Letter to the Editor

The Development of A Fluorescence Polarization Immunoassay for Aflatoxin Detection*

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A fluorescence polarization immunoassay (FPIA) was developed for the analysis ofaflatoxins (AFs) using an anti-aflatoxin B_1 (AFB_1) monoclonal antibody and a novel fluorescein-labeled AFB_1 tracer. The FPIA showed an IC_{50} value of 23.33 ng/mL with a limit of detection of 13.12 ng/mL for AFB_1. The cross-reactivities of AFB_1, AFB_2, AFG_1, AFG_2, AFM_1, and AFM_2 with the antibody were 100%, 65.7%, 143%, 23.5%, 111.4%, and 2%, respectively. The group-specificity of anti-AFB_1mAb indicated that the FPIA could potentially be used in a screening method for the detection of total AFs, albeit not AFG_2 and AFM_2. The total time required for analyzing 96 samples in one microplate was less than 5 min. This study demonstrates the potential usefulness of the FPIA as a rapid and simple technique for monitoring AFs.

Aflatoxins (AFs) are a group of toxic secondary metabolites that are mainly produced by *Aspergillusflavus*and *A. parasiticus*[1]. The four most important are AFB_1, AFB_2, AFG_1, and AFG_2 (Figure 1), which are often found in different types of matrices, such as maize, peanuts, cottonseed, fruit, and meat[2]. Of those compounds, AFB_1 and AFB_2 are the most commonly occurring. The hydroxylated forms of AFB_1 and AFB_2 are AFM_1 and AFM_2, respectively, which can sometimes be found in milk and dairy products. These compounds are generated when AFB_1 and AFB_2 are ingested from contaminated feed and metabolized into AFM_1 and AFM_2. Because of the widespread occurrence of AF-producing fungi and the occurrence of the AFs in a number of agricultural commodities, robust efforts have been made to develop new methods for AF detection[3-4]. The fluorescence polarization immunoassay (FPIA) is a homogeneous method that reaches equilibrium in minutes or even seconds, and involves no separation or washing steps. The principles and applications of FPIA to the detection of chemical contaminants in food have been reviewed previously[5]. FPIA is more suitable for use in the high-throughput screening of large numbers of samples than the conventional enzyme-linked immunosorbent assay (ELISA) method. To date, many FPIAs have been developed for the detection of food contaminants, the majority of which are pesticides[6-7]. However, few applications of FPIA for detecting mycotoxins have been reported, partly because of the unavailability of fluorescein-labeled mycotoxin tracers. Only Nasir and Jolley have previously reported on the use of FPIA for detecting AFs by a method that used of a tracer derived from AFB_1 and fluoresceinamine (isomers I and II)[8]. In this study, we report the synthesis of a novel AFB_1 tracer and describe our preliminary efforts towards developing a FPIA for the detection of AFB_1.

We developed a novel synthesis of the AFB_1 derivative (Figure 2). We added 20 mg (60 μmol) Carboxymethoxylamine (CMO,) 10 mg AFB_1 (30 μmol), and 5 mL pyridine to a round-bottom flask and maintained it for 24 h at room temperature. The pyridine was evaporated in a rotary evaporator at 50 °C, and the remaining 2 mL yellow-brown oil product was dissolved in 10 mL pure water. We added 0.1 mol/L NaOH (about 5 mL) drop-wise to adjust the solution to pH=8.0, completely dissolving the product. To this solution, 2×5 mL benzene was added to separate the organic and aqueous phases. The aqueous phase was acidified with 5 mol/L HCl to pH=2, anda white-brown precipitate formed. Then, the compound was extracted with ethyl acetate (3×20 mL) and then dried with 2 g anhydrous Na_2SO_4. The organic fraction was filtered through Whatman No. 1 filter paper. The solvent was evaporated and

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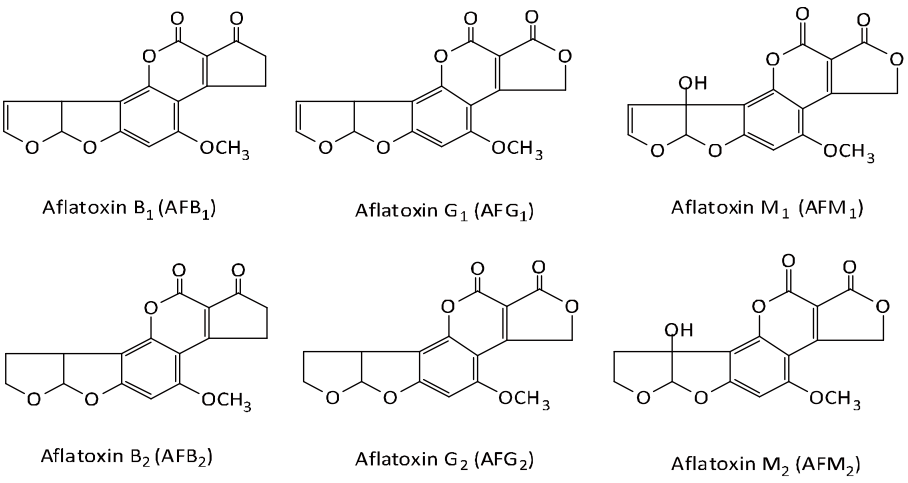

Figure 1. The chemical structures of the aflatoxins: AFB_1, AFB_2, AFG_1, AFG_2, AFM_1, and AFM_2.

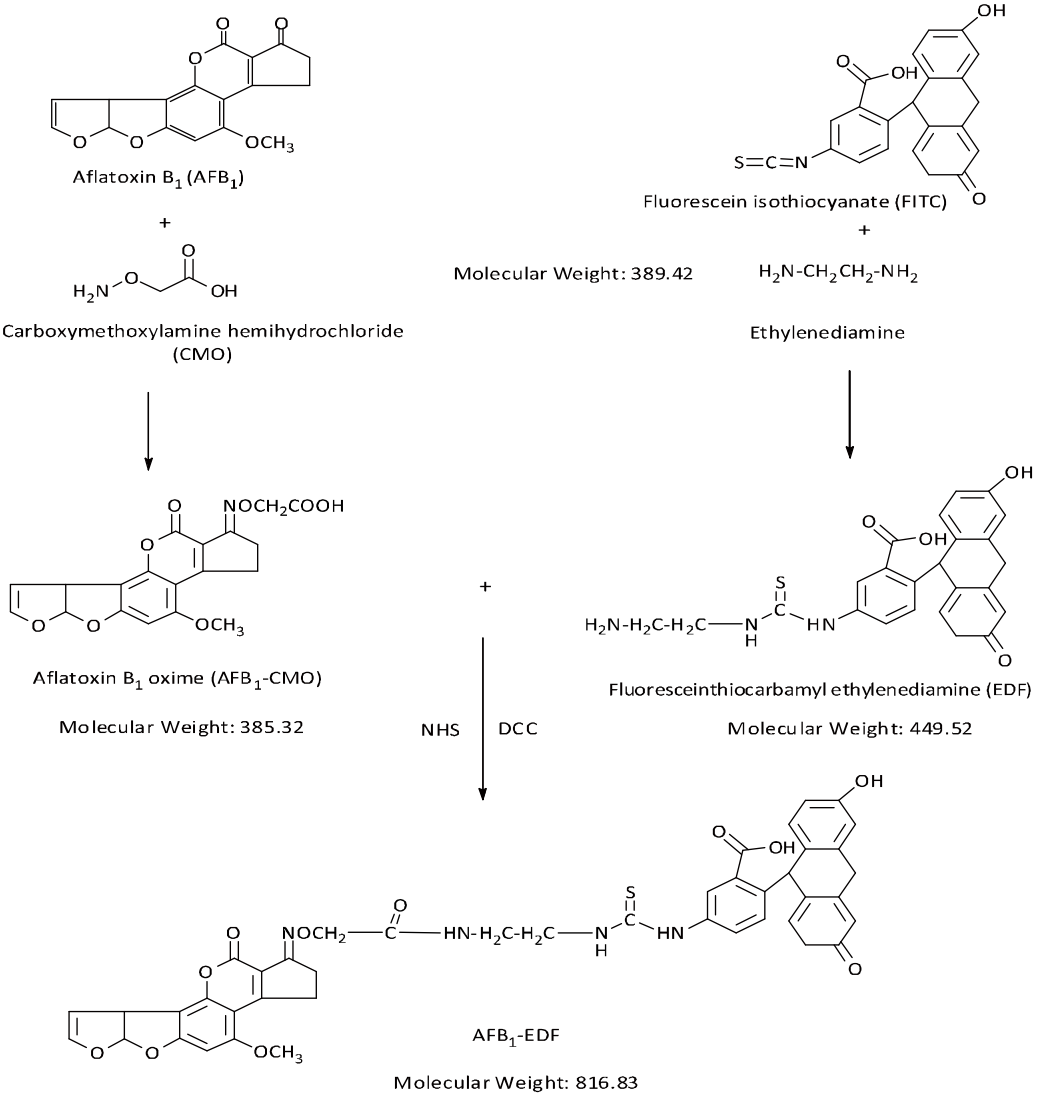

Figure 2. The chemical synthesis of AFB_1-EDF from AFB_1 and EDF.

9.5 mg (20 μmol) AFB_1-CMO was obtained. Next, 1 mL dicyclohexylcarbodiimide (DCC) in dimethylformamide (DMF) (50 μmol/L) and 1 mL *N*-hydroxysuccinimide (NHS) in DMF (50 μmol/L) were added to 9.5 mg AFB_1-CMO (ratio, AFB_1-CMO: DCC: NHS = 1: 2.5: 2.5). The mixture was stirred at room temperature overnight. Fluoresceinthiocarbamylethylenediamine (EDF) was synthesized from FITC and ethylenediamine as described previously[9], with modifications of the chemical reaction of EDF (Figure 2). Then, 2 mg EDF was added to activate AFB_1-EDF and the reaction mixture was kept at room temperature for 2 h before transfer to a refrigerator overnight at 4 °C. A small portion (about 10 μL) was purified by TLC using $CHCl_3$: methanol = 4:1. The main yellow band at R_f=0.7 was collected, eluted with 0.5 mL methanol, and stored at -20 °C in the dark.

The synthesis of the novel AFB_1-EDF tracer was confirmed by HPLC-MS/MS. The molecular weight of AFB_1-CMO and EDF are 385.32 and 449.52, respectively. We calculated a molecular weight of AFB_1-EDF of 816.83. The molecular ion peak (m/z) of AFB_1-EDF was 817.2 in positive ion mode, preliminarily indicating that the EDF was successfully conjugated with AFB_1. In addition, the synthesized AFB_1-EDF could specifically bind to anti-AFB_1mAb, which further supports the successful synthesis of the tracer.

The tracer solution was serially diluted to 1/100, 1/200, 1/400, 1/800, and 1/1 600 in Borate buffer (BB) (50 mmol/L, pH 8.5), measured by fluorescence, and selected according to the total final fluorescence intensity, which was 10 times higher than the background of BB. The anti-AFB_1mAb and a negative control antibody (specific to sulfamethazine) were serially diluted to 1/50, 1/100, 1/400, 1/800, 1/1 600, and 1/3 200 in BB to prepare antibody dilution curves. We added 50 μL of the diluted antibody to each microplate well (Corning Life Sciences, Kennebunk, ME, USA) with 50 μL tracer (350 ng/mL) and 50 μL BB to make a total volume of 150 μL. The reaction mixture was incubated for 5 min and then detected by FPIA. The FPIA was measured at λ_{ex}=485 nm and λ_{em}=530 nm (emission cutoff=515 nm, G factor=1.0) using a SpectraMax M5 microplate reader. We found that AFB_1-EDF did not bind to the negative control antibody, whereas it robustly bound to anti-AFB_1mAb, implying that the tracer was specifically recognized by its corresponding antibody. To improve analytical sensitivity, limiting concentrations of antibody and tracer were required. We determined the optimal antibody dilution (75% of the tracer binding to the mAb) for anti-AFB_1mAb to be 1/200, and this dilution was then used in the FPIA calibration curve for AFs.

To construct FPIA calibration curves, we sequentially added 50 μL mycotoxin standard solution, 50 μL of tracer in BB, and 50 μL of the optimal dilution of anti-AFB_1mAb to the microplate well. The reaction mixtures were mixed, incubated, and analyzed. Different concentrations of AFs and other related mycotoxin (fumonisin B_1, zearalenone, deoxynivalenol, and T2 toxin) solutions were prepared for analysis in a standard way. Working concentrations of AFB_2, AFG_1, AFG_2, AFM_1, and AFM_2 were calculated based on the calibration curves of AFB_1. Curve fitting and statistical analyses were performed using OrginPro 7.5 software (OriginLab Corporation, Northampton, MA). The IC_{50} value, detection range, and limit of detection (LOD) serve as criterions to evaluate the FPIA. These characteristics present the analyte concentrations that provide inhibition in mP values at 50%, between 20% and 80%, and at 90%, respectively. Curve fitting was performed using a four parameter logistic model as follows: $Y = (A-D)/[1 + (X / C)B + D]$; A = response at high asymptote, B = slope factor, C = concentration corresponding to 50% specific binding (IC_{50}), D = response at low asymptote, and X = calibration concentration. The cross-reactivity (CR) was calculated by dividing the IC_{50} of AFB_1 by the other AFs according to the following equation: CR (%) = [IC_{50} (AFB_1, ng/mL) / IC_{50} (analogs, ng/mL)]× 100[10].

The FPIA calibration curve for AFB_1 (Figure 3) indicated that the limit of detection (LOD) and IC_{50} were 13.12 and 23.33 ng/mL, respectively, and the detection range was 16.25-33.49 ng/mL. The sensitivity (as determined by the IC_{50}) of our FPIA is higher than that of the FPIA developed by Nasir and Jolley[8], partly because of the use of the novel tracer. The structure of tracer has a direct effect on the sensitivityof a FPIA. As the spacer arm of tracer AFB_1-EDF was longer than that of AFB_1-FITC, the fluorescent dye residue was farther from the antigen-binding site of the antibody and the steric hindrance of tracer binding was weaker.

The cross-reactivities of AFB_1, AFB_2, AFG_1, AFG_2, AFM_1, and AFM_2 with the antibody were 100%, 65.7%, 143%, 23.5%, 111.4%, and 2%, respectively. Cross-reactivity of other mycotoxins, including fumonisin B_1, zearalenone, deoxynivalenol, and T2 toxin, were also evaluated. Our results showed that the

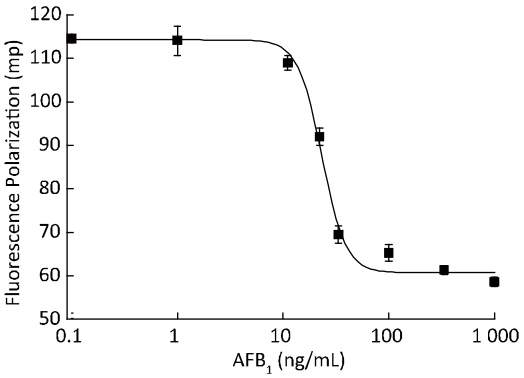

Figure 3. The FPIA calibration curves for AFB_1. Calibration curves were analyzed in triplicate with a coefficient of variation (CV) of 8.7%.

FPIA that we developed did not recognize these mycotoxinsat 1 000 ng/mL. The group-specificity of anti-AFB_1mAb indicated that the FPIA could be potentially used in a screening method for the detection of total AFs, albeit not AFG_2 or AFM_2.

A novel AFB_1-EDF tracer was synthesized, purified, and characterized to develop a FPIA for AFs. Prior to our study, Nasir and Jolley prepared a AFB_1 tracer using fluoresceinamine, which had a comparatively shorter bridge between fluorescein and AFB_1[8]. Here, we used a FITC derivative (EDF) to synthesize the tracer, thereby creating a novel tracer with a longer bridge. The novel tracer was expected to increase the flexibility of the tracer and to increase the sensitivity of the FPIA. In conclusion, we developed a FPIA method based on a novel tracer for the determination of AFs. The method is simple, homogenous, and does not require separations or multiple steps.

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