

Production of In-house ELISA Test Kit for Detection of Aflatoxin in Agricultural Commodities and Their Validations

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ABSTRACT

A specific microtest plate enzyme immunoassay was developed in Thailand for the rapid detection of aflatoxin B₁ (AFB₁) using Polyclonal antibody against AFB₁. Percentage of the antibody cross reaction to AFB₁, AFB₂, AFG₁ and AFG₂ were 100, 21.4, 25.0 and 2.5% respectively. AFB₁-HRP (Horseradish Peroxidase) conjugate was used as direct competitive test for AFB₁ detection.

The DOA- Aflatoxin ELISA Test Kit was produced for detection of both qualitative and quantitative results within 1 hr after sample preparation with the minimum of 0.4 ppb per assay. There was no significant difference in detection efficiency of this test kit compared to traditional methods of minicolumn, TLC and HPLC. Moreover, the kit obtained 82-100% AFB₁ recovery from the spike samples. Validation for the analysis of AFB₁ in sample matrices was also tested using peanut extract which had the virtually identical curves to the standard. Detection efficiency of DOA-Aflatoxin ELISA test kit was not different from those imported from the USA and Italy.

Key words: aflatoxin, antibody, ELISA test kit, validations

INTRODUCTION

Aflatoxins are a chemically diverse group of compounds produced as secondary metabolites of certain strains of *Aspergillus flavus*, *A. parasiticus*, *A. nomius*, and *A. tamaraii* that may be commonly found in/on foods and feedstuffs (Moss, 1998). The major aflatoxins of concern are Aflatoxin B₁ (AFB₁), AFB₂, AFG₁, AFG₂, AFM₁ and AFM₂. Aflatoxin B₁ which is the most toxic compound in this series and has been found to be one the most potent liver carcinogens occurring naturally. Because of frequent contamination of AFB₁ in agricultural commodities such as corn, peanut, cereal and animal feed stuffs, aflatoxin problems become a potential hazard to human and animal health. Furthermore, animal

products, meat and eggs may become contaminated with aflatoxins if contaminated feed is consumed by the producing animals (Bullerman, 1986; Pestka, 1988).

Generally, the occurrence of aflatoxins in food and feeds is unavoidable and unpredictable. It is influenced by certain environmental factors and vary not only from region to region but also from year to year. In addition, they can be presented in the absence of fungal contamination, a situation caused by death or removal of the fungi after toxin biosynthesis occurs (Morgan and Lee, 1990).

Since the aflatoxins problem is difficult to avoid, the most effective measure for their control depends on a program to monitor their presence in food and feeds. Consequently, sensitive and accurate

method for analysis of aflatoxins are essential for decreasing the risk of human exposure to aflatoxins (Mirocha *et al.*, 1979; Stoloff, 1980). Although TLC (Thin Layer Chromatography) and HPLC (High Performance Liquid Chromatography) may serve in this capacity, the instrumentation is costly, extensive clean up procedures is required and only single sample can be analyzed at a time (Chu, 1986). Therefore in an attempt to develop new methods for mycotoxin analysis, efforts should be made to overcome all those problems. A good method should be very specific, rapid, sensitive and relatively simple to operate. Recently, many laboratories have led to the development of specific antibodies for aflatoxin B₁ as well as the efficacy of their use in enzyme-linked immunosorbent assay (ELISA) for quantitating the aflatoxins in food and feed (Lawellin *et al.*, 1977). Moreover, the ELISA Test Kit which provides the reagents, materials and instruction to perform the test has been produced commercially for mycotoxins detection. In Thailand, most of the test kits used are also imported with high cost. The objective of this study is to reduce the cost of aflatoxin B₁ determination, through the production of ELISA Test Kit for inland use.

MATERIALS AND METHODS

Polyclonal antiserum production against AFB₁

Antibody against AFB₁ was produced in 4 New Zealand White rabbits by intradermal injection at the backs of rabbits with the mixture of 300 µg of AFB₁-BSA conjugate (Sigma Chemical Co.) and complete Freund's complete adjuvant (Sigma Chemical Co.) (ratio 1:3) for the first immunization. Booster injection was done one month later by intramuscular injection with the mixture of 250 µg of AFB₁-BSA and Freund's incomplete adjuvant (Sigma Chemical Co.) (ratio 1:2). First bleeding was done 3 weeks after initial immunization and continued bleeding for 21 weeks. The immunoglobulin G fraction of the rabbits' blood

was purified by the ammonium sulfate precipitation method of Herbert *et al.* (1973). Antibody titre was determined by ELISA. The specificity of the produced antibody to AFB₁, AFB₂, AFG₁ and AFG₂ was also tested by direct competitive ELISA.

Preparation of AFB₁-peroxidase conjugate

A modification of the method used for the conjugation of AFB₁ to BSA (Chu and Veno, 1977) was used for peroxidase. Fifty -two mg of AFB₁ was first converted to AFB₁-oxime, then AFB₁-oxime was conjugated to 2 mg of HRP (horseradish peroxidase) (Sigma Chemical Co.) in the presence of 1,3 diclohexyl carbodiimide (DCC) (Sigma Chemical Co.) and N-hydroxy succinimide (NHS). The reaction mixture was incubated at room temperature for 4 h then centrifuged at 15,000 rpm for 10 seconds. The supernatant was collected and dialyzed with 3 changes of 0.01M. Phosphate buffer (PBS) pH 7.2. AFB₁ - peroxidase conjugate was kept in vial and stored at -20°C.

Preparation of AFB₁ standard

AFB₁ standard (Sigma Chemical Co.) was used. One µg of the AFB₁ standard was dissolved in 1 ml benzene acetonitrile and 100 µl (100 ng) of the AFB₁ standard solution was transferred to each vial then nitrogen dried. Before used, 1 ml of methylalcohol (MeOH) was added to each vial to make up the concentration to 100 ng/ml of AFB₁. The concentrations of AFB₁ standard used in this test kit was diluted with sample diluent (7% MeOH + 92% 0.01 Phosphate buffer saline + 1% Dimethylformamide) to 5, 2, 1, 0.5, 0.2, and 0 ng/ml (ppb).

Microwell coated plate

The DOA-ELISA test kit was prepared using antiserum and AFB₁ - HRP conjugate produced from this experiment. Direct competitive ELISA was employed for this test kit. One hundred µl of the purified antibody was coated into the MicroELISA plate or the microwell strip at the concentration of

1:5000 then incubated overnight at 4°C. Brovine serum albumin in 0.01M phosphate buffer pH 7.2 was used as blocking solution. The coated microELISA plate kept at 4°C could be effective for 6 months. The procedures for determining aflatoxins using this test kit are described below.

Sample preparation : Weighed 20 gm ground sample into the blender jar and added 100 ml of 70% MeOH. Blended 2-3 min at high speed, left standing for 10 min and filtered upper phase through the filter paper. Diluted small portions of filtered extracts (1:20 or higher) with 0.01M Phosphate Buffer Saline (PBS). Sample was now ready for ELISA test. In case of many samples had to be done at a time, each sample could be put into the Elenyer flask and shaken at 300 rpm for 30 min until use.

ELISA protocol : Added 50 ul of AFB₁ standards into the antibody coated wells and 50 ul of diluted sample into the other wells followed by adding 50 ul of AFB₁-HRP conjugate to each well, slightly shaken then incubated at room temperature for 30 min. Dumped the contents of the well into the appropriate waste container and washed the plate 3-5 times by 0.01M phosphate buffer saline + 0.5% Tween 20 (PBS-T). Tapped out buffer on the absorbent paper towel. Added 100 ul of substrate (Tetramethylbenzidine)(KPL Inc.) to the wells, incubated 10 min at room temperature then added 100 ul of stopping solution (0.3M Phosphoric acid). Read the color at 450 nm using the MicroELISA Reader (Biotek EL 311).

Calculation: Qualitative result could be derived by visual comparison of the sample color to the standard wells. Samples containing less color than the standard well had greater concentration of aflatoxin than the standard well. In contrast, the sample containing more color had lower aflatoxin concentration. For quantitative result, plotting the standard curve on the semilogarithmic graph paper, placing the value of standards on x-axis and the corresponding absorbance value on Y-axis. Read AFB₁ concentration in the sample directly

from the standard curve (in ppb).

Effect of sample matrix on the ELISA

Different concentrations of the standard AFB₁ (Sigma Co.) at 0, 0.1, 1, 5, 10, 20 and 50 ppb were prepared in both diluent buffer and uncontaminated peanut extract. The prepared AFB₁ standard were then detected by DOA-ELISA test kit for the presence of aflatoxin.

Recovery of AFB₁ added in corn

The AFB₁ standard was added to the corn sample to receive the final concentrations of 10, 50 and 100 ppb. The DOA-ELISA test kit was used to determine the concentration of AFB₁ in spiked corn samples. Two trials were conducted.

Comparison of DOA-ELISA test kit with chemical methods

The effectiveness of the DOA-ELISA test kit for detection of aflatoxin in naturally contaminated corn samples was compared to those of Thin Layer Chromatography (TLC), Minicolumn and High Performance Liquid Chromatography (HPLC). The procedures involved in those three chemical methods were as described by the AOAC official methods. (A.O.A.C., 1995)

TLC, 50 gm ground sample was homogenized with 250 ml chloroform. The extract was cleaned up through silica gel column and concentrated by rotary evaporator. The concentrated extract was transferred to vial and nitrogen dried. Added 200 ul of benzene-acetonitrile into the dried extract vial, mixed well and then spotted on the TLC plate. TLC plate was developed using acetone- chloroform (1+9) as developing solution. For HPLC, silica gel column was used for clean-up and reversed-phase liquid chromatography (RPLC) with postcolumn derivatization. Fluorescence detector was used for separation and detection respectively.

Comparison of DOA- ELISA test kit with imported test kit

Two commercially imported test kits, Neogen (USA) and Techna (Italy) were used to detect aflatoxin in contaminated corn compared with the to DOA-ELISA test kit. The experiment was made twice for each test kit. The concentration of aflatoxin B₁ standard used in Neogen test kit were 0,5,15, and 50 ppb, whereas Techna test kit and DOA- test kit had aflatoxin B₁ standard concentrations of 0, 3, 6, 12, 25, 50, 100 ppb. and 4 0,20,40, 100 ppb respectively.

RESULTS AND DISCUSSION

Polyclonal antiserum production and their titre

Antibodies titre: Antibodies having sufficient titre were generally obtained 5-7weeks after initial immunization. However, antibodies harvesting continued up to 18-21 weeks.. Antibodies titration was performed by incubating dilution series of the antiserum with the AFB₁-HRP conjugate and then determining the total bound enzyme. The last well in the dilution scheme to give color visually distinct from the preimmune serum (control) was designated as the titre endpoint. The produced antiserum had the titre as high as 17,500. Chu and Ueno (1977) also reported that antiserum against AFB₁ was usable with the titers more than 1,000 in 5-8 weeks after immunization.

Cross reaction: The specificity of the AFB₁ antibody was determined for cross reaction with antigens AFB₁, AFB₂, AFG₁ and AFG₂. The relative cross – reactivity of different aflatoxins with the antibody produced as estimated from the amount of toxin necessary to cause 50 % inhibition of maximal conjugate binding were found to be 1.5, 7.0, 6.0 and 60.0 ng/ml or 100,21.42, 25.0 and 2.5 % cross - reaction, respectively.(Table 1) The antibody obtained in the present study affected their interaction to AFB₁ greater than other aflatoxins.

AFB₁-HRP conjugate : The prepared AFB₁- HRP conjugate had the optimum concentration of 1:20000 when tested by ELISA. A 100 ul of 10x conjugate was put in the small vial and 1 ml of conjugate buffer (0.01M PBS + 1% Brovine Serum Albumin) added in the vial prior to use.

DOA-ELISA test kit: A set of the test kit contained Ab-AFB₁ coated microwell plate or strips (96 well), 6 concentrations of AFB₁ standard (0, 4, 10, 20, 40 and 100 ppb), 6 vials of AFB₁-HRP conjugate, conjugate buffer, substrate, stopping solution, washing buffer and manual for using the test kit (Figure 2). The DOA-ELISA test kit for aflatoxin detection is the test kit firstly produced in Thailand. Generally, ELISA method for aflatoxin detection have been done in many countries such as USA, Japan, England, China and Italy. In Thailand this method has been used for aflatoxin detection in agricultural commodities at the laboratory of Plant

Table 1 Specificity of AFB₁ antiserum in binding to other aflatoxins.

Aflatoxins	50% inhibitory concentration (ppb)	% cross reaction
AFB ₁	1.5	100.00
AFB ₂	7.0	21.42
AFG ₁	6.0	25.00
AFG ₂	60.0	2.50

$$\% \text{ Cross Reaction} = \frac{X_1}{X_2} \times 100$$

X₁ = AFB₁ concentration (ppb) at 50% maximal binding

X₂ = other aflatoxins concentration (ppb) at 50% maximal binding

Pathology and Microbiology Division, Department of Agriculture since 1990 (Tanboon-Ek *et al.*, 1991) under the guidance of Prof. Dr. F.S.Chu, Wisconsin University. After that the ELISA method was extensively studied and developed into ELISA test kit to be used in other laboratories, feed factories and food factories. DOA-ELISA test kit was tested for their validation.

Effect of sample matrix on the ELISA

The result showed that the standard curve obtained from using peanut extract as diluent was

comparable to that obtained from the diluent buffer alone (Figure 3). The assay system of DOA-ELISA test kit appeared to have less sample matrix introduction and subsequently showed less interference. Hence, the test kit was effective in detecting aflatoxin in the sample matrix as well as in the buffer. Chu *et al.* (1987) were also studied the effect of sample matrix on the ELISA using extracts of peanut butter, white cornmeal and yellow cornmeal. They found out that absorbances of sample dilution were comparable to those obtained from the buffer alone.

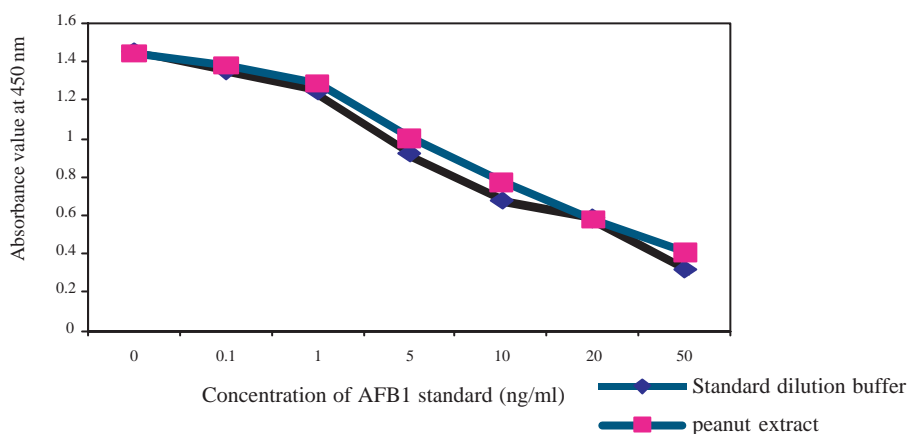


Figure 1 The efficiency of DOA -ELISA TEST KIT for AFB₁ standard detection in peanut extract and AFB₁ standard in dilution buffer.

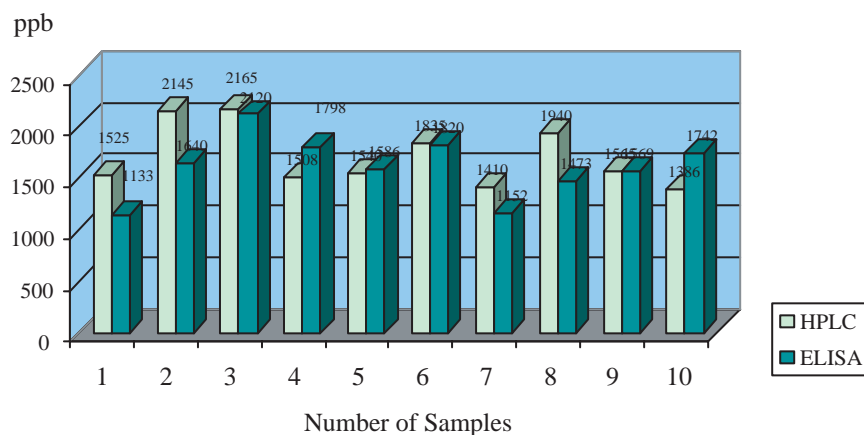


Figure 2 Comparison amount of aflatoxin (ppb) detected from naturally contaminated corn by ELISA and HPLC.

Recovery of AFB₁ added to corn

Recovery percentages of AFB₁ added (10,50 and 100 ppb) to corn were found to range from 83.6 to 128 % and 92.6 -100% in trail I and II respectively (Table 2). The result was comparable to the recovery studies of Chu *et al.* (1987) which received 91 and 95.4 % overall recoveries for all concentration tested on recovery of AFB₁ added to cornmeal and peanut butter. The DOA-ELISA test kit was noticed to be quite accurate in AFB₁ detection.

Comparison of DOA-ELISA Test Kit with chemical methods

To test the effectiveness of DOA-ELISA test kit, a number of naturally contaminated samples were analyzed in comparison to TLC and Minicolumn analysis. The aflatoxin in the sample which could not be detected by TLC, could be detected by ELISA at the lowest amount of 0.4 ppb. (Table 3). Only qualitative or approximate result

Table 2 Percent recovery of AFB₁ from spiked corn at various concentrations by DOA- ELISA test kit.

AFB ₁ added (ppb)	Amount of detected AFB ₁ (ppb)	% recovery
Trail I		
10	12.8	128
50	51.4	102.8
100	83.6	83.6
Trail II		
10	10.0	100
50	46.6	93.2
100	92.6	92.6

$$\% \text{ recovery} = \frac{\text{Amount of detected AFB}_1}{\text{Amount of AFB}_1 \text{ added}} \times 100$$

Table 3 Comparisons of the amount of Aflatoxin B₁ (ppb) in naturally contaminated corn when detected by ELISA , TLC method and Minicolumn method.

Sample	ELISA	TLC	ELISA	Minicolumn
1	60	41.3	< 10	> 30
2	0.4	ND	32	< 10
3	360	140	14	< 10
4	12.8	ND	17	15–20
5	160	87	14	< 10
6	> 200	141.7	75	70–90
7	24	11.5	95	20–30
8	> 200	219.1	7	< 10
9	> 200	370.2	75	20–30

ND = Not Detected

could be obtained by minicolumn. The result obtained from minicolumn was not comparable to ELISA in some samples which might be due to the misreading under the UV light. However, the correlation in the amount of AFB₁ detection from naturally contaminated corn by DOA- ELISA test kit and HPLC was found to be not significantly different. (Figure 4). Chu *et al.* (1987) also found out that data obtained from ELISA were more accurate than those obtained by TLC.

Comparison of DOA-ELISA Test Kit with Imported Test Kit

There were no differences in the amount of aflatoxin detected by DOA and NEOGEN ELISA test kits, yet both test kits showed different results from that of TECHNANA test kit with lower amount of aflatoxin. The steps in performing aflatoxin detection by NEOGEN test kit were quite rapid and caused tension to the analysts, whereas, DOA and TECHNANA test kits were quite easy to perform. Different results were sometimes obtained from two analysts when using the TECHNANA test kit in determining the same sample (Table 5). Variation in the results among three test kits was also due the different concentrations of the AFB₁ standard designed to be used in each test.

CONCLUSION

DOA- ELISA test kit is a direct competitive enzyme-linked immunosorbent assay that the user can obtain both quantitative and qualitative results in part per billion. AFB₁ in the samples and controls (free toxin) were allowed to compete with AFB₁-HRP conjugate for the antibodies binding sites. This ELISA test kit was successfully produced for inland-use with a sensitive, simple, cheap and rapid method for detection of aflatoxin in agricultural commodities used for human and animal consumption. The test would take only one hour to perform after sample preparation and provide the results of up to 40 samples with lower limit 0.4 ppb of detection. The simple and cheaper the test is, the greater the chances the producers could detect aflatoxin in their products. Consequently, the consumers health could be ensured.

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Table 4 Comparisons of the efficiency among DOA- ELISA test kit and imported test kits in detection of aflatoxin in corn.

Sample	Imported ELISA test kits					
	DOA		NEOGEN		TECHNA	
	R1	R2	R1	R2	R1	R2
C0	0	2.4	0	0.8	8.5	0
C1	19.0	15.2	8.3	11.7	12.4	12.4
C2	132.1	110.6	116.1	121.8	68.7	62.7

DOA = ELISA test kit produced by the Department of Agriculture

NEOGEN = ELISA test kit imported from USA.

TECHNA = ELISA test kit imported from Italy

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