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DEVELOPMENT OF ONE-STEP IMMUNOCHROMATOGRAPHIC STRIP FOR THE DETECTION OF TOTAL AFLATOXIN IN CORN SAMPLE BASED ON MONOCLONAL ANTIBODY CLONE 4G6

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ABSTRACT

Aflatoxins are cancer-causing chemicals produced primarily by *Aspergillus flavus* and *A. parasiticus*. Aflatoxin B1 (AFB1) is the most commonly found aflatoxin in improperly stored staple commodities such grain and feed. Its presence in the food supply, can be carried over to animal products such as meat, liver, kidney, pig blood and milk. A specific and sensitive detection method is required for preliminary screening of these samples. This research sought to develop a detection kit for total aflatoxin by immunochromatographic technique using monoclonal antibody (MAb) from the hybridoma cell line 4G6. The experiments were conducted at the Serology and Diagnostic Laboratory, Kasetsart University, Kamphaeng Saen Campus, Nakhon Pathom province during 2014-2016. The MAb is composed of IgG_{2b} isotype and lambda light chain. Its specificity recognized four aflatoxins including AFB_1, AFB_2, AFG_1 and AFG_2 with cross reactivity at 100%, 89.2%, 82.6%, and 72.7%, respectively by direct competitive enzyme-linked immunosorbent assay (dcELISA). *In vitro* propagation of the hybridoma was carried out using an Integra CELLine Culture System and the antibody was purified by affinity column chromatography. The conjugate probe was prepared by comparing two sizes of colloidal gold particles at 20 and 40 nm in diameter for the conjugation with the MAb. The MAb conjugate with 40 nm colloidal gold was selected and sprayed onto the conjugate release pad (CRP). The target cut-off value for the developed immunochromatographic strip (ICS) was 20 ng/mL according to a regulation limit in Thailand. The study on the appropriate conditions for this strip showed that aflatoxin B_1 conjugated to bovine serum albumin (AFB_1-BSA) and goat anti-mouse immunoglobulin (GAM) should be immobilized at the test line and control line at the same concentrations of 0.25 mg/mL. The testing sample was extracted with 70% methanol and further diluted 1:4 with Tris buffer saline with 0.05% Tween-20 (TBST) before application on the sample application pad (SAP) and the reaction could be visualized within 15 min. The analysis of 5 naturally contaminated corn samples (n=7) indicated that 2 samples contained $\geq$ 20 µg/kg and 3 samples contained < 20 µg/kg. Five samples, analyzed by dcELISA, showed contamination levels at <4, 9.6, 19.9, 10.5 and 39.7 µg/kg which delivered a good correlation to the results from ICS analysis.

Key words: rapid test kit, mycotoxin, toxin analysis, lateral flow assay

INTRODUCTION

Aflatoxins (AFT) are a group of carcinogenic secondary metabolites produced primarily by *Aspergillus flavus* and *A. parasiticus*. These are listed as group I carcinogens by the International Agency for Research on Cancer (IARC, 2002). Several types of aflatoxins are produced in nature, however, aflatoxin B1 (AFB_1) is the most common in food and feed as well as among the most potent genotoxic and carcinogenic aflatoxins. Therefore, exposure through food should be kept as low as possible. When the toxin enters human or animal bodies, it would be absorbed in the alimentary canal and spread rapidly through tissues but may take time to be excreted through urine and stool. For these reasons, AFT might be carried over to the animal product such as meat, liver, kidney, pig blood and milk (Kaushal and Bhatnagar, 1998).

European Communities (EC) have different regulations on AFT quantity in children's food and cow's milk, which should not exceed 4 µg/kg and 0.05 µg/L, respectively. In Thailand, the regulation limit of all food commodities is 20 µg/kg according to the Notification of Ministry of Public Health of 1986 (Ministry of Public Health, 1986), however, the percentage of major aflatoxin contamination observed in peanuts, dried seafood, poultry tissue and corn were reported to be 51.4%, 58.6%, 48.67%, and 54.39%, respectively (Tangmunkhong *et al*., 2011; Mahakarnchanakul *et al*., 2011; Anukul *et al*., 2013). These data indicate the need for more efforts to establish the control measures of the toxin in these commodities, especially for very low levels of AFT in food products, which requires a specific and sensitive method. Although the most acceptable and popular AFT analysis is high performance liquid chromatography (HPLC) with cleanup (Chu, 2004), it is expensive, time-consuming and requires highly skilled and professional personnel. Moreover, large quantities of chemicals are needed which poses extreme danger of used chemicals and cost of disposal. Currently, immunological methods such as quantitative enzyme-linked immunosorbent assay (ELISA) is acceptable to the Association of Official Analytical Chemists Research Institute (Lupo *et al*., 2010). Another method for rapid screening is an immunochromatographic assay (ICA) which is a qualitative analytical test recommended for preliminary screening. Its utilization has been increasing because it is convenient and allows qualitative or semi-quantitative determination of mycotoxin within a few minutes. In Thailand, the production of AFT-specific immunochromatographic strip (ICS) with the cut-off value at 40 ppb was reported and it took 20 min for color development (Biotechnology gallery, 2006). This research sought to develop the ICS for total AFT analysis, using monoclonal antibody from our previous research, with the required cut-off value at 20 ng/mL, according to Thailand regulation limit for food, by conjugating the antibody with nanogold particles. The success of this research would help to reduce import cost of commercial ICS and support agriculturist or trader needing raw material screening and decreasing the risk of consumption of food contaminated with aflatoxin.

MATERIALS AND METHODS

Materials

Hybridoma clone 4G6 from the previous research (Hongprayoon *et al*., 2009) producing specific antibody to AFT was used for the ICS development. The upscale production of the hybridoma was carried out in a CELLine CL1000 (INTEGRA Biosciences, Switzerland) as described by the manufacturer, with Complete medium (RPMI1640 medium + 15% fetal calf serum). The antibody was harvested and further purified by HiTrap Protein G together with AKTAprime plus chromatography system (GE Healthcare, Sweden). Nanogold particles were purchased from DCN Diagnostics (USA). Goat anti-mouse Ig (GAM), Aflatoxin B1-bovine serum albumin conjugate (AFB_1-BSA) and AFB_1, AFB_2, AFG_1, AFG_2 standards were purchased from Sigma-Aldrich Company (USA). High-flow nitrocellulose membrane, fiber glass and CF6 absorption material were purchased from GE Healthcare (Sweden). Phosphate buffer saline (PBS, pH 7.4) (137 mM NaCl, 27 mM KCl,

10 mM Na_2HPO_4, 1.8 mM KH_2PO_4) and Tris buffer saline (TBS, pH 7.2) (25 mM Tris base, 0.15 M NaCl) were prepared according to the standard protocols (Sambrook and Russell, 2001).

Nanogold particle conjugation with specific antibody against aflatoxins (MAb-gold conjugate)

Conjugation of the MAb with 20 nm and 40 nm nanogold particle was carried out according to Faulk and Taylor (1971). Briefly, 10 ml of the gold suspension was adjusted to pH 7.3 by 0.2M K_2CO_3. One hundred microliter of previously dialyzed MAb in 0.5x PBS (1 mg/mL) was gradually added to the suspension. The mixture was stirred slowly at room temperature (~25°C) for 1 h. One milliliter of 10% BSA in distilled water was prepared and filtered through a syringe filter (0.45 micropore size nylon membrane) prior to adding into the mixture. Stirring at room temperature was carried on for 30 min and the suspension was centrifuged at 10,000xg for 10 min. A total of 500 µL of gold dilution buffer, pH 7.4 (0.02 M Na_2HPO_4, 0.2% NaN_3, 1% BSA) was used to resuspend the MAb-gold conjugate precipitate in a microtube. One hundred milligram of sucrose was then added and mixed by inverting the tube. The concentration of MAb-gold conjugate was determined using NanoDrop 8000 (Thermo Scientific, USA) at 520 nm (O.D. 1 = 9.0 x 10^{10} particles/ml) (Haiss *et al.*, 2007).

Development of immunochromatographic strip and selection of a sample buffer

ICS components were layered on a protecting plastic sheet adhesive backing as follows; nitrocellulose membrane (NCM, Prima 40), conjugate releasing pad (CRP, 33 glass fiber), sample application pad (SAP, 33 glass fiber), and absorption pad (AP, CF6 cotton linter) with 1 mm overlapping for each component (Fig. 1). A test line and a control line were applied onto the NCM by immobilizing toxin conjugate and goat anti-mouse IgG (Sigma, USA), respectively. Five microliter per centimeter of MAb-gold conjugate ($O.D._{520}$ = 0.5) was applied to the CRP. The Biojet and Airjet: XYZ Dispensing System (BIODOT, USA) was used to manufacture the testing strips. After the reactive lines on NCM and the CRP had been conducted, it was allowed to dry at 37ºC for two hours.

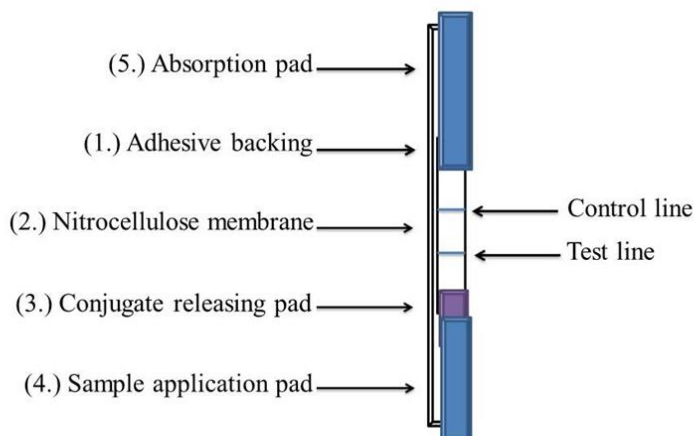

Fig. 1. Schematic diagram of the immunochromatographic strip (ICS). Numbers shown in the figure indicates orders of the component layers.

Four kinds of buffer solution including PBS, pH 7.4; PBST (PBS+ 0.05% Tween-20), pH 7.4; TBS, pH 7.4; and TBST (TBS + 0.05% Tween-20), pH 7.4 were compared to 70% (v/v) methanol (control) for their capillary action on the strip without non-specific reaction. The test was performed by dropping 50 µL of each buffer on the SAP and the reactions at both lines had been observed within 15 minutes.

Determination of the cut-off value of the ICS using aflatoxin standards dissolved in 70% methanol

Toxin standards for each aflatoxin (B_1, B_2, G_1 and G_2) were prepared in 70% methanol at the final concentrations of 10, 20, 50, and 100 ng/mL. Each standard preparation was diluted 1:4 with the sample buffer (TBST) before testing with the ICS. Completion of color development was determined 15 min after dropping the sample onto the SAP.

Analysis of spiked corn samples by the developed ICS

Spiked corn samples were prepared to evaluate the developed ICS. Dry ground corn was analyzed by HPLC at the Molecular Biology and Toxin Laboratory, Kasetsart University Research and Development Institute. The aflatoxin-negative sample was then spiked with each aflatoxin (B_1, B_2, G_1, and G_2) at the required concentrations; 0, 10, 20, 50, and 100 µg/kg, and left overnight at 4ºC. Twenty grams of the ground sample was extracted with 40 mL of 70% methanol for 20 min at room temperature and shaken at 200 rpm. To allow matrix precipitation, the extract was filtered through filter paper No.1 (Whatman, USA) after 5-10 min (Kladpan *et al.,* 2004). The sample extract was diluted 1:4 with TBST prior to analysis to decrease matrix interference and methanol concentration. Color development was determined 15 min after dropping the sample onto the SAP.

Analysis of naturally contaminated corn samples by the developed ICS

Five samples of animal feed raw material were received from the Veterinary Diagnostic Center, Faculty of Veterinary Medicine, Kasetsart University, Kamphaeng Saen Campus. The toxin was extracted as described above and diluted 1:4 with TBST. The sample solution from each preparation was applied to the sample pad until color development which was at least 15 min. The results of 7 replicates were compared with the results from direct competitive ELISA (dcELISA) using a commercial test kit, AgraQuant® ELISA Total Aflatoxin (4-40 ng/mL) (Romer Labs, Singapore) and operated by the Veterinary Diagnostic Center. The absorbance of the samples was compared to the absorbance of the standards and an interpretative result was determined. A dose-response curve was constructed using the five standards and the unknowns were measured by interpolation from this standard curve.

RESULTS AND DISCUSSION

Development of immunochromatographic strip (ICS) and selection of a sample buffer

The monoclonal antibody from hybridoma cell line 4G6 was used in the development of ICS, which was IgG_{2b} containing lambda light chain as characterized previously. The MAb specificity was evaluated by cross reactivity (CR) with four aflatoxins including AFB_1, AFB_2, AFG_1 and AFG_2 via dcELISA (Fremy and Usleber, 2003). Its specificity to aflatoxins were as follows; 100%, 89.2%, 82.6%, and 72.7% with B_1, B_2, G_1, and G_2, respectively without cross reaction with zearalenone and ochratoxin A. These characteristics supported that the MAb possesses good affinity to total aflatoxins and could be applied to develop the ICS for the detection of total aflatoxins. Twenty four milligrams of the purified antibody was obtained from the upscale production in the Integra CELLine system. The main purpose of this research was to develop an ICS which provides results that can be visualized by the naked eye, specifying a cut-off value at 20 ng/mL following Thailand regulation for food. It was important that the color intensity of the test line was visible enough to be seen and enable a clear distinction between negative and positive results. Therefore, the appropriate density of MAb-gold conjugate, toxin conjugate and anti-species antibody were major considerations. The conjugate probe was prepared by conjugating nanogold particles with the antibody because the particles are commonly used as an immunospecific probe with good biocompatibility and label of biomolecules (Liao and Li, 2010). Comparison of the two sizes of the colloidal gold particles at 20 and 40 nm in diameter showed the better signs on the test and control lines when using 40 nm size and offered maximum visibility owing to the least steric hindrance in the IgG conjugation (Chandler et al., 2000). Experimental results showed that the optimal amount of the MAb-gold conjugate applied onto the CRP was 5 µL/cm. The

optimal immobilized AFB_1- BSA was determined to be at the concentration of 0.25 mg/mL, which was the same concentration as the control line for anti-species antibody.

Optimization of the ICS assay condition

Even though the extraction of AFT from the sample can be performed in several solvents, such as water-saturated chloroform, aqueous methanol, acetonitrile, acetone as well as polar solvents, the most widely used has been 70% methanol which corresponded to our results. However, several obstacles can also be found in the extract interference with the binding between the toxin and the MAb conjugate (Kaushal and Deepak, 1998). Thus, other factors affecting the reaction on the assay strip such as sample buffer and methanol density in the system were also investigated, in order to get strong and clear reaction line. In the experiments, four kinds of buffer solutions were compared to 70% methanol and the results showed that TBST gave the best lines for both test and control zones when the analyte was absent in the buffer. We also compared three dilutions of methanol extract in TBST, e.g. 1:2, 1:3 and 1:4. The results showed that when 1:2 diluted extract was applied to the strip, signals at both lines were not observed whereas these could be clearly visualized at 1:3 and 1:4 dilutions (especially at 1:3 dilutions). Thus, 1:3 dilution was chosen for the succeeding experiments and the complete reaction could be achieved within 15 mins.

Determination of the cut-off value of the ICS using aflatoxin standards dissolved in 70% methanol

The cut-off value of the developed strip was determined by testing the strip with four aflatoxins in 70% methanol and diluting these at a ratio of 1:4 with TBST. In the absence of aflatoxin, the binding of the MAb-gold conjugate to the solid-phase AFB_1-BSA gave a strong red band. In contrast, the color band disappeared when the test solution contained any of AFB_1, AFB_2 and AFG_1 above 20 ng/mL except for AFG_2 where the test line would disappear at 50 ng/mL of the analyte (Fig. 2). This result corresponded to the specificity of the MAb mentioned above. Nevertheless, this characteristic did not affect the strip performance since AFG2 contaminated in a natural sample is a small ratio comparing with other aflatoxins (B1: B2: G1: G2 = 4:2:1:1) (Trucksess et al., 2008).

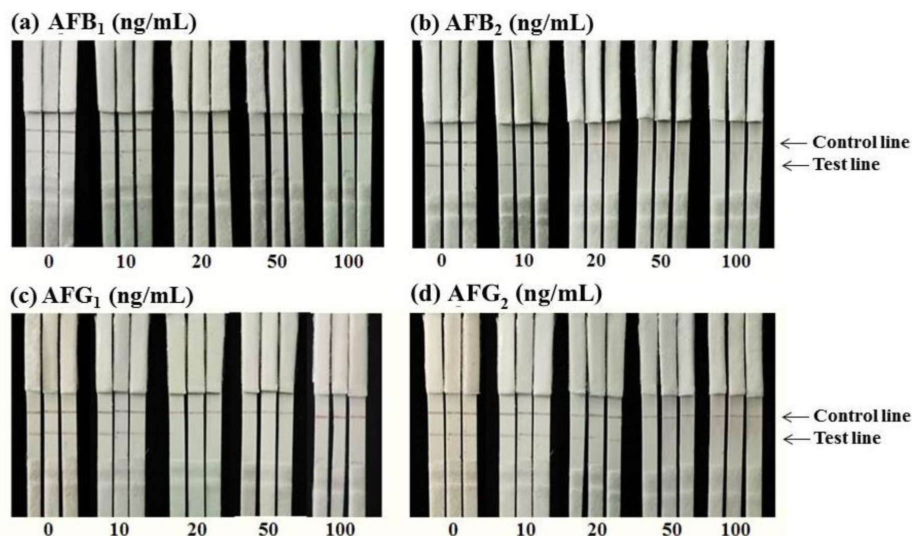

Fig. 2. Detection limit of standard aflatoxins (AFB_1, AFB_2, AFG_1 and AFG_2) dissolved in 70% methanol determined by the developed ICS. A concentration exceeding 20 ng/mL of AFB_1, AFB_2, AFG_1 led to disappearance of a red line at the test zone whereas the cut-off value for AFG_2 was at 50 ng/mL (*n*=3).

Analysis of spiked corn samples by the developed ICS

Corn samples were spiked with each aflatoxin at a concentrations of 10, 20, 50 and 100 µg/kg and analyzed. The results demonstrated that the strip successfully determined the cut off at 20 µg/kg for three aflatoxins including AFB_1, AFB_2 and AFG_1 whereas AFG_2 cut-off value was at 50 µg/kg. The disappearance of the colored line at the test line at 20 µg/kg and higher, for AFB_1, AFB_2 and AFG_1, showed the presence of the aflatoxin. For AFG_2, it was at 50 µg/kg (Fig. 3).

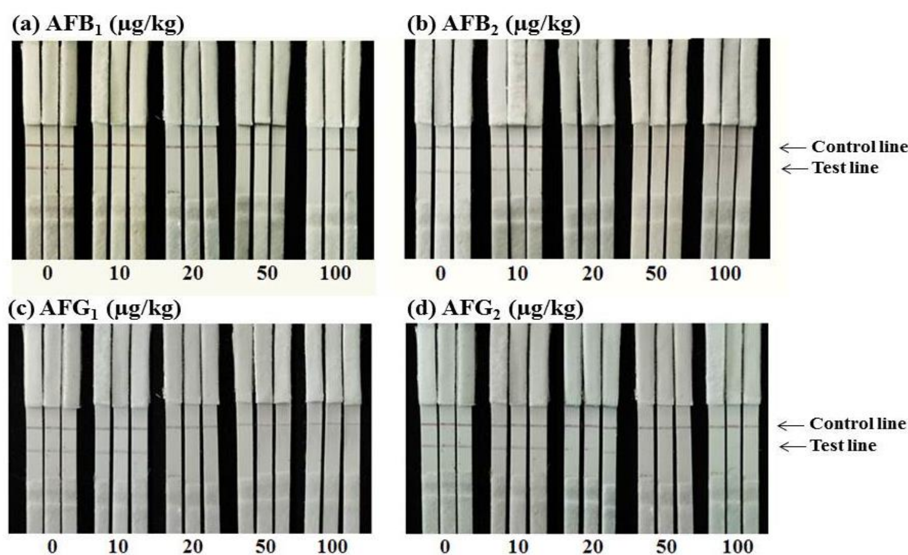

Fig. 3. Analysis of the spiked corn samples with each aflatoxin (AFB_1, AFB_2, AFG_1 and AFG_2) at the concentrations of 10, 20, 50 and 100 µg/kg, respectively (*n*=3). The strip successfully determined the cut-off value at 20 µg/kg for three aflatoxins including AFB_1, AFB_2 and AFG_1 whereas the cut-off value for AFG_2 was at 50 µg/kg where the test lines disappeared.

Analysis of naturally contaminated corn samples by the developed ICS

Five naturally contaminated corn samples were extracted and analyzed using the developed ICS and the results were compared with the values determined by dcELISA using the commercial test kit (Fig. 4). Comparative analysis by both methods conformed with each other (Table 1) where 2 samples contained ≥ 20 µg/kg and 3 samples contained < 20 µg/kg aflatoxin contamination.

Table 1. Aflatoxin analysis of five naturally aflatoxin-contaminated corn samples by the ICS compared to dcELISA.

Sample no.	ICS (*n*=7)	dcELISA (*n*=3)
1	(-)	<4 µg/kg
2	(-)	9.6 µg/kg
3	(+)	19.9 µg/kg
4	(-)	10.5 µg/kg
5	(+)	39.7 µg/kg

Remarks: (-) means less than 20 µg/kg aflatoxin contamination.
(+) means equal or more than 20 µg/kg aflatoxin contamination

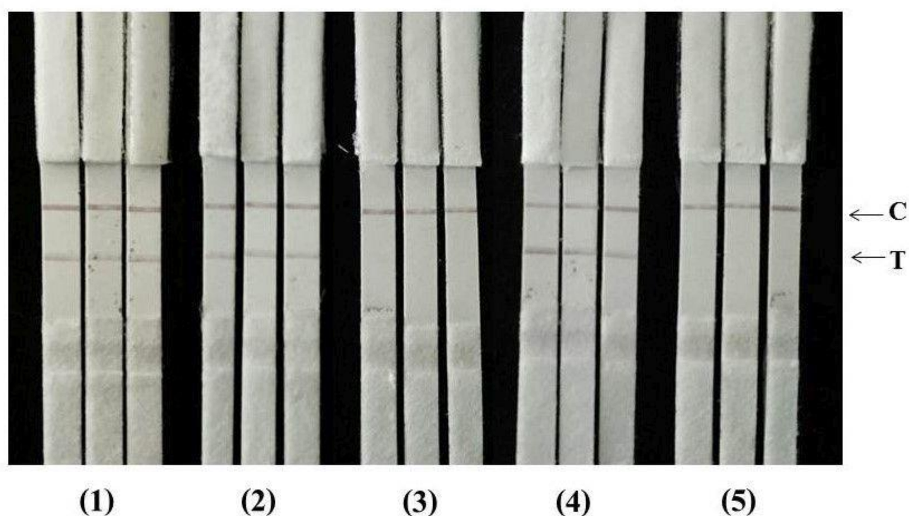

Fig. 4. Evaluation of the ICS reactivity (*n*=7) by analysis of five naturally aflatoxin-contaminated corn samples (1-5) at the concentrations of <4, 9.6, 19.9, 10.5 and 39.7 μg/kg determined by AgraQuant® ELISA Total Aflatoxin (4-40 ng/mL) test kit (*n*=3).

CONCLUSIONS

The ICS is a semi-quantitative assay based on color intensity of the lines and enables a clear distinction between negative and positive results through the naked eye. In the present research, the cut-off value of the developed ICS was achieved at 20 ng/mL for three aflatoxins including AFB_1, AFB_2 and AFG_1 while AFG_2 cut-off value was at 50 ng/mL. In addition, the specificity of our MAb to AFG2 is much higher than the previous report (Shim et al., 2007) which is good for the detection of total aflatoxins. To evaluate the performance of the ICS, five corn samples (n = 7) were analyzed using the developed ICS and compared to the commercial ELISA kit, which is officially approved by AOAC and GIPSA for its reliability. The results from both methods conformed with each other. The ICS developed in this study can be operated easily, conveniently, rapidly and the cut-off of 20 ng/mL is suitable for Thailand regulation. Therefore, it can be used as an on-site screening tool for detecting the contaminated aflatoxins in agricultural products at the primary screening step.

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