

Production and Characterization of Monoclonal Antibodies Against Aflatoxin M₁

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Abstract. Aflatoxin M₁ (AFM₁) is a hydroxylated metabolite of aflatoxin B₁ (AFB₁). It is found in milk from animals fed with AFB₁-contaminated feed. Since its toxicity is classified as Group 1 human carcinogen, USA and EU have regulated its maximum residue level (MRL) in milk at 0.5 ppb and 0.05 ppb, respectively. Therefore, the detecting kit to monitor the contamination of this toxin is necessary. The aim of this work was to generate monoclonal antibodies (MAbs) against AFM₁ for potential development of ELISA test kit. A monoclonal antibody (130F10) was established and characterized after fusion of P3X myeloma cell line with splenocytes from BALB/c mice that had been immunized with AFM₁-BSA. The isotype of this mAb was identified as IgG1. The 50% inhibition concentration (IC₅₀) value and the limit of detection (LOD) of AFM₁ were 0.07281 ppb and 0.035 ppb, respectively. Its cross reactivities to other aflatoxins, including aflatoxin B₁ (AFB₁), aflatoxin G₁ (AFG₁) and deoxynivalenol (DON) were 24,270%, 236.55% and 0.01%, respectively, as compared with that of AFM₁. However, its cross reactivities with other tested toxin were less than 0.01%. Therefore, the obtained mAb is suitable for immunoassay-based test kit development to detect AFM₁.

Keywords: Aflatoxin M₁, Monoclonal antibody, ELISA

1. Introduction

Aflatoxins are toxic metabolites produced by a variety of fungi such as *Aspergillus flavus* and *Aspergillus parasiticus*. They present in grains, nuts, cotton seeds and other commodities associated with human food or animal feeds. Crops may be contaminated by one or more of four of the following sub-types of aflatoxin: B₁, B₂, G₁, G₂. Aflatoxin B₁ (AFB₁) is the most hepatocarcinogen known in mammals [1]. It can cause severe illnesses, including acute liver damage, liver cirrhosis and tumor formation [2]. The International Agency for Research on Cancer of WHO (IARC) classified aflatoxins as a Group 1 carcinogen [3].

Aflatoxin M₁ (AFM₁) is a hydroxylated metabolite of aflatoxin B₁ (AFB₁) and can be found in milk from animals that are fed with AFB₁-contaminated feeds [4]. When the mammals are fed with feeds containing AFB₁, it is converted to AFM₁. The conversion rate of ingested AFB₁ to AFM₁ highly varies among animal species such as in the range of 0.5 to 5% [5], and 6% in dairy cows [6]. A daily intake of ≥ 70 mg in cows gives a mycotoxin level in milk greater than the accepted limit. Thus, AFM₁ concentration in milk and milk products depends on the amount of AFB₁ ingested [7]. Moreover, AFM₁ is highly stable during heat treatment like pasteurization process. AFM₁ intake, even at low concentrations, causes a significant risk to human health especially to children who are the major consumers of milk [8]. Thus, strict regulatory limits

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for aflatoxins are currently enforced in many countries. The European Commission announced that the maximum AFB₁ contents in feed for dairy animals has been set at 5 ppb, whereas the maximum levels of AFM₁ in liquid milk and dried or processed milk intended for adults was set at 0.05 ppb [9] and at 0.025 ppb for infants milk [10]. However, the maximum levels of AFM₁ in the United States and China are limited at 0.5 ppb. In Austria and Switzerland, the maximum level is even lower at 0.01 ppb for infant food commodities [11].

Various methods have been reported for AFM₁ detection including liquid chromatography/tandem mass spectrometry[12], high-performance liquid chromatography [13]. These techniques require expensive equipments and well-trained personnel. Immunoassays, especially enzyme-linked immunosorbent assay (ELISA), have been developed for AFM₁ detection. This method is not only a suitable tool for quick and sensitive analysis with high sample throughput [14] but also a cost-effective, fast method that requires only small sample volume for analysis [15]. In Thailand, AFM₁ ELISA test kit is mostly imported from abroad. Therefore, the domestic development of ELISA test kit is expected to reduce the amounts of imported kits. This study aimed at producing monoclonal antibodies (MAbs) against AFM₁ for future development of immunoassay-based detection test kit.

2. Methods

2.1. Immunization of mice

Initially, 8-week-old female BALB/c mice were immunized by intraperitoneal injection with 2.5 µg of AFM₁-BSA conjugate (Sigma-Aldrich) dissolved in normal saline solution and emulsified with an equal volume of Freund's complete adjuvant. Subsequent immunization (booster) at 2 weeks intervals were given with Freund's incomplete adjuvant. Blood samples were carried out by tail bleeding to determine antiserum titer using indirect and indirect competitive ELISA. The mice with high antiserum titer were given a final booster immunization 4 days before cell fusion.

2.2. Production of hybridoma

The mice were sacrificed and their spleens were aseptically removed, producing a single-cell suspension. The spleen cell suspension was washed with RPMI-1640 media and combined with P3X myeloma cells. The cell mixture was pelleted at 1,500 rpm and fused by the addition of warm 50% polyethyleneglycol (PEG) by gentle stirring for 1 min. The 30 ml of RPMI-1640 media was slowly added to the fused cell. The cell suspension was pelleted and diluted with HAT medium and then distributed into each well of 96-well microculture plate. After 7 to 10 days, hybridoma supernatant were screened for the present of antibodies against AFM₁. The positive clones were diluted to give approximately one cell per well by limiting dilution method. Monoclonal were stored in liquid nitrogen.

2.3. Screening for hybridoma

First round of screening by indirect ELISA

Plates were coated with 1 µg/ml of AFM₁-BSA (50 µl per well) and incubated at 4°C overnight. Plates were washed with PBS with 0.05% Tween20 and blocked to minimize non-specific binding with 0.5% skim milk in PBS. After washing, hybridoma cell culture supernatants or antiserum were added and plates were incubated at 37°C for 2 hr. After washing, horse radish peroxidase (HRP) conjugated goat anti-mouse IgG antibodies diluted 1:5,000 with PBS were added and incubated at 37°C for 1 hr. Plates were washed and then added substrate (TMB and H₂O₂ in 205 mM citrate buffer pH 4.0). The reaction was stopped after 10 min with 2.5 M H₂SO₄ and the absorption was measured at 450 nm using microtiter plate reader. Then the positive clones were further selected by second screening.

Second round of screening by indirect competitive ELISA

An indirect competitive ELISA was used to screen for antibodies specific for free AFM₁, sensitivity and cross reactivity. Plates were coated and blocked as described in indirect ELISA. After washing, competitors

and hybridoma cell culture supernatants were added and incubated at 37°C for 2 hr. The plates were washed afterward, and the similar procedures were performed as described above.

2.4. Characterization of monoclonal antibodies

Isotype determination

Isotype of monoclonal antibodies were determined by Sigma-Aldrich isotyping kit. Plates were coated with isotyping specific antibodies: IgG1, IgG2a, IgG2b, IgG3, IgM and IgA and incubated at 37°C for 1 hr. After washing, culture supernatant was added, plates were incubated at 37°C for 1 hr. After washing, HRP-goat anti-mouse IgG (Fab specific) were added and incubated at 37°C for 1 hr. The assay was then performed as previously described.

Sensitivity and cross reactivity

To assess the sensitivity and cross reactivity of the MAb, an indirect competitive ELISA was carried out using free AFM₁, AFB₁, AFG₁ and DON as potential binding competitors at various concentrations. The IC₅₀ was defined as the concentration at which 50% B/B₀ was obtained, Where B₀ and B are average of absorbance obtained from the indirect competitive ELISA without and with different concentrations of the competitors, respectively.

The percentage of cross reactivity was calculated by using the following formula:

$$\% \text{ cross reactivity} = \frac{\text{IC}_{50} \text{ of AFM}_1}{\text{IC}_{50} \text{ of competitor}} \times 100$$

Limit of detection (LOD) was calculated by subtracting three times of standard deviation from the AFM₁ concentration at B₀ (LOD = B₀-3SD). The standard deviation was obtained from indirect competitive ELISA without competitors with 6 replicates.

3. Results and discussion

3.1. Antiserum titer and establishment of Mab against AFM₁

The antiserum titer of blood samples of immunized mice was determined by indirect and indirect competitive ELISA with AFM₁ (2 µg) as a competitor. The titer of immunized mice was 1:32,768,000 (Figure 1) and the serum was specific to free AFM₁ (data not shown). During somatic cell fusion to produce hybridoma, the fusion efficiency (the number of wells showing cell multiplication) was 41.27%. The hybridoma supernatant were analyzed by first round of screening using indirect ELISA for the presence of antibodies against AFM₁. The result showed that 51.82% of the hybridomas gave positive result. The positive wells were further analyzed by second round of screening using indirect competitive ELISA. Eleven clones of the positive wells gave the positive result with specific binding to free AFM₁. Out of 11 clones, 5 stable clones were chosen to be subcloned for three cycles by limiting dilution to obtained 1 cell per well. Finally, a stable hybridoma, clone No. 130F10, was selected for future development of immunoassay-based detection test kit because of its highest sensitivity among the five clones obtained.

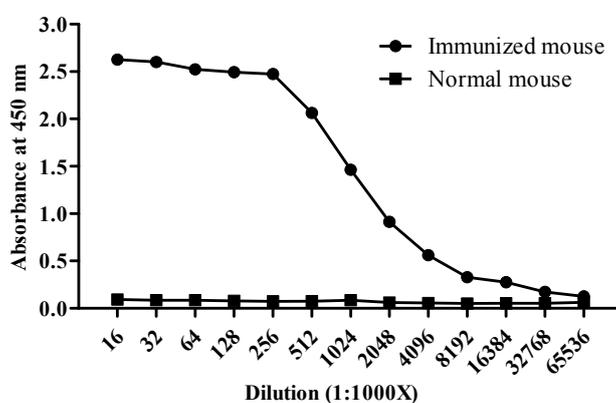


Figure1. Antiserum titers of AFM₁-BSA immunized mice determined by using indirect ELISA

3.2. Characterization of Mab against AFM₁

The preliminary study were performed to assess the optimal ratio for coating of antigen to antibodies. The optimized coating antigen concentration and antibody dilution were 0.2 µg/ml and 1:1,600 (v/v), respectively. Determination of 130F10 Mab isotype using isotyping kit was performed and revealed it to be an IgG1. The sensitivity and cross reactivity to AFM₁, AFB₁, AFG₁ and DON were analyzed by indirect competitive ELISA and the summary is shown in table 1.

Table 1. Specificity, sensitivity and cross reactivity of monoclonal antibody 130F10

| Competitors | | | | | | | | | |
|------------------|-----------|--------------------|------------------|-----------|--------------------|------------------|-----------|--------------------|--------------------|
| AFM ₁ | | | AFB ₁ | | | AFG ₁ | | | DON |
| IC50 (ppb) | LOD (ppb) | Cross Reaction (%) | IC50 (ppb) | LOD (ppb) | Cross Reaction (%) | IC50 (ppb) | LOD (ppb) | Cross Reaction (%) | Cross Reaction (%) |
| 0.07281 | 0.035 | 100 | 0.0003 | 0.00012 | 24,270 | 0.03078 | 0.012 | 236.55 | < 0.01 |

The concentration of AFM₁ with 50% inhibition of binding of antibody (IC₅₀) was found to be 0.07281 ppb and the limit of detection was 0.035 ppb. The antibody showed strong cross reactivity with AFB₁ and AFG₁ because of their closely related structures to AFM₁. However, these mycotoxins will have negligible effect on the detection of AFM₁ in milk product because AFB₁ in animal feed will be transformed to AFM₁ in milk. Importantly, cross reactivities (<0.01%) to other toxins are negligible. Thus, the antibody 130F10 was sensitive enough to detect AFM₁ at the regulatory limit in milk product as well as to detect AFB₁ and AFG₁ in animal feeds.

4. Conclusion

The monoclonal antibody against AFM₁ from this study can be used efficiently in the screening for aflatoxins by immunoassay-base method such as ELISA.

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6. References

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