Toxicol. Res.
Vol. 27, No. 2, pp. 125-131 (2011)
Open Access DOI:10.5487/TR.2011.27.2.125

Official Journal of
Korean Society of Toxicology
Available online at http://www.toxmut.or.kr

Production of Group Specific Monoclonal Antibody to Aflatoxins and its Application to Enzyme-linked Immunosorbent Assay

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(Received May 2, 2011; Revised May 6, 2011; Accepted May 11, 2011)

Through the present study, we produced a monoclonal antibody against aflatoxin B1 (AFB1) using AFB1-carboxymethoxylamine BSA conjugates. One clone showing high binding ability was selected and it was applied to develop a direct competitive ELISA system. The epitope densities of AFB1-CMO against BSA and KLH were about 1 : 6 and 1 : 545, respectively. The monoclonal antibody (mAb) from cloned hybridoma cell was the IgG1 subclass with λ-type light chains. The IC_{50}s of the monoclonal antibody developed for AFB1, AFB2, AFG1 and AFG2 were 4.36, 7.22, 6.61 and 29.41 ng/m*l*, respectively, based on the AFB1-KLH coated ELISA system and 15.28, 26.62, 32.75 and 56.67 ng/m*l*, respectively, based on the mAb coated ELISA. Cross-relativities of mAb to AFB1 for AFB2, AFG1 and AFG2 were 60.47, 65.97 and 14.83% in the AFB1-KLH coated ELISA, and 59.41, 46.66 and 26.97% in the mAb coated ELISA, respectively. Quantitative calculations for AFB1 from the AFB1-Ab ELISA and AFB1-Ag ELISA ranged from 0.25 to 25 ng/m*l* ($R^2 > 0.99$) and from 1 to 100 ng/m*l* ($R^2 > 0.99$), respectively. The intra- and inter-assay precision CVs were < 10% in both ELISA assay, representing good reproducibility of developed assay. Recoveries ranged from 79.18 to 91.27%, CVs ranged from 3.21 to 7.97% after spiking AFB1 at concentrations ranging from 5 to 50 ng/m*l* and following by extraction with 70% methanol solution in the Ab-coated ELISA. In conclusion, we produced a group specific mAb against aflatoxins and developed two direct competitive ELISAs for the detection of AFB1 in feeds based on a monoclonal antibody developed.

***Key words*: Aflatoxin B1, Monoclonal antibody, ELISA**

INTRODUCTION

Aflatoxin B_1 (AFB_1) is a secondary metabolite of the fungus *Aspergillus flavus,* and is well known as the most potent hepatocarcinogen in various animal species and human (Hinton *et al.*, 2003; Lu, 2003; Martinez Cerezo, 1994). Carcinogenicity of Aflatoxin B1 is occurred by the formation of Aflatoxin B1 guanine adducts (Bailey *et al.*, 1996; Nakatsuru *et al.*, 1990). Other toxicity of aflatoxin B1 are the reduced growth rate, lowered milk and egg production, reduced reproductivity, reduced feed utilization and efficiency, and anemia (WHO, 1998). Aflatoxin B1 can reversibly bind to serine proteases which represent profound implications in the manifiestation of aflatoxicosis (Cuccioloni *et al.*, 2009). Fish and poultry are known to be extremely sensitive to AFB1 and laboratory animals, especially mouse, showed very resistant to AFB1 (Rawal *et al.*, 2010). The immunosuppressive effects of aflatoxin B1, the most predominant aflatoxins, have been demonstrated in various livestock species and laboratory animals (Celik *et al.*, 2000; Hatori *et al.*, 1991; Hochstenbach *et al.*, 2010; Raisuddin *et al.*, 1994; Sharma, 1993). Most countries including Korea have been set for legal limit pertaining to AFB1 in foods and animal feeds due to the human health concern and the possibility of AFB1 contamination of internationally traded cereal grains. The establishing legal limit in food and feed triggered the development of analytical methods for AFB1.

Although high-performance liquid chromatography (HPLC) and liquid chromatography-tandem mass spectrometry (LC/MS-MS) (Goda *et al.*, 2001; Walton *et al.*, 2001) used to determine aflatoxins in sample quantitatively but these methods require time-consuming extractions, sophisticated equipment, and skilled technicians, so they are not suitable for the routine screening large numbers of samples in the field. Immunochemical techniques such as dipstick immunoassay and enzyme-linked immunosorbent assay (ELISA) are

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simple and less expensive methods for aflatoxin B determination. ELISA has advantages for rapid screening of aflatoxins from many kinds of matrix, and the detection limits of ELISA assay can be comparable with instrumental assay. The usefulness of these immunoassays is dependent on the specificity and sensitivity of antibody used, so the production of mAb and polyclonal antibodies to AFB1 and their application in immunoassay have been reported (Burkin *et al.*, 2000; Mortimer *et al.*, 1988; Saha *et al.*, 2007). Here, we produced a new anti-AFB1 mAb with high affinity to natural aflatoxin B1 and its analogs, then applied it to a direct competitive antibody coated ELISA (Ab-DC-ELISA) and a direct competitive antigen coated ELISA (Ag-DC-ELISA).

MATERIALS AND METHODS

Chemicals and reagents. Aflatoxin B1 (AFB1), pyridine, carboxymethoxylamine hemihydrochloride, dimethylformamide, N,N'-dicyclohexylcarbodiimide (DCC), casein, keyhole limpet hemocyanin (KLH), 8-azaguanine, hypoxanthine-aminopterin-thymidine medium (HAT/HT), Delbucco's Modified Eagle Medium (DMEM), bovine serum albumin (BSA), Tween 20, PEG1500, Freud complete adjuvant/ incomplete adjuvant, and N-hydroxysucciniimide (NHS) were purchased from Sigma-Aldirch (St Louis, MO, USA). Goat anti-mouse IgG and 3,3',5',5-tetramethylbenzidine (TMB) were purchased from KPL (Gaithersburg, MA, USA).

Experimental animals. Five female Balb/c mice (6 weeks old) were purchased from Orient Bio Incorporated (Sung-Nam, Republic of Korea). Mice were provided with tap water and a commercial diet ad libitum. The animal room was maintained at a temperature of $24 \pm 2^{o}C$, a relative humidity of $50 \pm 20\%$, and a 12 h light/dark cycle. All animals were cared for according to the Code of Laboratory Animal Welfare and Ethics of the National Veterinary Research and Quarantine Service (NVRQS). Experimental design was approved by the NVRQS Animal Welfare Committee.

Preparation of AFB1-CMO. AFB1 was first converted to AFB1-CMO to create reactive group for coupling according to the method described elsewhere (Kolosova *et al.*, 2006). Briefly, 4 mg of AFB1 and 6.36 mg of carboxymethoxylamine were dissolved in 3.2 m*l* of mixture solution (pyridine : water : methnol = 1 : 1 : 4, v/v/v). The mixture was maintained at 110°C for 2.5 h by agitation every 20 min. Then, the mixture was reacted for overnight in dark. Purple color residue obtained by drying at 70°C with vacuum evaporator was dissolved in 10 m*l* 0.1 N NaOH. The aqueous suspension was washed with 5 m*l* dichloromethane and adjusted pH 2.0 by adding HCl solution, and then extracted with 10 m*l* ethylacetate three times. All ethylacetate phases were pooled, filtered over anhydrous sodium sulfate and dried under vacuum. AFB1-CMO conjugate was further purified using preparative HPLC and fraction collector with Xetra PrepMS C_{18} column (Waters, 1.9 × 250 mm, 10 μm). The concentration of AFB1-CMO in purified fraction was determined by Beer-Lambert equation.

Preparation of AFB1-protein and AFB1-enzyme conjugate. AFB1-CMO-BSA was synthesized using the carbodiimide condensation principle (Cervino *et al.*, 2008). AFB1-CMO 2.0 mg was dissolved in dry dioxane 0.2 m*l*, and then added 20 μ*l* of NHS (34 mg/m*l* in dry dioxane) and 26 μ*l* DCC (38 mg/m*l* in dry dioxane). The mixture was reacted for overnight by stirring. BSA solution containing 10 mg of BSA (5 mg and 10 mg for KLH and HRP, respectively) in carbonate buffer (pH 7.5) was added drop by drop and carbonate buffer (1, 150 μ*l*, pH 7.5) by slow addition, drop by drop, and stirred at 4°C for 6 h. After desalting using PD-10 column (GE healthcare, Sweden) according to the instructions of the manufacturer, AFB1-conjugates were lyophilized and stored at −20°C before use. The numbers of haptenic groups per mole of each protein (epitope density) were calculated by dividing the mole of AFB1-CMO with those of each protein.

Production of monoclonal antibodies to AFB1. Five female Balb/c mice (6-week old) were acclimated for a week, and then immunized intraperitoneally with 100 μg of AFB1-BSA conjugate emulsified with an equal volume of Freund's complete adjuvant. Boosters with Freund's incomplete adjuvant were injected intraperitoneally 6, 8, and 10 weeks later. Four weeks after last injection, serum was collected from each mouse and antibody titers were measured by indirect competitive ELISA. Four days before cell fusion, a mouse with a high titer and good antibody affinity to AFB1 was given an intraperitoneal injection of 100 μg AFB1-BSA conjugate without adjuvant. The HAT selective SP2/0-Ag14 (ATCC) medium was prepared using 8-azaguanine-containg DMEM. The ratio of spleen cells from the immunized mouse and SP2/0 myeloma cells fused was about 5 : 1. After HAT selection, supernatant from the hybridoma cells was analyzed by indirect competitive ELISA. The isotype of the immunoglobulin secreted from the cloned cell was determined using a mouse monoclonal antibody isotyping kit (Roche, Switzerland). An indirect competitive ELISA for screening of mouse sera and culture supernatants was used to determine the presence of AFB1 antibodies. The immunoplates (Maxisorp, Nunc International, Rockilde, Denmark) were coated overnight at 4°C with 200 μ*l* of AFB1-KLH conjugate in 0.05 M carbonate buffer (pH 9.6) and then washed 3 times with 0.05% tween 20 in PBS (PBS-T). After blocking with PBS containing 1% casein (blocking buffer) for 2 h at RT, the plates were washed 3 times. A total of 100 μ*l* of serum (or culture supernatant) was placed

into the wells. Then, 100 μl of 20 ng/ml AFB1 diluted in blocking buffer was added and incubated at RT for 1 h. After washing 3 times, 100 μl of anti-mouse IgG-HRP conjugate (1 : 2,000) was added, incubated for 1 h, and the washing steps repeated. TMB substrate solution was added to the each well. After incubation for 15 min at RT, the reaction was stopped by adding of 2 N H_2SO_4 (100 μl per well). The absorbance was measured at 450 nm. For ascites fluid production, the cultured cells (2×10^6 cells) in DMEM were injected into the pre-injected mouse with 0.5 ml pristane. After 7~14 days, ascites fluid was collected and purified with Hitrap protein IgG column (GE Heathcare, USA) according to the manufacturer's instruction. An indirect competitive ELISA was used to determine the presence of AFB1 antibody in the purified ascites fluid.

Enzyme-linked immunosorbent assay. AFB1 Ab (2.5 μg/ml, 100 μl per well) for the AFB1 Ab-coated ELISA and AFB1-KLH (1 μg/ml, 100 μl per well) for the AFB1 Ag-coated ELISA in 0.05 M carbonate buffer and were immobilized on the immunoplate overnight at 4°C. After washing with PBS-T, a AFB1 standard (50 μl) or sample (50 μl) was diluted in PBS-MeOH (14% MeOH) buffer and 50 μl AFB1-HRP solution (1 : 500 in 1% casein) or 50 μl of AFB1 antibody-HRP conjugate solution (1 : 100 in 10% skim milk) were added. After incubation for 5 min at RT, the plates were washed and developed with 100 μl TMB solution for 5 min. To stop the color development, 100 μl of 2 N H_2SO_4 was added. The absorbance was measured at 450 nm.

Determination of cross-reactivity of both ELISA methods with AFB1 and its analogs. Cross-relativities (CR) of ELISA methods for AFB1 analogs such as aflatoxin B2, aflatoxin G1 and aflatoxin G2, were determined by dividing the 50% inhibitory concentration (IC_{50}) of AFB1 by the IC_{50} values of each analogs.

Feed sample preparation and recovery determination. A total of 5 g of feed was spiked with AFB1 at different concentrations (0, 5, 15, 50 ng/g), and extracted with 70% methanol/water (v/v) for 3 min. The extract samples were filtered through Whatman No. 1 filter paper. A total of 10 ml of filtrate was diluted with 40 ml of PBS (phosphate buffered saline, pH = 7.4). The concentration of AFB1 in the extracted samples was determined using an ELISA.

HPLC analysis. Before HPLC analysis, extracted samples were passed through an immunoaffinity column (R-biopharm rhone, Scotland) according to manufacturer's instruction. For HPLC, a reverse-phase C_{18} column (4.6 mm × 250 mm, 5.0 μm, Waters) was equilibrated with water-methanol-acetonitrile (60 : 20 : 20, v/v) at flow rate 1 ml/min. A total of 10 μl of each eluted sample was injected into HPLC system (Waters 2695) with fluorescence detector (Waters 2475) at wavelength of 365 nm excitation and 435 nm emission.

Safety notes. Aflatoxin B1 is a carcinogenic and should be handled with extreme care and aflatoxin-contaminated materials should be discarded into a aqueous solution of sodium hypochlorite.

RESULT AND DISCUSSION

Preparation of AFB1-protein/enzyme as immunogen. The small molecules like AFB1 cannot induce the production of antibodies by itself. Therefore, the conjugate of

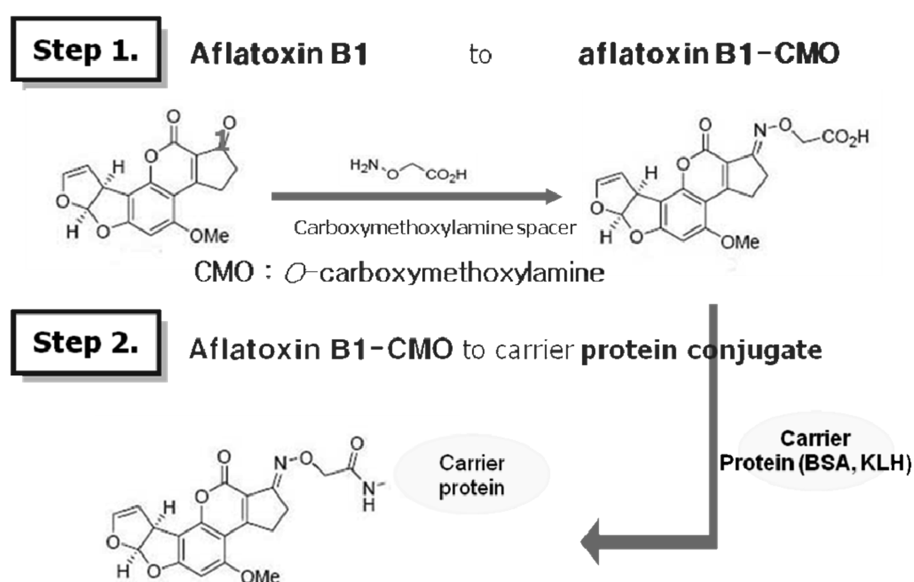

Fig. 1. The strategy for the preparation of AFB1-CMO conjugate and further conjugation with carrier proteins.

AFB1 and a carrier protein must be prepared for the production of desirable antibodies. We prepared AFB1-CMO conjugate by linking CMO to C1 carbon site of AFB1, and it was further conjugated with BSA by carbodimide condensation (Fig. 1). After haptenization of AFB1 with carboxymethoxylamine, the chromatographic mobility of the reaction product (AFB1-CMO) appeared earlier than that of the parent compound, as expected due to the increased polarity caused by obtaining a reactive group (Burkin *et al.*, 2002). The AFB1-CMO peak occurred before 6 min and AFB1 peak appeared around 12 min. The formation of conjugates was also confirmed by spectrophotometrical scanning between 190 and 340 nm. The UV spectra of AFB1-CMO and AFB1 alone showed three maxima at around 202, 268 and 360 nm, indicating that modification of AFB1 with CMO did not affect its chromosphere characteristics (Fig. 2).

The epitope densities of AFB1-CMO against BSA and KLH were about 1 : 6 and 1 : 545, respectively. The number of moles of conjugated protein was calculated based on the concentration of the carrier protein and the molecular weight (BSA: 66,430, KLH: 8,000,000). The number of moles of AFB1-CMO was calculated from the absorption at 362 nm using extinction coefficient ($\varepsilon = 20{,}950$). Coupling the density of the chemical with the carrier protein was reported to affect antibody production. Briefly, immunogens with higher coupling densities usually produce monoclonal antibodies with higher substrate affinities (Klaus and Cross, 1974; Nakagawa *et al.*, 1980). In the present study, coupling density AFB1-CMO to BSA was around 6.0 and represented a similar to that of previous report (Zhou *et al.*, 2007).

Production and characterization of AFB1 specific monoclonal antibody. The antisera from the five immunized mice each showed different specificities and affinities based on indirect competitive ELISA, and the mouse with the highest affinity was used for cell fusion with myeloma cells. Because the fused cells can produce many different antibodies, not only against AFB1, but against BSA and CMO, used as a linker, screening of these fused cells was performed by indirect-competitive ELISA, using AFB1-KLH as the coating antigen. Cloned cell produced the IgG1 subclass with λ-type light chains. We selected the clone with the highest specificity to AFB1, which had a similar affinity (sensitivity) to the commercial product (Santacruz, USA) based on indirect-competitive ELISA (Fig. 3). It was named kj-AFB1 and used for mass production of monoclonal antibody in mouse.

The IC_{50}s of the monoclonal antibody, Kj-AFB1, for AFB1, AFB2, AFG1 and AFG2 from the present study were 4.36, 7.22, 6.61 and 29.41 ng/m*l*, respectively, based

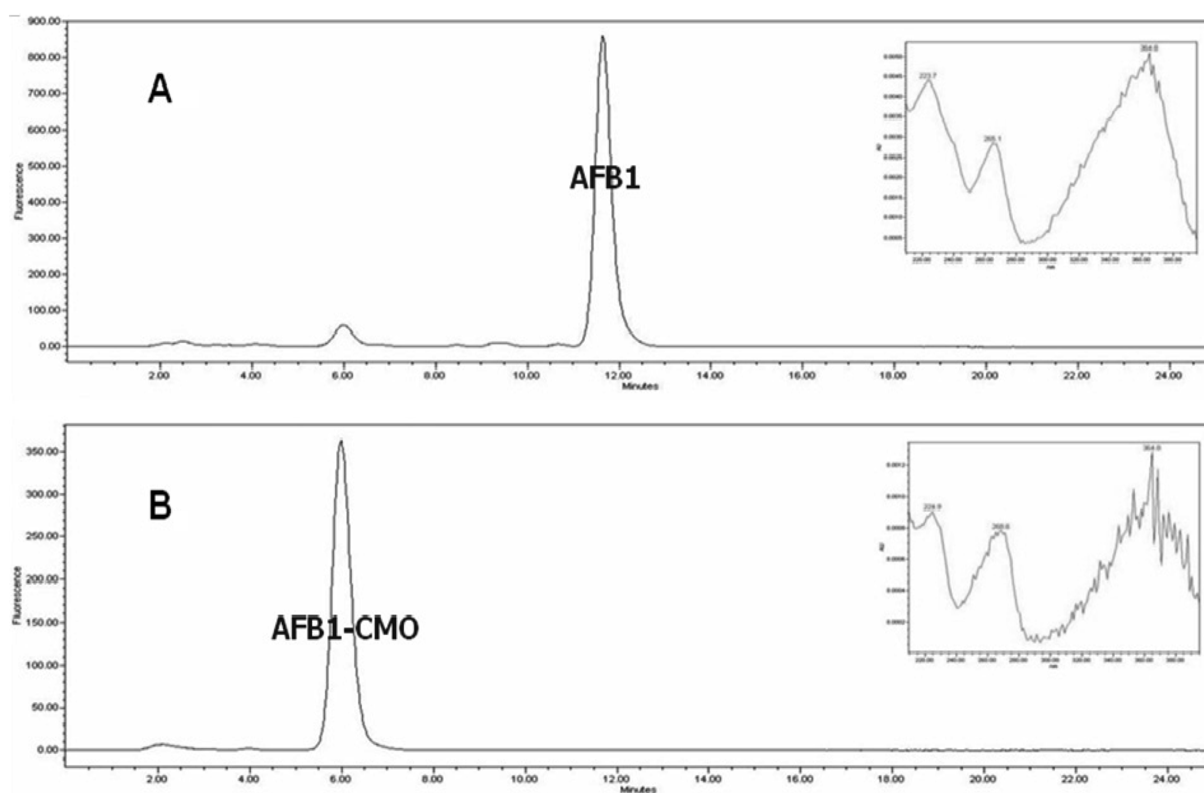

Fig. 2. HPLC chromatogram and UV scanning spectrum of aflatoxin B1 (A) and aflatoxin B1-CMO (B).

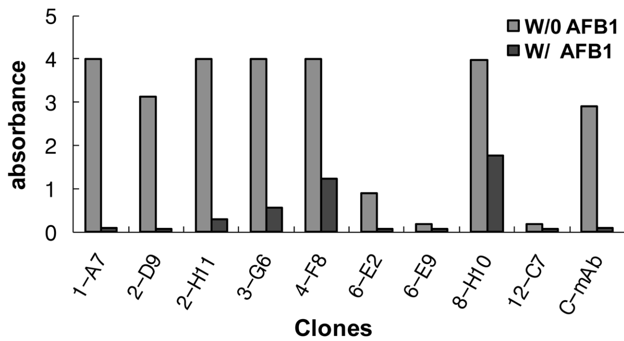

Fig. 3. Comparison of inhibition pattern of anti-AFB1 mAb with commercial anti-AFB1 mAb. Supernatant from the kj-AFB1 clones and the commercial mAb were diluted 10 fold with medium, and then inhibition was examined by adding 5 ng AFB1. The mAb represent the commercial monoclonal antibody to AFB1. Each point is the mean of three replicates.

on the AFB1-KLH coated ELISA system and 15.28, 26.62, 32.75 and 56.67 ng/m*l*, respectively, based on the mAb coated ELISA. Cross-reactivity of mAb to AFB1 for AFB2, AFG1 and AFG2 were 60.47, 65.97 and 14.83% in the AFB1-KLH coated ELISA, and 59.41, 46.66 and 26.97% in the mAb coated ELISA, respectively (Fig. 4). A lot of clones which can bind to aflatoxins can be prepared after immunization of AFB1, and mAb from this study, IgG1 (λ) type, represented more than 50% of cross-reactivity with AFB2 and AFG1, but it showed low cross reactivity to AFG2. Some mAb (IgG1, κ) showed low affinity to other aflatoxin analogs (Cervino *et al.*, 2008; Kolosova *et al.*, 2006) and other represent relatively high affinity to AFB1, AFB2 and AFG1 (Wang *et al.*, 1995). Based on the high affinity of this monoclonal antibody for both AFB1 and its analogs, it is likely that the monoclonal antibody developed in the present study can recognize common structure of aflatoxins.

Validation of AFB1 Ab/ or Ag coated ELISA. To obtain linear standard curves, a logit curve expressing the y-axis by calculating binding percentage to blank sample (B/B0) was made. Quantitative calculations for AFB1 from the AFB1-Ab ELISA and AFB1-Ag ELISA ranged from 1 to 100 ng/m*l* and from 0.25 to 25 ng/m*l* ($R^2 > 0.99$), respectively (Fig. 5). The intra-plate and inter-well assay variations for both AFB1-ELISAs were compared using coefficients variation (CV): each of two AFB1 standard concentrations, 10 and 20 ng/m*l* for the AFB1-Ab coated ELISA, and 15 and 75 ng/m*l* for the AFB1-Ag coated ELISA were analyzed. The intra- and inter-assay precision CVs were both < 10%, representing good reproducibility (Table 1). Because AFB1 can be extracted more easily from the solid matrices of foods and feeds with organic solvents like methanol, we

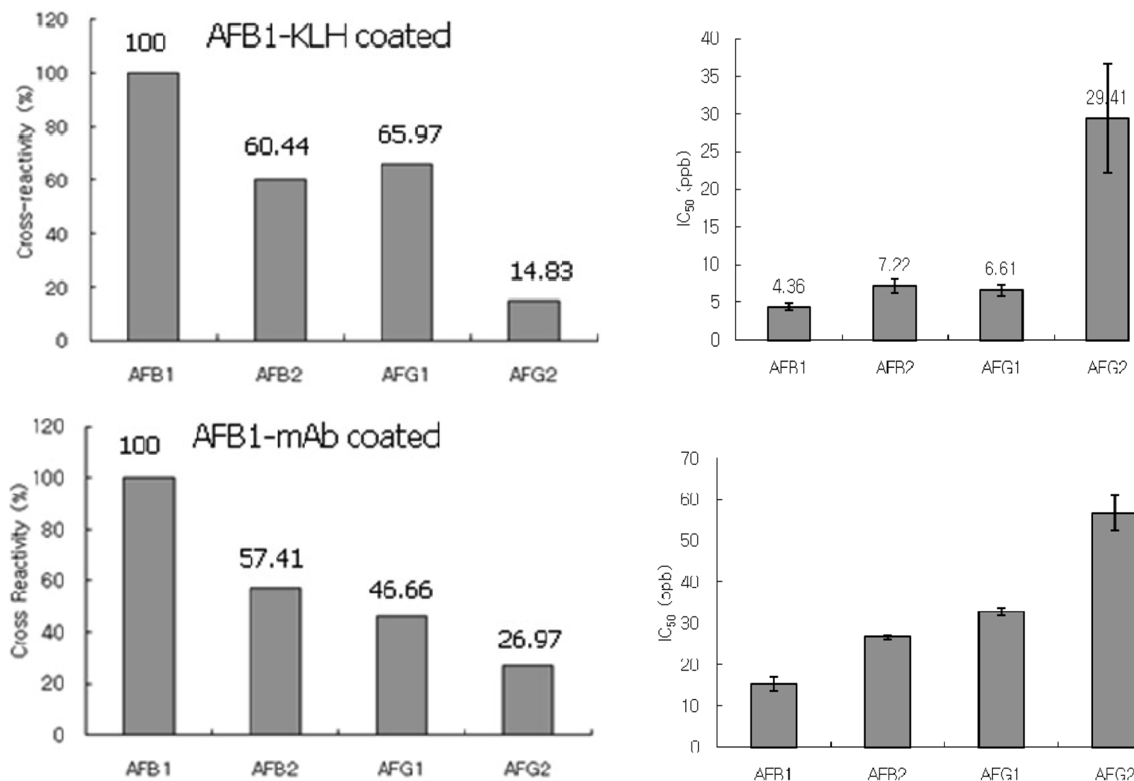

Fig. 4. Cross-reactivity and IC_{50} values of developed Mab for AFB1 with AFB2, AFG1 and AFG2.

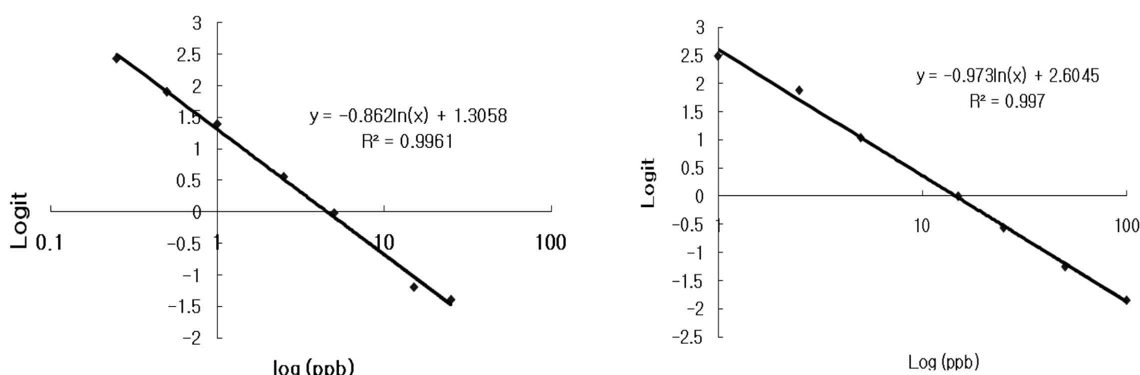

Fig. 5. Competitive direct ELISA standard curves for aflatoxin B1 by Ag-DC-ELISA (left) or Ab-DC-ELISA (right). Each B and B0 means value of absorbance at 450 nm in presence and absence of aflatoxin. Microplates with 100 ng AFB1-KLH/well of and 0.25 μg monoclonal antibody/well were prepared for each assay. Each point represents the mean of 3 duplicates.

Table 1. Recoveries and coefficient variations for the ELISA coated with AFB1 and monoclonal antibody

Coating materials	Assay	Added amount (μg/kg)	Detected amount (μg/kg)	Coefficient variation (%)
AFB1-KLH	Inter-well	10	12.36 ± 0.51	4.15
		20	20.97 ± 1.87	8.92
	Intra-plate	10	13.27 ± 0.53	4.04
		20	18.59 ± 1.38	7.44
Antibody	Inter-well	15	22.46 ± 1.81	8.06
		75	76.43 ± 3.03	3.97
	Intra-plate	15	17.91 ± 1.69	9.47
		75	80.03 ± 5.64	7.05

100 ng of AFB1-KLH and 0.25 μg of monoclonal antibody per well were coated (n = 5).

Table 2. Recoveries of AFB1 spiked in feed sample in mAb coated direct ELISA assay

AFB1 (μg/kg)		Recoveries (Mean ± SD)	CV (%)
Spiked	Founded		
5	4.45 ± 0.31	91.27 ± 6.12	6.71
15	11.88 ± 0.38	79.18 ± 2.54	3.21
50	44.13 ± 3.52	88.26 ± 7.03	7.97

0.25 μg of monoclonal antibody per well were coated and spiked each amount of AFB1 into feed sample. The amount of AFB1 in blank feed was deducted from those of each spiked samples.

used 70% methanol solution. The Ab-coated ELISA was validated by spiking AFB1 at concentrations ranging from 5 to 50 ng/m*l*, following by extraction with 70% methanol solution. Recoveries ranged from 79.18 to 91.27%, CVs ranged from 3.21 to 7.97% (Table 2). Based on CODEX alimentarius guideline (CAC/GL 71-2009) for quantitative analytical methods, acceptable CVs for intra-laboratory testing are < 30% and acceptable recoveries are 60~120% for samples containing 1 to 10 ng/m*l* analyte, and CVs are < 20% and recoveries are 70~120% for sample containing 10 to 100 ng/m*l*. The present data represents good repeatability of developed ELISA assay in the laboratory level.

In conclusion, we produced a group specific monoclonal antibody against aflatoxins and developed two direct competitive ELISAs for the detection of AFB1 in feeds based on a monoclonal antibody developed, and this assay also seems to be suitable for aflatoxins monitoring of grain samples without complicated purification steps.

ACKNOWLEDGEMENTS

This project was conducted as an international collaborative research project between National Veterinary Research and Quarantine Service (NVRQS), Republic of Korea and the Department of Population Medicine and Diagnostic Sciences of Cornell University, USA, and funded by NVRQS, Republic of Korea.

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