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Development of a Sensitive ELISA for the Determination of Microcystins in Algae

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Polyclonal antibodies for microcystin-leucine-arginine (MCYST-LR) were generated from rabbits after immunizing the animals with MCYST-LR conjugated with γ-globulin. 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 the toxin in algal cultures and dietary supplements. The concentrations causing 50% inhibition (IC_{50}) of binding of MCYST-horseradish peroxidase (MCYST-HRP) to the solid-phase antibodies by MCYST-LR, MCYST-arginine-arginine variant (MCYST-RR), MCYST-tyrosine-arginine variant (MCYST-YR), and nodularin (NODLN) in the cdELISA were found to be 0.10, 0.12, 0.14, and 0.20 ng/mL, respectively. In the presence of algae matrix, the detection limit is less than 10 ppb. The overall analytical recovery of MCYST-LR (25 to 500 ng/g) added to the algal dietary supplements and then extracted with 0.1 M ammonium bicarbonate in the cdELISA was found to be 83.7%. Analysis of MCYSTs in algal cultures and dietary supplements showed that six of eleven cultures produce MCYSTs, and five of the algal cultures were not MCYST producers. Eight of eleven tested commercial algal dietary supplements contained MCYSTs at a level lower than 100 ppb. The presence of MCYST-LR in the *Microcystis aeruginosa* culture was confirmed by high-performance liquid chromatography.

KEYWORDS: Enzyme-linked immunosorbent assay; ELISA; microcystins; antibodies

INTRODUCTION

Microcystins (MCYSTs) are a group of cyclic peptide hepatotoxins produced by several freshwater cyanobacteria including *Anabaena flos-aquae*, *Microcystis aeruginosa,* and *Oscillatoria agardhii* (*1*−*3*). Contamination of cyanobacteria in water has become a growing public health problem. Drinking of water containing MCYSTs has caused the death of wild and domestic animals worldwide (*4*), and has also been implicated in human fatalities (*5*). Toxicity of MCYSTs is associated with the specific inhibition of intracellular protein phosphatase 1 (PP1) and 2A both in vivo and in vitro. MCYSTs are found to specifically bind PP1 and PP2A in liver through both noncovalent and covalent binding (*6*−*10*). This group of low-molecular-weight cyclic peptides (824 to 1044 daltons) is made up of chemically stable molecules, and they cannot be destroyed or removed by conventional water purification methods (*1, 3*). However, a guideline for MCYSTs levels in drinking water has recently been introduced by the World Health Organization (*11*) with a recommended limit of 1 ppb of MCYST-LR equivalents for long-term exposure via drinking water. Potential health risks from exposure to toxins in dietary supplements made from algae have received little attention. The potential for MCYSTs exposure may be substantially greater for the consumers who use algae products (*12*). To help avoid the risks of human and animal exposure, it is essential to develop sensitive and specific methods for detection of MCYSTs.

Whereas a number of approaches have been developed for MCYST analysis, most methods have some disadvantages (*3*, *13*−*19*). Chromatographic methods provide good sensitivity, but they require highly qualified personnel, extensive sample cleanup, and expensive equipment (*20*). The protein phosphatase inhibition assay and mouse assay are toxicologically highly relevant, but they are not sensitive enough to detect toxin concentrations at ppb levels. Development of immunochemical approaches have led to more rapid and sensitive tools for monitoring, detection, and quantification of MCYSTs in contaminated algal foods and drinking water supplies (*3*, *14*−*16*, *19*, *21*−*23*). Nevertheless, the sensitivity and applicability of the immunodiagnostic approaches for MCYSTs still require further development to avoid algae matrix interferences and sample cleanup procedures. In the present study, a new method for the production of polyclonal antibodies against MCYSTs was developed, and a sensitive competitive direct ELISA (cdELISA) was established. Details for the production and characterization of these antibodies, as well as their use for

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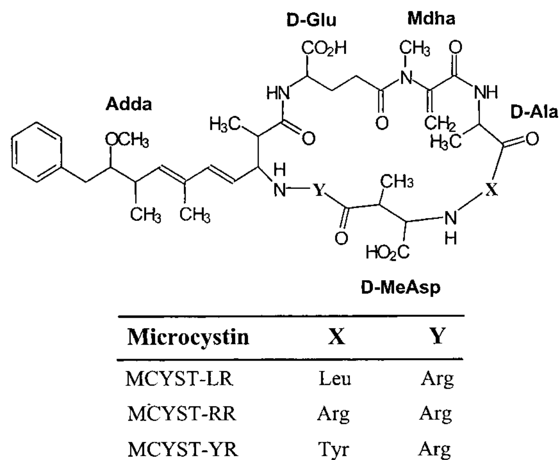

Microcystin	X	Y
MCYST-LR	Leu	Arg
MCYST-RR	Arg	Arg
MCYST-YR	Tyr	Arg

Figure 1. Chemical structures of microcystin-LR, -RR, and -YR. Mdha is the abbreviation for methyl-dehydro-alanine; and Adda is 3-amino-9-methoxy-2,6,8-trimethyl-10-penyl-deca-4,6-dienoic acid. The proposed site for conjugation of MCYST-LR to carrier proteins by carbodiimide is through the carboxylic group at D-MeAsp.

ELISA for MCYST in algae cultures and dietary supplements, are presented herein.

MATERIAL AND METHODS

Materials. MCYST-LR, MCYST-RR, MCYST-YR (**Figure 1**), NODLN, and okadaic acid were purchased from Calbiochem (San Diego, CA). Bovine serum albumin (BSA), keyhole limpet hemocyanin (KLH), gelatin, poly-lysine, γ-globulin, ovalbumin (OVA), ammonium bicarbonate, Tween 20, trifluoroacetic acid (TFA), *N,N*-dimethylformamide (DMF), 1-ethyl-3-[3-dimethyl-aminopropyl]-carbodimide (EDC), and *N*-hydroxysuccinimide (NHS) were obtained from Sigma Chemical Co. (St. Louis, MO). Goat anti-rabbit-peroxidase conjugate (ELISA grade) and horseradish peroxidase (HRP; ELISA grade) were obtained from Pierce Chemical Co. (Rockford, IL) and Boehringer Mannheim Biochemicals (Indianapolis, IN), respectively. HRP substrate solution 3,3′,5,5′-tetramethylbenzidine (TMB) was obtained from Kirkegaard & Perry Laboratories (KPL, Gaithersburg, MD). HPLC-grade acetonitrile and ammonium sulfate were obtained from Merck (Darmstadt, Germany). Female New Zealand white rabbits, 6−8 weeks old, 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 MCYST-LR Conjugates. *Conjugation of MCYST-LR to γ-Globulin and KLH*. MCYST-LR was conjugated to γ-globulin or KLH in the presence of a water-soluble carbodiimide, 1-ethyl-3[3-dimethylaminopropyl] carbodimide (EDC) and *N*-hydroxysuccinimide (NHS) under the following conditions. In a typical experiment, EDC solution (0.75 mg of EDC in 0.1 mL of *N,N*-dimethylformamide (DMF)) and NHS solution (0.75 mg of NHS in 0.05 mL DMF) were freshly prepared and added to a MCYST-LR solution (0.5 mg of MCYST-LR in 0.1 mL of DMF). The reaction was kept at room temp for 30 min and then kept at 4 °C overnight. The mixture was added slowly to either 2 mg of γ-globulin or KLH which was dissolved in 2 mL of 0.1 M pH 9.6 carbonate buffer. The reaction was carried out at room temperature for 2 h. Then the reacted mixture was dialyzed against 2 L of 0.01 M phosphate buffer pH 7.5 containing 0.15 M NaCl (PBS) for 72 h with two exchanges of this buffer, and then lyophilized.

Conjugation of MCYST-LR to Poly-Lysine for Indirect ELISA. MCYST-LR was conjugated to poly-lysine by the water-soluble carbodiimide (EDC) method for use as a solid-phase antigen for the indirect ELISA. In a typical reaction, 0.5 mg of MCYST-LR in 0.25 mL of 25% ethanol was mixed with 1.25 mg of poly-lysine to which 10 mg EDC in 0.2 mL of distilled water was 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 MCYST-LR-Peroxidase. Conjugation of MCYST-LR to peroxidase was achieved by a water-soluble carbodiimide (EDC) method similar to that described by Yu and Chu (*24*). Briefly, 0.4 mg of MCYST-LR in 0.8 mL of 25% ethanol was mixed with 8 mg of EDC. A horseradish peroxidase (HRP) solution (1 mg of HRP in 1 mL of 25% ethanol) was added dropwise to this solution, followed by the addition of 8 mg of EDC. After stirring this mixture at room temperature for 30 min, another 8 mg of EDC was added. The reaction was then carried out in a cold room (4 °C) overnight with stirring. The mixture was dialyzed as described above for 72 h against 0.01 M PBS and then lyophilized.

Production of Polyclonal Antibody. The schedule and methods of immunization were the same as those described previously (*25*). Two immunogens, MCYST-KLH and MCYST-γ-globulin, were tested in 4 rabbits, with 2 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 complete Freund adjuvant. For booster injections, the same amount of immunogen was mixed with an equal volume of incomplete Freund adjuvant and injected subcutaneously at 4 sites on the thigh of each rabbit. 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 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 ELISA (iELISA). The protocol for the indirect ELISA was similar to that described previously (*24*). In general, 0.1 mL of MCYST−polylysine conjugate (1 μg/mL in 0.01 M PBS) was added to wells of a microtiter plate (plate 2-69620; Nunc). The plate was kept at 4 °C overnight. After washing 4× with Tween−PBS (0.35 mL per well; 0.05% Tween 20 in 0.01 M PBS), 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 as described above, and 0.1 mL of diluted anti-MCYST-LR antiserum was added. After incubation at 37 °C for 1 h and washing of the plate, 0.1 mL of goat anti-rabbit IgG-HRP conjugate (1:20 000 dilution) was added. The plate was incubated at 37 °C for 45 min and then washed 4× with Tween−PBS and 0.1 mL of TMB substrate solution (1 mM 3,3′,5,5′-tetramethylbenzidine and 3 mM H_2O_2 per L of potassium citrate buffer, pH 3.9, a premixed solution supplied by KPL laboratory, Gaithersburg, MA) was added. The reaction was stopped by addition of 0.1 mL of 1 N hydrochloric acid after 10 min of incubation at room temperature. Absorbance at 450 nm was determined in an automatic ELISA reader (Vmax Microplate Reader, Molecular Devices Co., Menlo Park, CA).

Competitive Direct ELISA (cdELISA). The protocol for the competitive direct ELISA (cdELISA) was essentially the same as that described previously for fumonisin B_1 (*24*), except that in the antibody coating step, the antibody was diluted in 0.01 M PBS (1:1000 dilution, 10 μg/mL, with 0.1 mL coated onto each well). After the plate was incubated at 4 °C overnight, the plate was washed with PBS−Tween (0.35 mL per well; 0.05% Tween 20 in 0.01 M PBS) 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 4×, followed by incubation with different MCYST analogues (0.05 mL per well in 0.01 M PBS) at different dilutions or blank buffer together with the MCYST−HRP conjugate (1:1000 dilution, 25 ng/mL, in 0.01 M PBS, 0.05 mL per well) at 37 °C for 50 min. The plate was washed 4× 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 MCYST-LR Added to Algal Dietary Supplements by cdELISA. An analytical recovery study was carried out to test the efficacy of cdELISA for the analysis of MCYST in algal samples. In this study, 2 g each of ground algae dietary supplement products, which were purchased from health food stores in Taiwan and previously found negative for MCYST by ELISA, were spiked with MCYST-LR at concentrations ranging from 10 to 500 ppb. A control sample with no toxin added was used as the blank. After storage at 4 °C for 1 day, each of the samples was homogenized with 20 mL of 0.1 N ammonium bicarbonate for 3 min and then centrifuged at 10 000 rpm for 20 min (*15*). The supernatant solution, after dilution with 0.01 M PBS, was subjected directly to cdELISA. At least two analyses were performed for each sample, and triplicates were run for each analysis.

cdELISA of Cyanobacteria Cultures. Ten clones of *Microcystis aeruginosa* Kutzing and one of *Coleosphaerium kuetzingianum* Naegeli were cultured from single cells which were isolated from various freshwater ponds and reservoirs at different dates in Taiwan. They are identified according to the species described in *Plankton Algae of Reservoirs in Taiwan* (*26*), were cultured in modified Fitzgerald media (*27*) at 23 ± 1 °C, and illuminated with fluorescent light of 26.4 μEin·m^{-2}s^{-1} for 12 h/d. Cell masses were harvested in their late exponential phase of growth and lyophilized for toxin analysis. Briefly, 200 mg of lyophilized algal cultures (in 100 mL) were extracted with 2 mL of 0.1 N ammonium bicarbonate using the procedures described above. The supernatant solution was diluted up to 10 000-fold with 0.01 M PBS before cdELISA.

cdELISA of Algal Dietary Supplements Naturally Contaminated with MCYST. Eleven brand products of algal dietary supplement obtained from health food stores in Taiwan were used to test the efficacy of ELISA for MCYST. Briefly, each sample (2 g) was homogenized with 20 mL of 0.1 N ammonium bicarbonate for 3 min. After centrifugation at 10 000 rpm for 20 min, the supernatant solution was diluted with 0.01 M PBS and directly subjected to cdELISA.

HPLC of MCYSTs. For determination of MCYSTs, authentic MCYST-LR and a representative *Microcystis* culture extract were subjected to HPLC analysis according to Lee (*28*). A Beckman System Gold (Fullerton, CA) equipped with a 126 solvent module and a 168 photodiode array (PDA) detector was used. The MCYST-LR standard and the culture extract were diluted with an appropriate amount of 0.01 M PBS and passed

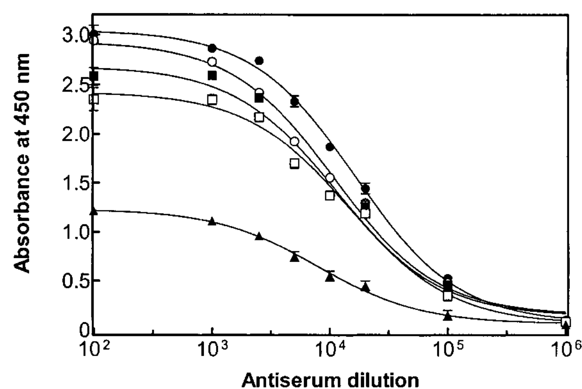

Figure 2. Determinant of antibody titers for a representative rabbit after immunization with MCYST-LR-γ-globulin by an MCYST-LR-PLL-based indirect ELISA. The antiserum were obtained 0 (▲), 6 (□), 9 (■), 12 (○), and 18 (●) weeks after immunization.

through a 0.45-μM filter (low protein binding; Gelman Science, MI) prior to HPLC. A Lichrospher C18 reverse-phase column (5-μm particle size, 4.0 mm × 250 cm; Merck) in conjunction with a Lichrospher C18 guard column (5-μm particle size, 4.0 mm × 4.0 mm; Merck) was equilibrated with a mobile phase consisting of solvents (acetonitrile/water/trifluoroacetic acid (TFA), 50:50:0.05, v/v) at a flow rate of 1 mL/min. After injection of 20 μL of samples, an isocratic elution with acetonitrile/water/TFA (50:50:0.05, v/v) was applied over a period of 20 min. The chromatograms were monitored at 238 nm by the PDA detector and the absorbance data were analyzed with Beckman System Gold Nouveau software.

RESULTS

Production of Polyclonal Antibodies. Sera collected from rabbits immunized with MCYST-γ-globulin or with MCYST-KLH were subjected to the indirect ELISA. Typical titration curves of antibody titers obtained from a MCYST-γ-globulin immunized rabbit over a period of 18 weeks are shown in **Figure 2**. Antibodies against MCYST-LR 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 around 18 weeks after immunization. The antibody titers of the rabbits immunized with MCYST-KLH were found to be considerably lower than those immunized with MCYST-γ-globulin (data omitted).

Characterization of Antibodies. Both the cdELISA and ciELISA were used to determine the specificity of antibodies. Because the antibody amount required to coat the microtiter plates in the cdELISA was much less for the antiserum obtained from rabbits immunized with MCYST-γ-globulin than those immunized with MCYST-KLH (data not shown), the antiserum obtained from rabbits immunized with MCYST-γ-globulin was used in the subsequent studies. In the cdELISA, the concentrations causing 50% inhibition (IC_{50}) of binding of MCYST-HRP with the antibodies by MCYST-LR, MCYST-RR, MCYST-YR, and nodularin were found to be 0.10, 0.12, 0.14, and 0.19 ng/mL, respectively (**Figure 3**). The relative cross-reactivity of the antibodies to MCYST-LR, MCYST-RR, MCYST-YR, and nodularin was calculated to be 100, 83, 71, and 53, respectively. Such results indicate that the antibody has similar affinity for MCYST-LR, MCYST-RR, and MCYST-YR. However, the affinity toward nodularin was less. Similar results were obtained in the ciELISA in which MCYST-polylysine was coated to the wells of ELISA plates to serve as solid-phase antigen (**Figure

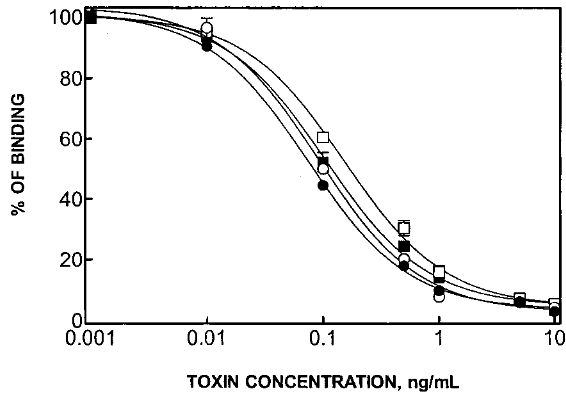

Figure 3. Cross-reactivity of anti-MCYST antibodies with MCYST-LR (●) MCYST-RR (○), MCYST-YR (■), and Nodularin (□) in a competitive direct ELISA (cdELISA). The microtiter plate wells were each coated with 0.1 mL of rabbit antiserum (bleeding at 18 weeks with 1:1000 dilution, 10 μg/mL). MCYST-LR-HRP (50 μL, 1:1000 dilution, 250 ng/mL) plus 0.05 mL of standard toxin was used in each assay. The concentration causing 50% inhibition of binding of MCYST-LR-HRP to the antibodies by MCYST-LR (●) MCYST-RR (○), MCYST-YR (■), and Nodularin (□) in the cdELISA were 0.10, 0.12, 0.14, and 0.19 ng/mL, respectively. All data were obtained from the average of three sets of experiments. The absorbance of the control, A_0, with no toxin present, was 1.2.

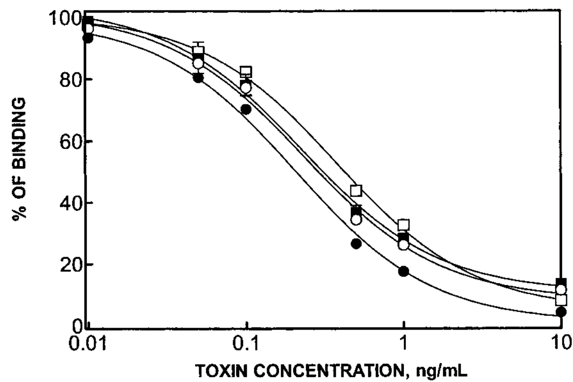

Figure 4. Cross-reactivity of anti-MCYST antibodies with MCYST-LR (●) MCYST-RR (○), MCYST-YR (■), and Nodularin (□) as determined by an competitive indirect ELISA (ciELISA). The microtiter plate wells were each coated with 0.1 mL of MCYST-LR-PLL (1 μg/mL). The binding of 0.05 mL of rabbit antiserum (1:5000 dilution, 1.5 μg/mL) to the solid-phase MCYST-LR-PLL in the presence of MCYST-LR, MCYST-RR, MCYST-YR, and Nodularin (0.05 mL per well) standard toxin was determined by goat anti-rabbit HRP conjugate (1:20 000 dilution, 0.1 mL per well). The concentration causing 50% inhibition of binding of MCYST-LR-PLL to the antibodies by MCYST-LR (●) MCYST-RR (○), MCYST-YR (■), and Nodularin (□) in the ciELISA were 0.21, 0.28, 0.30, and 0.42 ng/mL, respectively. 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.

4). The concentrations causing 50% inhibition of binding of antibodies to the solid-phase MCYST−polylysine by free MCYST-LR, MCYST-RR, MCYST-YR, and nodularin were found to be 0.21, 0.28, 0.30, and 0.42 ng/mL, respectively. The relative cross-reactivity of the antibodies to MCYST-LR, MCYST-RR, MCYST-YR, and nodularin in the ciELISA was calculated to be 100, 75, 70, and 50, respectively. Okadaic acid, a potent PP1 and PP2A inhibitor (*29*), at a concentration of 500 ng/mL did not inhibit the binding of the marker antigen with the antibodies in either ELISA.

Table 1. cdELISA Analysis and Recovery of MCYST-LR Added to Algal Samples

	analytical recovery			
MCYST-LR[a] added (ng/g)	ng/g	%	SD	CV%
10	12.4	124	2.1	16.8
25	23.1	92.4	2.9	12.5
50	41.3	82.6	4.7	11.4
100	84.5	84.5	7.8	9.2
500	376.8	75.4	24.7	6.6
overall[b]		83.7		9.9

[a] Each toxin level had two samples and each sample was run in triplicate. [b] Because high recovery was observed at 10 ng/g, this level was excluded from final calculation.

Table 2. ELISA Analysis of MCYSTs in *Microcystis aeruginosa* and *Coleosphaerium kuetzingianum* Cultures

cyanobacterial strain	MCYSTs (μg)[a]	MCYST (ppm)	ng/cell[b]
	Microcystis aeruginosa		
1. MTY-1	214.6	1073	2×10^{-4}
2. MTY-2	76.4	382	3×10^{-5}
3. MYL-1	ND[c]	ND	ND
4. MCY-1	29.8	149	3×10^{-5}
5. MTN-1	ND	ND	ND
6. MTN-2	293.2	1466	3×10^{-4}
7. MTN-3	111.0	555	8×10^{-5}
8. MTN-4	118.6	593	8×10^{-5}
9. MTN-5	ND	ND	ND
10. MKS-1	ND	ND	ND
	Coleosphaerium kuetzingianum		
1. CTN-1	ND	ND	ND

[a] 0.2 g of lyophilized algal cultures. [b] Algal cells were counted by cell counter at harvest time. [c] ND, not detectable.

Analytical Recovery of MCYST-LR Added to Algal Dietary Supplement by cdELISA. Results for the analytical recovery of MCYST-LR added to the algae samples by cdELISA are presented in **Table 1**. The analytical recovery for the sample spiked with MCYST-LR at a level of 10 ng/g was more than 100%. Therefore, data from this group was excluded from the calculation of the overall analytical recovery. In the concentration range of 25 to 500 ng/g, the overall average of analytical recovery for the samples was found to be 83.7% (CV, 9.9%).

Analysis of MCYSTs in Cyanobacteria Cultures with cdELISA. To test the efficacy of cdELISA for MCYSTs in cyanobacteria isolates and related algal products, various clones of *Microcystis* cultures were subjected to cdELISA. A wide range of levels of MCYSTs was detected in these cultures by cdELISA and the results are presented in **Table 2**. Six of the eleven strains tested were found to be MCYSTs producers, but 5 were not. Two of the heavy producers, MTY-1 and MTN-2, yielded as much as 1073 ppm (2×10^{-4} ng/cell) and 1466 ppm (3×10^{-4} ng/cell), respectively. MTN-3 and MTN-4 produce lower amounts of toxins with 555 ppm (8×10^{-5} ng/cell) and 593 ppm (8×10^{-5} ng/cell), respectively; MTY-2 and MCY-1 produce the least amounts of toxins at concentrations of 382 ppm (3×10^{-5}) and 149 ppm (3×10^{-5}), respectively.

Analysis of MCYSTs in Algal Dietary Supplement Products with cdELISA. Eleven algal dietary supplement products with *Spirulina*, blue-green algae, or *Chlorella* as major components were collected from health food stores and subjected to cdELISA for MCYSTs. The results are presented in **Table 3**. Eight of the eleven samples were found to be MCYSTs positive with the levels below 78 ppb. All of the examined

Table 3. ELISA Analysis of MCYSTs in Algae Dietary Supplements

major components	source	MCYSTs (ng/g)
Spirulina		
1. Spirulina powder	America	78 ± 9
2. Life Vital Spirulina (tablet)	Australasia	36 ± 6
3. Spirulina (capsule of powder)	America	28 ± 4
Blue-green algae		
4. Blue-Green Algae (powder)	America	48 ± 7
5. SPT-500 (blue green algae)	Asia	51± 10
Chlorella		
6. Algae tablet	Asia	36 ± 5
7. Bio-Green Algae (capsule)	Europe	<20
8. Green Gen (tablet)	Asia	< 20
9. Chlorella (tablet)	Asia	ND[a]
10. NaturaLife Chlorella (capsule)	America	ND
11. Green Shine (tablet)	Asia	ND

[a] ND, not detectable.

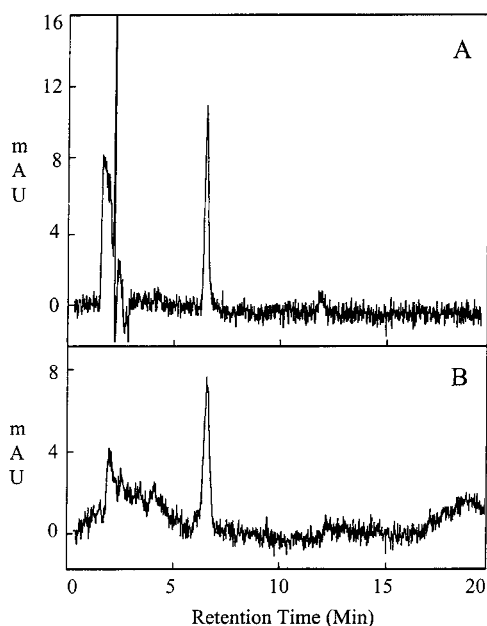

Figure 5. HPLC of a standard MCYST-LR and a *M. aeruginosa* culture (MTY-1) extract. Figure 5A was obtained from 0.2 μg of standard MCYST-LR, the peak at a retention time of 6.7 min was MCYST-LR. Figure 5B was obtained from culture MTY-1, the peak at a retention time of 6.6 min was identified as MCYST-LR (equal to 0.28 μg). The chromatographic conditions are isocratic acetonitrile/water/TFA (50:50:0.05, v/v) over a period of 20 min and monitored at 238 nm by a Beckman System Gold 168 photodiode array detector.

Spirulina and blue-green algae products contained MCYSTs in various amounts. Among them, sample 1 was found to have the highest level of MCYSTs at 78 ppb. Among the six *Chlorella* products, sample 6 contained measurable MCYSTs of 36 ppb, and two others, samples 7 and 8, had less than 20 ppb. Three of the remaining *Chlorella* products were found to be MCYSTs-free.

Confirmation of the Presence of MCYST-LR by HPLC. HPLC chromatograms for the analysis of MCYST-LR standard and MCYST-LR in *M. aeruginosa* culture (MTY-1) sample are shown in **Figure 5A** and **B**, respectively. MCYST-LR was well identified under the isocratic elution. The extraction of MTY-1 showed a major peak with a retention time of 6.6 min, which is comparable with that of standard MCYST-LR, which had a retention time of 6.7 min. Some small peaks, which may be associated with other MCYST-LR toxin analogues, were also detected.

DISCUSSION

Like other phycotoxins and mycotoxins, MCYSTs are low-molecular-weight nonimmunogenic toxins. To render them immunogenic, it is necessary to conjugate them to a protein carrier. Several approaches have been used to conjugate MCYSTs to protein carriers for immunizing animals (*3*). For the production of antibodies against MCYSTs, most immunogens were prepared via the carbodiimide method by linking MCYST-LR to KLH or BSA (*3*, *15*, *16*, *19*). MCYST-LR modified with 2-methyldehydroalanine was cross-linked to KLH via glutaraldehyde by Metcalf et al. (*18*). *N*-Methyldehydroalanine, a core amino acid of MCYST-LR, was chemically modified for linking to BSA or soybean trypsin inhibitor (*22*). The sensitivity of immunoassays by using antibodies developed from immunogens prepared via the carbodiimide method are in the nanogram range, whereas that of the immunoassays using antibody generated from immunogens via other methods are in the microgram range. It is known that carbodiimide catalyzes the formation of amide bonds between carboxylic group and amines, and NHS is often used to assist carbodiimide coupling (*30*). Therefore, in this study, the combination of carbodiimide and NHS greatly enhances the coupling efficiency between MCYST-LR and γ-globulin, which could then generate high-affinity antibodies for MCYST-LR. Because the basic structure of MCYST-LR contains one reactive carboxylic group at the MeAsp site, the carbodiimide coupling reaction includes the formation of the stable intermediate active ester by condensation of the carboxylic group at the MeAsp and NHS; and then this intermediate reacts with a primary amine on γ-globulin to form an amide bond (*30*).

The polyclonal antibodies produced from rabbits immunized with MCYST-γ-globulin in the present study have been shown to have high affinity to MCYST-LR. Using these antibodies, a highly sensitive immunoassay has been developed. We found γ-globulin to be a better carrier than KLH for generating antibodies against MCYST-LR. These antibodies also have a good cross-reactivity with MCYST-RR and MCYST-YR, both in cdELISA and ciELISA. The concentrations of MCYST-LR causing 50% inhibition of binding of the marker antigens in the cdELISA (MCYST-HRP) and ciELISA (MCYST-PLL) were found to be 0.10 and 0.21 ng/mL, respectively (**Figures 3** and **4**). The sensitivity of the present ELISA methods is about 2 orders of magnitude more than that of the monoclonal antibody-based ELISA reported by Mikhailov et al. (*22*) and Nagata et al. (*19*), but similar to that of Huang and Chu (*31*). In addition, we found that the polyclonal based ELISA established in this study is more tolerate to the interferences from algae matrix and extraction solvents.

Because the cdELISA is more sensitive and less time-consuming than the ciELISA, our studies were focused on the efficacy of cdELISA. Results from the recovery studies showed that the data at 10 ppb levels are questionable, because the analytical recoveries were more than 100%. Nevertheless, good recoveries were obtained at levels above 25 ppb, which suggests that the detection limit of the present method should fall between 10 and 25 ppb. In the absence of matrix interference, and based on 20% of inhibition of binding of MCYST-LR−HRP conjugate, the detection limit of MCYST-LR in buffer solution of the cdELISA was found to be around 20 pg/mL.

Because of the high sensitivity of the present cdELISA, extracts of algae cultures and algal dietary supplements can be used directly in the ELISA without any cleanup treatment. The ELISA results of *Microcystis* cultures showed that 6 of 10 clones were MCYSTs producers in the range of 149 to 1466 ppm. The

fact that MTY-1 and MTN-2 of the tested *Microcystis* cultures contain the highest amounts of MCYSTs is consistent with results obtained from toxicity bioassay and HPLC analysis by Lee (*28*). ELISA results of algal dietary supplement products indicate that most of the examined samples were contaminated with low levels of MCYSTs including the algae such as *Spirulina*, which normally is not a MCYST producer. The contamination levels and the frequency of MCYSTs described here were considerably lower than those documented by Gilroy et al. (*12*). They reported that MCYSTs were detected in 85 of 87 blue-green algae samples from Upper Klamath Lake (UKL) in southern Oregon, with 63 samples containing toxins at concentrations higher than 1 μg/g. Such discrepancy is not surprising because most of the samples collected by Gilroy et al. (*12*) were raw blue-green algae products, which were obtained at the peak of toxic blooms of *M. aeruginosa* at UKL during the harvesting of *Aphanizomenon flos-aquae* for dietary supplement products. However, the present results show that even the *Spirulina* products are not immune to the contamination of toxic blue-green algae that produce MCYSTs.

There is an increasing concern that chronic exposure to low levels of MCYSTs through dietary supplements and drinking water may pose a significant health risk to the general public. MCYSTs are considered to exert their toxic effects by promoting tumor formation through the inhibition of PP1 and PP2A, which are integrally involved in cell-cycle regulation (*32*, *33*). Falconer (*34*) has pointed out that consumption of MCYSTs by mice has produced progressive liver injury with leucocyte invasion and hepatocyte death. In addition, bioaccumulation of MCYSTs has also been demonstrated in laboratory animals (*35*). These results suggest that long-term exposure to even very low levels of MCYSTs may ultimately result in liver cancer and other liver diseases. Thus, a sensitive and specific monitoring method to evaluate the risks to humans and animals from exposure to these toxic compounds is urgently required. In conclusion, a sensitive and effective cdELISA for MCYSTs was developed for determination of the levels of MCYSTs in cyanobacteria cultures and algal dietary supplements; as low as 20 ppb of MCYSTs could easily be detected. This ELISA method could also be applied in large numbers of samples, including drinking water, without sample cleanup treatment.

ABBREVIATIONS USED

Adda, 3-amino-9-methoxy-2,6,8-trimethyl-10-penyl-deca-4,6-dienoic acid; BSA, bovine serum albumin; DMF, *N,N*-dimethylformamide; 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; HPLC, high-performance liquid chromatography; KLH, keyhole limpet hemocyanin; MCYST-LR, microcystin-leucine-arginine; MCYST-RR, MCYST-arginine-arginine; MCYST-YR, MCYST-tyrosine-arginine; Mdha, methyl-dehydro-alanine; NHS, *N*-hydroxysuccinimide; NODLN, nodularin; OVA, ovalbumin; PP1, protein phosphatase 1; TFA, trifluoroacetic acid; TMB, 3,3′,5,5′-tetramethylbenzidine.

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