JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Development of a Sensitive Enzyme-Linked Immunosorbent Assay for the Determination of Domoic Acid in Shellfish

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Polyclonal antibodies for domoic acid were generated from rabbits after the animals had been immunized with either domoic acid−keyhole limpet hemocyanin (KLH) or domoic acid−bovine serum albumin (BSA). A competitive direct enzyme-linked immunosorbent assay (cdELISA) and a competitive indirect ELISA (ciELISA) were used for the characterization of the antibodies and for analysis of domoic acid in blue mussels and clams. The antibody titers in the serum of rabbits immunized with domoic acid−KLH were considerably higher than those in rabbits immunized with domoic acid−BSA. The antibodies from the rabbits immunized with domoic acid−KLH were further characterized. In the cdELISA, the concentrations causing 50% inhibition (IC_{50}) of binding of domoic acid−horseradish peroxidase to the antibodies by domoic acid and a domoic acid analogue, kainic acid, were found to be 0.75 and 200 ng/mL, respectively. In the presence of blue mussel matrix, the detection limit of domoic acid was <25 ng/g. The overall analytical recovery of domoic acid (25−500 ng/g) added to the blue mussels and then extracted with 50% aqueous methanol in the cdELISA was found to be 81.1%. The efficacy of cdELISA was also confirmed by the high-performance liquid chromatography method. Analysis of domoic acid in shellfish samples showed that 10 of the 15 shellfish examined were contaminated with domoic acid at levels of <50 ng/g.

KEYWORDS: Domoic acid; antibodies; ELISA; shellfish

INTRODUCTION

Domoic acid, a naturally occurring neuroexcitatory toxin produced primarily by the marine diatom *Pseudonitzschia pungens*, is accumulated in shellfish by filter-feeding during *Pseudonitzschia* blooms (*1*−*4*). Ingestion of domoic acid-contaminated shellfish leads to amnesic shellfish poisoning (ASP), which is characterized by both gastrointestinal and neurological symptoms, including severe headache, seizures, and either temporary or permanent memory loss (*2*, *4*). ASP has been found to cause the death of sea birds, sea lions, and human consumers in some severe cases (*4*−*8*). To protect consumers from ASP, most countries have set a regulatory limit for domoic acid in shellfish of 20 μg/g and also established extensive shellfish sampling and analysis programs to monitor the levels of domoic acid (*9*).

Domoic acid, like its analogue, kainic acid, is an excitatory amino acid and a potent agonist of glutamate receptors in the dorsal hippocampus of brain (*1*, *3*). However, the toxicity of domoic acid is 3 times greater than that of kainic acid (*2*, *10*). After exposure to domoic acid, it binds predominately to *N*-methyl-D-aspartate (NMDA) receptors in the central nervous system and causes depolarization of the neurons (*11*). Because the affected neurons are mainly located in the hippocampus, ∼25% of the affected persons in the 1987 domoic acid-contaminated mussel event showed the syndrome of short-term memory loss (*2*).

To help minimize the risk of human and animal exposure to ASP, extensive research has been conducted to develop sensitive and specific methods for the detection of domoic acid in shellfish (*9*, *12*−*18*). High-performance liquid chromatography (HPLC) with good accuracy and reproducibility is most widely employed for monitoring domoic acid. However, HPLC methods require highly qualified personnel and extensive sample cleanup as well as expensive equipment (*9*, *15*, *17*). Other assays available for domoic acid include a modification of the PSP mouse bioassay capable of detecting domoic acid at a concentration of 40 μg/g, and a receptor binding assay with a detection limit of 0.1 ng/g (*19*). These two assays are highly relevant to domoic acid toxicity, but the use of animals or radioisotopes restricts their applications to research laboratories. Development of immunochemical approaches has led to more rapid and sensitive methods for monitoring and quantification of domoic acid in contaminated shellfish. Although several groups have developed immunoassays for domoic acid (*13*, *14*, *18*, *20*, *21*), those assays rely on the competitive indirect enzyme-linked immunosorbent

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10.1021/jf049303t CCC: $27.50
Published on Web 07/30/2004

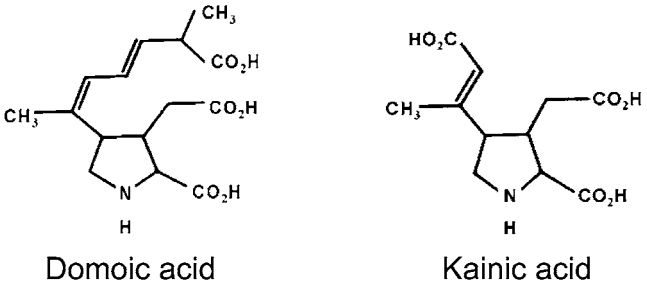

Figure 1. Structures of domoic acid and kainic acid.

assay (ciELISA) in which domoic acid−protein conjugates were coated onto microplates. Compared with the competitive direct ELISA (cdELISA), the ciELISA is more time-consuming. To effectively detect domoic acid levels in shellfish, in the present study a new method for the production of polyclonal antibodies against domoic acid was developed, and a sensitive cdELISA was established to analyze shellfish samples.

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, A7511), gelatin (G-2500), polylysine, ovalbumin (OVA, A5503), ammonium biocarbonate, Tween 20, trifluoroacetic acid (TFA), 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-rabbit−peroxidase conjugate (no. 31460) and keyhole limpet hemocyanin (KLH, no. 77600) were obtained from Pierce Chemical Co. (Rockford, IL). Horseradish peroxidase (HRP, no. 814407) was obtained from Boehringer Mannheim Biochemicals (Indianapolis, ID). HRP substrate solution 3,3′,5,5′-tetramethylbenzidine (TMB, no. 507604) was obtained from Kirkegaard and Perry Laboratories (Gaithersburg, MD). Ammonium sulfate and HPLC grade acetonitrile were obtained from Merck (Darmstadt, Germany). Seven-week-old female New Zealand white rabbits were obtained from Deer-Ho farm (Taichung, Taiwan). Freund's complete adjuvant containing *Mycobacterium tuberculosis* (H37 Ra) and Freund's incomplete adjuvant were obtained from Gibco BRL (Grand Island, NY). 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 and BSA.* Domoic acid was conjugated to KLH or BSA in the presence of water-soluble carbodiimide, EDC, and NHS under the following conditions (*13*). In a typical experiment, EDC solution (1.2 mg of EDC in 0.02 mL of DMSO) and NHS solution (0.8 mg of NHS in 0.02 mL of DMSO) were freshly 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 either KLH or BSA, which was dissolved in 1.5 mL of 0.1 M carbonate buffer (pH 9.6) and kept at room temperature for another 2 h. After reaction, the mixture was dialyzed against 2 L of 0.01 M phosphate buffer containing 0.15 M NaCl (PBS, pH 7.5) for 72 h with two exchanges of buffer and then lyophilized.

Conjugation of Domoic Acid to Polylysine for Indirect ELISA. Domoic acid was conjugated to polylysine by the water-soluble carbodiimide method and used as a solid-phase antigen for the indirect ELISA. In a typical reaction, 0.5 mg of domoic acid in 0.1 mL of DMSO was mixed with 2.5 mg of polylysine 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. 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 being stirred at room temperature for 2 h, the mixture was dialyzed against 0.01 M PBS for 72 h and then lyophilized.

Production of Polyclonal Antibody. The schedule and methods of immunization were the same as those described previously (*22*). Two immunogens, domoic acid−KLH and domoic acid−BSA, were tested in four rabbits, with two rabbits for each immunogen. Each rabbit was injected intradermally at multiple sites on the shaved back (30 sites) with 500 μg of the immunogen in 1 mL of 0.01 M PBS mixed with 1 mL of Freund's complete adjuvant. For booster injections, the same amount of immunogen in PBS solution was mixed with an equal volume of Freund's incomplete adjuvant and injected subcutaneously at four sites on the thigh of each rabbit at the 5th and 12th weeks. Antisera were collected from the ears of the rabbits from the fifth week after the initial injection. The antisera were precipitated twice with $(NH_4)_2SO_4$ to a final saturation of 35% using a 100% saturated $(NH_4)_2SO_4$ solution. The precipitate was redissolved in distilled water equal to half of the original serum volume and then dialyzed against 2 L of PBS for 72 h at 4 °C with two changes of buffer.

Monitoring of Antibody Titers by Indirect (iELISA). The protocol for the iELISA was similar to that described previously (*23*). In general, 0.1 mL of domoic acid−polylysine conjugate (1 μg/mL in 0.01 M PBS) was added to each well of microtiter plate (Nunc) 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. Then the plate was washed as described above, and 0.1 mL of diluted anti-domoic acid antiserum was added. After incubation at 37 °C for 1 h and washing with PBS−Tween, 0.1 mL of goat anti-rabbit 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 (1 mM 3,3′,5,5′-tetramethylbenzidine and 3 mM H_2O_2 per liter of potassium citrate buffer, pH 3.9, a premixed solution supplied by KPL, Gaithersburg, MD) 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 (Molecular Devices Co., Menlo Park, CA).

cdELISA. The protocol for cdELISA was essentially the same as previously described (*23*). The antibody collected from the 20th week was diluted in 0.01 M PBS, pH 7.5 (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 100 ng/mL or sample together with the domoic acid−HRP conjugate (1:3000 dilution, 10 ng/mL, in 0.01 M PBS, 0.05 mL per well) was 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.

Analytical Recovery of Domoic Acid Added to Blue Mussels by cdELISA. An analytical recovery study was carried out to test the efficacy of cdELISA for the analysis of domoic acid in blue mussel samples. Four grams each of the blue mussel product shown to be domoic acid-free by ELISA was spiked with domoic acid at concentrations ranging from 25 to 500 ng/g. A control sample with no toxin added was used as the blank. Each of the spiked samples was homogenized with 16 mL of extract solvent (methanol/water, 50:50, v/v) for 3 min and then centrifuged at 10000 rpm for 10 min (*9*). The supernatant solution, 1 mL of the clear extract, was diluted with 4 mL of 0.01 M PBS and subjected directly to cdELISA. Further dilutions with PBS were made for samples with high levels of domoic acid before the cdELISA. At least two separate extracts were taken for each sample, and analysis on each extract was determined in triplicate.

cdELISA of Shellfish Samples Contaminated with Domoic Acid. Ten blue mussel (*Mytilus edulis*), three clam (*Meretrix lusoria*), and two oyster (*Crassostrea gigas*) samples purchased from local food stores

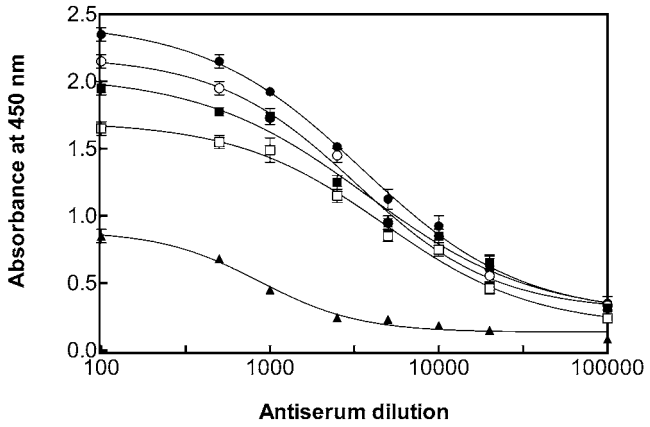

Figure 2. Determination of antibody titers for a representative rabbit after immunization with domoic acid–KLH by a domoic acid–polylysine-based iELISA. The antiserum was obtained 0 (▲), 6 (□), 9 (■), 12 (○), and 20 (●) weeks after immunization.

in Taiwan were used to determine their 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. After centrifugation at 10000 rpm for 10 min, 1 mL of the supernatant solution was aspirated and diluted with 4 mL of 0.01 M PBS and directly subjected to cdELISA.

HPLC of Domoic Acid. Authentic domoic acid, a negative blue mussel extract spiked with domoic acid standard, and a naturally domoic acid contaminated mussel sample were subjected to HPLC analysis according to the procedure of Quilliam et al. (*24*). A Beckman System Gold instrument (Fullerton, CA) equipped with a 126 solvent module and a 168 photodiode array (PDA) detector was used. The domoic acid standard and the sample extracts obtained as described above were passed through a low protein binding 0.45 μM filter (Gelman Science, Ann Arbor, MI) prior to HPLC. A 25 cm × 4.0 mm, 5 μm, Lichrospher C18 reverse phase column (Merck) in conjunction with a 4 cm × 4.0 mm, 5 μm, Lichrospher C18 guard column (Merck) was equilibrated with a mobile phase consisting of 5% acetonitrile in 0.05% TFA/water at a flow rate of 1 mL/min. After the sample was injected, domoic acid was eluted from the column with a linear gradient of acetonitrile in 0.05% TFA from 5 to 35% acetonitrile in 15 min and then 35% acetonitrile for another 5 min. The chromatograms were monitored at 242 nm, and the absorbance data were analyzed with Beckman System Gold Nouveau software. A calibration curve was generated using domoic acid standards of 0.5, 1.0, 2.5, 5.0, and 12.5 μg/mL ($R^2 = 0.99$); each injection was 20 μL. The lowest detectable standard was 0.5 μg/mL, which corresponded to 3 times the standard deviation of the signal from the instrument.

RESULTS

Production of Polyclonal Antibodies. Sera collected from rabbits immunized with domoic acid−KLH or with domoic acid−BSA were subjected to the iELISA. Typical titration curves of antibody titers obtained from a domoic acid−KLH immunized rabbit over a period of 20 weeks are shown in **Figure 2**. Antibodies against domoic acid were detected in the sera of rabbits as early as 6 weeks after initial immunization. The antibody titer increased progressively with time, and the highest titer was found in the sera of rabbits at the 20th week after two subsequent immunizations. The antibody titers of the rabbits immunized with domoic acid−BSA were found to be considerably lower than those of the rabbits immunized with domoic acid−KLH (data not shown).

Characterization of Antibodies. Both the cdELISA and ciELISA were used to determine the specificity of antibodies. Because the amount of antibody required to coat microtiter plates in the cdELISA was much less for the antiserum from rabbits immunized with domoic acid−KLH (1:1000 dilution) than those

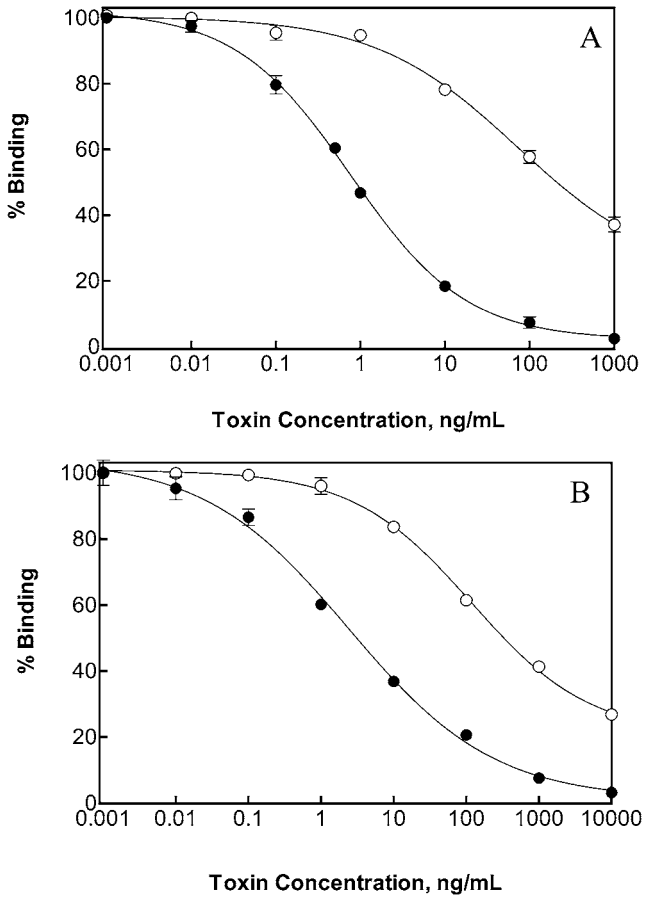

Figure 3. (**A**) Cross-reactivity of anti-domoic acid antibodies with domoic acid (●) and kainic acid (○) in a cdELISA. All data were obtained from the average of three sets of experiments. The absorbance of the control, A_0, with no toxin present, was 1.6. (**B**) Cross-reactivity of anti-domoic acid 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, A_0, with no toxin present, was 1.4.

immunized with domoic acid−BSA (1:250 dilution), the 20th antiserum from rabbits immunized with domoic acid−KLH was used in the subsequent studies. In the cdELISA, the concentrations causing 50% inhibition (IC_{50}) of binding of domoic acid−HRP with the antibodies by domoic acid and kainic acid were found to be 0.75 and 200 ng/mL, respectively **(Figure 3A**). The relative cross-reactivities of the antibodies to domoic acid and kainic acid were calculated to be 100 and 0.37, respectively. Similar results were also obtained in the ciELISA, in which domoic acid−polylysine was coated to the wells of ELISA plates to serve as solid-phase antigen. The concentrations causing 50% inhibition of binding of antibodies to the solid-phase domoic acid−polylysine by free domoic acid and kainic acid were found to be 2.0 and 286 ng/mL, respectively **(Figure 3B)**. The relative cross-reactivities of the antibodies to domoic acid and kainic acid in the ciELISA were calculated to be 100 and 0.2, respectively. Glutamic acid, an analogue of domoic acid and kainic acid (*1*), at a concentration of 100 μg/mL, did not inhibit the binding of the marker antigen with the antibodies in either ELISA system.

Analytical Recovery of Domoic Acid Added to Blue Mussels by cdELISA. Results for the analytical recovery of domoic acid added to the blue mussel samples by cdELISA are presented in **Table 1**. Recoveries for domoic acid spiked into blue mussel at 25−500 ng/g ranged from 73.8 to 92.8%.

Table 1. Analytical Recovery of Domoic Acid Added to Blue Mussel Samples by cdELISA

domoic acid[a] added (ng/g)	analytical recovery ng/g	%	SD	CV%
25	23.2	92.8	3.4	14.6
50	42.3	84.6	4.3	10.1
100	76.6	76.6	7.2	9.4
200	147.5	73.8	7.8	5.2
500	387.6	77.5	12.7	3.3
overall	81.1	8.5		

[a] Each toxin level had two samples, and each sample was analyzed in triplicate.

Table 2. ELISA Analysis of Domoic Acid in Shellfish Samples[a]

sample	source area	domoic acid (ng/g ± SD)
blue mussel		
1	New Zealand	48 ± 6
2	New Zealand	36 ± 8
3	New Zealand	<20
4	New Zealand	ND[b]
5	Thailand	<20
6	Philippines	<20
7	Taiwan	40 ± 8
8	Taiwan	<20
9	Taiwan	ND
10	Taiwan	ND
clam		
11	Taiwan	ND
12	Taiwan	ND
13	Taiwan	<20
oyster		
14	Taiwan	<20
15	Taiwan	<20

[a] Each sample was extracted twice, and each extract was analyzed in triplicate. [b] Not detected.

The overall average of recovery for all of the spiked samples was found to be 81.1% (CV = 8.5%).

Analysis of Domoic Acid in Shellfish with cdELISA. Ten blue mussel samples, three clam samples, and two oyster samples were collected from local food stores and subjected to cdELISA for domoic acid determination; the results are presented in **Table 2**. Ten of the 15 examined samples were found to be domoic acid positive with levels below 50 ng/g. Sample 1, imported from New Zealand, was found to have the highest level of domoic acid at 48 ng/g; samples 2 and 7 had domoic acid levels of <40 ng/g. The remaining four positive mussel samples were lower than 20 ng/g. Two examined oyster samples were found to contain domoic acid at <20 ng/g, and two of three examined clam samples were domoic acid-free.

HPLC of Spiked Domoic Acid in Mussel Sample. To test the efficacy of the cdELISA for domoic acid determination in shellfish samples, 100 ng of domoic acid standard and the negative mussel extract spiked with 200 ng of domoic acid were analyzed with an HPLC method and further by cdELISA. The HPLC results are shown in **Figure 4**. Domoic acid standard was well identified with a retention time of 12.6 min under the linear gradient elution (**Figure 4A**). The spiked mussel sample also shows a domoic acid peak with a retention time of 12.6 min (**Figure 4B**), and the peak area was calculated to be 196 ng on the basis of the calibration curve. In the cdELISA, the domoic acid level in the spiked sample was found to be 175 ng. However, the mussel extract with a naturally contaminated domoic acid level at 48 ng/g (sample 1) did not show any major peak similar to the domoic acid standard in the HPLC chromatogram (data not shown), because the detection limit for

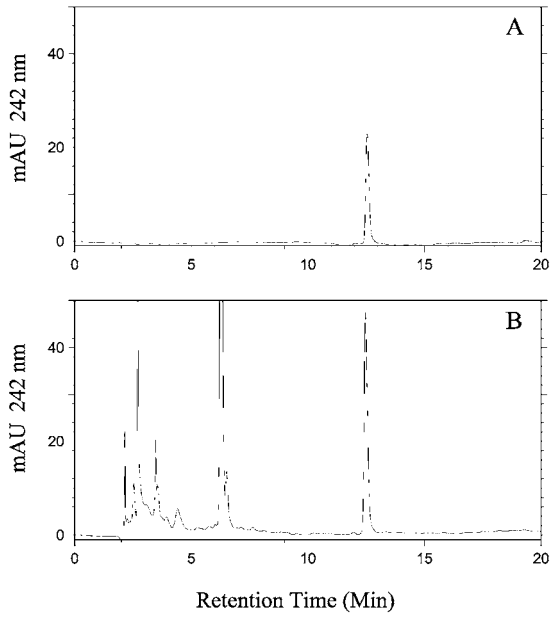

Figure 4. HPLC chromatograms of (**A**) 100 ng of domoic acid standard and (**B**) a mussel sample spiked with 200 ng of domoic acid.

domoic acid with HPLC is 10 ng, which is equivalent to 20 μL of 0.5 μg/mL per injection.

DISCUSSION

Domoic acid is a low molecular weight nonimmunogenic toxin similar to most phycotoxins and mycotoxins. To render it immunogenic, it is necessary to conjugate domoic acid to a protein carrier. Several approaches have been used to conjugate domoic acid to protein carriers for immunizing animals (*12*, *14*, *18*). Smith and Kitts (*12*) first reported the generation of polyclonal antibodies against domoic acid in mice; domoic acid was conjugated to either OVA or KLH via the carbodiimide method by linking the carboxyl groups of domoic acid to the amino groups of carrier proteins. Their ELISA method based on mouse polyclonal antibodies had a low sensitivity for domoic acid with a detection limit at 500 ppb (*13*). The carbodiimide method was initially used by us to conjugate domoic acid to KLH for antibody production in rabbit, but this approach was not successful. We demonstrate herein that domoic acid was conjugated to KLH by a combination of carbodiimide and NHS as immunogen for generating specific polyclonal antibodies for domoic acid. It is known that carbodiimide catalyzes the formation of amide bonds between carboxylic groups and amines, and NHS often assists carbodiimide coupling (*25*). Therefore, in this study the combination of carbodiimide and NHS greatly enhanced the coupling efficiency between domoic acid and KLH, which could then help generate high-affinity antibodies for domoic acid (*14*). Because the basic structure of domoic acid contains three reactive carboxylic groups, the carbodiimide coupling process includes the formation of stable intermediate active ester by condensation of the carboxylic groups at the domoic acid with the assistance of NHS, and this intermediate then reacts with a primary amine on KLH to form a stable amide bond (*23*, *25*).

The polyclonal antibodies produced from rabbits immunized with domoic acid−KLH have been shown to have a high affinity to domoic acid. Using these antibodies, a highly sensitive immunoassay was also developed. We found KLH to be a better carrier for generating antibodies against domoic acid than BSA. Antibodies using domoic acid−KLH as immunogen have a

weak cross-reactivity with kainic acid in both cdELISA and ciELISA (**Figure 3**). The result was similar to that of Kawatsu et al. (*18*), who reported that their domoic acid antibody had a cross-reactivity of 0.2 with kainic acid compared to a cross-reactivity of 100 with domoic acid.

The concentration of domoic acid causing 50% inhibition of binding of the marker enzymes (domoic acid−HRP) in the cdELISA was found to be 0.75 ng/mL. In the absence of matrix interference and based on 10% of inhibition of binding of domoic acid−HRP conjugate, the detection limit of domoic acid in the buffer solution of the cdELISA was found to be 0.02 ng/mL (**Figure 3A**). The cdELISA developed here showed a better sensitivity than that of Osada et al. (*26*), with a detection limit of 0.1 ng/mL, but similar to those of Kawatsu et al. (*21*) and Garthwaite et al. (*14*). The group of Smith and Kitts (*13*) was the first to demonstrate the utility of ciELISA to detect domoic acid levels in shellfish extracts. However, their assay relied upon the limited resource of serum from mice, and the detection limit of the assay is in the range of 500 ng/g. To develop an assay that could be widely used in shellfish screening, our studies are focused on the development of a cdELISA, which is more rapid and sensitive and less time-consuming than ciELISA. Results from the recovery studies of domoic acid in blue mussel showed that good recoveries were obtained at domoic acid levels >25 ng/g, suggesting that the detection limit of the present method is <25 ng/g.

The cdELISA established is tolerant of the interferences from shellfish matrix and extraction solvents. In our assay, shellfish extracts prepared in 50% methanol were diluted only 5-fold with 0.01 M PBS to sufficiently avoid both matrix and solvent effects. The ELISA results of mussel, clam, and oyster samples purchased from food stories in Taiwan showed that 7 of 10 mussel samples contained <50 ng/g of domoic acid, which is 400-fold lower than the regulatory limit of 20 μg/g (*2*, *3*). Clam and oyster samples examined were contaminated with domoic acid at <20 ng/g, which could be considered as domoic acid-free. However, mussel samples obtained from Portugal were reported to be contaminated with domoic acid at levels as high as 90 μg/g in whole mussel and 325 μg/g in digestive glands (*27*). Lefebvre et al. (*5*) also reported that humpback and blue whales were exposed to the toxin via consuming domoic acid-contaminated prey; fecal samples of whales were found to contain domoic acid at levels ranging from 10 to 207 μg/g using the HPLC-UV method.

Scholin et al. (*7*), who investigated the mortality of sea lions linked to domoic acid, found that the blue mussels collected during domoic acid outbreak contained no domoic acid or only trace amounts, suggesting that monitoring of mussels alone does not necessarily provide adequate warning of domoic acid entering the food chain at levels sufficient to harm marine wildlife and even humans. Dolphin and whale mortalities along the western coast of Taiwan are reported occasionally, but the cause of death has not been established conclusively. Collection of fecal or blood samples for domoic acid analysis may confirm whether these marine animals are exposed to domoic acid or not. Because only 15 shellfish samples were tested in the present study, further studies with a large number of samples harvested in different seasons are needed to evaluate the existence of domoic acid. In conclusion, a sensitive and effective cdELISA for domoic acid was developed for determination of the levels of domoic acid in shellfish samples; as low as 25 ng/g of domoic acid could be easily detected. This ELISA method could also be applied to screen a large number of 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; TMB, 3,3′,5,5′-tetramethylbenzidine.

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Received for review May 3, 2004. Revised manuscript received June 22, 2004. Accepted June 23, 2004. This work was supported by Grants DOH 92-TD-1083 from the Department of Health and NSC 90-2313-B-040-005 from the National Science Council of the Republic of China.

JF049303T