JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Development of a Monoclonal Antibody against Domoic Acid and Its Application in Enzyme-Linked Immunosorbent Assay and Colloidal Gold Immunostrip

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A monoclonal antibody (mAb) specific to domoic acid was produced from a stable hybridoma cell line, 9F1F11, generated by the fusion of P3/NS1/1-AG4-1 myeloma cells with spleen cells isolated from a Balb/c mouse immunized with domoic acid−keyhole limpet hemocyanin. The 9F1F11 mAb belongs to the immunoglobulin G1 (κ-chain) isotype. A competitive direct enzyme-linked immunosorbent assay (cdELISA) and a competitive indirect ELISA were established for antibody characterization. In the cdELISA, the concentration causing 50% inhibition (IC_{50}) of binding of domoic acid−horseradish peroxidase to the antibody by domoic acid was found to be 0.58 ng/mL. A sensitive and rapid mAb-based colloidal gold immunostrip was also developed. The immunostrip assay, which has a detection limit of 5 ng/mL for domoic acid, can be completed in 10 min. Analysis of domoic acid in blue mussel samples revealed that data obtained from immunostrip were in a good agreement with those obtained from cdELISA. The mAb-based cdELISA and immunostrip assay established in this study were sensitive and accurate for rapid screening of domoic acid in shellfish samples.

KEYWORDS: Domoic acid; monoclonal antibodies; ELISA; colloidal gold immunostrip

INTRODUCTION

Domoic acid is a naturally occurring neuroexcitatory toxin produced primarily by the marine diatom *Pseudonitzschia multiseries.* It has been found as a contaminant in blue mussels by filter feeding during *Pseudonitzschia* blooms (*1*−*3*). The first human intoxication case was reported in 1987 by ingestion of domoic acid-contaminated blue mussels, which led to amnesic shellfish poisoning (ASP) (*4*); ASP is characterized by both gastrointestinal and neurological symptoms, including severe headache, confusion, and either temporary or permanent memory loss (*2*−*4*). Several outbreaks of deaths of brown pelicans, sea birds, sea lions, and mammals were documented to be associated with ASP (*5*−*8*). To protect consumers from ASP, most countries have set a regulatory guideline of 20 μg/g for domoic acid in shellfish and also established extensive shellfish sampling and analysis programs to monitor the levels of domoic acid (*9*).

Several research efforts have been conducted to develop sensitive and specific methods for the detection of domoic acid in blue mussels (*9*−*17*). High-performance liquid chromatography (HPLC) is the most widely employed method for monitoring domoic acid contamination. However, HPLC methods require extensive sample cleanup, highly trained personnel, and expensive equipment (*9*, *10*, *13*, *15*). Other assays available for domoic acid include surface plasmon resonance biosensors with a detection limit of 3.0 ng/g (*18*, *19*), a receptor binding assay with a detection limit of 0.1 ng/g, and a modification of the paralytic shellfish poisoning (PSP) mouse bioassay, which is capable of detecting toxins at a concentration of around 40 μg/g (*20*, *21*). Although the results of receptor binding assays and PSP mouse assays are closely correlated with the toxicity of domoic acid, the use of radioisotopes and animals restricts their applications in general laboratories.

The development of immunochemical analysis has led to many rapid and sensitive methods for monitoring and quantifying domoic acid in contaminated shellfish. Several groups have established immunoassays for domoic acid (*11*, *12*, *17*, *22*−*24*), but most of the assays are not suitable for on-site detection because of the long incubation time, the washing steps, and the application of instrumentation. Recently, colloidal gold particles have been generally applied in immunocytochemistry, biosensors, and immunostrips (*25*, *26*). The performance of immunostrips relies on the migration of test samples and antibody−colloidal gold conjugates along membrane strips on which the binding interactions take place. Most immunostrip tests for mycotoxins based on the use of red-colored antibody−colloidal gold conjugates as a label can be detected visually, thus providing rapid one-step detection in less than 10 min without the need for any instruments (*27*−*30*). An effective on-site method of detecting domoic acid levels in shellfish is needed.

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10.1021/jf0708140 CCC: $37.00 © 2007 American Chemical Society
Published on Web 06/02/2007

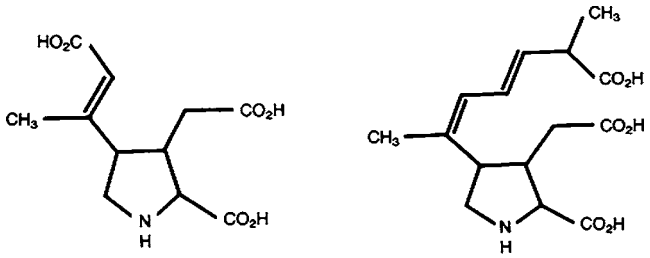

Figure 1. Chemical structures of kainic acid and domoic acid.

In the present study, a mAb against domoic acid was generated and a sensitive competitive direct enzyme-linked immunosorbent assay (cdELISA) and a mAb-based colloidal gold immunostrip were established for such purposes.

MATERIALS AND METHODS

Materials. Domoic acid (**Figure 1**) was purchased from Calbiochem (San Diego, CA). Kainic acid (**Figure 1**), glutamic acid, bovine serum albumin (BSA), gelatin, polylysine (PLL), ovalbumin (OVA), ammonium biocarbonate, Tween 20, trifluoroacetic acid, dimethyl sulfoxide (DMSO), 1-ethyl-3-(3-dimethylaminopropyl)carbodimide (EDC), and *N*-hydroxysuccinimide (NHS) were obtained from Sigma Chemical Co. (St. Louis, MO). Goat anti-mouse−peroxidase conjugate and rabbit anti-mouse−IgG-Fc and keyhole limpet hemocyanin (KLH) were obtained from Pierce Chemical Co. (Rockford, IL). Horseradish peroxidase (HRP) was obtained from Boehringer Mannheim Biochemicals (Indianapolis, IN). HRP substrate solution 3,3′,5,5′-tetramethylbenzidine (TMB) was obtained from Neogen Corp. (Lexington, KY). Ammonium sulfate and HPLC grade acetonitrile and methanol were obtained from Merck (Darmstadt, Germany). Polyethylene glycol (PEG 1500), hypoxanthine (H), aminopterin (A), and thymidine (T) were purchased from Boehringer Mannheim Biochemicals. Freund's incomplete adjuvant, Dulbecco's modified Eagle's medium (DMEM), fetal calf serum, and penicillin−streptomycin were obtained from Gibco Laboratories (Grand Island, NY). Isostrip was obtained from Boehringer Mannheim. Virus-free, 9−10 week old, female BALB/c mice were obtained from the National Animal Research Center (Taipei, Taiwan). The murine myeloma cell line P3/NS-1/1-AG4−1 (NS-1) was obtained from Bioresources Collection and Research Center in Taiwan. Colloidal gold (10 and 40 nm in diameter) was obtained from BBInternational (Cardiff, United Kingdom). An Easypack Developer's Kit was purchased from MDI Membrane Technologies (Ambala, India). The 0.45 μm syringe filter was obtained from Gelman Science (Ann Arbor, MI). All other chemicals and organic solvents used were of reagent grade or better.

Preparation of Various Domoic Acid Conjugates. *Conjugation of Domoic Acid to KLH.* Domoic acid was conjugated to KLH by adding water-soluble carbodiimide, EDC, and NHS under the following conditions (*31*). Freshly prepared EDC (1.2 mg of EDC in 0.02 mL of DMSO) and NHS (0.8 mg of NHS in 0.02 mL of DMSO) solutions were prepared and added to a domoic acid solution (1.0 mg of domoic acid in 0.2 mL of DMSO). The reaction was kept at room temperature for 30 min and then at 4 °C overnight. Then, the mixture was added slowly to 2 mg of KLH, which was dissolved in 1.5 mL of 0.1 M carbonate buffer (pH 9.6), and was kept at room temperature for another 2 h. After the reaction, the mixture was dialyzed against 2 L of 0.01 M phosphate buffer containing 0.15 M NaCl [phosphate-buffered saline (PBS), pH 7.5] for 72 h with two exchanges of buffer and then lyophilized.

Conjugation of Domoic Acid to PLL or OVA for Indirect ELISA (iELISA). Domoic acid was conjugated to PLL or OVA by the water-soluble carbodiimide method and used as a solid-phase antigen for the iELISA (*31*). In a typical reaction, 0.5 mg of domoic acid in 0.1 mL of DMSO was mixed with 2.5 mg of PLL or OVA first, and then, 1 mg of EDC and 0.75 mg of NHS in 0.05 mL of DMSO were added dropwise with constant stirring. The coupling reaction was carried out at 25 °C for 2 h. The mixture was dialyzed as described above for 72 h against 0.01 M PBS and then lyophilized.

Preparation of Domoic Acid-Peroxidase. Conjugation of domoic acid to HRP was achieved by the combination of water-soluble carbodiimide and NHS (*31, 32*). Briefly, 0.2 mg of domoic acid in 0.04 mL of DMSO was mixed with 0.4 mg of EDC and 0.3 mg of NHS, and then, a HRP solution (0.5 mg of HRP in 0.1 mL of 0.1 M, pH 9.6, carbonate buffer) was added. After it was stirred at room temperature for 2 h, the mixture was dialyzed against 0.01 M PBS for 72 h and then lyophilized.

Production of Monoclonal Antibody (MAb). *Immunization.* For generating mAbs against domoic acid, four female BALB/c mice (9−10 weeks of age) were each immunized with 40 μg of domoic acid−KLH in 0.01 M PBS that had been emulsified with an equal volume of Freund's complete adjuvant. Four weeks after the initial immunization, weekly booster injections were made with the same amount of immunogen in PBS containing no adjuvant. Blood samples were collected from the tail of each mouse at weekly intervals after each booster injection. A competitive indirect ELISA (ciELISA) as described below was used to determine the antibody specificity in the serum.

Fusion and Cloning. The mouse with the highest antibody specificity (8 weeks after the initial immunization, including three booster injections) was selected for fusion reaction. Four days before fusion, the mouse was primed with a total of 50 μg of immunogen. The mouse was sacrificed 3 days after the final immunization, and the entire spleen was aseptically removed and mashed with a glass pestle. The spleen cells were then passed through a tissue collector sieve to produce a single-cell suspension, which was then mixed with 1×10^7 of myeloma cells. The cells were centrifuged, suspended in 0.2 mL of HT medium, and then fused by gradually adding 1 mL of PEG 1500 in 1 min into the cell pellet. After fusion, the cells were pelleted again, resuspended in hypoxanthine, aminopterin, and thymidine (HAT) medium plus normal mouse erythrocytes to a final concentration of 0.5%, and plated into 96 well tissue culture plates (Corning plate, Corning, NY). The colonies were nourished every fifth day with freshly prepared HAT medium. When the colonies reached at least half confluence in the well, hybridomas were screened for specific antibodies against domoic acid using the ciELISA described later. One hybridoma cell line from the mouse immunized with domoic acid−KLH was obtained. Wells containing positive cells were cloned by the limiting dilution method into 96 well tissue culture plates (*33*).

Production of Ascites Fluid. Female BALB/c mice, 10 weeks old, were injected intraperitoneally with 0.5 mL of pristane 7 days before receiving an intraperitoneal injection of 2×10^6 hybridoma cells suspended in DMEM. Ascites fluid developed 2−3 weeks after the injection of the cells and was collected every other day for 6 days. The ascites fluid was centrifuged at 7000 rpm (5900*g*) for 5 min to remove cell debris. The cleared ascites fluid was purified by ammonium sulfate precipitation (50% saturation for the final solution) twice and then stored at −70 °C.

Characterization of Monoclonal Antibodies. *Determination of Isotype.* A mouse mAb isotyping kit (Isostrip; Boehringer Mannheim Co.) was used to determine the isotypes of mAb. Identification of specific immunoglobulin was carried out according to the manufacturer's protocol.

ciELISA. A ciELISA was used to characterize mAb. Briefly, each well of a microtiter plate was coated with 0.1 mL of the domoic acid−PLL and kept at 4 °C overnight. After the plate had been washed four times with PBS-Tween (0.35 mL per well; 0.05% Tween 20 in 0.01 M PBS) using an automated ELISA washer (Elx 50, Bio-Tek), 0.17 mL of gelatin−PBS (0.17 mL per well; 0.1% gelatin in 0.01 M PBS) was added and allowed to incubate at 37 °C for 30 min. The plate was washed again, and 0.05 mL of domoic acid standard with concentrations from 0.01 to 1000 ng/mL or extracted samples (0.05 mL per well in 0.01 M PBS) were added to each well, and then, the anti-domoic acid mAb (1:5000 dilution, 20 ng/mL in 0.01 M PBS, 0.05 mL per well) was added to all wells and incubated at 37 °C for 50 min. After incubation, the plate was washed four times with Tween−PBS, and 0.1 mL of goat anti-mouse IgG−HRP conjugate (1:20000 dilution) was added and incubated at 37 °C for 45 min. The plate was washed four times with Tween−PBS again, and 0.1 mL of TMB substrate solution was added. After 10 min of incubation at room

temperature, 0.1 mL of 1 N hydrochloric acid was added to stop the reaction. Absorbance at 450 nm was determined in a Vmax automatic ELISA reader.

cdELISA. The rabbit anti-M-IgG-Fc antibody was diluted in 0.01 M PBS (1:5000 dilution, 2 μg/mL), and 0.1 mL of the diluted form was coated onto each well. After incubation at 37 °C for 1 h, the wells were washed with PBS−Tween. The anti-domoic acid mAb was diluted in 0.01 M PBS (1:1000 dilution, 10 μg/mL), and 0.1 mL of the diluted form was coated onto each well. After the plate had been incubated at 4 °C overnight, it was washed with PBS−Tween followed by blocking with BSA−PBS (0.17 mL per well; 0.1% BSA in 0.01 M PBS) at 37 °C for 30 min. The plate was washed again with PBS−Tween four times, and then, domoic acid standard (0.05 mL per well in 0.01 M PBS) concentrations from 0.01 to 1000 ng/mL or samples together with the domoic acid−HRP conjugate (1:1000 dilution, 30 ng/mL, in 0.01 M PBS, 0.05 mL per well) were added and incubated at 37 °C for 50 min. The plate was washed four times with PBS−Tween, and 0.1 mL of TMB substrate solution was added. After incubation at room temperature in the dark for 10 min, the reaction was terminated by adding 0.1 mL of 1 N HCl. The absorbance at 450 nm was determined in the Vmax automatic ELISA reader.

cdELISA of Shellfish Samples Contaminated with Domoic Acid. Twelve blue mussel (*Mytilus edulis*) samples purchased from local food stores were used to determine the domoic acid levels. Briefly, each sample (4 g) was homogenized with 16 mL of extract solvent (methanol/water, 50/50, v/v) for 3 min (*34*). After centrifugation at 10000 rpm for 10 min, the extract solution was passed through a 0.45 μm syringe filter. One milliliter of the supernatant solution was aspirated and diluted with 4 mL of 0.01 M PBS and directly subjected to cdELISA.

Preparation of Antibody-Colloidal Gold Probe. Domoic acid mAbs were dialyzed against boric acid−borax buffer for 24 h at 4 °C and then centrifuged at 10000 rpm for 10 min to get clear supernatant for conjugation. The pH of the colloidal gold (40 nm in diameter) solution was adjusted to pH 9.0 with 0.1 M K_2CO_3 (pH 11.5) for conjugation with domoic acid mAb (*26*). The 120 μg of anti-domoic acid mAb was added dropwise to the 8 mL of pH-adjusted colloidal gold solution with gentle stirring. The mixture was reacted for 1 h at room temperature and blocked by 10% (w/v) filtered BSA for 30 min. This mixture was centrifuged at 14000 rpm for 30 min at 4 °C, and then, the supernatant was discarded, and the gold pellets were resuspended by adding 450 μL of 20 mM Tris-buffered saline (pH 8.0) with 1% BSA and 0.1% sodium azide. These domoic acid mAb−colloidal gold probes were stored at 4 °C until use.

Preparation of Immunostrip. An immunostrip was prepared as follows: 1 μL of domoic acid−OVA (0.8 mg/mL) and 0.5 μL of rabbit anti-mouse IgG antibody (1 mg/mL) were coated on to nitrocellulose membrane with a plastic backing plate and dried at room temperature. The rabbit anti-mouse IgG antibody and domoic acid−OVA were immobilized on the control and test zones, respectively. The domoic acid mAb−colloidal gold conjugate (10 μL/strip) was added to the conjugate release pad and air-dried. The release pad was pasted on the plate by over-crossing 4 mm with the NC membrane. The sample pad was also pasted on it by over-crossing 6 mm with the release pad. The absorbent pad was passed on the other side of the plate. The whole assembled plate was cut lengthways and divided into strips (5 mm × 75 mm) (*26*).

Assay of Domoic Acid in Blue Mussel by Immunostrip. The assay was carried out by adding sample extraction or the standard domoic acid solution by serial dilutions (0−10 ng/mL) into the microplate wells. Subsequently, the immunostrips were dipped into the wells vertically. The extract samples or standard domoic acid solution (0.2 mL) were applied to the sample pad and migrated up the membrane. After 5−10 min, test results were observed and judged by eyes.

RESULTS

Production of Monoclonal Antibodies. Several mice were injected with domoic acid−KLH, and then, the mouse with the serum of highest affinity to domoic acid was tested by ciELISA and selected for hybridoma screening. After cell fusion and cloning, a ciELISA in which domoic acid−PLL was a coated reagent was used for screening the hybridoma cell that produces monoclonal antibodies specific to domoic acid. Of the 532 wells tested, only two clones gave a strong positive signal, and the clone 9F1 showed the highest affinity for domoic acid. Therefore, the supernatant of the 9F1 culture was aspirated from the fusion well and subjected to limiting dilution. After limiting dilution, one of the positive clones, 9F1F11, was selected for production of culture supernatant and ascites fluid against domoic acid. Both antibodies obtained from supernatant and ascites fluid could be used directly in the ELISA without further affinity column purification.

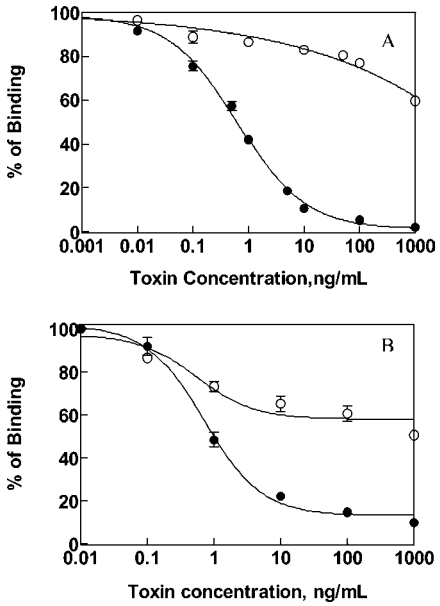

Figure 2. (**A**) Cross-reactivity of anti-domoic acid monoclonal antibodies with domoic acid (●) and kainic acid (○) in a cdELISA. All data were obtained from the average of three sets of experiments. The absorbance of the A_0, with no toxin present, was 1.8. (**B**) Cross-reactivity of anti-domoic acid monoclonal antibodies with domoic acid (●) and kainic acid (○) as determined by a ciELISA. All data were obtained from the average of three sets of experiments. The absorbance of the control, A0, with no toxin present, was 1.7.

Characterization of Antibodies. *Determination of Isotypes.* The isotype of mAb produced by cell line 9F1F11 was found to be immunoglobulin G1, κ-light chain.

cdELISA and ciELISA. Both the cdELISA and the ciELISA were used to determine the specificity of 9F1F11 mAb. The results of cdELISA are shown in **Figure 2**. The concentrations causing 50% inhibition (IC_{50}) of binding of domoic acid−HRP with the mAb by domoic acid were found to be 0.58 ng/mL (**Figure 2A**). A similar result was also found in the ciELISA, in which domoic acid−PLL was coated onto the wells of ELISA plates to serve as solid-phase antigen. The IC_{50} of binding of mAb to domoic acid−PLL by free domoic acid was found to be 0.71 ng/mL in the ciELISA. (**Figure 2B**). Kainic acid is known to be an analogue of domoic acid (*2*), but both cdELISA and ciELISA showed a very weak cross-reactivity with it (**Figure 2A**,**B**). Similarly, glutamic acid, another analogue of domoic acid, did not inhibit the binding of antibody to the marker antigen in either ELISA system even at a concentration as high as 10 μg/mL (data not shown).

Analysis of Domoic Acid in Shellfish with mAb-Based cdELISA. Twelve blue mussel samples were collected from local food stores and subjected to cdELISA for domoic acid determination; the results are presented in **Table 1**. Eleven of the examined samples were found to be domoic acid positive

Table 1. ELISA and Immunostrip Analysis of Domoic Acid in Blue Mussel Samples

samples	ELISA[a] (ng/mL ± SD)[b]	immunostrip assay[a]
1	3.64 ± 0.19	±
2	6.99 ± 0.79	+
3	3.02 ± 0.54	−
4	3.25 ± 0.27	−
5	3.94 ± 0.21	−
6	4.25 ± 0.18	−
7	10.35 ± 0.49	+
8	5.4 ± 0.30	+
9	2.32 ± 0.68	−
10	2.91 ± 0.44	−
11	3.60 ± 0.67	−
12	ND[c]	−

[a] Each sample was extracted twice, and each extract was analyzed in duplicate. [b] One milliliter extract solution contains 0.25 g of mussel tissue. [c] ND, not detected.

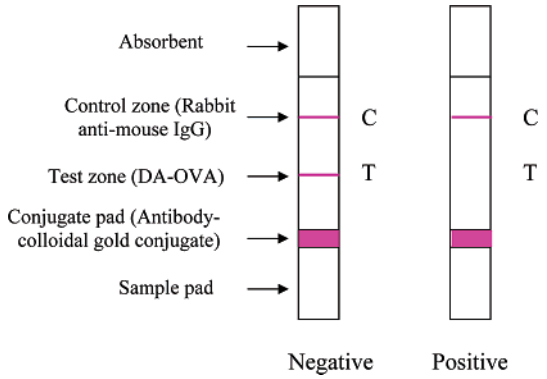

Figure 3. Schematic illustration of Immunostrip. C, control zone (rabbit anti-mouse IgG); T, test zone (DA-OVA).

with levels below 20 ng/mL. The extract of sample 7 showed the highest level of domoic acid at 10.35 ng/mL (41.4 ng/g). Samples 2 and 8 had domoic acid levels higher than 5 ng/mL, and the remaining seven positive samples were lower than 5 ng/mL. Sample 12 was found to be domoic acid-free.

Characterization of Immunostrip Assay. Because immunostrip was considered as a one-step rapid immunoassay, for toxin determination, the above MAb was applied to construct an effective immunotrip, in which the domoic acid−OVA conjugate competes with domoic acid in the sample solution for the antibody−colloidal gold label. A schematic description of the immunostrip test format is shown in **Figure 3**. When the sample pad of immunostrip was dipped into the sample solutions, the sample solution moved upward to the conjugate pad containing red-colored antibody−colloidal gold conjugate by capillary action across the nitrocellulose membrane. This combination then continuously migrates upward to the test zone with immobilized domoic acid−OVA conjugate. In the absence of domoic acd in the sample solution, the antibody−colloidal gold conjugate was bound and trapped by the domoic acid−OVA conjugate to form a visible red line on the test zone (**Figure 3**). In contrast, when a sufficient concentration of domoic acid was present in the sample solution, it would occupy the antigen binding sites on the antibody−colloidal gold conjugate; consequently, the limited antibody−colloidal gold conjugate failed to bind with the domoic acid−OVA conjugate on the test zone. The absence of a color line on the test zone indicates a positive result (**Figure 3**). The control zone with rabbit anti-M secondary antibody absorbed on it was used to verify whether the assay has been performed properly. This

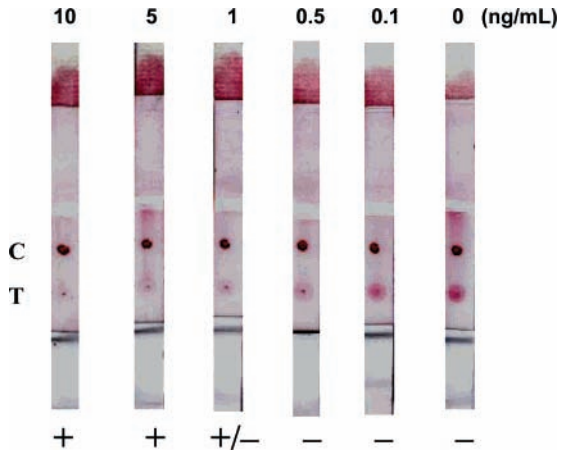

Figure 4. Detection limit of domoic acid with immunostrip. A series of dilution (0–10 ng/mL) of standard domoic acid was dissolved in PBS. A concentration higher than 5 ng/mL of domoic acid was found to cause a disappearance of the red spot at the test zone.

control zone should always show a red color line under an accurate operation regardless of the presence of domoic acid. Briefly speaking, a domic acid-free sample shows two red spots, whereas a positive sample with domoic acid presents only one red spot on the membrane, indicating that the immunostrip has been completely conducted.

Detection Limit of Domoic Acid Test Strip. Various concentrations of standard domoic acid solution (0−10 ng/mL) were subjected to the immunostrip assay. The whole assay could be completed in less 10 min, and the detection limit of immunostrip test for domoic acid was about 5 ng/mL (**Figure 4**). Domoic acid at a concentration above 5 ng/mL could occupy all of the antibody−colloidal gold conjugates and prevent the antibody−colloidal gold conjugates from binding with the domoic acid−OVA on the test zone, which resulted in only one spot on the control zone.

Analysis of Domoic Acid in Shellfish by Immunostrip Assay. Immunostrip was used for domoic acid analysis in blue mussel samples. The results are shown in **Table 1**. According to the ELISA data, samples 2, 7, and 8 were known to have domoic acid levels more than 5.0 ng/mL, and each of them gave a positive result with only one spot on the immunostrip membrane (**Figure 5**). Sample 1 containing 3.64 ng/mL of domoic acid gave a weak spot in the test zone, showing a positive/negative symbol. The remaining eight mussel samples with toxin levels lower than 5.0 ng/mL were found to be negative with two red spots in the immunostrip assay.

DISCUSSION

Domoic acid, like most phycotoxins and mycotoxins, is a low molecular weight nonimmunogenic toxin. To render it immunogenic, it is necessary to conjugate domoic acid to a protein carrier as an immunogen. Using domoic acid−KLH conjugate as an antigen, polyclonal antibodies were produced in our laboratory, and a sensitive ELISA for domoic acid was also established (*31*). In the present study, a stable hybridoma cell line generating mAb specifically against domoic acid was obtained. Thus, there will be an unlimited supply for mAb for domoic acid in the future. Although mAb obtained from both culture supernatant and ascites could be used in the cdELISA of domoic acid, a secondary antibody of rabbit anti-mouse Fc

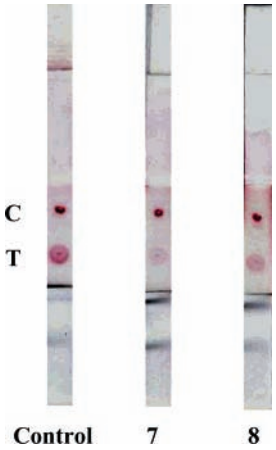

Figure 5. Immunostrip detection of domoic acid in control and two representative blue mussel samples. The control strip containing no domoic acid shows two red spots on the membrane. Sample 7 or 8 containing domoic acid was found to cause a disappearance of the red spot at the test zone.

was necessary to coat onto the microplate before the supernatant was added. In contrast, the mAb from ascites can be directly coated onto the microplate to run the cdELISA.

The IC_{50} value of the cdELISA was found to be 0.58 ng/mL. On the basis of 10% inhibition of binding of domoic acid−HRP conjugate, the detection limit of domoic acid in buffer solution of the cdELISA was around 0.01 ng/mL (**Figure 2A**). The mAb-based cdELISA developed here showed a better sensitivity than that developed by Osada et al., showing a detection limit of 0.1 ng/mL (*35*), but similar to the polyclonal-based cdELISA systems established by us or Garthwaite et al. (*12*, *31*). Both cdELISA and ciELISA showed a very weak cross-reactivity with kainic acid (**Figure 2A**,**B**); kainic acid is an excitatory amino acid and a potent agonist of glutamate receptors in the dorsal hippocampus of brain, with a chemical structure similar to domoic acid (*36*).

The ELISA results obtained from blue mussel samples purchased from food stores in Taiwan showed that 11 of the 12 mussel samples contained less than 15 ng/mL (equal to 60 ng/g) of domoic acid, which is 333-fold lower than the regulatory limit at 20 μg/g of wet weight tissue established by the Canadian Department of Health and Welfare (*2*, *3*). Although the samples that we examined showed very low levels of domoic acid, as high as 90 μg/g of domoic acid in whole mussel was reported by Vale and Sampayo (*37*). Scholin et al. (*7*), who investigated the mortality linked to domoic acid of sea lions, found that the blue mussel collected during a domoic acid outbreak contained no domoic acid or only trace amounts of it; it suggests that monitoring of mussels alone does not provide adequate warning of domoic acid entering the food chain at levels sufficient to harm marine wildlife and even humans.

To develop an assay that could be widely used in on-site domoic acid shellfish screening, our study focused on the development of a mAb−colloidal gold-based rapid immunostrip assay, which is simpler and less time-consuming than cdELISA. Colloidal gold particles have been generally used in immunocytochemistry, biosensors, and immunochromatographic test strips for mycotoxins (*25*, *26*). In this study, the domoic acid mAb was conjugated to 40 nm gold particles and 10 nm gold particles, respectively. The 40 nm gold particles offered a better visibility than 10 nm gold particles (data not shown), probably due to the lower steric hindrance between conjugation of 40 nm gold particles and antibody. Before the conjugation, we also conducted an antibody titration experiment and determined that 15 μg of mAb was the most suitable amount for stabilizing 1 mL of gold particles. The required amount of antibody conjugated to gold particle varied a lot according to the types and sources of antibodies. In addition, nitrocellulose membranes with various pore sizes, including 5, 10, and 15 μm, were also tested before they were assembled into the immunostrip. We found that the red gold particles showed an obvious visual effect and reasonable migration rate on the membrane with a pore size of 5 μm. Therefore, the 5 μm membrane was chosen for all the immunostrip studies herein.

Both domoic acid−OVA and domoic acid−PLL were coated on the test zone of the membrane, respectively, to test for their effectiveness in competing for binding domoic acid in the sample with the antibody−colloidal gold conjugate. Domoic acid−OVA was found to be a better carrier protein than domoic acid−PLL. Because domoic acid−PLL contains many lysine residues, it is likely that these residues cause nonspecific binding in the test zone in both polyclonal and mAb-based immunostrips. The polyclonal-based immunostrip when coated with domoic acid−OVA on the test zone had a detection limit of 100 ng/mL (data not shown). Nevertheless, when mAb was used in the test strip, a better specificity and sensitivity were also achieved with domoic acid−OVA. Both immunostrips are sensitive enough to inspect the domoic acid present in samples because the recommended regulatory limit for the toxin is about 20 μg/g.

Results obtained from colloidal gold immunostrip assay were in a good agreement with those obtained from cdELISA. Because immuostrip is less time-consuming and needs neither expensive instruments nor well-trained personnel, it could be used for on-site toxin detection. Domoic acid was extracted from mussels with 50% methanol. We found that the cd-ELISA was more susceptible to solvent interference and that the assay solution should be diluted to less than 10% of methanol before analysis. In contrast, no interference was found for immunostrips when the assay solution contained less than 30% methanol. The immunostrip can be dipped into 200−300 μL of mussel extraction without further sample cleanup. The disadvantage for the immunostrip is that it can only be used for one sample and large amounts of antibody and domoic acid conjugates are required for each sample detection as compared to ELISA.

The colloidal gold is very stable in storage conditions that make the immunostrip a reliable reagent. In the present study, we first developed the domoic acid immunostrip assay, which used the colloidal gold nanoparticles as a tracer to provide visual evidence of the presence of domoic acid in a shellfish sample within 10 min. This immunostrip could be applied to on-site detection of domoic acid in shellfish samples without sample cleanup.

ABBREVIATIONS USED

BSA, bovine serum albumin; DMSO, dimethyl sulfoxide; EDC, 1-ethyl-3[3-dimethylaminopropyl]carbodimide; ELISA, enzyme-linked immunosorbent assay; cdELISA, competitive direct ELISA; ciELISA, competitive indirect ELISA; iELISA, indirect ELISA; HRP, horseradish peroxidase; KLH, keyhole limpet hemocyanin; NHS, *N*-hydroxysuccinimide; OVA, ovalbumin; PLL, polylysine; TMB, 3,3′,5,5′-tetramethylbenzidine.

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Received for review March 20, 2007. Revised manuscript received April 19, 2007. Accepted April 19, 2007. This work was supported by Grant NSC 95-2313-B-040-004 from the National Science Council of the Republic of China, Taiwan, and by funding from Tian-Sheng Memorial Hospital, Tong Kong, Ping-Tong, Taiwan.

JF0708140