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Analytical Methods

Characterisation of monoclonal antibody against aflatoxin B_1 produced in hybridoma 2C12 and its single-chain variable fragment expressed in recombinant *Escherichia coli*

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ABSTRACT

An anti-aflatoxin B_1 monoclonal antibody (anti-AFB_1 mAb) from the hybridoma 2C12 was established and its inhibition concentration fifty (IC_{50}) for AFB_1 and relative cross-reactivities (CRs) to other mycotoxins were estimated to be 8 ng/mL and less than 4% compared with AFB_1 by a competitive direct enzyme-linked immunosorbent assay. For production of anti-AFB_1 single-chain variable fragment (anti-AFB_1 scFv) in recombinant *Escherichia coli*, its scFv-coding genes were cloned from the hybridoma 2C12. The anti-AFB_1 scFv formed inclusion bodies in the cytoplasm of *E. coli* required *in vitro* refolding process and hence recovered to retain binding activity successfully. Surface plasmon resonance analysis resulted that anti-AFB_1 scFv possessed 1.16×10^{-7} M of equilibrium dissociation constant (K_D), which was about 17 times higher than the parental anti-AFB_1 mAb of 6.95×10^{-9} M.

1. Introduction

Aflatoxin B_1 (AFB_1) is a fungal metabolite produced by *Aspergillus flavus* and *Aspergillus parasiticus*. It possesses hepatotoxic, teratogenic and mutagenic properties and causes toxic hepatitis, hemorrhage, immunosuppression and hepatic carcinoma (Reddy, Reddy, & Muralidharan, 2009). Many reports have warned severe human aflatoxicosis caused by international consumption of heavily contaminated foods such as maize, cacao, and peanuts (Asis, Di Paola, & Aldao, 2002; Fernández-Ibañeza, Soldado, Martínez-Fernándeza, & de la Roza-Delgado, 2009; Var, Kabak, & Gök, 2007). Especially, International Agency of Research on Cancer (IARC) categorised AFB_1 into a group I carcinogen for humans (IARC (International Agency for Research on Cancer)). European Union established the action levels between 2 and 50 ppb for AFB_1 present in all feed materials (Zheng, Richard, & Binder, 2006).

AFB_1-contaminated raw and processed foods are monitored through several screening and analytical methods which are based on chromatography or antibody platforms. Liquid chromatography (LC) is generally used for quantification of AFB_1, and LC-tandem mass spectrometry and gas chromatography were introduced to analyse AFB_1 (Akiyama, Goda, Tanaka, & Toyoda, 2001; Monbaliu et al., 2009). Chromatographic technologies are generally improper for real-time assay in actual fields and incompatible with demands of most of food industries because they want to manage the large quantity of raw materials and products in a short period. For easy and rapid screening of AFB_1, antibody-based techniques including enzyme-linked immunosorbent assay (ELISA) and immuno-chromatographic test have been used in place of acceptances. (Lee & Rachmawati, 2006; Li et al., 2009). ELISA technology requires high sensitive and specific polyclonal antibody, monoclonal antibody (mAb) or recombinant antibody against target antigens for their accurate detection. In spite of complexity in the construction of hybridoma cell line, once hybridoma is constructed, mAb can be produced stably and consistently. Recombinant antibodies have wide applications to therapeutics and diagnostics agents and have been developed for analysing foods contaminants such as ampicillin, domoic acid and sulfamethazine (Burmester et al., 2001; Finlay, Shaw, Reilly, & Kane, 2006; Yang et al., 2007).

Single-chain variable fragment (scFv) is one of small recombinant antibody (approximately one-fifth size of full-length immunoglobulin G (IgG) ⩽30 kD) and constructed by fusing two variable regions of the heavy and light chains (V_H and V_L) of antibodies via a short peptide linker. It can be produced highly in recombinant microorganisms such as *Escherichia coli* (*E. coli*),

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Saccharomyces cerevisiae and *Pichia pastoris*. It was reported that some small-size recombinant antibodies retained better properties in binding affinity and thermal stability than their parent mAbs (Burmester et al., 2001).

In this study, a hybridoma cell line immunized with bovine serum albumin conjugated to AFB_1 (AFB_1–BSA conjugate) was screened to produce anti-AFB_1 mAb, of which isotypes, sensitivity and specificity were characterized. For the production of anti-AFB_1 scFv in recombinant *E. coli*, two genes coding for the V_H and V_L of anti-AFB_1 mAb were PCR-amplified and cloned. After overproduction of anti-AFB_1 scFv as intracellular inclusion body, optimal refolding conditions were determined using a 96 well-based refolding screening module. Anti-AFB_1 mAb and anti-AFB_1 scFv were characterized at a protein level by competitive direct ELISA (cdELISA) and surface plasmon resonance (SPR).

2. Materials and methods

2.1. Materials

AFB_1, aflatoxin B_2 (AFB_2), aflatoxin G_1 (AFG_1), aflatoxin G_2 (AFG_2), aflatoxin M_1 (AFM_1), aflatoxin M_2 (AFM_2), ochratoxin (OTA), deoxynivalenol (DON), fumonisin B_1 (FMB_1), fumonisin B_2 (FMB_2), T-2 toxin, AFB_1–BSA conjugate, horseradish peroxidase (HRP), *N*-(3-dimethylaminopropyl)-N′-ethylcarbodiimide hydrochloride (EDAC) and myeloma cells (Sp2/0-Ag14) were bought from Sigma Chemical Co. (St. Louis, MO, USA.). A rabbit anti-mouse IgG Fc specific antibody, anti-mouse IgG-HRP conjugate, and T-Gel™ purification kit were purchased from Pierce (Rockford, IL, USA.). The Mouse Immunoglobulin Isotyping ELISA kit and eight-week-old female BALB/c mouse were purchased from BD PharMingen (San Diego, CA, USA.) and Biogenomics (Washington, DC, USA.), respectively. AFB_1–horseradish peroxidase conjugate (AFB_1-HRP conjugate) was prepared as described previously (Chu, Lau, Fan, & Zhang, 1982). Serum-free Dulbecco's Modified Eagle medium (DMEM) was purchased from Invitrogen Co. (MD, U.S.A.) which was formulated to contain L-glutamine (2 mM), penicillin (100 U/mL) and streptomycin (100 μg/mL). HT medium consists of serum-free DMEM with 10% (v/v) fetal calf serum (FCS), sodium hypoxanthine (100 μM) and thymidine (16 μM), which was heated at 56 °C for 30 min. HTA indicates HT medium with aminopterin (0.4 μM). Modified HTA medium was composed of HTA medium containing 2% (v/v) HFCS, 10% (v/v) BM Condimed H1, L-glutamine (2 mM) and β-mercaptoethanol (24 μM). Polyethylene glycol 1500 (PEG), BM Condimed H1, and hybridoma fusion and cloning supplement (HFCS) were purchased from Roche Applied Science (Indianapolis, IN, USA). One milligram of lyophilised AFB_1 was dissolved in 10 mL of pure methanol in dark room and 100 μL of the AFB_1 solution was distributed into a capped brown vial for storage at −20 °C. Phosphate buffed saline (PBS, 50 mM sodium phosphate, 0.9% (v/v) NaCl, pH 7.2) was used for the dilution of the AFB_1 standard solution .

2.2. AFB_1 immunization

Six eight-week-old BALB/c mice was immunized by intraperitoneal (i.p.) injection of 0.1 mg of the AFB_1–BSA conjugate which was diluted in PBS and emulsified with an equal volume of Freund's complete adjuvant (FCA). Each mouse was immunized three times in every two weeks and subsequent boosting injection was carried out with Freund's incomplete adjuvant (FIA) instead of FCA until a strong immune response to AFB_1 was perceived. Blood was collected periodically from a tail vein of each immunized mouse to determine the antibody titer.

2.3. Construction of hybridoma cell

The lymphocytes collected from the spleens were fused with the Sp2/0-Ag14 myeloma cell using HFCS. The lymphocytes (1×10^8 cells) and myeloma cell (2×10^8 cells) were mixed in serum-free DMEM and spun down at 1000 rpm for 10 min, followed by complete removal of the supernatant. The mixed cells were disrupted gently by tapping and incubated in a water bath at 37 °C during cell fusion. PEG 1500 (1.5 mL) pre-warmed at 37 °C was dropped into the cell pellet slowly for 1 min, followed by gentle stirring for 1 min. The cell suspension was also mixed in DMEM complete and centrifuged at 1000 rpm for 10 min, followed by incubation for 15 min at 25 °C. After removal of the supernatant, the cells were resuspended gently in 10 mL FCS. About 0.2 mL of the cell suspension was inoculated into 1 mL of modified HTA medium in a 96-well cloning plate and incubated at 37 °C and for 7 days in order to remove non-fused cells. About 50% of modified HTA medium initially used for selection of the fused cells was exchanged to fresh modified HTA medium, and the cells was cultured in a CO_2 incubator at 37 °C. For screening positive hybridomas, the supernatant of the culture was subjected to modified cdELISA (Cho et al., 2005). One hundred microliter of 2 μg/mL rabbit anti-mouse IgG Fc specific antibody dissolved in 50 mM carbonate–bicarbonate buffer (pH 7.8) was coated onto each well and the treated 96-well plate was incubated at 37 °C for 1 h. After three times washing with PBST, the rabbit anti-mouse IgG Fc specific antibody-coated 96-well plate was blocked with 200 μL of blocking buffer (PBST buffer with 5% skim milk). The blocked plate was incubated at 37 °C for 1 h and washed with PBST three times. The culture supernatants from each hybridoma were diluted serially with buffer and 100 μL of the suspension was transferred to the blocked 96-well plate. After its incubation at 37 °C for 1 h, the plate was washed three times with PBST, and both 50 μL AFB_1 (0–2 μg/mL) and 50 μL AFB_1–HRP conjugate (1:1000 in PBST) were loaded onto the well prior to 1 h incubation. AFB_1 standard solution was prepared by dilution of AFB_1 stock solution (0.1 mg/mL prepared in pure methanol) with PBS and stored at 4 °C. The treated plate was washed with PBST and 100 μL of fresh substrate solution (319 μM 3,3′,5,5′-tetramethylbenzidine (TMB) and 60 ppb H_2O_2 in 10 mL of 50 mM phosphate–citrate buffer at pH 5.0) was added. After stopping the reaction by adding 50 μL of 2 M H_2SO_4, the absorbance at 450 nm of measuring wavelength was monitored using a ThermoMax™ microtiter plate reader (Molecular Devices Co., Menlo Park, CA, USA). Single hybridoma was cloned by limiting dilution as the previous research (Cho et al., 2005). The screened cells with inhibitory response by AFB_1 were transferred to 24-well plates and positive hybridomas were grown in HT medium. Final selection was made on the basis of antibody titer, sensitivity, and specificity. The cloning experiment for the selection of hybridoma clone was repeated three times.

2.4. Production of anti-AFB_1 mAb

To obtain concentrated anti-AFB_1 mAb, 0.5 mL of 2,6,-10,14-tetramethylpentadecane (pristane) was injected i.p. into mature female BALB/c mice. Pristane-primed BALB/c mice were injected i.p. with 3×10^6 hybridoma cells suspended in DMEM. Ascitic fluid was developed for 2 weeks after the injection, collected 3 and 4 weeks later, centrifuged and stored at −20 °C (Cho et al., 2005). The anti-AFB_1 mAb was obtained from the ascetic fluid and purified using a T-Gel™ purification kit (Pierce, U.S.A.).

2.5. Characterisation of anti-AFB_1 mAb

The cdELISA was performed to determine the sensitivity and specificity of anti-AFB_1 mAb secreted from the constructed