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EFFICIENT CONJUGATION OF AFLATOXIN B1 WITH BOVINE SERUM ALBUMIN FOR THE DEVELOPMENT OF AFLATOXIN B1 QUICK TEST

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ABSTRACT

Among several methods to detect the toxic and cancerous aflatoxins in agricultural products used in foods and animal feeds, only the lateral flow immunodipstick method is suitable for field usage. The aim of this study is to determine optimal conditions for the conjugation of aflatoxin B1 (AFB1) with bovine serum albumin (BSA) to develop a lateral flow immunoassay test strip for detection of AFB1. Optimal conditions for the generation of the intermediate compound AFB1-CMO were: AFB1/CMO ratio of 1:2, AFB1 concentration of 8 mM, reflux temperature of 80 ^{o}C, reaction time of 1 hour. The optimal conditions for further conjugation of AFB1-CMO with bovine serum albumin (BSA) were: AFB1-CMO/BSA ratio of 40:1 in bicarbonate buffer pH 9.5, reaction temperature of 25 ^{o}C, reaction time of 2 hours. The generated conjugate AFB1-BSA was used to construct a lateral flow immunoassay test strip for the detection of aflatoxin B1.

Keywords: aflatoxin B1, aflatoxin B1- BSA conjugate, quick test.

1. INTRODUCTION

Aflatoxins are carcinogen secondary metabolites produced mainly by the fungi *Aspergillus flavus* and *Aspergillus parasiticus* [1]. These fungi contaminate a wide range of agricultural products, such as peanuts, corn, rice, and animal feed [2]. Due to appropriate climate for fungi growth, the rate of aflatoxin contaminated agricultural products and food in Vietnam is very high (95.4 %). Twenty three to sixty seven percent of them exceeded the maximum levels set by the Ministry of Health [3-5]. Aflatoxins are classified as group I carcinogens by the International Agency for Research on Cancer (IARC) [6]. Aflatoxins are difuranocoumarines synthesized through the polyketide pathway [7]. There are 4 types of aflatoxins commonly found in food, including B1, B2, G1, and G2. Aflatoxin B1 (AFB1) is the most common compound and shows the highest toxicity [8].

Several methods have been proposed for the detection and determination of AFB1 in agricultural food crops and feeds, including thin layer chromatography (TLC) [9, 10], high performance liquid chromatography (HPLC) [11, 12], and immunoassays [13-16]. TLC method is simple, easy and economic to perform, but the sensitivity and accuracy of TLC is low. HPLC is widely accepted as an official method for aflatoxin detection. However, HPLC method is expensive and time-consuming. Both methods require skilled technician, pretreatment of sample and well-equiped laboratory. Furthermore, they are unsuitable for the routine screening of large sample numbers and on-site analysis. Immunoassay, especially the rapid test strip is becoming more widespread because of its sensitivity, specificity, rapidity, simplicity and cost-effectiveness. The rapid test strip is suitable for the on-site application in detection of aflatoxin. The test strip is based on immunochromatographic principle and often called lateral flow assay. This technique ultilizes antigen and antibody properties to rapidly detect an analyte. For the development of a test strip to detect aflatoxin B1, two crucial components are requested, an AFB1-carrier conjugate and an anti-AFB1 antibody – gold nanoparticle conjugate. This paper reports the generation of the AFB1-BSA carrier conjugate and application of this conjugate for the development of a test strip.

2. MATERIALS AND METHODS

2.1. Materials

Aflatoxin B1 (AFB1) was purchased from Cayman Chemical, USA. O-(Carboxymethyl) hydroxylamine hemihydrochloride (CMO), Pyridine anhydrous, Bovine serum albumin (BSA), goat anti-mouse IgG antibody alkaline phosphatase conjugate, 1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC), sulfo-N-hydroxysuccinimide (sulfo-NHS), 5-bromo-4-chloro-3-indolyl phosphate (BCIP) and nitro blue tetrazolium (NBT) were purchased from Sigma-Aldrich, USA. Monoclonal anti-Aflatoxin B1 antibody [AFA-1] was purchased from Abcam, USA. Other chemicals and solvents were purchased from Merck and Sigma-Aldrich.

2.2. Synthesis of the aflatoxin B1-carboxymethyl hydroxylamine hemihydrochloride hapten (AFB1-CMO)

The AFB1-CMO hapten was generated according to the method of Wicaksono with some modifications [17]. Briefly, 60 µl of a mixture of methanol/pyridine/water (4:1:1) containing 16 µg of AFB1 and 16.8 µg of CMO was refluxed at 90 ^{o}C in the dark for 2 hours, followed by evaporation at 65 ^{o}C, 150 mbar until dry to remove pyridine. The obtained brown yellow pellet was dissolved in 200 µl of 0.1 M NaOH and neutralized by 20 µl of 5 M HCl. The AFB1-CMO was extracted by adding 220 µl of ethyl acetate. The extraction was repeated for three times and the ethylacetate phase was collected. The extract was evaporated until dry to remove ethylacetate. The pellet was dissolved in DMF/H_2O (6:9) and characterized using TLC and FT-IR method.

2.3. Synthesis of AFB1-BSA conjugate

The conjugate AFB1-BSA was prepared by mixing 54 µg of AFB1-CMO with 80 µl of DMF in phosphate buffer pH 5.7. (6:9). Then 8 µl of 0.1 M EDC and 8 µl of 0.1 M sulfo-NHS was added, followed by incubation for 15 min. Then the solution was added dropwise into the BSA solution (1 mg/ml in carbonate buffer pH 9.5) and incubated at 25 ^{o}C for 2 hours. The

conjugate AFB1-BSA was washed three times with PBS pH 7.4 and stored at -20 ^{o}C. The result was checked by dot-blot method.

2.4. Dot-blot method

One µg of AFB1-BSA conjugate was dotted on the nitrocellulose membrane. The membrane then was bloked with 1 % non-fat dry milk for 1 hour and washed three times with PBS pH 7.4. After washing, the membrane was incubated in solution of monoclonal anti-AFB1 antibody for 2 hours. After three washes, the membrane was incubated in solution of second antibody of anti-mouse IgG antibody alkaline phosphatase conjugate, followed by three washes. The signal was developed by adding the solution of NBT and BCIP and quenched by adding H_2SO_4.

2.5. Thin layer chromatography

Thin layer chromatography - TLC was used to analyze aflatoxin B1 and AFB1-CMO. TLC was performed by spotting the product and standard aflatoxin at position of 2 cm from the base of a silica gel plate followed by running the TLC plate in a mixture of chloroform and acetone (9:1). The result was visualized by UV exposure.

3. RESULTS AND DISCUSSION

3.1. Synthesis of the AFB1-CMO hapten

3.1.1. Optimizing the AFB1 to CMO ratios and concentration of AFB1

AFB1 is a low molecular weight compound (312.27 g/mol) and considered as a hapten. Therefore, in order to recognize this toxin by specific antibody, aflatoxin B1 needs to be conjugated with a protein carrier. Since AFB1 lacks a reactive group for the coupling of the toxin to a protein carrier, AFB1 needs first to react with *O*-(Carboxymethyl) hydroxylamine hemihydrochloride (CMO) to generate the AFB1-CMO hapten (Fig. 1).

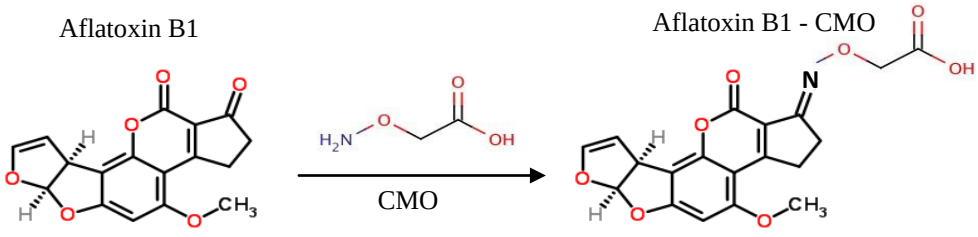

Figure 1. The formation of the AFB1-CMO hapten.

Theoretically, aflatoxin molecule will be coupled to CMO with a molar ratio of 1:1. In this study, four different molar ratios (AFB1/CMO 1:1, 1:2, 1:3, 1:4) were investigated. The results obtained from TLC showed that the product (AFB1-CMO) was more polar than aflatoxin B1. TLC results showed that there were two spots on the TLC pattern of reaction with molar ratio of 1:1, whereas there was only one spot on the others (Fig. 2). The spot in the upper part of the lane 2 (ratio 1:1) had the same Rf value as the standard aflatoxin B1 (Fig. 2A, lane 1), and the spots with lower Rf value were the product AFB1-CMO (Fig. 2A, lane 3-5). Obtained result indicated that AFB1 was completely converted to AFB1-CMO with a minimum AFB1/CMO ratio of 1:2.

Furthermore, four different concentrations of AFB1 were investigated, namely 1, 2, 4 and 8 mM. The results showed that complete coupling reaction was observed at concentration of 8 mM (Fig. 2B).

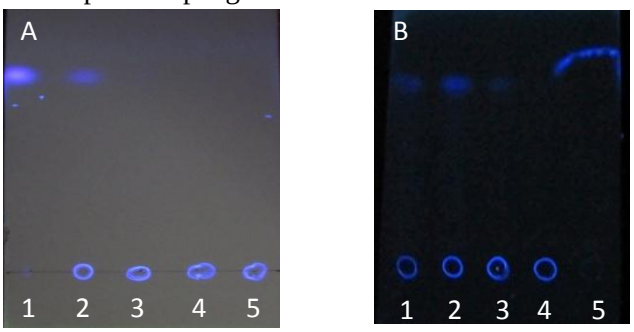

Figure 2. (A) TLC analysis of the products from reaction between AFB1 and CMO. Lane 1: Standard aflatoxin B1; Lanes 2-5: AFB1/CMO ratios were 1:1, 1:2, 1:3 and 1:4, respectively. (B) Effect of AFB1 concentration on the reaction between AFB1 and CMO. Lanes 1-4, reaction products at AFB1 concentrations of 1, 2, 4, 8 mM. Lane 5, standard aflatoxin B1.

3.1.2. Optimizing the reaction temperatures and times

In the present study, the reaction of AFB1 with CMO was performed at five different temperatures of 70, 80, 90, 100 and 110 ^{o}C. The reaction was incomplete at lower temperature (70 ^{o}C) (Fig. 3, lane 2) and complete at temperature higher than 80 ^{o}C (Fig. 3A, lane 3-6). This reaction was carried out at 86 ^{o}C by Wicaksono et al. [17] and at 110 ^{o}C by Kim et al. [18]. We observed that the spot intensity of AFB1-CMO was decreased at 110 ^{o}C. Therefore, suitable temperature for coupling reaction of AFB1 and CMO should be in a range of 80 - 100 ^{o}C.

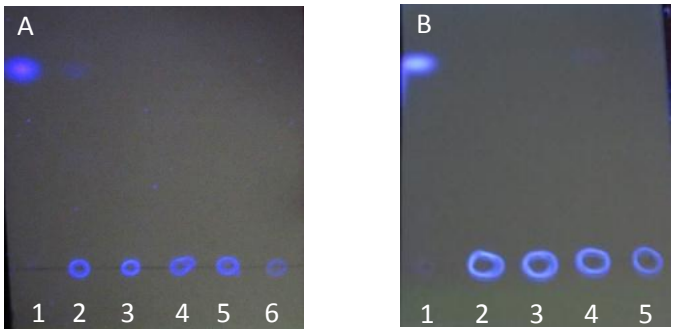

Figure 3. (A) Effect of temperatures on the reaction between AFB1 and CMO. Lane 1, standard aflatoxin B1; lanes 2-6, coupling products at different temperatures of 70, 80, 90, 100 and 110 ^{o}C. (B) Reaction time of AFB1 and CMO. Lane 1, standard aflatoxin B1; lanes 2-5, coupling products at different times of 1, 2, 3, 4 hours.

Four different reaction times of 1, 2, 3 and 4 hours were investigated to identify the suitable reaction time for coupling AFB1 and CMO. The results showed that AFB1 has completely reacted with CMO after one hour at 80 ^{o}C (Fig. 3B). This reaction was carried out for 3 hours by Khademi et al. [19] and even for 5 hours by Wicaksono et al. [17].

3.1.3. Characterization of the hapten AFB1-CMO

In order to confirm the coupling of AFB1 to CMO, the reaction product was analyzed by TLC, HPLC and FT-IR. The TLC results showed spots of the hapten seem to be not moving in chloroform – acetone (9:1) solvent system, while the standard aflatoxin B1 was reached near the

solvent front (Fig. 3A). In addition, the product of the coupling reaction was analyzed by HPLC (Fig. 4). The results showed that the free AFB1 peak appeared at 13.15 min, AFB1-CMO peak appeared at 29.33 min.

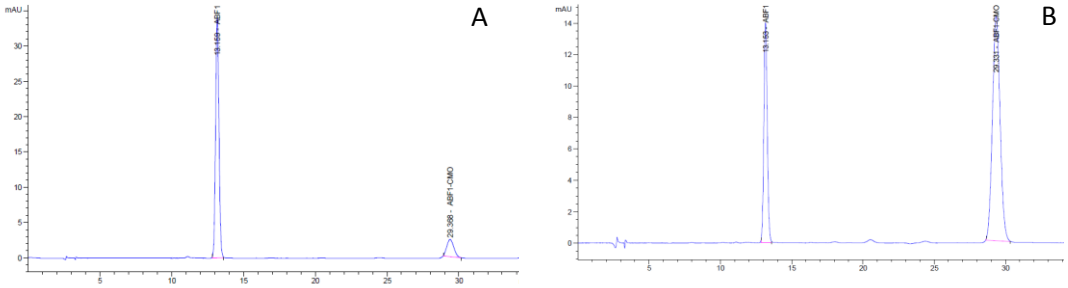

Figure 4. HPLC chromatogram of standard AFB1 (A) and AFB1-CMO (B).

Characterization using FT-IR spectroscopy showed a peak at 1634 cm^{-1}, indicating the absorption of the C=N groups (Fig. 5). The peak was also observed in spectra of AFB1 due to C=C bonds. The intensity of this peak increase as the C=N group was created after coupling CMO to AFB1. Furthermore, the presence of a broad peak at 3433 cm^{-1} indicated the absorption of the O-H groups. The increase of peak intensity is due to the presence of carboxylic group after coupling CMO to AFB1.

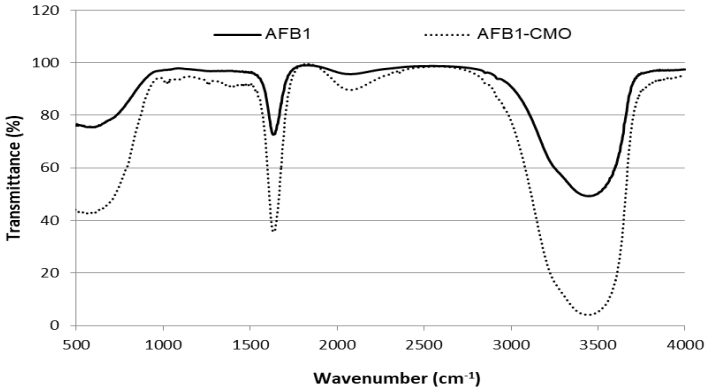

Figure 5. Fourier-Transform Infrared Sprectra of the aflatoxin B1 (solid line) and the hapten of AFB1-CMO (dotted line).

3.2. Conjugation of AFB1-CMO to Bovine Serum Albumin (BSA)

3.2.1. Optimizing the AFB1-CMO to BSA ratios

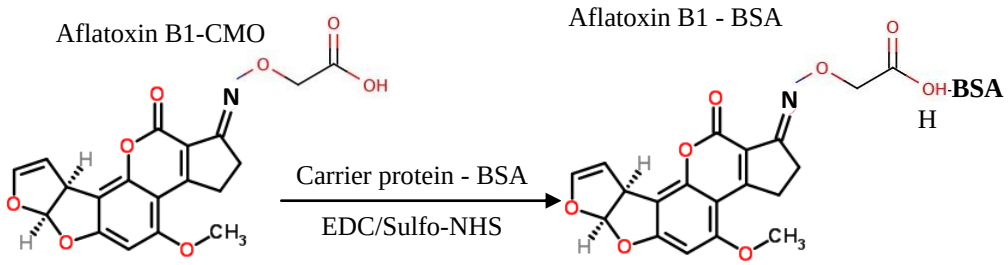

Figure 6. Reaction scheme of AFB1-BSA synthesis.

The AFB1-CMO hapten is conjugated to the protein carrier BSA as shown in Fig. 6. Theoretically, one BSA molecule can couple with a maximum of 35 molecules of AFB1-CMO based on the number of lysine and Ɛ-amine residues of BSA. In order to determine the optimal ratio of AFB1-CMO to BSA, five molar ratios of 10:1, 20:1, 30:1, 40:1, 50:1 were investigated. The conjugation efficiency was checked by dot blot method. The result showed that the increase of molar ratio of AFB1-CMO/BSA resulted in the increase of conjugation efficiency (Fig. 7). The significant efficiency was obtained at the molar ratio of 40:1. The results obtained from spectrometric method by Khademi *et al*. showed that 12 moles of AFM1-CMO were successfully conjugated to 1 mole of BSA [19].

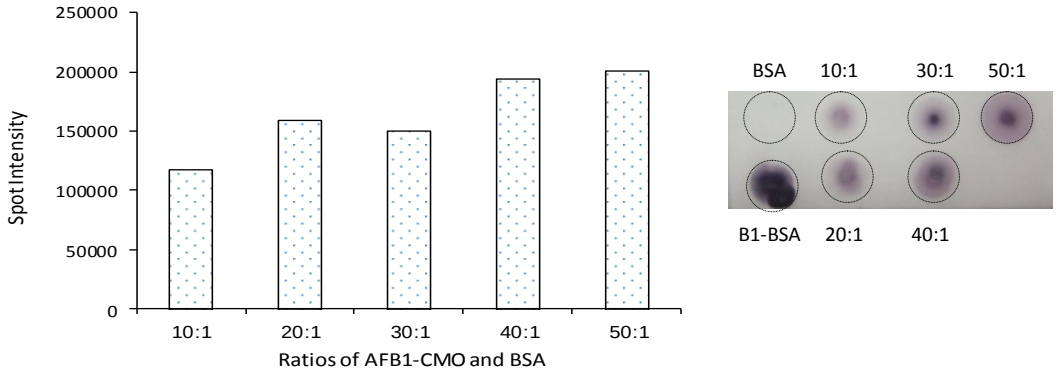

Figure 7. Dot blot analysis of products from conjugation reactions of AFB1-CMO and BSA with different ratios (10:1, 20:1, 30:1, 40:1, 50:1). BSA, bovine serum albumin; B1-BSA, standard conjugate.

3.2.2. Effect of pH, temperature and time on the conjugation efficiency

The conjugation reaction of AFB-CMO and BSA is catalyzed by 1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) and sulfo-N-hydroxysuccinimide (Sulfo-NHS). EDC reacts with carboxylic acid group of CMO moiety of AFB1-CMO to form reactive intermediate follows by reacting with Sulfo-NHS to form a stable active ester. In the presence of amine nucleophiles from protein (BSA), the sulfo-NHS ester is rapidly hydrolyzed, allowing the formation of an amide bond between BSA and AFB1-CMO. The activation reaction with EDC and Sulfo-NHS is pH dependent. In this study, the reaction was carried out at three different pH values of 4.5, 7.4 and 9.5. The result showed that the highest conjugation efficiency was observed in carbonate buffer pH 9.5 (Fig. 8).

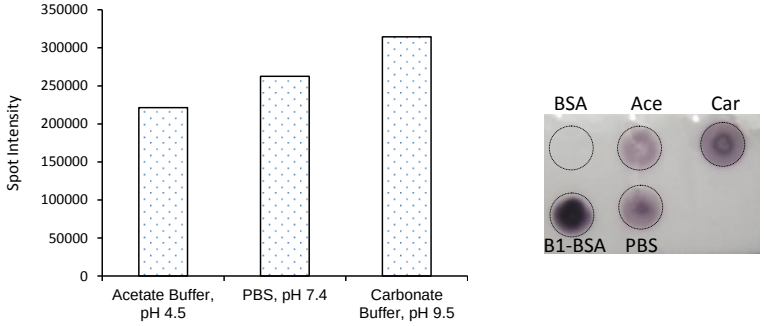

Figure 8. Dot blot analysis of products from conjugation reactions of AFB1-CMO and BSA in different buffers: acetate buffer pH 4.5 (Ace), phosphate bufferred saline (PBS) pH 7.4, carbonate buffer pH 9.5 (Car). BSA, bovine serum albumi; B1-BSA, standard conjugate.

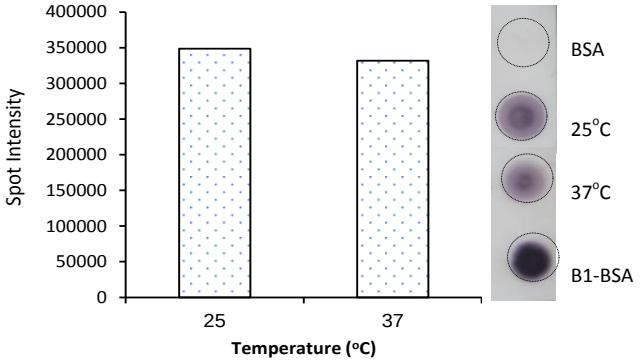

Figure 9. Dot blot analysis of products from conjugation reactions of AFB1-CMO and BSA at 25°C and 37°C. BSA, bovine serum albumin; B1-BSA, standard conjugate.

The conjugation reaction was carried at 25 °C and 37 °C and showed that the conjugation reaction at 25 °C was better than 37 °C (Fig. 9).

The conjugation reaction was carried out for 0.5, 1.0, 2.0, 3.0 and 4.0 hours. The results indicated that the highest conjugation efficiency was obtained for 2 hours incubation (Fig. 10).

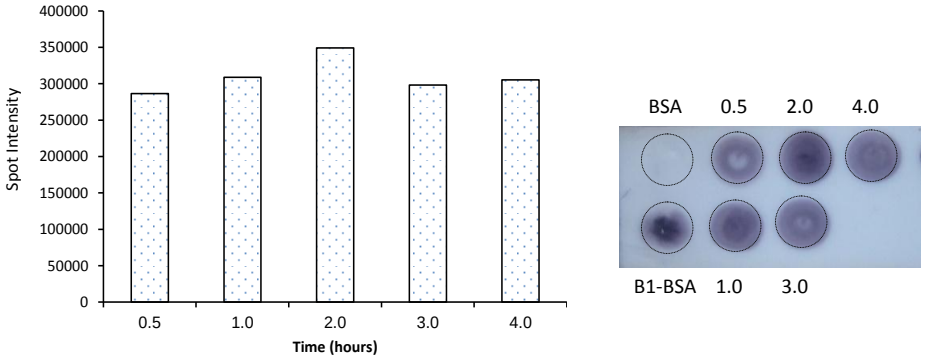

Figure 10. Dot blot analysis of products from conjugation reactions of AFB1-CMO and BSA for 0.5, 1.0, 2.0, 3.0 and 4.0 hours. BSA, bovine serum albumin; B1-BSA, standard conjugate.

3.3. Preliminary application of AFB1-BSA for generating a lateral flow immunoassay test strip

Figure 11. A format of the lateral flow test strip generated by the combinantion of AFB1-BSA immobilized membrane and anti-AFB1 antibody – gold nanoparticles conjugate. Signal band is indicated by arrow.

The generated AFB1-BSA conjugate was immobilized on a nitrocellulose membrane and assembled with anti-AFB1 antibody – gold nanoparticles conjugate to prepare a lateral flow

immunoassay test strip. The result showed there was a clear band on the observation window of the test strip (Fig. 11). The presence of a signal band indicated that AFB1-specific antibody in conjugate with gold nanoparticles recognized the hapten of AFB1 on the surface of protein carrier BSA which was immobilized on the nitrocellulose membrane.

4. CONCLUSIONS

Aflatoxin B1 (AFB1) was shown to form the hapten AFB1-CMO by condensation with O-(carboxymethyl) hydroxylamine hemihydrochloride (CMO). The optimal reaction conditions were found as follows: AFB1/CMO ratio of 1:2, AFB1 concentration of 8 mM, reflux temperature of 100 ^{o}C, reaction time of 2 hours. Successful condensation of AFB1 with CMO was confirmed by TLC, HPLC and FT-IR methods. The optimal conditions for further conjugation of AFB1-CMO with bovine serum albumin (BSA) were found as follows: AFB1-CMO/BSA ratio of 40:1 in bicarbonate buffer pH 9.5, reaction temperature of 25^{o}C, reaction time of 2 hours. Generated conjugate AFB1-BSA was successfully applied to construct a lateral flow immunoassay test strip for detection of aflatoxin B1.

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