RESIDUES AND TRACE ELEMENTS

Enzyme Immunoassay for Direct Determination of Microcystins in Environmental Water

SATOSHI NAGATA [1], TOMOAKI TSUTSUMI, AKIHIRO HASEGAWA, FUYUKO YOSHIDA, and YOSHIO UENO [2]
Science University of Tokyo, Faculty of Pharmaceutical Sciences, Department of Toxicology and Microbial Chemistry, 12 Ichigaya Funagawara-machi, Shinjuku-ku, Tokyo 162, Japan
MARIYO F. WATANABE
Tokyo Metropolitan Research Laboratory of Public Health, 3-24-1, Hyakunin-cho, Shinjuku-ku, Tokyo 160, Japan

An enzyme-linked immunosorbent assay (ELISA) was developed for direct quantitation of microcystins (MCs), a group of freshwater cyanobacterial toxins. An anti-MC monoclonal antibody exhibiting broad cross-reactivity to major MC derivatives was used. The detection limit and linear range of the ELISA standard curve with microcystin-(leucine–arginine) (MCLR), a variant of MCs, were 20 and 20–500 pg/mL, respectively. For analysis of MC released from cyanobacterial cells, water sample filtered through a glass fiber filter was applied directly to ELISA. For analysis of total MC (released MC plus intracellular MC), intracellular toxin was extracted by freeze-thawing twice before filtration. Mean recovery of MCLR added to tap water and toxin-free environmental water was 101%, with a coefficient of variation (CV) of 7.3% at toxin levels of 20–500 pg/mL. Mean recovery of MCLR added to toxin-free cyanobacterial extracts was 93%, with a CV of 12.5% at toxin levels of 50–500 pg/mL. At 20 pg/mL, an increasing matrix effect on assay variance was observed; therefore, both released MC and total MC were measured in the range 50–500 pg/mL. Comparative studies with a liquid chromatographic (LC) method showed that the ELISA gives a reliable correlation with LC for analysis of MC in water extracts of natural blooms and cultured cyanobacterial cells (r = 0.98). The ELISA was applied to water samples collected from lakes and ponds in Japan. In 4 of 13 and 12 of 17 samples, 81–800 pg released MC/mL and 64–94 000 pg total MC/mL were detected, respectively. By LC separation followed by the ELISA analysis, the presence of MCLR, microcystin-arginine–arginine, and microcystin-tyrosine–arginine were confirmed in 4 ELISA-positive samples selected randomly. The newly developed ELISA is a reliable and powerful method for mass monitoring of MC levels in environmental water.

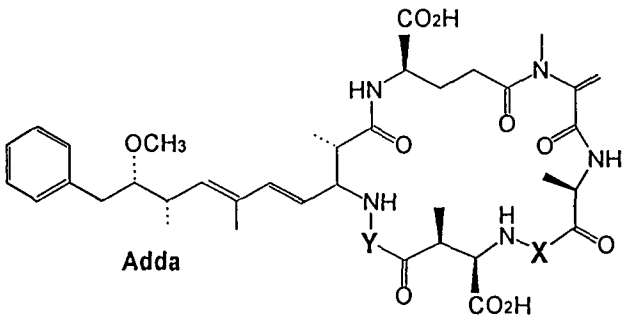
Various microbial secondary metabolites have acute or chronic toxic effects on humans and animals (1, 2). We focused our attention on such natural toxic compounds, especially on food-born mycotoxins, and reported novel immunological assays for aflatoxins (3, 4), ochratoxin A (5), trichothecenes (6), and fumonisins (7). In this paper, we deal with a cyanobacterial toxin, microcystins (MCs).

MCs are a group of hepatotoxic cyclic heptapeptides produced by bloom-forming cyanobacteria such as *Microcystis aeruginosa*. With the increasing cultural eutrophication of environmental waters, cyanobacterial blooms containing MCs often represent health hazards to animals and humans in many regions of the world (8–10). Over 50 MCs isolated so far consist of a common moiety composed of 7 amino acids, including 2 variable L-amino acids and a unique amino acid called Adda (3-amino-9-methoxy-2,6,8-trimethyl-10-phenyldeca-4,6-dienoic acid; 8, 10, 11). Structures of microcystin-LR (MCLR), an MC containing leucine (L) and arginine (R); microcystin-RR (MCRR), and microcystin-YR (MCYR; MC containing tyrosine [Y] and R) are shown in Figure 1.

Recently, MCLR was found to inhibit protein phosphatases 1 and 2A (12, 13) and to exhibit tumor-promoting activity in rats (14). Thus, MCs are possible environmental tumor promoters, as reported in Qidong and Haimen County in China (15, 16). At present, tentative long-term guideline levels of MCLR in drinking water of 0.1 μg/L (17) and 0.5 μg/L (18) were proposed. Actual and potential hazards of MCs emphasize the need for methods to detect this toxin in water supplies (9, 19). Liquid chromatography with UV detection (LC-UV) is now most widely used to determine MCs. However, its low sensitivity restricts analytical samples to extracts of cyanobacterial cells or

Received August 16, 1996. Accepted by JS November 16, 1996.
Some results were presented at the 20th Symposium on Toxicology and Environmental Health, September 21–22, 1994, Tokyo, Japan; the 1st International Congress on Toxic Cyanobacteria (Blue-green Algae), August 20–24, 1995, Rønne, Denmark; and the 3rd International Conference on Food Safety and Quality Assurance, November 7–9, 1995, Canberra, Australia.

[1] Present address: Laboratory of Biological Chemistry, Department of Applied Biological Chemistry, Faculty of Agricultural and Life Science, University of Tokyo, 1-1-1 Yayoi, Bunkyo-ku, Tokyo 113, Japan.
[2] Author to whom correspondence should be addressed.



Microcystins	X	Y
Microcystin LR	Leu	Arg
Microcystin RR	Arg	Arg
Microcystin YR	Tyr	Arg

Figure 1. Structures of microcystins.

highly concentrated water (20, 21). Other physicochemical methods (22–25) are available, but they are not suitable for direct quantitation of MCs in natural water. In addition, the methods are time-consuming and require expensive equipment.

Highly sensitive, rapid, and inexpensive methods that can screen for MC in environmental water must be developed. Two methods have been reported as potential approaches: enzyme-linked immunosorbent assay (ELISA) using anti-MC antibodies (26, 27) and protein phosphatase inhibition assay (28–31).

Recently, we produced novel monoclonal antibodies (MAbs) against MCLR. Of these MAbs, M8H5, which shows the highest affinity to MCLR (affinity constant, 3.1×10^{10}), exhibits wide cross-reactivity with MC derivatives (32). In this paper, we report a highly sensitive and reliable ELISA using this widely reactive MAb.

Experimental

Apparatus

(a) *ELISA washer.*—96-well automatic washer, ImmunoWasher NK-300 (Japan InterMed, Tokyo, Japan).

(b) *ELISA reader.*—Microplate reader MR5000 (Dynatec Laboratories, Inc., Chantilly, VA).

(c) *Adjustable pipettes.*—Pipetman P-10, P-20, P-100, P-200, P-1000 (Gilson, Middleton, WI); Finnpipette 12-channel, 50–250 μL (Labsystems, Helsinki, Finland).

(d) *ELISA plates.*—Polystyrene 96-well plates, Immunoplate Maxi Sorp (Nunc, Inc., Roskilde, Denmark).

(e) *Plastic tubes.*—Various sizes of polypropylene tubes for sample preparation and stock, 1.5 mL microcentrifuge test tubes (Treff Lab., Schweiz, Switzerland), 2 mL Slim tubes (Sumitomo Bakelite Co., Ltd., Tokyo, Japan), and 15 and 40 mL sample cups (Corning, Corning, NY).

(f) *Filter paper.*—Glass microfiber filters GF/C, 2.5 cm id (Whatman International Ltd., Maidstone, UK).

(g) *Liquid chromatograph.*—Type L-6000 pump (Hitachi, Ltd., Tokyo, Japan), L-4200 UV-Vis detector (Hitachi), D-2500 Chromato-Integrator (Hitachi).

(h) *Chromatographic column.*—Capcell Pak C_{18} UG120, 4.6 × 250 mm (Shiseido Corp., Ltd., Tokyo, Japan).

Chemicals

MCLR was isolated with 95% purity by LC analysis from natural blooms of *M. aeruginosa* collected from Lake Suwa, Japan, according to a method reported previously (20). MCRR, MCYR, and 7-desmethyl MCLR (7-D MCLR) were provided by Ken-Ichi Harada, Meijo University, Japan. Gelatin, 3,3′,5,5′-tetramethylbenzidine (TMBZ), Tween 20, and 30% hydrogen peroxide were from Wako Pure Chemical Co. (Tokyo, Japan). Bovine serum albumin (BSA) was from Sigma Chemical Co. (St Louis, MO). 1-Ethyl-3,3′-diethylaminopropylcarbodiimide (EDPC) was from Pierce Chemical Co. (Rockford, IL). Octadecyl silanized (ODS) silica gel cartridge (Waters Sep-Pak Plus C_{18}) was obtained from Millipore Corp. (Milford, MA). Horseradish peroxidase (HRP)-labeled affinity-purified goat anti-mouse immunoglobulin G (IgG) (Tago 6450) was from Tago, Inc. (Burlingame, CA). Methanol (LC grade) was from Kanto Chemical Co., Inc. (Tokyo, Japan). All other chemicals were reagent grade.

Immunoassay Reagents

(a) *Water.*—Deionized and run through a MilliQ reagent water system (Millipore Corp.).

(b) *Phosphate-buffered saline (PBS).*—Phosphate buffer (1.5 mM KH_2PO_4, 8.1 mM Na_2HPO_4), pH 7.4, containing 0.137M NaCl and 2.7 mM KCl.

(c) *Coating antigen.*—MCLR–BSA conjugate was prepared with EDPC as described earlier (32); the previously determined molar ratio of MCLR to BSA was 1.3. The conjugate solution (3 mg/mL in PBS) was stored at 4°C.

(d) *PBS–Tween.*—0.05% (v/v) Tween 20 in PBS.

(e) *Blocking solution.*—0.5% (w/v) gelatin in PBS, containing 0.1% (w/v) NaN_3.

(f) *Sample diluent.*—0.005% (w/v) gelatin in water, containing 0.1% (w/v) NaN_3.

(g) *Standard solutions.*—About 10 mg MCLR was dissolved in 10 mL methanol, and the concentration was adjusted to 1 mM spectrophotometrically (log ε = 4.60; 238 nm) (33). This solution was diluted to 10 μM in blocking solution and stored at −80°C. MCRR, MCYR, and 7-D MCLR standards in methanol were prepared in the same manner. Working standards of 0, 2, 20, 50, 100, 200, 500, and 2000 pg MCLR/mL were prepared on the day of assay by diluting the stock solution in sample diluent. Methanol concentrations in standard solutions were <0.0002%.

(h) *Monoclonal antibody to microcystin.*—Hybridoma cells producing the anti-MCLR MAb M8H5 had been established previously (32). Cell culture supernatant was used as the MAb stock. Hybridoma cells were grown in Iscove's modified Dulbecco's medium supplemented with 10% (v/v) fetal bovine serum; culture supernatant containing MAb (100 μg IgG/mL) was stored at 4°C after addition of 0.1% (w/v) NaN_3. This MAb

shows extensive cross-reactivity to various related compounds; on the basis of concentrations able to cause 50% inhibition of antibody binding in a competitive ELISA, cross-reactivities were 100, 109, 44, 26, 51, 48, and 20% for MCLR, MCRR, MCYR, MC-(leucine–alanine), 3-desmethyl MCLR, 7-D MCLR, and nodularin, respectively (32).

(**i**) *Monoclonal antibody dilution buffer.*—Phosphate buffer (3.0 mM KH_2PO_4, 16.2 mM Na_2HPO_4), pH 7.4, containing 0.276M NaCl, 5.4 mM KCl, 1% (w/v) gelatin, and 0.1% (w/v) NaN_3.

(**j**) *Enzyme conjugate dilution buffer.*—0.5% (w/v) gelatin in PBS–Tween.

(**k**) *Substrate buffer.*—0.1M Sodium acetate buffer, pH 5.0.

(**l**) *Enzyme substrate solution.*—Stock solutions of TMBZ (10 mg/mL in dimethyl sulfoxide) and H_2O_2 [0.5% (v/v) in water] were stored at −80°C. Substrate solution was prepared just before use by dissolving both 100 μL TMBZ and 100 μL H_2O_2 in 10 mL substrate buffer.

(**m**) *Stopping solution.*—2N H_2SO_4.

Sampling and Sample Preparation

(**a**) *Environmental freshwater.*—Water (40 mL) was collected in a polypropylene tube (Corning 1730) from the surface of various Japanese lakes or ponds, and 0.1% (w/v) NaN_3 was added as a preservative. A part of the sample (5 mL) was separated, and 2% (v/v) formaldehyde was added for microscopic examination of cyanobacterial cells. To determine MC released into the water, a part of the sample (5 mL) was filtered through a glass fiber filter just after sampling. To determine total MC (released MC plus intracellular MC), remaining samples were freeze-thawed twice to extract intracellular MC into the surrounding water, which was then filtered through a glass fiber filter. Water samples except those fixed with formalin were stored at −20°C.

(**b**) *Artificial MC-contaminated water.*—To compare ELISA with LC-UV, water samples contaminated with microgram levels of MCs were artificially prepared by water extraction of cyanobacterial cells. Natural bloom samples collected from Japanese lakes and ponds by plankton net during 1989–1994 and cultured cells of *Microcystis* strains (34) were lyophilized and stored at −20°C prior to extraction. MCs in 100 mg dried samples were extracted by rehydration in 10 mL water followed by 2 cycles of freeze-thawing. Some samples were extracted also with 10 mL 5% (v/v) acetic acid instead of water. After filtration through a glass fiber filter, 10 μL extract was appropriately diluted with sample diluent (>2000-fold) and analyzed by ELISA. Remaining extracts were cleaned up for LC analysis.

(**c**) *Sample cleanup for LC.*—For LC-UV, artificial MC-contaminated waters were cleaned up with an ODS cartridge (20, 35). Each extract (10 mL) was adjusted to pH 6.0 by addition of 2 mL 0.1M citric acid—0.2M Na_2HPO_4 buffer (pH 6.0, 37 + 63) and applied to the ODS cartridge preconditioned with methanol. Cartridges were washed with water (10 mL) and 20% (v/v) methanol (15 mL). The material eluting from the cartridge with 90% (v/v) methanol was evaporated to dryness, and the residue was dissolved in 0.5 mL methanol. Prepared samples were applied directly to the liquid chromatograph or diluted with sample diluent (>28000-fold) and then analyzed by ELISA.

Indirect Competitive ELISA

(**a**) *Plate coating.*—MCLR–BSA conjugate at 100 pg/mL in PBS (1:30 000 dilution of stock) was absorbed onto microtiter plates (50 μL/well) at 4°C overnight. The plates were then washed twice with PBS–Tween and treated with blocking solution (150 μL/well) for at least 30 min at room temperature. These antigen-coated plates could be stored for up to 3 weeks at 4°C.

(**b**) *Assay procedure.*—Samples or standards (100 μL) were incubated with 100 μL M8H5 MAb diluted 1:20 000 with MAb dilution buffer in microtubes for 1 h at room temperature. The antigen-coated plates were washed 3 times with PBS–Tween, and the sample–MAb mixtures were added to the washed plates (50 μL/well). A series of standards was placed on each plate, and 3 replicate wells with each standard and samples were prepared on each plate. After overnight incubation at 4°C, the plates were washed 4 times with PBS–Tween and incubated at 50 μL/well with HRP-labeled goat anti-mouse IgG (at a dilution of 1:7500 in enzyme conjugate dilution buffer) for 2 h at room temperature. Plates were then washed 5 times with PBS–Tween, and enzyme substrate solution (100 μL/well) was added. The enzyme reaction was allowed to proceed at room temperature until control wells showed 0.25–0.30 optical density at 630 nm (usually within 15–30 min). Stopping solution was added (50 μL/well), and the absorbance at 450 nm was measured.

(**c**) *Calculation.*—Average absorbance was calculated from individual absorbances obtained from triplicate wells. Results were corrected for background contribution of blank by subtraction and were expressed as the percentage of binding:

$$\text{Binding, \%} = \frac{A^+}{A^-} \times 100$$

where A^+ is the mean absorbance in the presence of each sample or standard and A^- is the mean absorbance in their absence (MCLR-free wells of standards). The standard curve was obtained by plotting percent binding against log concentration of MCLR. We developed computer software in Microsoft Excel (Microsoft Corp., Redmond, WA) to calculate MC concentration. By using the software, second degree polynomial curve-fitting after logit–log transformation was applied to 20, 50, 100, 200, and 500 pg/mL data points of the standards. Correlation coefficients of the fitting range of standard curves should be ≥0.99. When a sample resulted in complete inhibition, it was diluted with sample diluent to give ca 60% inhibition in ELISA.

Recovery Tests

(**a**) *Recovery from environmental water.*—Tap water and environmental water used in recovery tests had been estimated to be negative for MCs (<20 pg/mL) by the present ELISA. To water samples and MC-free algal extracts was added 20–500 pg MCLR/mL (n = 4 at each concentration). The mix-

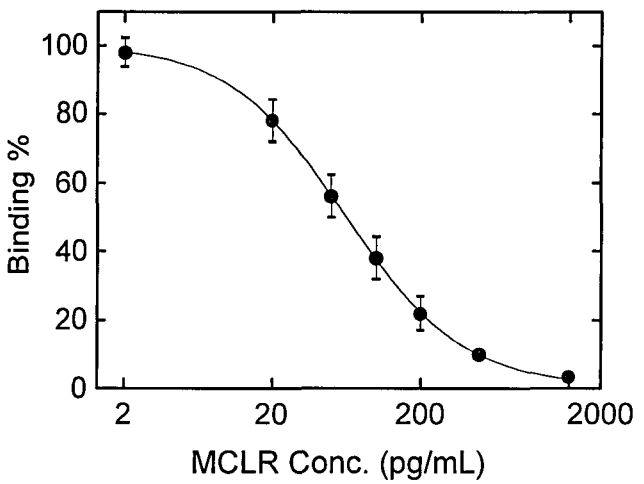


Figure 2. Standard curve of competitive ELISA for MCLR. Circles represent the mean binding from 10 standard curves performed on different days over a 6-month period. In each case, absorbance values for blank (no toxin added) and background wells (no MAb added) were 0.6–0.8 and 0.04–0.1, respectively. Bars represent standard deviations, which are missing when interfering with symbol.

ture was taken through freeze-thawing twice, filtered, and analyzed by ELISA.

(b) *Recovery from water extracts of cyanobacterial cells.*—Two strains of *M. aeruginosa* were used to prepare MC-free cyanobacterial extracts. Strain TAC 51, a nonproducer of MC (34), was freeze-dried and then extracted with water by rehydration (1 mg dried cells/mL, corresponding to ca 10^8 cells/mL) and 2 cycles of freeze-thawing. A confluent culture of strain M230-4, a producer of MC (36), was diluted with PBS to 10^6 cells/mL and freeze-thawed twice. These extracts were filtered through glass fiber filters. The M230-4 extract was then passed through a M8H5 MAb-combined affinity column (37) to selectively remove endogenous MCs. From 20 to 500 pg MCLR/mL ($n = 4$ at each concentration) was added to the MC-free extracts, which were then measured by ELISA.

(c) *Recovery of intracellular MC.*—Strain M230-4 and its subculture M230-4L, which produces lower amounts of MCs than the original, were used. These cells, suspended in water at 2×10^4 to 1×10^6 cells/mL, were subjected to multiple freeze-thaw cycles (up to 3 times), filtered through a glass fiber filter, and then analyzed by ELISA. If necessary, samples were diluted with water before or after filtration.

LC Analysis with UV Detection

The LC system was operated in an isocratic mode with methanol–0.01M phosphate buffer (pH 3.0, 6 + 4) at a flow rate of 1 mL/min and with UV detection at 238 nm (20). Each sample (10 μL) was injected to an ODS column maintained at 42°C, and the peaks obtained were compared with those of MCLR, MCRR, MCYR, and 7-D MCLR standards. Concentrations of these MCs in samples were determined by comparing the peak area for the sample with a linear regression plot established with consecutive dilutions of MCLR (1–100 μg/mL).

LC/ELISA

To confirm ELISA results, a limited number of ELISA-positive water samples were separated by LC, and each eluted fraction was measured by ELISA (LC/ELISA). Each water sample (50 mL) was treated with an ODS cartridge according to the same protocol described for sample cleanup, except that the residue of methanol elution was dissolved in 100 μL LC mobile phase. The sample (20 μL) was injected into the LC system, and the eluate was collected in 0.3 mL fractions. To each fraction was added 4 μL 2N NaOH to adjust the pH to 7.0, and then the mixture was evaporated to dryness in a centrifuge evaporator. Each residue was redissolved in 0.3 mL water and analyzed by ELISA. If necessary, an appropriate dilution with water was done before ELISA.

Results

ELISA Standard Curve

The anti-MCLR MAb used in this study shows extensive cross-reactivity to various MCs (32). In the present ELISA, total MCs were measured, and amounts were calculated as MCLR equivalents. The ELISA standard curve of MCLR (Figure 2) shows a typical sigmoidal response for MCLR concentrations of 2–2000 pg/mL. The curves are highly reproducible, as indicated by low standard deviations (SDs). A nonlinear regression was adopted to maximize assay range and to maintain a high degree of sensitivity. The ELISA's minimum detectable concentration was 20 pg/mL, assuming the MCLR concentration causes 20% inhibition of binding as the detection limit. This value corresponds to the mean plus 3–10 SDs of absorbance from 3 replicate wells of the zero standard.

To determine accuracy and precision of ELISA quantitation, MCLR standards at concentrations of 2–2000 pg/mL were evaluated repeatedly with different sets of wells of the same plate (intraassay) or different plates in different days (interassay). As shown in Table 1, at 20–500 pg/mL, residual errors are small and randomly distributed, indicating the absence of significant assay bias. In addition, coefficients of variations (CVs) in the same range are low, 3.3–13.6% and 4.4–17.6% for intra- and interassay, respectively. From these results, the ELISA's quantitation range was selected to be 20–500 pg/mL.

Recovery Tests

To examine potential matrix interference, 3 series of recovery tests were done. Table 2 shows the results of the first series of experiments in which known quantities of MCLR were added to tap water and MC-negative environmental water. Prior to ELISA, samples were treated with the same procedure for total MC analysis. Recoveries of 9 experiments over the detection range (20–500 pg/mL) were 90–107% (mean, 101%) with CVs of 3.5–10.5% (mean, 7.3%). Thus, recoveries are satisfactory and highly reproducible. These results suggest that natural substances commonly present in environmental water

Table 1. Intra- and interassay variations of ELISA assessed over a range of concentrations of MCLR standard[a]

MCLR standard, pg/mL	MCLR found			
	Intraassay (n = 5)		Interassay (n = 10)	
	Mean ± SD, pg/mL	CV, %	Mean ± SD, pg/mL	CV, %
2	2.8 ± 2.6	92.9	5.5 ± 7.2	130.9
20	25.0 ± 3.4	13.6	21.6 ± 3.8	17.6
50	53.2 ± 3.4	6.4	51.7 ± 3.0	5.8
100	98.9 ± 3.3	3.3	100.6 ± 7.3	7.3
200	192.0 ± 14.5	7.6	198.1 ± 8.7	4.4
500	503.7 ± 18.5	3.7	496.0 ± 48.0	9.7
2000	1974.0 ± 384.6	19.5	3095.2 ± 1644.3	53.1

[a] Intra- and interassay variations were determined by replicate analyses of each concentration of standard run in the same plate (n = 5) and by replicate analyses of a set of standards run in different plates on different days (n = 10), respectively.

do not interfere with the assay and that the sample preparation does not cause any loss of MC.

In practical analysis, samples from bloom-forming water are expected to contain a large number of cyanobacterial cells. Therefore, the second series of recovery tests was performed in which known quantities of MCLR were added to water extracts of cyanobacterial cells. Because toxin levels of natural blooms are generally too high for direct ELISA, 2 kinds of MC-free extracts were prepared artificially from cultured cyanobacterial cells. Table 3 shows recoveries of MCLR added to these extracts. In both extracts, the toxin spiked at levels ranging from 50 to 500 pg/mL was detected with recoveries of 77–115% (mean, 93%) and CVs of 3.8–32.6% (mean, 12.5%). These recoveries and precisions are acceptable, but at a toxin level of 20 pg/mL, precisions are poor, as shown by large CVs (47.5 and 62.5%). Therefore, for samples containing considerable amounts of cyanobacterial cells, we selected 50–500 pg MC/mL as the ELISA's quantitative range.

Table 2. Recovery of MCLR added to tap water and environmental water[a]

Source of water	MCLR added, pg/mL	MCLR found, pg/mL	Recovery, %
Tokyo tap water	25	25.4	102 ± 9.8
	50	47.2	94 ± 4.4
	100	102.2	102 ± 8.6
	200	191.6	96 ± 3.5
	500	449.0	90 ± 9.3
Moat Sotobori	50	52.4	105 ± 8.6
Pond Zenpukuji	50	53.6	107 ± 5.9
Pond Inokashira	50	53.6	107 ± 10.5
Lake Shiroyama	50	53.6	107 ± 5.2

[a] Known quantities of MCLR were added to tap water and environmental waters that had been estimated to be negative for MCs by ELISA (<20 pg/mL). Samples were freeze-thawed twice, filtered through Whatman GF/C, and then analyzed by ELISA. Four examinations were done. Recoveries are means ± SDs.

Table 3. Recovery of MCLR added to water extracts of cyanobacterial cells[a]

Cyanobacterial extract	MCLR added, pg/mL	MCLR found, pg/mL	Recovery, %
TAC51[b]	0	34.1	—
	20	59.6	128 ± 47.5
	50	83.6	99 ± 17.0
	100	133.9	100 ± 8.8
	200	264.5	115 ± 3.8
	500	528.7	99 ± 14.3
M230-4[c]	20	20.4	102 ± 62.5
	50	41.8	84 ± 32.6
	100	77.1	77 ± 9.8
	200	180.0	90 ± 8.5
	500	402.1	80 ± 5.4

[a] Known quantities of MCLR were added to water extracts of cultured *Microcystis aeruginosa* strains. Samples were analyzed by ELISA. Four examinations were done. Recoveries are means ± SDs.

[b] Cultured cells were freeze-dried and then extracted with water by rehydration (1 mg dried cells/mL) and 2 cycles of freeze-thawing. This strain is a nonproducer of MCs, but a trace amount of MC (34 pg/mL) was found by ELISA. To calculate recoveries, this level of toxin was subtracted from assayed values.

[c] The confluent cell culture was diluted with PBS to 10^6 cells/mL, freeze-thawed twice, and filtered through Whatman GF/C. Before addition of MCLR, the extract was pretreated with anti-MC MAb-combined affinity column to remove original MCs.

In the third series of recovery tests, extraction of intracellular MC was assessed with MC-containing cyanobacterial cells. The effects of cell concentrations and number of cycles of freeze-thawing were examined. Results (Table 4) reveal that >80% of MCs is extracted in the first freeze-thawing cycle regardless of cell concentration. Repeating the freeze-thawing cycle twice is sufficient to extract almost all intracellular MC, and extraction is highly reproducible. In addition, efficiencies of filtration are independent of cell concentrations.

Comparison of ELISA with LC

The correlation between ELISA and LC-UV was determined with water extracts of natural blooms and cultured cyanobacterial cells. Before LC analysis, extracts were treated with an ODS silica gel cartridge. The standards used were MCLR, MCRR, MCYR, and 7-D MCLR, which are the major MCs in Japanese freshwaters (10, 38). ELISA was applied to cleaned-up sample as well as to original extract to take into account the efficiency of the cleanup step. To fit in the detection range, samples were diluted by 2000–1 600 000 fold; therefore, matrix effects were neglected in the ELISA.

Table 5 lists results of ELISA and LC analyses of lyophilized materials of 10 natural blooms from 4 Japanese lakes or ponds with 3 strains of *M. aeruginosa* cells. LC analysis revealed that the samples contain several MC variants at various concentrations. However, total MCs determined by LC highly agreed with values obtained by ELISA for both cleaned up and original samples. Amounts of total MCs determined by ELISA

Table 4. Recovery of intracellular MCs by freeze-thawing followed by filtration from cyanobacterial cells[a]

Cyanobacteria	Cell concn before freeze-thawing, cells/mL	No. of freeze-thawing cycles	Dilution factor		MC detected, pg/mL	Recovery, %[b]
			Before filtration	After filtration		
M230-4L[c]	10^6	0	1	1	48.4 ± 0.9	3
		1	1	10	140.3 ± 14.6	100
		2	1	10	140.9 ± 6.4	100
		3	1	10	154.4 ± 14.0	110
		2	10	1	155.1 ± 19.7	110
	10^5	1	1	1	158.6 ± 7.5	98
		2	1	1	161.5 ± 0.8	100
M230-4	2×10^4	0	1	1	31.6 ± 2.3	3
		1	1	10	72.4 ± 4.4	80
		2	1	10	91.0 ± 15.8	100
		3	1	10	93.6 ± 1.7	103

[a] *Microcystis* cells diluted in water at indicated concentrations were subjected to multiple freeze-thaw cycles, filtered through Whatman GF/C, and then analyzed by ELISA. Samples containing high amounts of MCs were diluted with water before or after filtration as indicated. Two examinations were done. Amounts of MC detected are means ± SDs.

[b] MC concentrations detected after standard sample preparation (2 cycles of freeze-thawing and filtration) were assumed as the expected recovery (100%).

[c] This subculture derived from M230-4 produces lower amounts of MCs than the original.

relative to amounts determined by LC were 76–122% (mean, 93%) with cleaned-up sample and 66–159% (mean, 103%) with original extracts. The larger variance in the latter samples seems to be due to the error of the cleanup step. Excellent correlation (r = 0.98) between LC and ELISA was observed for cleaned-up samples. The slope of 0.97 and y-intercept of 0.26 were very near to 1 and zero, respectively, indicating that the ELISA's accuracy is comparable with that of LC. By contrast, ELISA of original extracts gave slightly higher values than LC at relatively high concentrations of MCs. This may be due to loss of MCs during cleanup at high concentrations. Nevertheless, the correlation (r = 0.94) between LC and ELISA for original extracts was still acceptable.

ELISA for MC in Environmental Water

Japanese freshwaters sampled during the summer seasons of 1993 and 1994 were analyzed by ELISA. Table 6 shows the total and released MCs. Twelve of 17 samples collected from 12 Japanese lakes and ponds contained total MC levels ranging from 64 to 94 000 pg/mL. Released MC at 81–800 pg/mL were detected in 4 of 13 samples. The concentrations of released MC were equivalent to 3–21% of total MC. Microscopic identification of phytoplankton species revealed that all MC-positive waters except one were dominated by *M. aeruginosa* L-type or S-type (39), which probably is responsible for the MCs found. We could not find a species responsible for the positive result in a sample from Pond Syakujii in the present experiment.

Confirmation of ELISA Results by LC/ELISA

To confirm ELISA results and to identify the MC species showing reactivity in ELISA, a limited number of ELISA-positive samples were separated by LC. Each eluted fraction was measured by ELISA (LC/ELISA). In this analysis, a mixture of standards containing 3 μg each of MCLR, MCRR, and MCYR resulted in 3 clearly separated peaks in ELISA, each peak corresponding to the retention time of a standard determined by UV detection (Figure 3A). Immunoreactivities were similarly separated even when only 50 pg of each standard was injected (data not shown). The sensitivity of LC/ELISA surpassed that of standard UV detection by ≥200-fold. Quantities of MCs assessed by LC/ELISA were roughly correlated to those obtained by LC/UV, although exact quantitation was not validated. Figure 3B shows an LC/ELISA analysis of natural water from Pond Syakujii on 08/11/93 (collection dates indicate month/day/year) after freeze-thawing and 500-fold concentration with an ODS cartridge. Three clear peaks with the same retention windows for MCLR, MCYR, and MCRR were identified by ELISA. By contrast, large quantities of impurities were observed with UV detection and the MC peaks could not be defined because of the relatively small amounts of MCs. In addition, 3 randomly selected positive samples—Lake Tsukui 08/01/93 and 09/21/93 and Pond Ishigaki 09/11/93—were also found positive for MCLR, MCRR, and MCYR by similar analyses (data not shown).

Discussion

ELISA Characteristics

The ELISA method we developed is reliable and practical for direct determination of MCs in environmental waters. The remarkable characteristics of the ELISA are wide cross-reactivity with various MCs, high sensitivity, and no requirement for sample cleanup. Therefore, the ELISA is well suited for mass screening of environmental waters.

Over 50 MC analogues have been isolated, and most of them are toxic (10, 11). The Adda, a commonly shared structure of MCs, is considered to play an important role in MC toxicity (40, 41). By our ELISA, almost all the principal MCs can be detected at once because of the ELISA's selective reactivity to Adda (32). This wide cross-reactivity is a great advan-

Table 5. Comparison of LC and ELISA quantitation of microcystins in water extracts of natural cyanobacterial blooms and cultured cyanobacteria[a]

	MC concentration, μg/mL[c]						
	Extract cleaned up with ODS cartridge						
	LC						
Sample[b]	RR	YR	LR	7D-LR	Total	ELISA	Original extract by ELISA
Pond Senzoku	0.64	ND[d]	0.24	0.23	1.11	1.11 (100)[e]	1.07 (96)[e]
Lake Teganuma	ND	ND	0.62	ND	0.62	0.56 (91)	0.64 (103)
Lake Suwa No. 1	0.48	ND	0.67	ND	1.15	1.07 (93)	1.83 (159)
Lake Suwa No. 2	2.36	ND	1.07	ND	3.43	2.93 (85)	3.23 (94)
Lake Kasumigaura No. 1	2.85	ND	3.47	4.40	10.72	9.79 (91)	11.52 (107)
Lake Kasumigaura No. 2	4.48	ND	2.00	1.44	7.92	6.65 (84)	7.57 (96)
Lake Kasumigaura No. 3	3.37	ND	2.81	11.92	18.10	13.69 (76)	14.56 (80)
Lake Kasumigaura No. 4	2.65	ND	2.78	14.09	19.52	15.57 (80)	12.92 (66)
Lake Kasumigaura No. 5	3.90	ND	2.03	7.12	13.05	11.87 (91)	10.01 (77)
Lake Kasumigaura No. 6	0.33	ND	ND	ND	0.33	0.30 (91)	0.30 (91)
TAC 63	15.96	0.46	7.32	0.83	24.57	26.94 (110)	36.29 (148)
TAC 70	15.88	0.33	9.19	1.05	26.45	24.34 (92)	35.20 (133)
TAC 70[f]	12.52	ND	5.54	ND	18.06	21.99 (122)	23.38 (129)
TAC 76	4.23	2.14	2.27	0.74	9.38	9.17 (98)	7.45 (79)
TAC 76[f]	4.18	1.39	1.57	0.43	7.57	7.44 (98)	6.70 (89)
Average recovery relative to LC (%)[g]						93	103

[a] Freeze-dried cells of natural cyanobacterial blooms or cultured *Microcystis* were extracted with water by rehydration (10 mg dried cells/mL) and 2 freeze-thawing cycles. Water extracts were filtered through Whatman GF/C, diluted with sample diluent at least 2000-fold, and subjected to ELISA. Parts of the filtered samples were treated with an ODS cartridge. The ODS extracts (5× concentrates) were analyzed by LC, with MCRR, MCYR, MCLR, and 7-D MCLR as standards, or by ELISA after at least 28 000-fold dilution with sample diluent.

[b] Sampling dates of natural blooms are as follows: Pond Senzoku, 07/25/91; Lake Teganuma, 09/12/89; Lake Suwa No. 1, 07/21/94; No. 2, 07/26/94; Lake Kasumigaura No. 1, 07/31/91; No. 2, 08/21/91; No. 3, 08/27/91; No. 4, 08/28/91; No. 5, 09/12/91; No. 6, 09/01/94. TAC, Tsukuba Algal Collection.

[c] MC concentrations in the original water extracts were shown.

[d] ND, not detected (<0.2 μg/mL).

[e] Values are means of 2 determinations. Values in parentheses are recoveries relative to LC (%).

[f] Extracted with 5% (v/v) acetic acid instead of water.

[g] Linear regression equations $y = 0.97x - 0.26$ (r = 0.98) and $y = 1.23x - 1.73$ (r = 0.94) were obtained for LC vs ELISA of ODS extracts and LC vs ELISA of original extracts, respectively.

tage for screening of whole MCs in environmental water. In our ELISA, the configuration of the double bond in Adda is critical for reactivity, and geometric isomers in the Adda unit are not detected (32). However, these isomers usually are minor components in environmental samples and their toxicities are much less than those of the other MCs (14, 33, 40); therefore, in most cases, the absence of reactivity of these compounds can be neglected for environmental monitoring.

One possible drawback of the wide cross-reactivity of ELISA is the false positive for chemically related compounds, such as MC precursors or metabolites. However, false positives almost never occur in practice, as evidenced by the excellent correlation between ELISA and LC-UV analyses with natural bloom and cyanobacterial extracts (Table 5). The ELISA's reliability is supported by positive results of confirmation test by LC/ELISA (Figure 3).

The ELISA is sensitive enough to detect relevant concentrations of MCs in environmental waters. Quantitation limits for MCLR are 20 pg/mL in tap water and 50 pg/mL in environmental freshwater, both of which are ≥1000-fold higher than the quantitation limit of the standard LC-UV method. In previous reports, minimum levels of toxins directly determined in water were 200 pg/mL by ELISA with rabbit anti-MCLR antiserum (26), 90 pg/mL by ELISA with chicken antibodies (27), and 100 pg/mL by a protein phosphatase inhibition assay (30). Thus, our ELISA is more sensitive than previously reported immunoassays or protein phosphatase inhibition assay. The higher sensitivity is not only helpful for survey studies but also important in regulatory work. The proposed guideline values for MCs in drinking water are 100–500 pg/mL for long-term exposure (17, 18).

The present ELISA requires no cleanup or concentration of water samples. Despite omission of cleanup, the ELISA's precision is sufficient. Insignificant interference from the matrix is an important advantage of our ELISA compared with protein phosphatase inhibition assay. In the latter assay, nonspecific phosphatases or endogenous protein phosphatases can lead to an underestimation of the MC content (29). Omitting cleanup also ensures timely and accurate assay. The small volume of samples needed for ELISA (<0.1 mL) enables us to handle a great number of samples at relatively low costs.

Table 6. ELISA analysis of microcystins and identification of dominant phytoplankton species in Japanese environmental freshwaters

Sampling site	Date	Microcystins, pg/mL[a]		Dominant species of phytoplankton[b]
		Total	Released	
Lake Shiroyama	08/01/93	ND[c]	—	—
Lake Inbanuma	07/21/94	94000	800	M.a.L. (3.4×10^6), M.w., A.a.
	08/14/94	560	120	—
	08/26/94	2500	81	—
Lake Tsukui	08/01/93	300	—	—
	09/21/93	870	ND	M.a.S. (3.2×10^7), M.g.
Lake Suwa	09/21/93	170	ND	M.a.S. (3.0×10^4), O.l. (1.5×10^4)
Reservoir Yamamura	09/12/93	2200	ND	M.a.S. (3.8×10^4), M.w., G.sp.
Reservoir Isaka	09/12/93	730	ND	M.a.L. (6.0×10^3), Co.sp., P.sp.
Pond Kasato	09/11/93	ND	ND	—
Pond Ishigaki	09/11/93	2600	85	M.a.L. (6.2×10^4), M.a.S. (7.0×10^3), Ch.sp.
Pond Syakujii	08/11/93	520	—	—
	09/15/93	64	—	—
	09/21/93	75	ND	M.g.s., P.s.d., L.l.
Pond Zenpukuji	08/01/93	ND	ND	—
Pond Inokashira	08/01/93	ND	ND	—
Moat Sotobori	08/03/93	ND	ND	—

[a] Values are means of 2 determinations.
[b] A.a., *Anabaena affinis*; Ch.sp., *Chroococcus* species; Co.sp., *Coelastrum* species; G.sp., *Gomphosphaeria* species; L.l., *Lyngbia limnetica*; M.a.L., *Microcystis aeruginosa* L-type; M.a.S., *M. aeruginosa* S-type; M.g., *Melosira granulata*; M.g.s., *Melosira granulata* V. *angustissima* f. *spiralis*; M.w., *M. wesenbergii*; O.l., *Oscillatoria limnetica*; P.s.d., *Pediastrum symplex* v. *duodenarium*; P.sp., *Peridinium* species. According to Komarek (45), *M. aeruginosa* L-type corresponds to typical *M. aeruginosa*. Numbers in parentheses are number of cells/mL.
[c] ND, not detected (<50 pg/mL).
[d] —, not determined.

Analytical Target of ELISA

Cyanobacteria grown in water produce MCs in the cells and release the toxin into surrounding water by the cell lysis. In previous reports, intracellular MC and released MC were analyzed separately after filtration or centrifugation of water samples (21, 23, 27, 30, 35). However, measurement of both MCs together is probably important, because levels of total MC indicate the potentially highest levels of released toxins that can be taken up by animals or human. Therefore, we measured total MC in addition to released MC.

The density of cyanobacteria in environmental water often reaches as high as 10^6 cells/mL. When the cells form a mat at the water surface, the density can be $>10^8$ cells/mL (42). The ELISA's sufficient performance in recovery tests with such high concentrations of cyanobacterial suspension (Table 3) suggests that environmental water can be analyzed by ELISA without any determination of cell concentrations.

Future Prospects for Microcystin Analysis

The present ELISA is sufficiently selective for screening but not definitive as a physicochemical method. The LC/ELISA described here is a novel means to identify the molecular species of the trace amounts of MCs in environmental water. A similar technique, LC combined with a protein phosphatase bioassay, has been used to determine MCs in natural water (28, 30). Identification of MCs in environmental water has been achieved also by improving or optimizing sample concentration techniques (21, 23, 35). Further work will be needed to define a method for practical use.

The present ELISA can be used to monitor MCs, especially in freshwater used for recreation and drinking. We recently used the ELISA to survey MCs in more than 1000 samples of drinking water in Haimen and Fusui, areas where primary liver cancer is endemic (15, 16), and demonstrated a wide but intermittent MC contamination (several hundreds of picograms per milliliter) of drinking waters from ponds, ditches, and rivers (43). Another survey with the present ELISA revealed that MCs are also present in environmental water in Thailand, Germany, Portugal, and Japan (44). The survey data will help in estimating the hazards of MCs.

Drinking water appears to be a major intake route for MCs, however, there remains the possibility of MC exposure through the food chain. Appropriate detection methods are required for MCs in tissues. Because of the presence of massive interferences, effective cleanup methods will be necessary. We recently used the MAb-combined immunoaffinity column for cleanup prior to LC analysis, and detection of MCs and their metabolites in mice liver was successful (37).

Acknowledgments

We thank Ken-Ichi Harada, Meijo University, Nagoya, Japan, for providing MC standards. This work was partly supported by Asahi Glass Foundation (Tokyo), Nippon Life Insur-

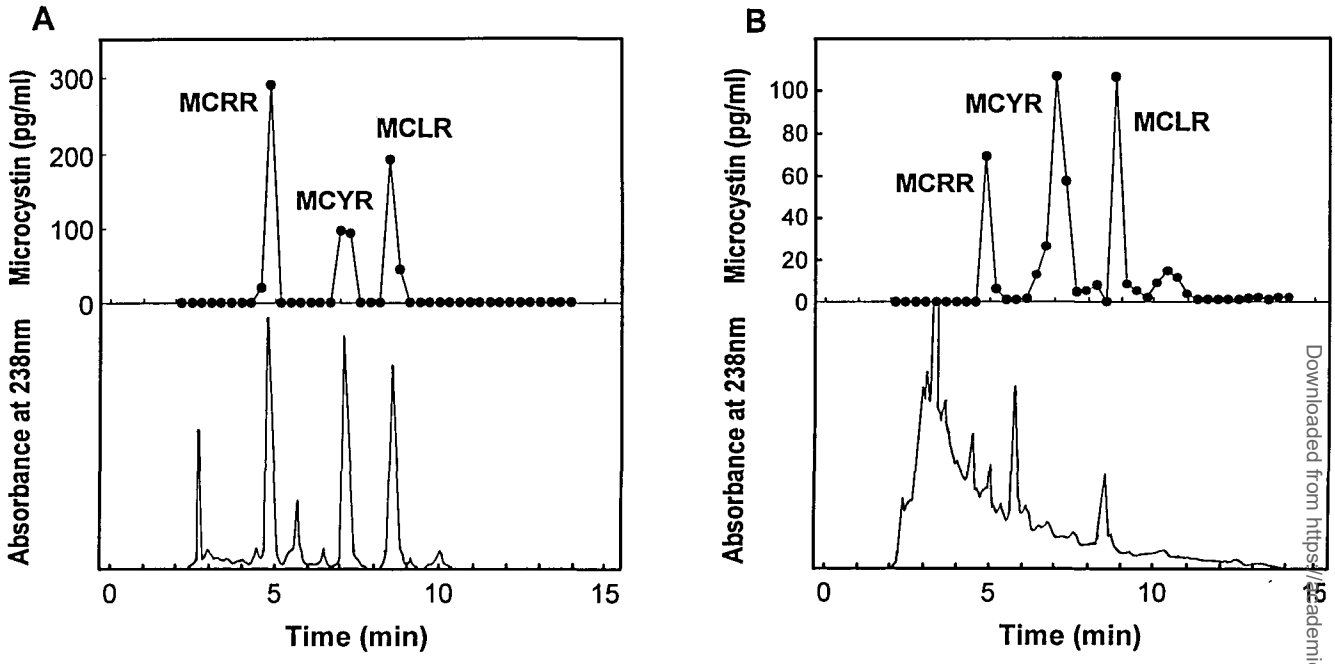


Figure 3. LC/ELISA analysis of MCs of standards and environmental freshwaters. Samples were resolved by LC with an ODS column. Then the MCs in each fraction were measured by ELISA. ELISA results and UV absorbance at 238 nm are shown in upper and lower panels, respectively: (A) Analysis of standard mixture containing 3 μg each of MCLR, MCRR, and MCYR. Each fraction was diluted 1/300 for ELISA; (B) Analysis of natural freshwater collected in Pond Syakujii (08/11/93). Before analysis, 50 mL water sample was concentrated 500-fold with an ODS cartridge. Each fraction was diluted 1/20 for ELISA.

ance Foundation (Osaka), and the Ministry of Education, Science and Culture of Japan.

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