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Application of Mimotope Peptides of Fumonisin B_1 in Peptide ELISA

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ABSTRACT: Anti-fumonisin B_1 (FB_1) McAb 1D11 was used as the target for biopanning from a phage random loop-constrained heptapeptide library. After three cycles of panning, seven phages with three mimotope peptides were selected to mimic the binding of FB_1 to 1D11. After the identification of phage ELISA, the phage clone that showed the best linear range of detection was chosen for further research. One peptide with the inserted peptide sequence of the phage was synthetized, named CT-452. An indirect competitive ELISA (peptide ELISA) for detecting FB_1 was established using the CT-452-bovine serum albumin conjugate as coating antigen. The linear range of the inhibition curve was 1.77−20.73 ng/mL. The half inhibitory concentration (IC_{50}) was 6.06 ng/mL, and the limit of detection was 1.18 ng/mL. This method was compared with conventional indirect ELISA (commercial ELISA kit) and high-performance liquid chromatography (HPLC), and the results showed the reliability of the peptide ELISA for the determination of FB_1 in cereal samples. The relationship between the CT-452 and FB_1 standard concentrations in peptide ELISA was evaluated. The results indicated that synthetic peptide CT-452 can replace the FB_1 standard to establish an immunoassay free of FB_1.

KEYWORDS: *mimotope, phage display, fumonisin B_1, peptide, ELISA*

INTRODUCTION

Fumonisins are a group of structurally related mycotoxins mainly produced by *Fusarium verticillioides* and other *Fusarium* species.[1,2] These mycotoxins parasitize crops, especially maize. Fumonisin B_1 (FB_1), whose structure is shown in Figure 1, is predominant in most fusaria. FB_1 has the highest toxicity among this class of toxins,[3] usually representing about 70% of the total fumonisin content.[4] FB_1 is a potent toxin that can be neurotoxic, immunosuppressive, genotoxic, carcinogenic, and cytotoxic.[5−9] FB_1 contamination has occurred worldwide, mainly in maize and maize-based products.[2] Hence, the routine screening of raw agricultural commodities consumed by humans and animals is urgently needed.

Many methods of detecting FB_1 have been developed, including thin-layer chromatography, high-performance liquid chromatography (HPLC), gas chromatography, immunoassays, and combined methods.[10−13] Immunoassays such as ELISAs are widely applied in the routine screening of FB_1 contamination.[14−16] However, the immunoassay for FB_1 involves using the mycotoxin itself, in both free and conjugated forms, which may be toxic to manufacturers and users.

To overcome this challenge, proteins or peptides that mimic the function of mycotoxins should be developed by panning from phage display libraries. Phage display libraries have been successfully used to identify antigenic eptopes and screen mimotopes by panning against antibodies and other receptor molecules,[17−24] of which the loop-constrained peptide library is the special one. The randomized segment of a loop-constrained peptide library is flanked by a pair of cysteine residues, which are oxidized during phage assembly to a disulfide linkage, resulting in the displayed peptides being presented to the target as loops. The use of the constrained peptide library can strengthen the revelation of the antibody's preference to longer sequences.[25] In this study, we described the loop-constrained heptapeptide mimotopes of FB_1 isolated from a phage display random peptide library and evaluated the use of synthetic peptides with mimotope sequences in an analysis of FB_1 by peptide ELISA.

MATERIALS AND METHODS

Chemicals and Reagents. All inorganic chemicals and organic solvents were of reagent grade or higher. FB_1, fumonisin B_2 (FB_2), ochratoxin A, zearalanone, deoxynivalenol, aflatoxin B_1, bovine serum albumin (BSA, fraction V), goat anti-mouse IgG-horseradish peroxidase (HRP) conjugate, and glutaraldehyde were from Sigma-Aldrich Chemical Co. A RIDASCREEN fumonisin ELISA kit was obtained from R-Biopharm. Synthetic peptides CT-452 (ACWELPT-LACGGGS) and CT-453 (ACWELATLACGGGS) were synthetized from China Peptides Co., Ltd. Anti-FB_1 McAb 1D11 was prepared in the laboratory. HRP-conjugated anti-M13 antibody was obtained from Pharmacia Inc. A Ph.D.-C7C phage display peptide library kit was purchased from New England Biolabs.

Monoclonal Antibody Production and Characterization. FB_1 was conjugated to KLH and OVA for use as immunogen and coating antigen, respectively. The preparation of FB_1 conjugates was performed as described by Xu et al.[26] After immunization and cell fusion process performed according to the methods described previous by Liu et al.,[27] the competitive ELISA with FB_1-OVA as coating antigen was used for screening the hybridoma cells, and one clone named 1D11 was found to show the highest affinity for FB_1.

Mimotope Biopanning and Identification. Anti-FB_1 McAb 1D11, which served as the target in mimotope biopanning, was first purified using protein G resin and then subjected to three cycles of panning following the method described by Liu et al.[18] Afterward, individual plaques were collected from LB/IPTG/Xgal plates to infect *Escherichia coli* ER2738 cells for phage amplification and isolation. Binding to McAb 1D11 was determined by ELISA for each individual phage clone.[19] A competitive ELISA (phage ELISA) was set up to

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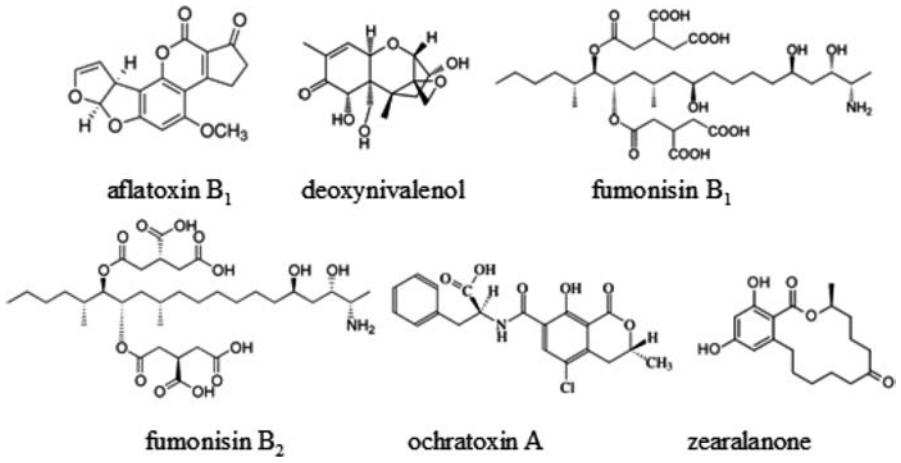

Figure 1. Chemical structures of fumonisin B_1, fumonisin B_2, ochratoxin A, zearalanone, deoxynivalenol, and aflatoxin B_1.

screen phages capable of mimicking FB_1.[19] Briefly, for each phage ELISA, the concentration of McAb 1D11 and phages was optimized by a checkerboard titration.[28] Microtiter wells were coated with McAb 1D11 (10 μg/mL, 100 μL/well) and blocked with 3% BSA (300 μL/well). Various concentrations of FB_1 (0−50 ng/mL in PBS solution) were mixed with equal volumes of phages. The mixture was then added to wells (100 μL/well) and incubated at 37 °C for 1 h. After the wells were washed 6 times with PBST (PBS, pH 7.2, containing 0.05% Tween-20), ELISA was performed as described above.

DNA Sequencing. Phage particles with specific binding to McAb 1D11 were used for single-stranded DNA isolation as described in the Ph.D.-C7C peptide library kit instruction manual. Single-stranded DNA was sequenced with -96 gIII sequencing primer 5′ d(CCCTCA-TAGTTAGCGTAACG) 3′ at GenScript Biotechnology Co., Ltd. The amino acid residue sequences of the phage display peptide were translated from the inserted DNA sequences.

Peptide Conjugate Preparation. After the identification of phage ELISA, the phage clone that showed the best linear range of detection (IC_{20}−IC_{80})[29] was chosen for further research. One peptide with the inserted peptide sequence of the phage was synthetized, named CT-452. The CT-452−BSA conjugate was prepared with glutaraldehyde as described by Carter.[30] In brief, 1 mg of BSA and 1 mg of peptide were weighed out and dissolved in PBS and acetonitrile, respectivity. Then 1 mL of glutaraldehyde solution was added dropwise, mixing gently with a magetic stir bar. Mixing was continued, and the mixture was allowed to react for 1 h at room temperature. The product was collected and dialyzed in PBS at 4 °C for 48 h.

Sample Preparation. Sample preparation for measurement with both peptide ELISA and commercial ELISA kit was performed as described by He et al.[31] with adjustment. In brief, a ground sample (5 g) was weighed into a container and extracted with 25 mL of 60% methanol−PBS solution by shaking vigorously for 5 min with a shaker. Then the extract was filtered through a Whatman no. 1 filter and 1 mL of filtrate was mixed with 1 mL of PBS for measurement. If the FB_1 content of the filtrate was higher than the calibration range, the supernatant was diluted with 30% methanol−PBS and peptide ELISA analysis was repeated.

Development of the Peptide ELISA. An indirect competitive ELISA (peptide ELISA) was developed based on the CT-452−BSA conjugate, which served as coating antigen. The concentrations of anti-FB_1 McAb 1D11 and CT-452−BSA conjugate were optimized by checkerboard titration.[28] The peptide ELISA can be described as follows: microtiter wells were coated with 100 μL/well conjugate (3 μg/mL) and incubated at 4 °C overnight. Then blocking was done with 300 μL/well blocking solution (PBS, pH 7.2, containing 3% skimmed milk powder). Several concentrations of FB_1 (0−50 ng/mL) in PBS were mixed with equal volumes of McAb 1D11 diluent (1.5 μg/mL, diluted 1:3000 in PBS). The mixture was then added to wells (100 μL/well) and incubated at 37 °C for 30 min. After being washed six times with PBST, the plates were incubated with 100 μL/well goat anti-mouse IgG-HRP conjugate (diluted 1:2000 in PBS, pH 7.2) at 37 °C for 30 min and washed again. The wells were then incubated with 100 μL/well 3,3′,5,5′-tetramethylbenzidine substrate at 37 °C for 10 min. The absorbance at 450 nm was determined by a microplate reader after stopping the reaction by adding 50 μL of 2 M H_2SO_4 per well.

The standard curve was obtained by plotting binding (%) against the standard FB_1 concentrations. Binding (%) were calculated as follows: binding (%) = $(OD/OD_0) \times 100$, where OD is the absorbance in the presence of FB_1 and OD_0 is the absorbance in the absence of FB_1. The Origin 8.0 software package (OriginLab, U.S.) was used to fit the sigmoidal curve. The correlation among the results from the peptide ELISA, commercial ELISA kit, and HPLC was evaluated by means of one-way analysis of variance for repeated measure design.

In order to determine the selectivity of peptide ELISA, a cross-reactivity was carried out under optimum conditions. The cross-reactivity of the McAb 1D11 against FB_1, FB_2, ochratoxin A, zearalenone, deoxynivalenol, and aflatoxin B_1 dissolved in 30% methanol−PBS was calculated using the following equation:

$$\text{cross-reactivity (\%)} = \frac{IC_{50} \text{ of } FB_1}{IC_{50} \text{ of other analytes}} \times 100$$

HPLC for FB_1 Determination. HPLC conditions were as follows. The mobile phase consisted of methanol/0.1 M NaH_2PO_4 (77 + 23, v/v) at pH 3.35 (pH was adjusted with H_3PO_4). The HPLC system flow rate was 0.8 mL/min. The column temperature was 25 °C, and the injection volume was 20 μL. The variable wavelength detector was set at 335 nm (excitation) and 440 nm (emission). The extraction procedure was performed as described by Visconti et al.[32] Briefly, a ground sample (10 g) was weighed into a 100 mL plastic centrifuge tube and extracted with 25 mL of acetonitrile−methanol−water (25 + 25 + 50, v/v/v) solution by shaking vigorously for 20 min with a shaker. Centrifugation was for 10 min at 2500g, and filtration of the supernatant was through Whatman no. 4 filter paper. The remaining solid material was again extracted as described above. The two filtrates were mixed with 20 mL of PBS, and filtration was through a microfiber filter. The filtrate was collected for cleanup through immunoaffinity column, and the elute was evaporated under stream of nitrogen at 60 °C. The dried residue was retained at 4 °C for derivatization and HPLC analysis. If the FB_1 content of derivatized extract was higher than the calibration range, the purified extract was diluted with acetonitrile−water (50 + 50, v/v) and derivatized with *o*-phthalaldehyde reagent and HPLC analysis was repeated.

Assessment of the Peptide ELISA Effectiveness. The assay assessment of peptide ELISA was performed according to the related content of Commission Decision 2002/657/EC[33] and Commission Regulation 401/2006.[34] The limit of detection (LOD) was the lowest amount of analyte that was based on the mean value of 20 blank

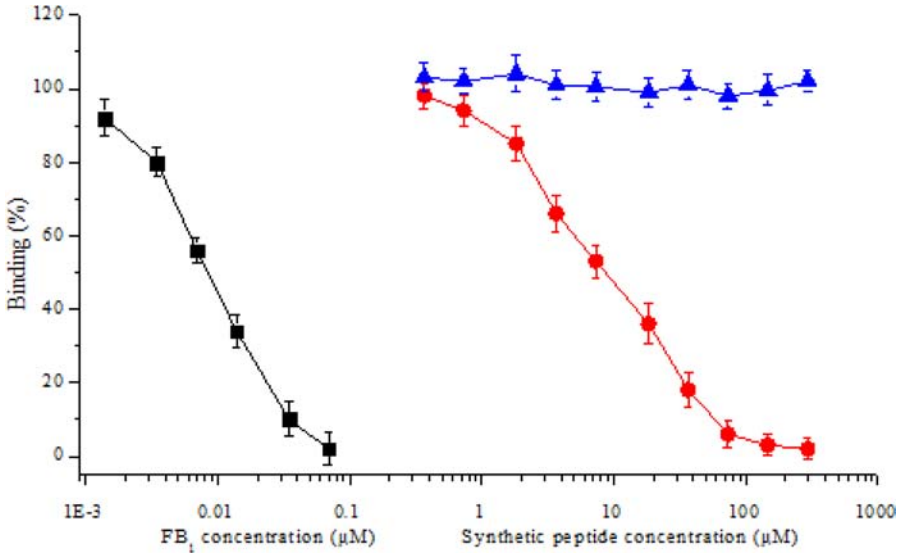

Figure 2. Competitive inhibition curves of peptide ELISA established with FB_1 (■), CT-452 (red circle), and CT-453 (blue triangle), respectively. The tests were run four times using various concentrations of FB_1 (0.0014, 0.0035, 0.0069, 0.0139, 0.0347, 0.0694 μM), CT-452 (0.37, 0.73, 1.83, 3.67, 7.33, 18.33, 36.67, 73.34, 146.67, 293.35 μM), and CT-453 (0.37, 0.73, 1.83, 3.67, 7.33, 18.33, 36.67, 73.34, 146.67, 293.35 μM) to compete binding McAb 1D11 (1.5 μg/mL) against coating antigen CT-452−BSA conjugates (3 μg/mL) at 37 °C for 30 min, respectively.

samples plus 3 times the mean standard deviation (SD). The accuracy and precision of peptide ELISA were represented by recovery and coefficient of variation (CV), respectively. The mean recovery and CV values were calculated by repeated analysis (n = 3) of the spiked FB_1-free maize powder confirmed by HPLC (50, 100, 200, 500 ng/g). A total of 32 random cereal samples collected from markets and feedstuff factories were used to compare the developed peptide ELISA with the commercial ELISA kit and HPLC method and to calculate correlation. Sample preparation was performed as described above.

Synthetic Peptides Experiment. To determine whether the peptide sequence binding, especially to McAb 1D11, was independent of the phage structural context, the synthetic peptide CT-452 was used to test for its capability to bind to McAb 1D11 by peptide ELISA. To evaluate the effectiveness of the common sequence, another peptide CT-453 was tested with the identical method except that a common amino acid residue proline (P) of CT-452 was replaced with an alanine (A). To avoid the toxicity of the FB_1 standard substance, a nontoxic substitute of FB_1 (CT-452) was used to replace FB_1 in the peptide ELISA. The concentration of CT-452 was determined by referring to the same binding (%) as an FB_1 standard substance, and the correlation between concentrations of both reagents was tested.

RESULTS

McAb Production and Characterization. The subclass of anti-FB_1 McAb 1D11 was identified as IgG_1, and the light chains were identified as κ-chains by a mouse immunoglobulin isotyping ELISA kit. The specificity of 1D11 was determined in the indirect ELISA using FB_1-OVA as the coating antigen. The cross-reactivity of 1D11 was 8.61% with FB_2 and was <1% with FB_2, ochratoxin A, zearalanone, deoxynivalenol, and aflatoxin B_1. The IC_{50} of binding of 1D11 to FB_1-OVA by free FB_1 was found to be 10.2 ng/mL in the indirect competitive ELISA, and the range of detection was 1.7−61.7 ng/mL.

Table 1. IC_{50} and Cross-Reactivity of the McAb 1D11 in Peptide ELISA Based on CT-452−BSA Conjugate

analyte	IC_{50} (ng/mL)	cross-reactivity (%)
FB_1	6.05	100
FB_2	>1000	<1
ochratoxin A	>1000	<1
zearalanone	>1000	<1
deoxynivalenol	>1000	<1
aflatoxin B_1	>1000	<1

Table 2. Recoveries and CV Values for the Peptide ELISA Based on CT-452−BSA Conjugate

	intra-assay (n = 3)		interassay (n = 3)	
spiked concn (ng/g)	mean ± SD (%)	CV (%)	mean ± SD (%)	CV (%)
50	98 ± 8	9	96 ± 13	14
100	103 ± 10	10	105 ± 11	12
200	90 ± 7	8	92 ± 9	10
500	104 ± 8	8	101 ± 12	12

Table 3. FB_1 Content of Cereal Samples Detected with Three Methods

	peptide ELISA (n = 3)		commercial ELISA kit (n = 3)		HPLC (n = 3)	
sample[a]	mean ± SD (μg/kg)	CV (%)	mean ± SD (μg/kg)	CV (%)	mean ± SD (μg/kg)	CV (%)
M1	123 ± 4	3	128 ± 2	2	110 ± 3	3
M3	586 ± 6	1	588 ± 4	1	553 ± 6	1
M5	509 ± 11	2	543 ± 8	2	527 ± 5	1
M6	515 ± 5	1	541 ± 10	2	499 ± 11	2
M8	55 ± 8	15	60 ± 5	8	68 ± 7	10
M11	789 ± 9	1	817 ± 12	2	821 ± 9	1
M12	1047 ± 7	1	1053 ± 9	1	1411 ± 8	1
M13	335 ± 3	1	384 ± 6	2	354 ± 5	1
F1	729 ± 7	1	789 ± 5	1	715 ± 6	1
F3	66 ± 5	8	62 ± 8	13	68 ± 5	7
F4	138 ± 7	5	134 ± 4	3	117 ± 9	8
W1	123 ± 5	4	110 ± 11	10	115 ± 6	5
W5	87 ± 11	13	89 ± 8	9	106 ± 6	6
W6	413 ± 9	2	433 ± 5	1	408 ± 12	3

[a]M, F, and W stand for maize, feedstuff, and wheat samples, respectively.

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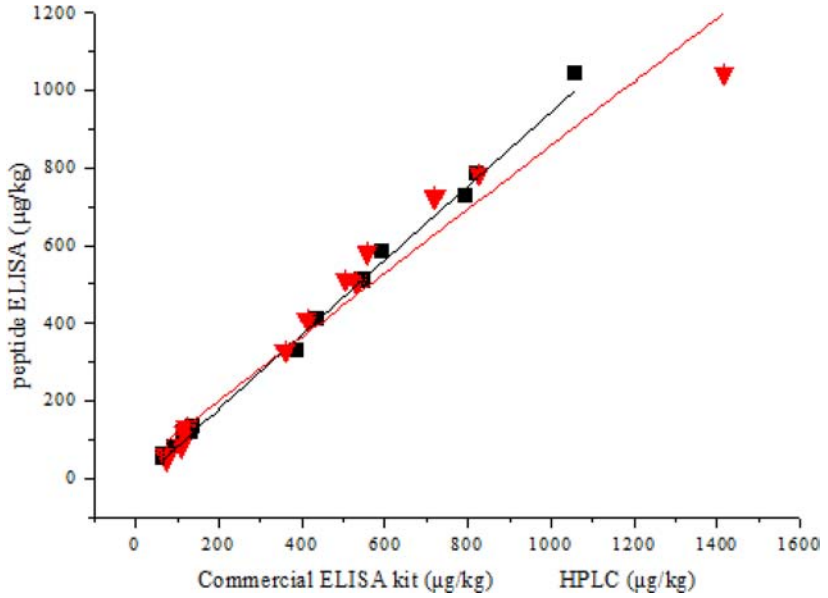

Figure 3. Correlation analysis between the developed peptide ELISA and commercial ELISA kit, and HPLC, respectively. The content of FB_1 in 32 cereal samples was measured by peptide ELISA, commercial ELISA kit, and HPLC, respectively, and the correlation among the results were evaluated: (■) FB_1 content measured by commercial ELISA kit and peptide ELISA, respectively; (red triangle) FB_1 content measured by HPLC and peptide ELISA, respectively; (−) linear fit of results from HPLC and peptide ELISA; (red line) linear fit of results from commercial ELISA kit and peptide ELISA.

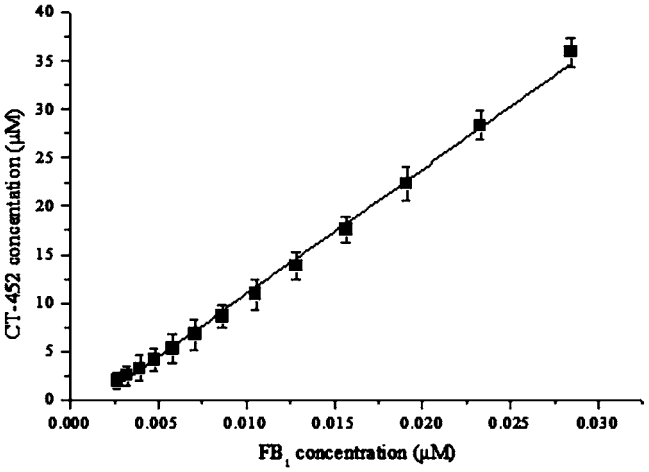

Figure 4. Correlation analysis between the FB_1 concentration and CT-452 concentration in peptide ELIS:. (■) concentrations of FB_1 and CT-452 at different inhibition rates (20%, 25%, 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%), respectively.

Mimotope Biopanning and DNA Sequencing. Enrichment for specific phages with affinity to anti-FB_1 McAb 1D11 was observed after one cycle of biopanning. The phages with affinity to McAb 1D11 were enriched from an initial 1.1×10^6 pfu to 2.0×10^7 pfu and 2.1×10^8 pfu in the subsequent two cycles of biopanning. A total of 20 individual phage plaques from the third cycle selected phages were randomly isolated to infect *E. coli* ER2738 cells for amplification. About 18 of 20 phages were found to be capable of binding to 1D11, and 7 phages were capable of mimicking FB_1 by competitive ELISA.

The DNA sequencing results indicated that the seven phages were virtually three clones designated as P1, P2, and P3. The inserted peptide sequence of P1, P2, and P3 was FELPTLA, YELPTLL, and WELPTLA, respectively. Their common sequence was E-L-P-T-L. The IC_{50} and linear range of detection[29] for P1, P2, and P3 exhabited in the phage ELISA were 1.3, 1.2, and 1.2 ng/mL, respectively, and 0.5−5.3, 0.2−6.6, and 0.2−7.2 ng/mL, respectively. Phage clone P3 that showed the best linear range of detection was preferentially chosen for further research on peptide ELISA.

Peptide ELISA Based on Synthetic Peptide−BSA Conjugate. Peptide ELISA was performed with CT-452−BSA conjugate as the coating antigen. Checkerboard titration was performed to determine the optimal dilution of coating antigen CT-452−BSA and McAb 1D11, and the results were 3 and 1.5 μg/mL, respectively. As exhibited in Figure 2, IC_{50} of the assay was 6.06 ng/mL, and the competitive inhibition curve of FB_1 showed a linear range of detection (IC_{20}−IC_{80}) from 1.77 to 20.73 ng/mL. The specificity of the peptide ELISA was evaluated by determining the cross-reactivity with a set of structurally related mycotoxins. The IC_{50} and cross-reactivities were summarized in Table 1. The McAb 1D11 showed negligible cross-reactivity with FB_2, ochratoxin A, zearalanone, deoxynivalenol, and aflatoxin B_1.

Assay Validation. The assay validation was performed by investigating LOD, recovery, and CV. On the basis of the determination of 20 blank samples, the LOD of the peptide ELISA was 1.18 ng/mL. As shown in Table 2, the average recovery values ($n = 3$) ranged from 90 ± 7% to 104 ± 8% and from 92 ± 9% to 105 ± 11% during intra-assay and interassay, respectively, with CV values of less than 15%.

A total of 32 domestic cereal samples were analyzed using three different methods. As shown in Table 3, FB_1 was detected in 14 samples. The results measured by peptide ELSIA were compared to commercial ELISA kit and HPLC analysis using a correlation test, respectively. As can be observed from Figure 3, results determined by the peptide ELISA were compatible with that of commercial ELISA kit and HPLC, and the coefficients of correlation R^2 were 0.95 and 0.90, respectively. It demonstrated that the developed peptide ELISA can be used

for real sample analysis before confirmation by an instrumental method.

Synthetic Peptides Test Results. To determine whether the peptide sequences can specifically bind to 1D11 independent of the phage structural context, the binding characteristics of two synthetic peptides (CT-452 and CT-453) used as substitutes for FB_1 standard substance were investigated by peptide ELISA. As can be observed from Figure 2, CT-452 can bind to 1D11 and inhibit the binding of conjugate to 1D11, whereas CT-453, in which the P residue was replaced by A, showed no binding. Comparison between the concentrations of FB_1 and CT-452 under the same binding rate revealed a good correlation as shown in the equation $Y = 1290.84X - 1.74$ ($R^2 = 0.996$), where X and Y are the concentrations of FB_1 and CT-452, respectively (Figure 4). In other words, 1289 μM CT-452 equals replacement of 1 μM FB_1.

DISCUSSION

This study demonstrated that peptide mimics of FB_1 can be selected from a phage display random peptide library. An immunoassay for detecting FB_1, in which a synthetic peptide with the sequence of FB_1 mimotope served as the substitute for FB_1, was also described.

The structures of the mimopeptides of nonproteinaceous molecules, especially those of carbohydrates, reportedly contain aromatic side chains and proline residues.[35] Two mimotope peptides of deoxynivalenol,[23] SWGPFPF and SWGPLPF, show this preference. In the present research, we found that this preference was also true for the mimotope peptides of FB_1, namely, FELPTLA, YELPTLL, and WELPTLA.

The mechanism of peptide-mimicking nonproteinaceous chemicals still needs further investigations. Liu[18] deduced that hydrophobic interactions are vital and that mimicking of such interactions between anti-OTA McAb and OTA may contribute to the mechanism of peptide-mimicking nonproteinaceous OTA. In the current study, the amino acid sequences of three mimotope peptides of FB_1 mostly consisted of nonpolar amino acid residues. These residues may have mainly contributed to the mimicry of the binding interaction of anti-FB_1 McAb to FB_1, although further research is necessary.

The synthetic peptide tests suggested that FB_1 standard substance can be replaced by synthetic peptide CT-452. Compared with the conventional assays for FB_1, the use of a nontoxic peptide CT-452 as an immunochemical reagent to replace FB_1 can make the peptide ELISA free of mycotoxin, which provides a new way for FB_1 detection.

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Notes

The authors declare no competing financial interest.

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