-cyclized Ph.D.-C7C phage display library. Among them, peptide with the amino acid sequence CVPSKPGLC was synthesized and biotinylated to develop an immunoassay based on biotinylated mimotopes (known as biotinylated peptide–based ELISA, bp-ELISA) with higher sensitivity. Biotin-streptavidin complexes were also used in this assay, because they are easy to chemically conjugate with a tracing enzyme, which is helpful to improve the assay sensitivity owing to the extraordinarily high affinity between them (dissociation constant $\approx 10^{-15}$ M) [33, 34]. To our knowledge, very few peptide-based assays have been developed for mycotoxins, especially based on cyclic peptide.

Materials and methods

Chemicals

AFB_1, B_2, G_1, and G_2, ochratoxin A (OTA), zearalenone (ZEN), citrinin (CIT) standards, bovine serum albumin (BSA), polyethylene glycol 8000 (PEG 8000), Tween 20, and 3,3,5,5-tetramethylbenzidine (TMB) were obtained from Sigma (St. Louis, MO, USA). New Zealand white rabbit was immunized by AFB_1-BSA conjugation and anti-AFB_1 polyclonal antibody (PAb) was collected by centrifugation and ammonium sulfate precipitation. BALB/c mice were also immunized by AFB_1-BSA conjugation and anti-AFB_1 monoclonal antibodies (MAb) 2F5 and 2E6 were produced by hybridoma techniques in our laboratory. Both AFB_1-BSA and AFB_1-coating antigen (AFB_1-OVA) were formed by AFB_1-oxime derivative coupling with carrier proteins in our laboratory. Mouse anti-M13 MAb-horseradish peroxidase (anti-M13 HRP) was purchased from GE Healthcare (Piscataway, NJ, USA). The phage display library on the coat protein pIII expressing a loop-constrained heptapeptide (Ph.D.-C7C) and *E. coli* ER2738 host strain were purchased from New England Biolabs (Ipswich, MA, USA). Streptavidin-HRP and EZ-Link® NHS-LC-Biotin Reagents were obtained from Thermo Fisher Scientific Inc. (Rockford, IL, USA).

Phage biopanning and identification

The competitive panning method employed in this study was similar to that reported by Wang et al. [35], with slight modifications. In brief, one well of a microtiter plate was coated with 100 μL of MAb 2F5 in phosphate-buffered saline (PBS) and four wells were coated with 100 μL of 1% BSA and incubated overnight at 4 °C. The next day, following blocking with 200 μL of 3% BSA for 1 h at 25 °C, the phage library (1×10^{13} pfu/mL) diluted with PBS was first added to the MAb 2F5-coated well and incubated for 1 h at 25 °C. After washing with PBST (PBS with 0.05% Tween 20) ten times, the combined peptide was competitively eluted using 500 ng/mL AFB_1 in 10% methanol PBS. The supernatant was then transferred in equal amounts into the other four wells and incubated for 1 h at 25 °C. Subsequently, the supernatants of the four wells were collected and infected into *E. coli* ER2738 for amplification by mixing with PEG solution (20% [*w*/*v*] PEG 8000 and 2.5 M NaCl). The entire panning procedure was repeated twice, except for the use of concentrations of

AFB_1, MAb 2F5, and Tween 20 in PBST (0.1%, 0.15%) in the second and third round of panning, respectively.

After each round of panning, the eluted phage titer was determined according to the manufacturer's protocol. The clones were picked from the plate after the third round of panning to determine their binding ability to MAb 2F5 by using competitive phage ELISA [35]. Briefly, the phage ELISA was performed by adding the phage supernatant with or without AFB_1 (10% methanol in PBS) to wells with 1 μg/well of anti-AFB_1 MAb. Anti-M13 HRP (100 μL/well) were incubated and used as secondary antibody. Positive clones were selected for DNA extraction (Omega Bio-Tek, Inc. Norcross, GA, USA). The phage DNA was sequenced by Sangon Biotech (Shanghai, China) using 96 gIII primer (CCCTCATAGTTAGCGTAACG).

Biotinylated peptide

The positive peptide was synthesized by Sangon Biotech. An aliquot of 2 mg of peptide was dissolved in 50 μL of N,N-dimethylformamide (DMF) and 4.6 mg of EZ-Link® NHS-LC-Biotin were dissolved in 50 μL of DMF. Then, the biotin solution was mixed dropwise into the prepared peptide, and stirred gently for 2 h on ice. Following dialysis (MWCO 1000) by 0.01 M PBS (pH 7.4) for 24 h, the biotinylated peptide was stored at − 20 °C until use. The ratio of peptide and NHS-LC-biotin was set at 1:1 and 1:2.

Bp-ELISA

The amount of MAb 2F5 and dilutions of the biotinylated peptide were optimized by using checkerboard titration method. The MAb 2F5 coating was diluted in PBS, and 100 μL/well was pipetted into a microtiter plate and held overnight at 4 °C. The next day, the wells were washed thrice with PBST (0.05% of Tween 20 in PBS) and blocked with 1% BSA in PBS for 1 h at 25 °C. After another washing step, equal volumes of a series of concentrations of AFB1 and biotinylated peptide were mixed and pipetted (100 μL) into the blocked plate. After incubation for 1 h at 25 °C and another wash step, 100 μL of streptavidin-HRP (1:2000 dilution in PBST) were added and incubated for 1 h at 25 °C. Then, 100 μL of TMB solution (400 μL of 0.6% TMB in DMSO and 100 μL of 1% H_2O_2 diluted with 25 mL of 0.1 mol/L citrate-acetate buffer, pH 5.5) was dispensed into each well. The reaction was stopped by the addition of 50 μL of 2 M H_2SO_4 and absorbance was measured at 450 nm. The IC_{50}, the expression of the assay sensitivity, the limit of detection (LOD, IC_{10}), and linear detection range (IC_{20}–IC_{80}) were determined from the four-parameter logistic calibration curve using SigmaPlot 10.0.

Indirect competitive ELISA

The amount of AFB_1-OVA-coating antigen and dilutions of the primary antibodies (anti-AFB_1 MAb 2F5, 2E6, and PAb) were optimized by the checkerboard titration method. AFB_1-OVA (0.05 μg/well) was coated on plate with coating buffer (pH 9.6) and 100 μL/well was added and incubated for coating overnight at 4 °C. The next day, the plate was washed and blocked as described earlier. A series of AFB_1 (50 μL) standard solution was then added to the wells, followed by the addition of equal volume of primary antibody (1 μg/mL, 0.2 μg/mL, and 0.2 μg/mL for PAb, 2E6, and 2F5, respectively). After further incubation and washing, the bound PAbs and MAbs were detected with goat anti-rabbit antibody-HRP and goat anti-mouse antibody-HRP, respectively. All the subsequent steps were similar to those employed for bp-ELISA.

Characteristics of biotinylated peptide

To study the stability of the biotinylated peptide, both the phage particles displaying peptide and biotinylated peptide were stored at 4 °C and 25 °C, and their binding abilities to anti-AFB_1 MAb 2F5 were evaluated by phage ELISA and bp-ELISA, respectively.

To study the binding activities of the cyclic peptide, anti-AFB_1 PAb and MAb 2E6 [36] were coated onto a 96-well plate at a concentration of 2 μg/mL. Both the bp-ELISA and indirect competitive ELISA (icELISA) were conducted for each antibody, and the IC_{50} and linear detection ranges were calculated.

Bp-ELISA optimization

To optimize the bp-ELISA conditions, the organic solvents (methanol, DMSO, and DMF) and their concentrations (5%, 10%, 15%, and 20%) were varied. The effects of pH were evaluated using PBST with pH ranging from 4.0 to 10.0.

Cross-reactivity

The specificity of the optimized bp-ELISA was determined by measuring cross-reactivity (CR) using a group of common mycotoxins. The CR was calculated as follows: CR (%) = [IC_{50} (AFB1)/IC_{50} (tested compound)] × 100.

Sample preparation

Rice and corn used for sample spike-and-recovery assessment were purchased from a local market in Wuhan, China. For matrix assay, 5 g of ground sample was mixed with 15 mL of methanol/water (8:2, *v*/*v*) and vortexed for 20 min, followed by centrifugation at 4000×*g* for 10 min. The supernatant was

collected and diluted 10-fold with PBS-BSA solution before bp-ELISA.

For the recovery study, 5 g of rice and corn samples were thoroughly dried, ground, and spiked with AFB_1 in methanol (100 μg/mL) to reach a final concentration of 10, 20, and 40 ng/g, followed by mixing thoroughly by vortexing to homogeneity and extracted as above. The recovery AFB_1 concentration was also validated by HPLC system with a fluorescence detector ((HPLC-FLD); excitation wavelength = 365 nm, emission wavelength = 436 nm). Before loading to HPLC system, the samples were cleaned using an AFB_1 immunoaffinity column (VICAM, Watertown, MA, USA). All other conditions for HPLC were in accordance with the standard Chinese detection method for AFB_1 [37].

Results and discussion

Isolation of MAb 2F5-binding phage-displayed peptides

In the present study, the commercial library cyclized 7-mer peptides displayed on phage particles with CX_7C format were employed for biopanning with anti-AFB_1 MAb 2F5. Screening from a linear 7-mer and linear 12-mer peptide library was also conducted in the presence or absence of AFB_1 in our previous work; however, none of the clones represented a binding epitope for antibodies identical to AFB_1. These prepared libraries have complexities on the sequences of 10^9 independent clones, which is sufficient to encode almost of possible 7-mer peptide sequences ($20^7 = 1.28 \times 10^9$). Together with the disulfide linkage oxidized by a pair of cysteine residues, the cyclized 7-mer peptide displayed on phage would be one of the ideal libraries for isolating ligands against target molecules. Generally, the differences on constrained ring size and flanking residues around the sequence motif would be a factor that influences the selected peptide sequences [32]. Owing to disulfide bridge, the cyclic peptides has more rigidity in conformation and the entropy term of the Gibbs free energy decreased, allowing the enhanced binding toward target molecules [30]. Therefore, such structurally constrained phage display library is advantageous in yielding high-affinity ligands and screening successfully for targets which cannot be obtained from the linear peptide libraries [38]. In the present study, the initial input phage for each round of selection was around 1×10^{11} pfu/well phage. With a decreasing concentration of coating antibody and increasing concentration of Tween 20, the titers of the output phage during the successive panning procedure did not obviously vary (7×10^7, 2.24×10^6, and 3.2×10^7 pfu/mL for the first, second, and third panning round, respectively). After the third round of biopanning, 47 clones were picked from the plate and verified by phage ELISA. Among them, 12 clones presented higher binding abilities with the coating MAb as competitive mimotopes and lower binding abilities in PBS-coated wells (Fig. 1). Clone C43 was chosen and sequenced as CVPSKPGLC, which exhibited an IC_{50} of 60 ng/mL in phage ELISA (see Electronic Supplementary Material (ESM) Fig.S1).

Two prolines (P) were found in clone C43, similar to that determined in previous studies [18, 39]. It must be noted that a disulfide closed loop rich in Pro (P), Val (V), or Ile (I) is more constrained than sequences that are rich in Gly (G), Ala (A), or Ser (S) [40]. Moreover, Pro is considered as one of the most common amino acids in the interaction between anti-AFB_1 antibodies and aflatoxins [18].

Optimization of the biotinylated peptide

The peptide CVPSKPGLC was synthesized and chemically labeled with biotin. Typically, a flexible linker (Gly_4Ser) is inserted or synthesized to modify the coupling protein characteristics [26]. Instead of that, in the present study, NHS-LC-biotin (spacer arm length, 22.4 Å) was used as a biotinylating reagent with longer spacer arm to reduce steric hindrance and ameliorate the biotin residue for avidin/SA-based reagents [41]. Furthermore, lysine (K) residues and N-terminus of each peptide with $-NH_2$ residue were used as labelling targets. The ratio of the peptide and NHS-LC-biotin was set as 1:1 and 1:2, respectively. Higher absorption in the absence of AFB_1 and lower absorption in the presence of 50 ng/mL of AFB_1 were observed at the ratio of 1:2, which was set as the ratio for further study (see ESM Fig. S2).

Optimization of bp-ELISA

The concentrations of MAb 2F5 and biotinylated peptide were optimized by checkerboard assay. The results revealed that 2 μg/mL anti-AFB_1 MAb 2F5 and 20 μg/mL biotinylated peptide were the optimal concentrations (see ESM Table S1). The performance of bp-ELISA was improved by several factors, such as organic solvents and pH (Fig. 2). It must be noted that owing to the lipophilic nature of AFB1, organic solvents are commonly used as water-miscible solvents to dissolve AFB_1 and as extract solvents in sample matrix. In the present study, the effects of methanol, DMSO, and DMF on bp-ELISA performance were determined (Fig. 2a–c). PBS buffer with 10% methanol was found to be the most suitable for bp-ELISA owing to more competitive abilities with 1 ng/mL AFB_1 and acceptable absorbance in the absence of AFB_1. As shown in Fig. 2 d, the buffer pH varied in the range of 4.0–9.0 and had a slight influence on bp-ELISA performance. Thus, under the optimized conditions (10% MeOH in PBS, pH 7.4), a typical calibration curve of bp-ELISA for AFB1 was obtained (Fig. 3). Moreover, bp-ELISA presented a linear range (IC_{20}–IC_{80}) of 0.23–3.36 ng/

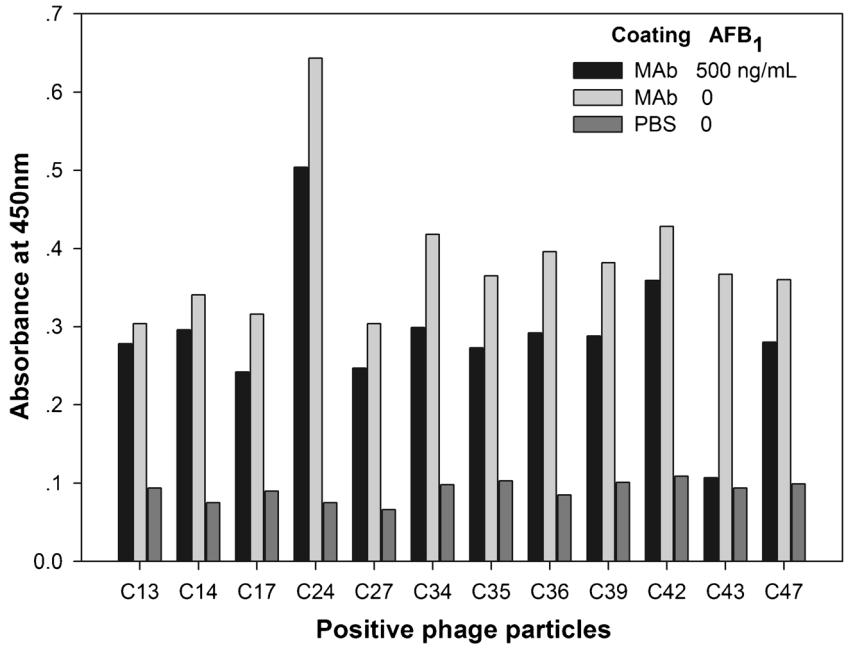


Fig. 1 Screening of positive phage clones specific to anti-AFB_1 MAb 2F5 by competitive phage ELISA. Only PBS-coated wells were used to test the non-specific binding of phage particles to blocking proteins

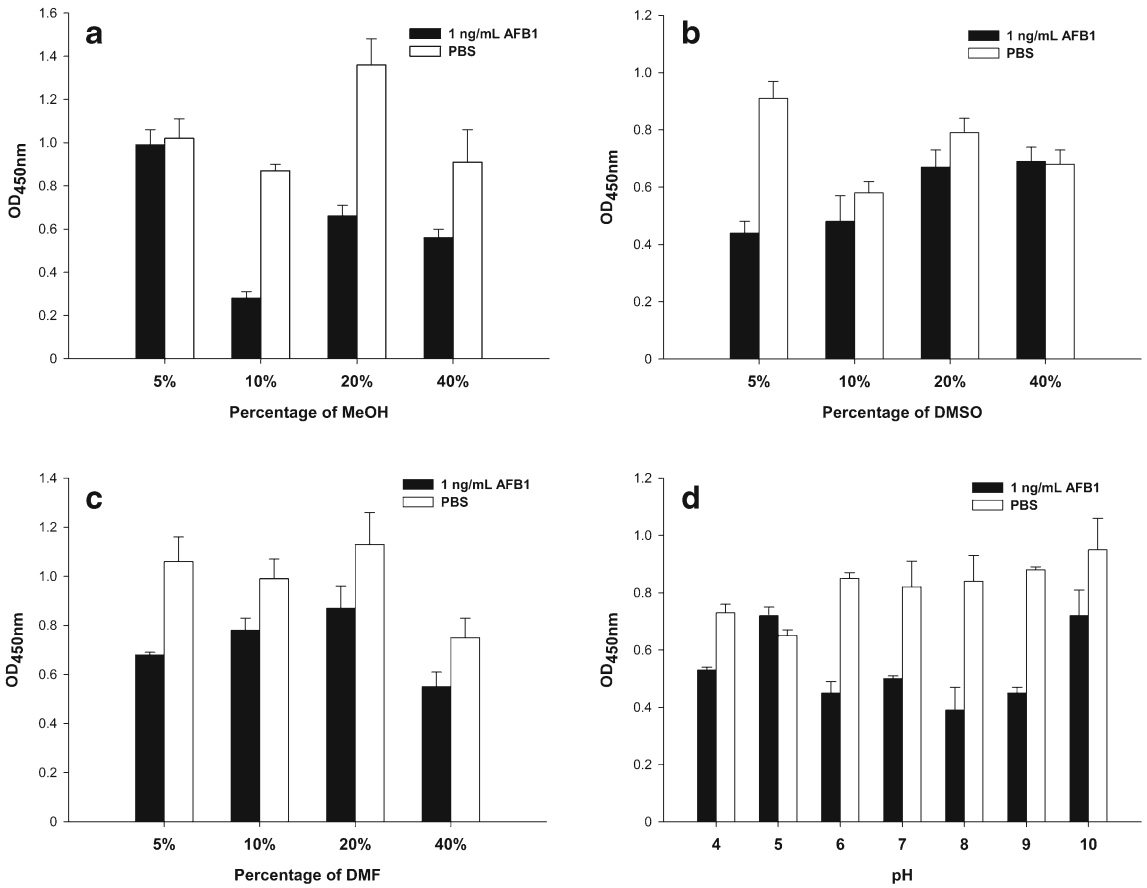


Fig. 2 Effects of methanol (**a**), DMSO (**b**), DMF (**c**), and pH (**d**) on the performance of bp-ELISA for AFB_1. The black column represents measurements in the presence of 1 ng/mL AFB_1. The white column denotes measurements in the absence of AFB_1. The data shown are the average of triplicates and standard deviations

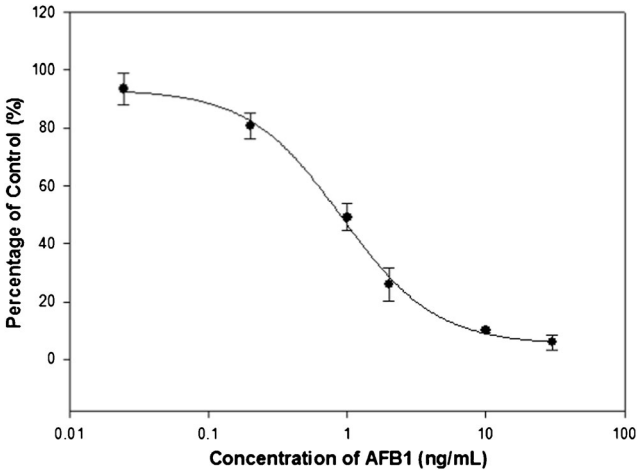


Fig. 3 Calibration curve of bp-ELISA for AFB_1. Plates were coated with 2 μg/mL purified MAb 2F5. Error bars indicate standard deviations of triplicate measurements

mL, with LOD of 0.09 ng/mL and an IC_{50} value of 0.92 ng/mL. Compared with the sensitivity of the same phage clone C43 by competitive phage ELISA under its optimal conditions (IC_{50} = 60 ng/mL), the assay sensitivity of bp-ELISA was demonstrated around 60-fold higher (IC_{50} = 0.92 ng/mL). Besides, there was a reduction in the cost of biotinylated peptide–based assay, because SA-HRP is a common and inexpensive reagent compared with anti-M13 antibody-HRP for phage ELISA. In addition to HRP, streptavidin also has the ability to bind to various visible tracers, such as fluorescent proteins, quantum dots, and nanoparticles.

Comparison of bp-ELISA and icELISA

Anti-AFB_1 MAb 2F5, MAb 2E6, and PAb were used as coating antibodies to test their binding activities with the cyclic peptide in the absence or presence of AFB_1. All the antibodies examined were used to develop icELISA for AFB_1 detection. By coating with AFB_1-OVA, the IC_{50} values for AFB_1 icELISA with MAb 2F5, MAb 2E6, and PAb were 40.1, 85.8 [36], and 35.5 ng/mL, respectively (Fig. 4). The linear detection range for AFB_1 analysis using MAb 2F5, MAb 2E6, and PAb was 1.5–535, 9.1–545, and 1.5–536 ng/mL, respectively. The cyclic peptide CVPSKPGLC represented a competitive binding epitope with all these antibodies similar to that with AFB_1, with IC_{50} ranging from 0.92 to 1.21 ng/mL in bp-ELISA (Fig. 4). The linear detection range for AFB_1 bp-ELISA coated with MAb 2F5, MAb 2E6, and PAb was 0.23–3.36, 0.3–2.75, and 0.49–2.43 ng/mL, respectively (Fig. 4). While the cyclic peptide was presumed to mimic the antigenic determinant of AFB_1 that interacts with primary antibodies, the cyclic peptide antibody showed less binding ability than AFB_1 antibody complexes, thus leading to higher assay sensitivity. However, the linear range for bp-ELISA was noted to be much narrower than that for conventional icELISA, which may be attributed to the

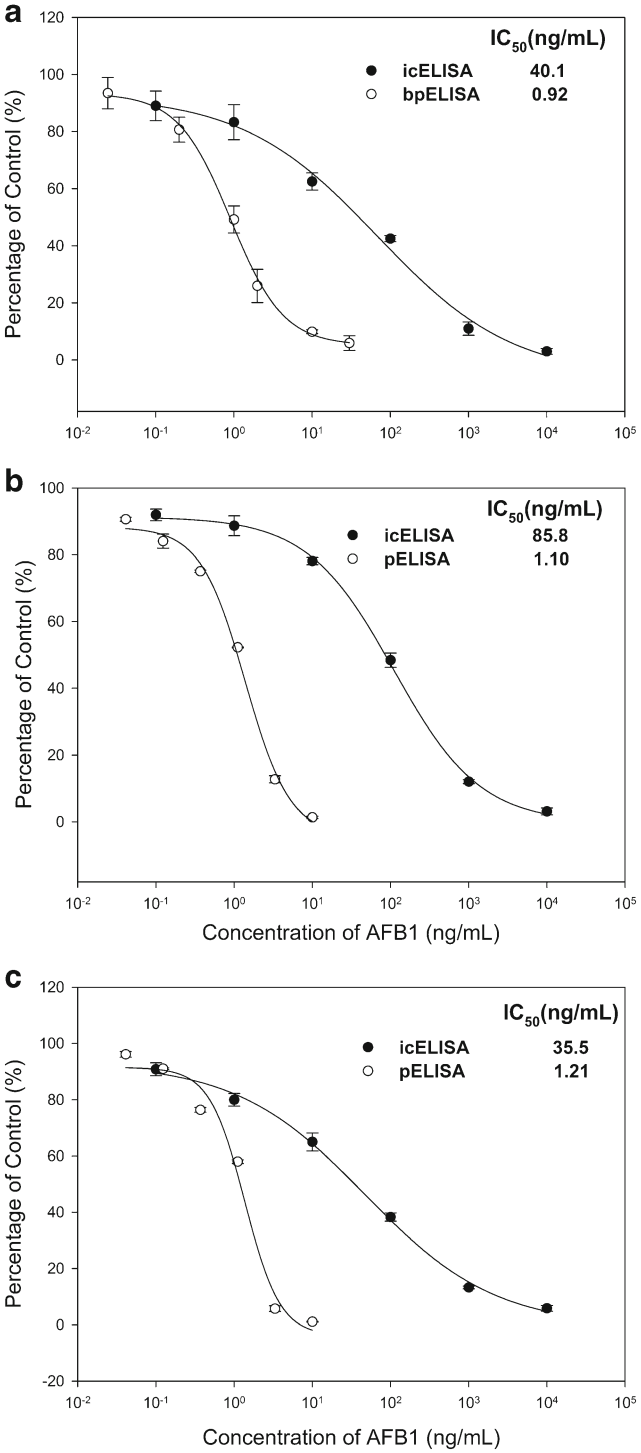
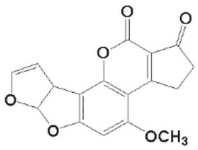
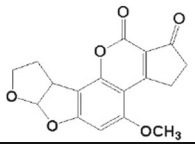
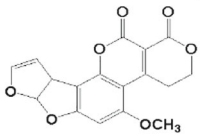
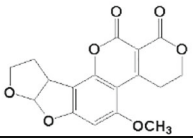
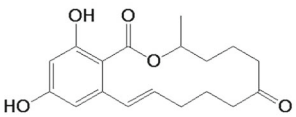
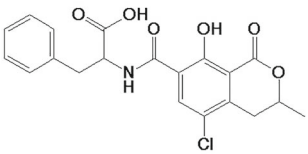
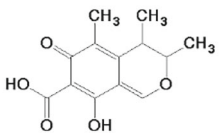


Fig. 4 Calibration curves for AFB_1 by bp-ELISA (white circle) (coating with anti-AFB_1 antibody MAb 2F5 (**a**), MAb 2E6 (**b**), and PAb (**c**)) and conventional icELISA (black circle) (coating with competitive antigen (AFB_1-OVA), followed by primary antibody MAb 2F5 (**a**), MAb 2E6 (**b**), and PAb (**c**)). Error bars indicate standard deviations of triplicate measurements

biotin-streptavidin-HRP detection system. A chemiluminescent or fluorescent detection system using directly coupled peptide may enable linear detection in a broader range [42].

Table 1 Cross-reactivity of the bp-ELISA with aflatoxins and other mycotoxins

Type of mycotoxins	Structure	Cross-reactivity
AFB_1		100%
AFB_2		<0.1%
AFG_1		<1%
AFG_2		<1%
ZEN		<0.1%
OTA		<0.5%
CIT		<0.1%

The peptide was proven to be an excellent substitute for competing antigen in competitive immunoassay for small-sized compounds (MW < 1500 Da). In general, in icELISA, a large amount of haptens are designed and synthesized not

Table 2 Recovery analysis of AFB_1 in rice and corn samples by bp-ELISA and HPLC-FLD

Samples	Spiked level (ng/g)	bp-ELISA			HPLC-FID		
		Measured AFB_1 (ng/g)	Mean recovery (%)	CV (%)	Measured AFB_1 (ng/g)	Mean recovery (%)	CV (%)
Rice	10	9.7±0.4	97%	4.1	8.3±1.2	84%	14.4
	20	16.8±0.6	84%	3.6	17.3±0.9	86%	4.9
	40	36±1.1	90%	3.1	37.3±2.4	93%	6.4
Corn	10	8.3±2.3	83%	27.1	8.9±0.9	89%	10.1
	20	16.5±3.6	83%	21.4	19.0±1.4	95%	7.4
	40	40.7±1.2	102%	3.0	35.7±1.9	89%	5.3

only to mimic the different structural elements of the analyte (such as its carboxylic acid derivatives) to improve assay sensitivity, but also to provide a group that allow coupling to a carrier protein [43]. Only antibodies that preferentially recognize the target analyte over competitive antigens can be used for assay development [44]. Even so, in some cases, antibodies only recognize the carrier protein or have higher affinity for competitive antigen than for free analyte. Moreover, the commonly used competitive antigen AFB_1-OVA/AFB_1-BSA was prepared by first derivating AFB_1 into AFB_1-oxime, which inevitably exposed operators to the harmful mycotoxin. However, in the present study, the peptide is a prospective reagent to be employed as a substitute for AFB_1-competitive haptens, avoiding the complex steps of hapten synthesis and AFB_1 exposure to manufacturers.

Characterization of the biotinylated peptide

Owing to their small molecular weight, phage-free peptides are more stable and less prone to losing their activity [27]. In the present study, phage particles and biotinylated peptides were stored at 4 °C and 25 °C to check their stability, because 4 °C and 25 °C are the temperatures for solution transportation and assay work, respectively. The results obtained showed that the biotinylated peptide maintained 40% of its binding activity with anti-AFB_1 MAb 2F5 after incubation for 52 h at 4 °C; however, the phage particles retained only 20% of their binding activity (see ESM Fig.S3A). Similar results were also obtained following incubation at 25 °C (see ESM Fig.S3B). Even though peptide shows a little more stability than phage particles, it should be optimized to retard the loss of peptide activities before putting them into use.

Cross-reactivity

The specificity of bp-ELISA was confirmed by assessing cross-reactivity with toxins structurally similar to AFB_1 such as AFB_2, AFG_1, AFG_2, ZEN, OTA, and CIT. The cross-reactivity with AFB_2, AFG_1, AFG_2, ZEN, OTA, and CIT was less than 1% (Table 1). Thus, the bp-ELISA developed in this study can be applied to test the AFB_1 content, but not total aflatoxin content in agricultural samples.

Assay validation

Owing to the components present in the specimen, matrix effect is one of the major causes of interference in an analysis. Dilution of the sample or sample extraction is an effective method to reduce the possibility of false-positive results of an assay. In this study, employing rice and corn samples, methanol was used as the extraction solvent, and the matrix effect was evaluated by assessing the response of the working calibrations in specimen and blank matrix extracts [27]. The results showed that 10-fold dilution of both rice and corn extracts hardly affected the sensitivity of the assay and presented acceptable absorption in the absence of AFB_1 (see ESM Fig. S4). Taking into account of the dilution factor to remove matrix interference, the sensitivity of this method for AFB_1 in rice and corn samples was approximately 27.6 μg/kg, with a linear range of 6.9–100.8 μg/kg.

The accuracy and precision of bp-ELISA with rice and corn samples were evaluated by spike-and-recovery test and validated by HPLC-FLD [45]. The absence of AFB_1 has been determined in rice and corn blank samples by HPLC-FLD before AFB_1 spiking performance. Table 2 shows the results of the recovery analysis of AFB_1-spiked rice and corn samples by bp-ELISA and HPLC-FLD. The average recoveries of AFB_1 in rice samples by bp-ELISA and HPLC-FLD were in the range of 84–97% and 84–93%, respectively, while those of AFB_1 in corn samples were in the range of 83–102% and 89–95%, respectively. Both the methods showed good recoveries; however, in corn samples, the coefficient of variation (CV, %) at lower concentrations of AFB_1 in samples exceeded 20%, which was acceptable when quantifying samples at the end concentration of the assay's range [46]. These findings indicate that the biotinylated peptide is an excellent alternative reagent for AFB_1-competing antigen, with advantages of being a synthetic-competitive antigen and preventing from toxic AFB_1 exposure, and therefore, the bp-ELISA is a promising method to detect AFB_1 contamination in agricultural products.

Conclusions

In this study, cyclic peptide that mimic the antigenic determination of AFB_1 was synthesized chemically and biotinylated for immunoassay development for AFB_1. Screening a library of disulfide-cyclized peptide displayed on phage particles has been found to be a valid tool in the discovery of ligands for a receptor or enzyme. The isolated cyclized peptide CVPSKPGLC was easily synthesized chemically showing improved assay sensitivity. Taking the place of a chemical-competitive antigen and avoiding toxic AFB_1 exposure, the peptide is an excellent alternative reagent for AFB_1-competing antigen. Due to peptide cyclization and the biotin-streptavidin complex, the biotinylated peptide–based assay for AFB_1 showed a significant improvement in sensitivity (IC_{50} = 0.92 ng/mL), almost 60-fold better than phage ELISA (IC_{50} = 60 ng/mL). Thus, this study demonstrates that cyclic peptides derived from a phage library are suitable candidates for AFB_1 non-toxic detection and monitoring in food and the environment.

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Compliance with ethical standards

This article does not contain any studies with human participants or animals performed by any of the authors.

Conflict of interest The authors declare that they have no conflict of interest.

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