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Original article

Detection of aflatoxins in tea samples based on a class-specific monoclonal antibody

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Summary An enzyme-linked immunosorbent assay (ELISA) using a monoclonal antibody was established to detect aflatoxin B_1 (AFB_1) in tea. The antibody was prepared from a hybridoma derived by fusing Sp2/0-Ag14 myeloma cells and immunised spleen B cells. The effects from pH, ionic strength, and organic solvents on immunoassay were optimised and the 50% inhibition (IC_{50}) value was 0.057 ± 0.007 ng mL^{-1}. Spiked black and green tea samples at 10, 20 and 50 ng g^{-1} levels of AFB_1 were detected with this proposed ELISA. The recoveries for black tea samples ranged from 68.5% to 117.7% and 73.5 to 114.3% for green tea samples. This immunoassay showed no cross-reactions with other mycotoxin family but good recognition with related aflatoxins. These results indicate that the ELISA assay could be used as a screening method for aflatoxin detection in tea samples.

Keywords Aflatoxin B_1, black tea, ELISA, green tea, monoclonal antibody.

Introduction

Aflatoxins (AF) are extremely toxic secondary metabolic products mainly produced by *Aspergillus flavus* and *A. parasiticus* (Reddy *et al.*, 2011). Nearly 18 different types of AF (e.g. B_2, G_1, G_2, M_1) have been identified (Arzandeh & Jinap 2011); however, aflatoxin B_1 (AFB_1), which is generally present in the largest quantities, is the most toxic form (Bircan *et al.,* 2008) and is regarded as a Class 1 human carcinogen by the International Agency for Research on Cancer (Ghali *et al.*, 2008).

Due to widespread presence of aflatoxins in food and extreme toxicity (Bircan *et al.,* 2008; Reddy *et al.* 2009), many detection methods have been developed, such as thin layer chromatography (TLC) (Var *et al.*, 2007), high performance liquid chromatography (HPLC) (Bircan, 2005; Ventura *et al.,* 2006) mass Spectrometry (Han *et al.*,2012; Liu *et al.*, 2013) and sensor methods (Tan *et al.,* 2009). However, immunological analysis is still the most common rapid method for screening a large number of samples, due to its low-cost and high throughput. Ye carried out latex agglutination tests based on antibody against AFB_1 and the method was successfully applied in the detection of peanut or rice samples (Ye *et al.*, 2011). Based on ELISA, AFB_1 presence in feed, cereals were screened with high sensitivity (Khan *et al.*, 2012; Rossi *et al.*, 2012). Li developed ELISA experiments to detect AFB_1 with a high sensitivity (visual limit of detection of 0.03 ng mL^{-1}) and he explained the potential mechanism of the binding between antibody and AFB_1 using homology modelling and molecular docking(Li *et al.*, 2012).

The European Commission set tolerance levels at 2 μg kg^{-1} for AFB1 and 4 μg kg^{-1} for total aflatoxins in certain species (EC No.1525/98). The tolerance levels are typically ranging from 5 to 20 μg kg^{-1} but 0.5 μg kg^{-1} for aflatoxin M1 in milk (Chinese National Standard GB2761). China is a main provider of tea in the world. With the growing concern over pollutes in tea, some importers have proposed the detection of aflatoxins in tea. In this study, we developed a rapid ELISA based on a sensitive monoclonal antibody for the detection of AFB_1 in tea samples without extra-purification steps.

Materials and methods

Materials

The AFB_1, haemocyanin from *Megathura crenulata* (KLH), polyethylene glycol solution (PEG,

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Hyb-ri-Max, 50% (w/v)), N-(3-dimethylaminopropyl)-N′-ethylcarbodiimide hydrochloride (EDC) and N-hydroxysuccinimide (NHS) were purchased from Sigma (St. Louis, MO, USA). HAT supplement (**h**ypoxanthine-**a**minopterin-**t**hymidine medium), HT supplement (**h**ypoxanthine-**t**hymidine medium), 1640 cell culture medium and foetal calf serum were obtained from Gibco BRL (Paisley, Scotland). The Sp2/0-Ag14 murine myeloma cell line was obtained from the cell bank of the Chinese Academy of Sciences (Shanghai, China). Bovine serum albumin (BSA) was purchased from BoAo Co. (Shanghai, China). QuickAntibody™ adjuvant was purchased from Kang Biquan Biotechnology Co. (Beijing, China). Goat anti-rabbit immunoglobulin conjugated to horseradish peroxidase was purchased from Hua Mei Co. (Shanghai, China). 3, 3, 5, 5-Tetramethylbenzidine (TMB) was from Fluka (Buchs, Switzerland). BALB/c mice were provided by the Comparative Medicine Centre of Yangzhou University. Aflatoxin standard solutions (AFB_1, AFB_2, AFG_1, AFG_2, AFM_1, OTA, ZEN and DON) were provided by Jiangsu Import and Export Commodity Inspection and Quarantine Bureau. The IsoQuick™ Kit for Mouse Monoclonal Isotyping was purchased from Youlong-Bio Co. (Shanghai, China). All other chemicals and reagents (analytical grade or higher) were purchased from East China Chemicals Co., Ltd. (Shanghai, China).

Antigen synthesis

A hapten (AFB_1-oxime) was synthesised by inserting a carboxyl group to AFB_1,which was used to react with the lysine groups on the carrier protein by a previously described method (Chu *et al*., 1977; Acharya & Dhar, 2008). A total of 8 mg carboxymethoxylamine hemihydrochloride (0.073 mmol) and 5 mg AFB_1 (0.015 mmol) were dissolved in mixture of methanol-water-pyridine (volume ration, 4:1:1). The solution was refluxed at 75 °C for 3 h, and then cooled at room temperature overnight. After the solvents were evaporated, the yellow-oil was then separated from unreacted AFB_1 by TLC (20 × 20 cm, GF254 plate, mobile phase: chloroform: methanol = 9:1).

The AFB_1-carrier protein conjugates (KLH and BSA) were prepared using the activated ester method (Acharya & Dhar, 2008). A total of 5 mg AFB_1-oxime (0.013 mM), 2.7 mg EDC (0.013 mM) and 1.5 mg NHS (0.013 mM) were dissolved in 2 mL anhydrous dimethyl formamide (DMF). The mixture was stirred at room temperature overnight, and then centrifuged at 4000 ***g*** for 15 min. The supernatant was dropped into pre-cooled BSA, which was dissolved in 0.13 mol L^{-1} $NaHCO_3$ containing 10% DMF. The reaction was maintained at room temperature for 4 h, and then the mixture was dialysed against PBS for 2 days. The conjugates were analysed by ultraviolet spectroscopy.

Immunisation

The AFB_1-KLH conjugate was used as the immunogen. The solution of 1 mg mL^{-1} immunogen was mixed with an equal volume of Quick Antibody adjuvant. Four six-week-old female Balb/c mice were injected intramuscularly with a 50-μg dose per mouse. Three weeks later, a booster injection was given and subsequent injections were given at 2-week intervals. One week after each booster injection, blood was drawn from each mouse and tested by indirect competitive ELISA. The mouse that produced higher titre antibody against AFB1 was selected for cell fusion.

Screening of positive hybridoma

The spleen from immunised mouse was separated aseptically and was disaggregated into a single cell suspension. Myeloma cells and spleen cells were mixed at a ratio of 1:5 in a 50 mL centrifuge tube and centrifuged at 200 ***g*** for 10 min. The supernatant was discarded. Then, 1 mL 50% PEG was added into the tube drop by drop. One minute later, the fusion cycle was stopped by adding cell culture solution.

The produced hybridoma cells were suspended in RPMI 1640 with 20% FCS (v/v) and HAT solution, and then distributed to six 96-well microtitre plates (200 μL per well). One week later, the supernatants of each well were tested by ELISA and the positive cells were selected for subcloning using a limiting dilution measure.

Production and isotype of antibodies

Mice were pre-treated with 1 mL paraffin liquid and then, the hybridoma cells (2×10^6 cells in PBS per mouse) were injected intraperitoneally into mice. Ten days later, ascites were collected and purified by the caprylic acid-ammonium method (Temponi *et al*., 1989). The isotype of mAb was tested with the IsoQuickKit

ELISA development

The concentrations of the coating antigen and mAb were optimised using chess-board method. Coating antigen (AFB_1-BSA, 1.25 mg mL^{-1}) was diluted with carbonate-bicarbonate buffer (CBS, pH 9.6, Na_2CO_3 1.59 g, $NaHCO_3$ 2.93 g, diluted with 1 L H_2O) 1 000, 2 000, 4 000, 8 000 and 16 000 times, while the mAb (5.18 mg/mL) was double diluted with PBS (containing 0.05% gelatin, w/v) and diluted from 1000 to 128 000 times. The effects of methanol concentration (0, 10%, 20%, 30%, 40% and 50%, v/v), ionic

strength (0, 1, 5, 10, 20 and 50 mM PBS) and pH (5.3, 6.2, 7.0, 7.4 and 8.0) of the assay buffer were tested.

Different levels of AFB_1 (0, 0.01, 0.02, 0.05, 0.1, 0.2, 0.5 and 1 ng mL^{-1}) standards were detected with the indirect competitive ELISA. A standard curve was constructed as a plot of the absorbance vs. AFB_1 concentrations and the IC_{50} value was calculated based on the standard curve.

Cross-reactivity determination

Cross-reactivities (CR) for different mycotoxins [AFB_2, AFG_1, AFG_2, AFM_1, ochratoxin A (OTA), zearalenone (ZEN) and deoxynivalenol (DON)] were determined. CR was calculated with the formula:%CR = IC_{50} for AFB_1 / IC_{50} for analyte × 100% (Kuang *et al.,* 2009).

Sample extraction and analysis

Tea samples were obtained from Jiangsu Entry-Exit Inspection and Quarantine Bureau, (Nanjing, China) and their residue-free status were confirmed by HPLC-MS/MS.

The tea samples were ground into powder, filtered through a 40-mesh sieve. One-gram aliquots of tea powder (green or black tea) were spiked with AFB_1 at 10, 20 and 50 ng. Samples were extracted with 5 mL 80% methanol (80:20, v/v) by ultrasonic extraction for 10 min. The extracted solution was then centrifuged at 4000 ***g*** for 10 min. 0.1 mL supernatant was taken out and diluted into 10 mL with distilled water before detection by ELISA. Inter and intraday coefficients of variation were calculated based on determinations on three consecutive days.

Results and discussion

Antigen characterization

The modification of AFB_1 by oximation reaction was verified with TLC. It was found that the AFB_1-oxime showed a smaller R_f (~0.20) value compared with AFB_1 (~0.9).The AFB_1-oxime was used as hapten to conjugate with carrier protein. Ultraviolet spectroscopy showed that AFB_1-oxime showed absorption peaks at 268 nm and 363 nm (Fig. 1). The typical protein absorption peak was at 277 nm. The conjugate of AFB_1-oxime and BSA showed both adsorption nature of hapten and protein, which indicated successful preparation of antigen. Similar analysis was carried out for AFB_1-OVA and AFB_1-KLH conjugates.

Antibody production

The immunised mouse with appropriate positive antisera titre (>1:9 000) was used for cell fusion. A cell line 11A9, which steadily secreted monoclonal antibody against AFB_1, was obtained and used to inject mice for ascites production. Ascites were collected from mice and purified by the caprylic acid-ammonium method.

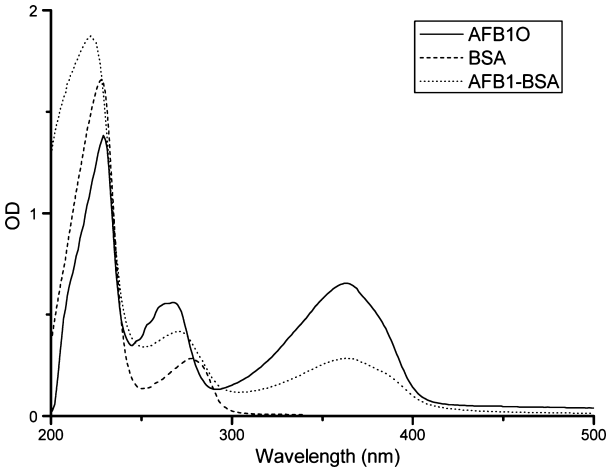

Figure 1 The ultraviolet spectrum of AFB1-oxime (AFB1O), bovine serum albumin (BSA) and their conjugate AFB1–BSA.

The mAb isotype characterisation was carried out using a commercial kit and a colloidal gold strip. The results (Fig. 2) indicated that the produced mAb belonged to IgG1 family and that the light chain isotype was lambda.

Optimisation of ELISA

The checkerboard method showed that optimal concentration of coating antigen and the antibody was 0.8 μg mL^{-1} and 0.3 μg mL^{-1}. Methanol, as an organic co-solvent is widely used in immunoassay. Amax (the maximum optical density value) and IC_{50} value were the key parameters to evaluate the performance of ELISA. The data in Table 1 showed that the Amax was slightly reduced as the methanol concentration increased. The IC_{50} values were between 0.069 and 0.097 ng mL^{-1} when methanol content increased from 0% to 50%.

To evaluate potential interferences that may be encountered in real sample analysis, the effects of pH and ionic strength on the ELISA system were tested in this study. The Amax value is sensitive to ionic concentration and it decreased to 0.965 from 1.650 (Table 2) with increasing ionic strength (0, 1, 5, 10, 20 and 50 mM PBS). No significant effects on the IC_{50} value were found at tested salt concentrations. Therefore, water was selected for dilution of sample solution in subsequent experiments.

With different pH values (pH 5.3–8) in assay buffer, no obvious changes in Amax values (from 1.181 to

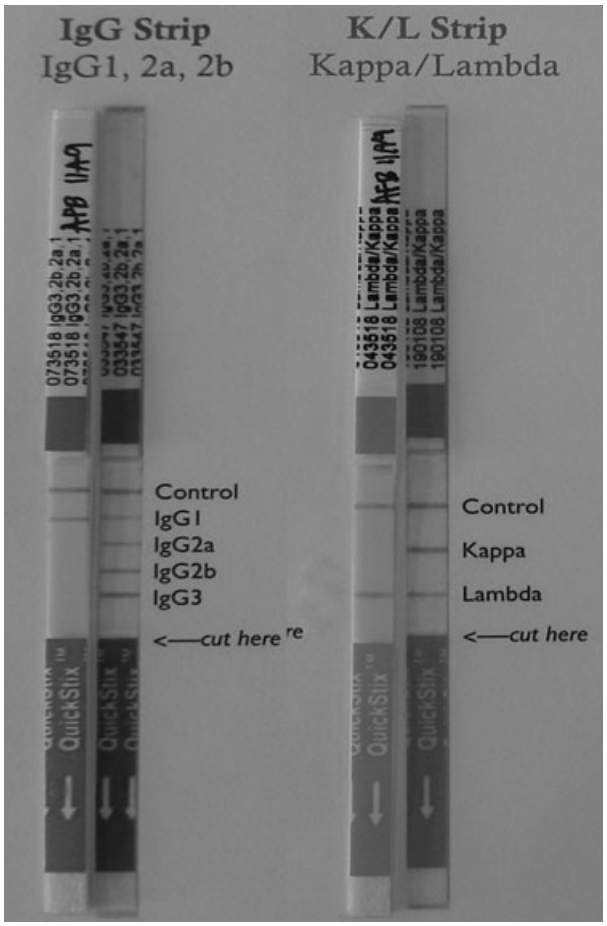

Figure 2 The result of the IgG isotype analysis. Compared with the standard control (right), the IgG isotype is IgG1 and the light chain isotype is lambda.

Table 1 The effects of methanol concentration on the immunoassay

Methanol concentration (v/v), %	Amax	IC_{50} (ng mL^{-1})	Amax/ IC_{50}
0	1.217 ± 0.033	0.069 ± 0.005	17.6
10	1.155 ± 0.033	0.077 ± 0.001	15.0
20	1.176 ± 0.037	0.085 ± 0.000	13.8
30	1.096 ± 0.044	0.081 ± 0.004	13.5
40	1.065 ± 0.030	0.092 ± 0.001	11.6
50	0.974 ± 0.001	0.097 ± 0.002	10.0

1.372) were seen in this experiment (Table 3). So, PBS buffer at pH 7.4 was used for assay solution.

Under the optimised results, a standard curve was established (Fig. 3). The IC_{50} calculated using a logistic function was 0.057 ng mL^{-1}, with a limit of detection (LOD) of 0.019 ng mL^{-1} (15% inhibition). Cross-reactions with other mycotoxins were showed in Table 4.

Table 2 The effects of ionic strength on the immunoassay

Concentrations of PBS (mM)	Amax	IC_{50} (ng mL^{-1})	Amax/ IC_{50}
0	1.650 ± 0.061	0.079 ± 0.01	20.91
1	1.370 ± 0.090	0.094 ± 0.01	14.54
5	1.376 ± 0.002	0.080 ± 0.00	17.18
10	1.286 ± 0.091	0.077 ± 0.00	16.67
20	1.147 ± 0.035	0.063 ± 0.01	18.09
50	0.965 ± 0.061	0.059 ± 0.00	16.32

Table 3 The effects of pH on the immunoassay

pH	Amax	IC_{50} (ng mL^{-1})	Amax/ IC_{50}
5.3	1.181 ± 0.057	0.088 ± 0.008	13.5
6.2	1.283 ± 0.010	0.087 ± 0.003	14.8
7	1.372 ± 0.055	0.091 ± 0.002	15.1
7.4	1.217 ± 0.010	0.075 ± 0.006	16.2
8	1.312 ± 0.035	0.096 ± 0.003	13.7

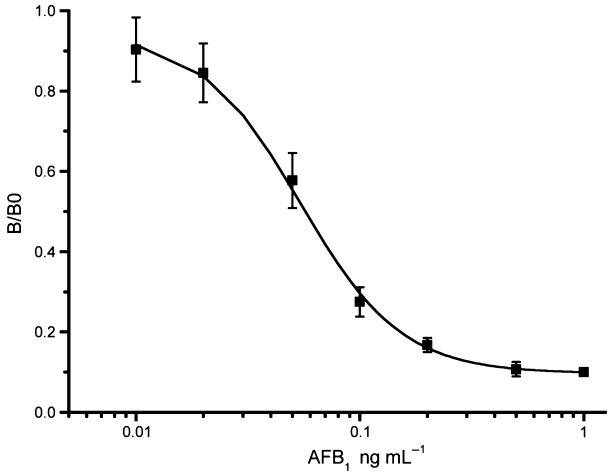

Figure 3 The standard curve of AFB1 derived from ELISA. Each point of the curve represents the mean of three determinations, and the error bar indicates standard deviations (SD). IC_{50} = 0.057 ± 0.007 ng mL^{-1}.

Table 4 Determination of cross-reactivity with other mycotoxins

Mycotoxins	IC_{50} (ng mL^{-1})	CR (%)
AFB_1	0.059 ± 0.007	100.00
AFB_2	0.079 ± 0.005	74.15
AFG_1	0.139 ± 0.004	42.16
AFG_2	0.248 ± 0.022	23.64
AFM_1	0.794 ± 0.041	7.37
OTA	N	N
ZEN	N	N
DON	N	N

N, no 50% inhibition was observed for OTA, ZEN and DON.

Table 5 The AFB_1 analysis of spiked tea samples with ELISA

		Intra-assay(n = 5)			Inter-assay (n = 15)		
Sample	Spiked sample (ng g^{-1})	Measured	CV(%)	Recovery(%)	Measured	CV(%)	Recovery (%)
Black tea	10	11.77	1.8	117.7	11.50	3.5	115.0
	20	17.34	3.4	86.7	16.86	4.5	84.3
	50	34.25	7.50	68.5	36.10	8.7	72.2
Green tea	10	11.43	1.7	114.3	10.94	5.5	109.4
	20	17.3	3.4	86.5	15.98	5.4	79.9
	50	36.75	7.7	73.5	40.00	12.0	80.0

Besides AFB1, the monoclonal antibody shows identification to AFB_2 (CR 74.15%), AFG_2 (23.64%) and AFM_1 (7.37%). No obvious CR with other mycotoxins family such as OTA, ZEN or DON was found.

Recovery studies for ELISA

Three spiked levels (10, 20, 50 ng g^{-1}) were tested for black and green tea samples. Recoveries data were presented in Table 5. Intra- and inter-assay recoveries for black tea ranged from 68.5% to 117.7% and from 72.2% to 115% respectively. For green tea, intra- and inter-assay recoveries ranged from 73.5% to 114.3% and from 79.9% to 109.4% respectively.

Tea contains many active substances, such as pigments, sugars, biological proteins, alkaloids, lipids, steroids and volatile oils. The use of ELISA for the detection of other substances in tea has been rarely reported (Lu *et al*., 2010; Song *et al*., 2011). In the previous study (Nakata *et al*., 2001), solid-phase extraction was used for purification in tea immunoassay. Dilution is one of the best methods of eliminating matrix interference, in our study, matrix effects were substantially removed by simple dilution with water at a ratio of 1:100.

Conclusion

Immunoassays are rapid and convenient for the detection of harmful substances in food samples due to their little requirements in pre-treatment of samples and advantages of high throughput. But, complex food matrices may affect the binding of the antibody and coating antigen, which results in false-positive results in detections.

Thanks to the monoclonal antibody with low IC_{50} value (0.057 ± 0.007 ng mL^{-1}), the developed ELISA method here applied a simple dilution of up to 100 times to successfully detect low concentrations of AFB1 in tea samples. The recovery tests exhibited the potential of this proposed immunoassay in tea analysis. However, due to high dilution, the sensitivity of the antibody becomes a major limiting factor in trace analysis.

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Conflicts of interest

Authors have no conflict of interest.

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