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Development of an immuno-affinity column for ochratoxin analysis using an organic solvent-tolerant monoclonal antibody

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ABSTRACT

Two kinds of monoclonal antibodies (MoAbs), OCA-10A and OCA-1B, were prepared based on their specificity to ochratoxin A (OTA) and ochratoxin B (OTB) and on their tolerance to 40% methanol. In an indirect competitive enzyme-linked immunosorbent assay, the half maximal inhibitory concentration (IC_{50}) value of OCA-10A was 27 ng/mL for OTA and 17 ng/mL for OTB, and that of OCA-1B was 28 ng/mL for OTA and 13 ng/mL for OTB. Immuno-affinity columns (IACs) using these MoAbs were prepared with agarose gel beads. The IAC with OCA-1B showed a NaCl-dependent binding ability to OTA and OTB, while interestingly, the IAC with OCA-10A bound to them without NaCl. The IAC with OCA-10A showed a high methanol tolerance when compared with existing IACs, as expected from the high methanol tolerance of OCA-10A itself. Such tolerance was maintained for the application of the cocoa extract with 70% methanol and the wheat extract with 60% acetonitrile, while the tolerance was slightly altered by interference from the cocoa extract. Examinations with organic solvents at higher concentrations than the allowable level in existing IACs showed that OTA and OTB spiked with wheat, cocoa and red wine could be purified with high recovery. The newly developed IAC is expected to show sufficient clean-up ability for food analyses.

1. Introduction

Ochratoxins (OTs) such as ochratoxin A (OTA) and ochratoxin B (OTB), shown in Fig. 1, are secondary metabolites that are produced by several species of *Penicillium* and *Aspergillus* genera, which commonly exist in the world [1]. OTA is known as the most harmful agent among OTs, being nephrotoxic and carcinogenic [2]. OTA can especially contaminate a wide range of foods such as cereal grains, raisin, wine, cocoa and green coffee beans by the invasion and growth of the fungi [3,4]. The monitoring and regulation of OTA are, therefore, important for human safety. Thus, the Codex Alimentarius Commission has adopted a maximum OTA level (5 μg/kg) for raw wheat, barley and rye. Increasingly more countries will introduce regulations for OTA in the near future, including Japan [5].

OTAs in foods are generally analyzed by thin-layer chromatography (TLC) or high-performance liquid chromatography (HPLC) using a fluorescence detector or by liquid chromatography using a mass-spectrometric detector [6,7]. For the measurement, food samples are initially extracted using aqueous solutions containing high concentrations of organic solvents such as acetonitrile or methanol. The extracts of food samples must be purified by removing matrix substances by multifunctional column (MFC) chromatography or by immuno-affinity column (IAC) chromatography [8]. IAC generally has an extremely high purification performance because the immobilized antibody can specifically bind to OTA with high affinity [9]. It is expected that IAC will become a major technique for the efficient purification of trace amounts of OTA.

An OTA-specific antibody was initially prepared by Chu et al. [10], and since then, a number of specific antibodies have been prepared [11–14]. IACs for OTA were developed using such antibodies and are currently used for food analyses. The IACs, however, tend to have low organic solvent tolerance because the antibodies generally interact weakly with the solvents. Authors previously reported that an organic solvent-tolerant monoclonal antibody (MoAb) could be prepared for total aflatoxins, and that an IAC with the MoAb showed high organic solvent tolerance and high purification performance for aflatoxin in organic solvent extracts from food samples [15]. The high organic solvent tolerance has some advantages for the purification step, such as a smaller sample volume and less insolubilization of the extract by dilution with water.

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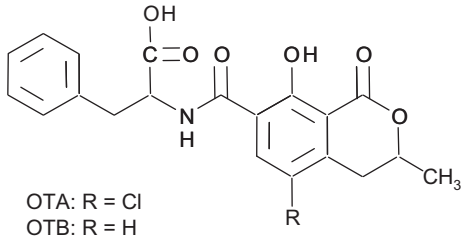

Fig. 1. Structures of OTA and OTB.

We therefore worked to develop a new IAC for OTs through a similar approach as that in the previous work.

This paper describes MoAbs preparation, the performance of IACs prepared using the MoAbs, and their characteristics and applicability.

2. Materials and methods

2.1. Reagents and apparatus

OTA, OTB and keyhole limpet hemocyanin (KLH) were purchased from Wako Pure Chemical (Tokyo, Japan). Bovine serum albumin (BSA) was purchased from Sigma–Aldrich (St. Louis, MO, USA). Freund's complete adjuvant and incomplete adjuvant were obtained from Difco Laboratories (Detroit, MI, USA). Horseradish peroxidase (HRP)-labeled rabbit anti-mouse IgG antibody, 96-well microplates used for cell cultures, and 96-well microtiter plates used for enzyme-linked immunosorbent assay (ELISA) were purchased from Thermo Fisher Scientific (Rockford, IL, USA). Agarose gel activated with cyanogen bromide and the protein G column were purchased from GE Healthcare UK (Buckinghamshire, England). Existing IACs for ochratoxin purification were purchased from Waters (Milford, MA, USA) and R-Biopharm (Darmstadt, Deutschland). All of the other chemicals and reagents were of analytical grade and were purchased from Wako Pure Chemical or Nacalai Tesque (Kyoto, Japan). The ELISA absorbance was measured using a microplate reader (MPR-01, HORIBA, Kyoto, Japan).

2.2. OTA–protein conjugate preparation

OTA was conjugated with KLH and BSA according to the activated ester method, as described previously [16].

In brief, the OTA (25 μmol) was dissolved in dried dimethyl sulfoxide (1.0 mL), and then *N*-hydroxysuccinimide (50 μmol) and 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (50 μmol) were added to the solution. The solution was stirred at room temperature for 1.5 h. The stirred solution (220 μL) was added to KLH (25 mg) or BSA (9 mg) dissolved in 1 mL of borate buffered saline (100 mM sodium borate, 150 mM NaCl, pH 8.0), and the mixture was gently stirred at room temperature for 1.5 h. After dialysis against 10 mM phosphate buffered saline (PBS; 10 mM sodium phosphate, 150 mM NaCl, pH 7.2) for 3 days at 4 °C, the prepared OTA–KLH conjugate was used for mouse immunization and the OTA–BSA conjugate was used as an ELISA antigen.

2.3. Monoclonal antibody preparation

The monoclonal antibody (MoAb) was prepared as described previously [17]. In brief, 7-week-old female BALB/c mice from Nippon SLC (Shizuoka, Japan) were intraperitoneally immunized with 50 μL of the OTA–KLH conjugate (100 μg/mouse) after it had been emulsified with an equal volume of Freund's complete adjuvant. Booster injections (25 μg/mouse) were performed three times using the emulsion with Freund's incomplete adjuvant at intervals of 2 weeks. Three days after the last injection, spleen cells from the mouse (5×10^8 cells) were fused with PSU1 myeloma cells (5.5×10^7 cells) using polyethylene glycol (MW 1500). The fused cells were suspended at 2.5×10^6 cells/mL as a number of spleen cells in a hypoxanthine–aminopterin–thymidine medium, transferred to the wells of a 96-well microplate, and incubated at 37 °C in 5% CO_2. Ten days after the start of incubation, the cultured fluids in which a hybridoma had formed a colony were screened by reactivity with the OTA–BSA conjugate in 40% methanol in a direct-bind ELISA (db-ELISA), and the fluids were subjected to secondary screening of their reactivity with OTA and OTB in an indirect competitive ELISA (ic-ELISA), as described below. The hybridoma grown in the positive well was cloned twice by the limiting dilution technique, and the representative cell clones were used for the preparation of the MoAb.

For MoAb preparation, BALB/c mice were pretreated by intraperitoneal injection with 0.5 mL of pristane, and 1 week after the pretreatment the mice were inoculated with 2×10^7 viable cells. Seven to ten days after the inoculation, the ascite fluids that were produced were collected from the mice, and the MoAb in the fluid was purified on a protein G column. The MoAb concentration was determined from the extinction coefficient (1.4 for 1 mg/mL of IgG).

2.4. db-ELISA and ic-ELISA

A db-ELISA was used to screen hybridomas producing methanol-tolerant MoAbs, as described previously [16]. In brief, 100 μL of the OTA–BSA conjugate (1 μg/mL) in PBS was coated to each well of the 96-well microtiter plates. Following three washes, the wells were blocked by the addition of 300 μL of 1% BSA in PBS. Each 50 μL of cultured fluids of the hybridomas mixed with an equal volume of 80% methanol was added to the wells, and the plate was incubated for 1 h at 25 °C. After the plate had been washed three times, 100 μL of HRP-labeled rabbit anti-mouse IgG antibody (1 μg/mL) in PBS modified with 0.3% BSA were added to each well. The plate was incubated for 1 h at 25 °C and then washed three times with PBS. The HRP substrate solution based on 3,3′,5,5′-tetramethylbenzidine was added to each well, and the plate was incubated for 10 min at 25 °C. Color development with the HRP reaction was stopped by the addition of 100 μL of 0.5 M sulfuric acid, and the absorbance at a wavelength of 450 nm was measured using a microplate reader.

An ic-ELISA was used to screen and to confirm the reactivity of the antibodies with OTA and OTB. The OTA and the OTB were dissolved at the concentration (100 ng/mL) for the screening and at the concentrations (0.064–1000 ng/mL) for the confirmation of the reactivity in 1% methanol. Cultured fluids of the hybridomas were diluted with PBS modified with 0.3% BSA. The dilution was twice the rate at which the absorbance of the above db-ELISA fell to 50% of the maximum absorbance. The OTA or OTB solution (50 μL) was added to the above blocked wells, and an equal volume of the diluted cultured fluid was immediately added to the wells. The plate was incubated for 1 h at 25 °C. Then, the HRP-labeled rabbit anti-mouse IgG antibody was added, followed by incubation, washing and substrate addition steps as described before for the db-ELISA.

2.5. Preparation of IAC and its conditions for application to samples

IAC was prepared as described previously [15]. In brief, MoAb (40 mL, 0.5 mg/mL) dissolved in PBS was mixed with 20 mL of activated agarose gel, and the mixture was gently agitated for 2 h at 25 °C. The gel coupled with the MoAb was added to 40 mL of a blocking buffer (1 M monoethanolamine modified with 0.5 M