Synthesis of Polyclonal Antibodies against Aflatoxin B1

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Abstract

Polyclonal antibodies of aflatoxin B1 were successfully produced from New Zealand White female rabbits after immunization by the hapten of aflatoxin B1-carboxymethyl hydroxylamine hemihydrochloride (AFB1-CMO) conjugated with bovine serum albumin (BSA) as the antigen. The hapten was synthesized using the carbodiimide method with CMO as a linker. Absorption peaks at 362, 264, and 218 nm were observed as a result of characterization with UV-Vis spectroscopy, while IR spectroscopy showed peaks at 3448 cm⁻¹ and 1642 cm⁻¹ attributable to the hydroxyl and nitrile groups, respectively. Furthermore, mass spectrometry showed fragmentation at the m/z of 386, 368.2, and 310, which confirms that the hapten of AFB1-CMO was successfully synthesized. The hapten was then conjugated with BSA to serve as an antigen of AFB1 when it was injected into the rabbits. The specificity of the antigen towards its antibody and the confirmation of hapten-BSA conjugation were characterized using the dot blot immunoassay, which showed a BSA concentration of 1.74 mg/mL. Two weeks after the primary immunization by its antigen, agar gel precipitation testing showed that the rabbit blood serum had positive results for polyclonal antibodies against AFB1 with the highest concentration of antibody of 2.19 mg/mL.

Introduction

Fungal contamination is often found in agricultural products, and fungi can produce toxic metabolites called mycotoxins. Aflatoxin is the mycotoxins produced by Aspergillus flavus and A. parasiticus. Aflatoxin is suspected to damage human health, livestock, and agricultural products [1]. Aflatoxin B1 (AFB1) is known to be toxic and carcinogenic to humans [2-3]. It is also reported to be teratogenic and mutagenic, and to cause toxic hepatitis, bleeding, immunosuppression, and liver carcinoma [4]. It has been reported that agricultural products, such as corn, cocoa, and peanuts are commonly highly contaminated by AFB1 [5-7]. Accordingly, International Agency of Research on Cancer (IARC) classifies AFB1 as a Group 1 carcinogen in humans [8].
Various analytical methods have been developed for the detection of AFB1. One example is thin-layer chromatography [9], which has less sensitivity and specificity than other methods. The use of high-performance liquid chromatography [10] or as liquid chromatography-tandem mass spectrometry [11] is popular since they offer high sensitivity and specificity. However, these methods require special expertise, a long time analysis, and very expensive instrumentation.

On the other hand, immunoassays and related immunochromatographic procedures, such as the enzyme-linked immunosorbent assay (ELISA) and immunochromatographic strip-tests offer several advantages for easy and fast aflatoxin-screening tests [12-14]. Immunoassay methods are highly dependent on the quality of its detection agent, i.e. antibodies, which are often conjugated with a tracer, such as enzymes (for ELISA), radioactive (for radioimmunoassay), and or nanoparticles. Therefore, the ability to produce sensitive and specific antibodies to AFB1 has become very important [12]. Furthermore, although monoclonal antibody can be produced and they provide more specificity, the production of polyclonal antibody is simple and can be performed with adequate specificity when coupled with lateral immunosensors [15,16].

AFB1 is a non-protein compound with a low molecular weight. Therefore, when it enters a living system, it is not recognized as an antigen and as a result an antibody will not be produced. In order to act as an antigen, AFB1 needs to be combined with a larger protein. Since AFB1 has no reactive group for a coupling reaction with a protein, a linker is required. Chu and Ueno reported on the use of carboxymethyl hydroxylamine hemihydrochloride (CMO) as a linker [17] and bovine serum albumin (BSA) as the protein. The reaction scheme is shown in Figure 1.

CMO has three atoms as a long arm space in which this linker maintains the specific groups of AFB1, and thus it has a high antigenicity [18]. Another type of linker, dicyclohexylcarbodiimide, has been commonly used, but it was reported to produce N, N’-dicyclohexylurea [12], which is insoluble and difficult to remove. Immunization with about 50 to 210 µg of hapten emulsion in NaCl, given to rabbit intradermally, was reported to initiate antibody production after three weeks [17].

In this work, the synthesis and characterization of the hapten and the antigen of AFB1 is explained. The hapten was the injected subcutaneously to increase the amount of antibody production. Subcutaneous injection is reported to offer some advantages, including the less painful felt by the rabbit as well as a greater injection volume: 250 µg hapten could be injected into each rabbit two times during the first week. It was found that 11 days after the primary immunization with the antigen, the rabbit blood serum showed a positive result for the polyclonal antibodies against AFB1. Furthermore, booster immunization of 500 µg of antibodies at the third week produced the highest antibody concentration at 2.19 mg/mL.

**Materials and Methods**

**Chemicals.** AFB1 was purchased from Romer Labs, while 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC), dimethylformamide (DMF), and carboxymethyl hydroxylamine hemihydrochloride (CMO) were supplied by Merck. N-hydroxysuccinimide (NHS) was purchased from Wako Pure Chemical Industries Ltd., while bovine serum albumin (BSA), Freund’s complete adjuvant (FCA), Freund’s incomplete adjuvant (FIA), succinic anhydride, anti-IgG-HRP (goat anti-rabbit enzyme conjugate), 3,3’-diaminobenzidine (DAB), and other chemicals were supplied by Sigma Aldrich. Female New Zealand White rabbits were supplied by the Faculty of Veterinary Medicine, Bogor Agriculture University, Bogor, Indonesia.
Synthesis of the hapten of aflatoxin B1-carboxymethyl hydroxyalmine hemi-hydrochloride (AFB1-CMO).

The hapten of AFB1-CMO was prepared according to the method of Chu [17]. Briefly, 2 mg of AFB1 was added into a 3 mL solution containing 0.5 mL of pyridine anhydrous, 2 mL of methanol, 0.5 mL of distilled water, and 4 mg of CMO. The solution was refluxed at 86 °C in the dark while being constantly stirred for 5 h. The pyridine was removed from the solution with N₂ evaporator. Then the solution was extracted using 10 mL of ethyl acetate and 10 mL of distilled water. The extraction was repeated for three times. Anhydrous Na₂SO₄ and N₂ evaporator were used to absorb the remaining water and organic solvent, respectively. The synthesized hapten, which appeared as a yellow powder, was then characterized using thin layer chromatography (TLC), UV-Vis and IR spectrophotometers, and a mass spectrometer.

**Synthesis of the hapten-protein (antigen of AFB1).**

The antigen of AFB1 was prepared by mixing 1 mg of AFB1-CMO with 2 mL of DMF in distilled water (6 : 9). Then, 0.598 mg of NHS and 1.073 mg of EDC were added, followed by incubated shaking at 37 °C for 24 h. The solution was centrifuged (5 min at 4000 rpm) and the filtrate was added by 8.6 mg of BSA in 5 ml of 0.13 M NaHCO₃ dropwise, followed by slow stirring for 1 h. The solution was then incubated and shaken in the dark at 37 °C for 3 h. Next, the supernatant was dialyzed for 24 h in distilled water, then dialyzed for 3 days in PBS at pH 7.6. The antigen was characterized using the UV-Vis spectrophotometer and the dot-blot immunoassay technique.

**Production of polyclonal antibody against AFB1 (pAb AFB1) in rabbits.**

The polyclonal antibody of AFB1 was produced using female New Zealand White rabbits by injecting 250 µg of antigen in 150 µL of FCA two times in the first week. At the second week, the serum was extracted and examined with the agar gel precipitation test (AGPT). To increase the production of antibodies, at the third week, each rabbit was injected with 500 µg of the AFB1 antigen in 300 µL of FIA. The same steps were repeated to extract a large amount of blood serum harvested from rabbits.

**Result and Discussion**

**Characterization of the hapten of AFB1-CMO (AFB1-CMO).** The TLC results showed spots of the hapten with Rf values of 0.39 (1 µL) and 0.40 (5 µL), while the AFB1 standard sample had spots with Rf values of 0.77 (5 µL) and 0.76 (10 µL). The Rf values for the hapten were lower than the standard due to the increased numbers of carboxylic groups, which are more polar than aflatoxin B1, after the haptenization of aflatoxin B1 using CMO.

Meanwhile, UV-Vis spectra (Figure 2) showed that both aflatoxin B1 and its hapten have the same absorption peaks with λ_max at 218, 264 and 362 nm. This confirms to the results previously reported by Kim et al. [18]. The intensity of the peaks at 264 and 362 nm were not relatively changed, as the haptenization of AFB1 using CMO did not damage its chromophore characteristics. However, the peak intensity at 218 nm decreased after haptenization due to the chromophore changing from C=O to be C=N.

Mass spectrometry (Figure 3) showed the fragmentation at m/z 386, 368.2, and 310, confirming the formation of AFB1-CMO.

Theoretically, the molecular structures of the hapten and AFB1 are similar. The only difference is in the carboxymethoxylamine (−NOH−COOH) groups, which binds to ketone groups of aflatoxin B1 through condensation to form oxime (C=N) after haptenization. Characterization using IR spectroscopy (Figure 4) showed wide peak at 3448 cm⁻¹, indicating the absorption of the O-H groups. The intensity of the peak increase as the carboxylic group was performed after haptenization. The peak was also observed in spectra of AFB1 indicated the presence of water molecules in the sample.

Furthermore, a peak at 1632 cm⁻¹ related to the ketone groups as well as small absorption peaks in the hapten spectrum were observed at 1642 cm⁻¹ and 1075 cm⁻¹ related to the C≡N and C=O groups, respectively. The absence of the peak around 2100 cm⁻¹ is attributable to N=C=O groups, confirming that the modification by CMO occurred at the C≡O in the cycloheptene.

**Characterization of the hapten-protein (antigen of AFB1).** Dot blot immunoassay was then used to characterize the hapten-BSA conjugation. The positive result indicated that the hapten of AFB1-CMO was successfully conjugated to BSA. Meanwhile, the concentration of the antigen was determined using a linear calibration curve created from different concentrations of BSA. A concentration of 1.74 mg/mL was obtained.
Production of polyclonal antibodies against AFB1 (pAb AFB1). Production of pAb AFB1 was performed by immunizing the rabbits with the AFB1 antigen. The AGPT assay was performed to characterize the produced antibodies to AFB1. While the control showed negative results (Figure 5(a)), the blood serum harvested at the second week (11th day) after the primary immunization with the antigen of AFB1 using FCA showed a thin line, indicating that it contained a low concentration of pAb AFB1 (Figure 5(b)). After three weeks, the AGPT result showed a bold line, which indicated the rabbit serum contained a high concentration of pAb AFB1 (Figure 5(c)).

In order to purify the pAb AFB1, a protein column (Hi Trap) was used. Protein A consists of six different regions, five of these are strong enough to specifically bind to the Fc portion of IgG antibodies, and to then isolate the IgG polyclonal antibodies from the serum. Immobilization protein A binds to at least two molecules per molecule of IgG antibody [19]. After purification, the UV-Vis spectrum showing the highest concentration of pAb AFB1 was 2.19 mg/mL.

Conclusions

The polyclonal antibody of aflatoxin B1 was successfully produced in female New Zealand White rabbits after 2 subcutaneous injections with 250 µg AFB1-CMO hapten conjugated to BSA. Eleven days after the primary immunization, the rabbit blood serum
began to contain pAb AFB1. The booster injection with a 500 µg of the hapten at the third week showed the highest concentration of polyclonal antibodies against AFB1 at 2.19 mg/mL.

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References


