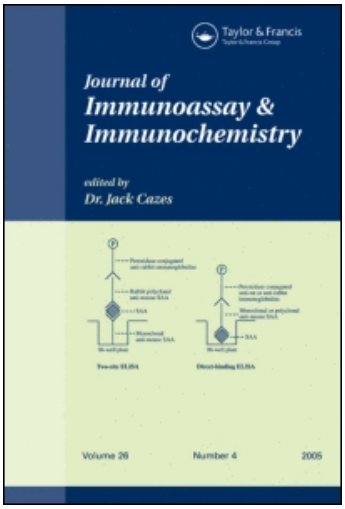

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Determination of Trace Amount of Cyanobacterial Toxin in Water by Microchip Based Enzyme-Linked Immunosorbent Assay

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Abstract: Routine monitoring of microcystin in natural waters is difficult because the concentration of the toxin is usually lower than the detection limits. As a more sensitive detection method for microcystin, we developed a microchip based enzyme-linked immunosorbent assay (ELISA) based on monoclonal antibodies. New monoclonal antibodies against the microcystin leucine-arginine variant (MCLR), a cyclic peptide toxin of the freshwater cyanobacterium *Microcystis aeruginosa*, were prepared from cloned hybridoma cell lines. We used keyhole limpet hemocyanin(KLH)-conjugated MCLR as an immunogen for the production of mouse monoclonal antibody. The immunization, cell fusion, and screening of hybridoma cells producing anti-MCLR antibody were conducted. Since the ELISA test was highly sensitive, the newly developed microchip based ELISA can be suitable for the trace analysis of cyanobacterial hepatotoxins, microcystins in water. The linear responses of monoclonal antibodies with different concentrations of microcystin LR were established between 0.025 and 0.3 ng/mL.

Keywords: ELISA, Microcystins, Monoclonal antibodies, Trace analysis

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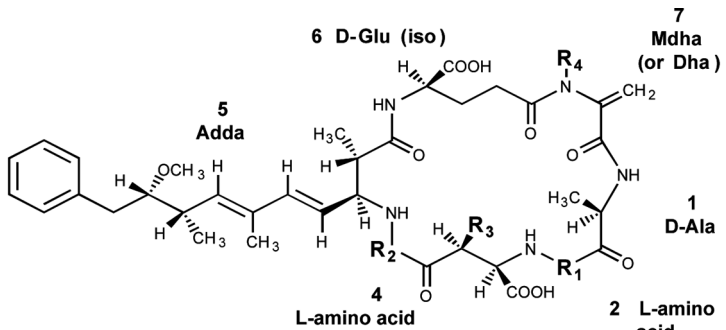
INTRODUCTION

Algal blooms, in particular cyanobacterial blooms, are a major issue for water authorities, causing significant taste and odour problems. The knowledge that many of these blooms are toxic has changed the concern from a purely aesthetic problem to one that affects human health.

Over 20 different cyclic peptide hepatotoxins termed microcystins[1] have been isolated from cyanobacteria (blue-green algae).[2,3]

While Microcystis is the most studied genus, species in the genera Anabaena, Nodularia, Nostoc, and Oscillatoria also contain these toxins.[4–6]

Microcystins (Fig. 1) are characterized as monocyclic heptapeptides containing a common moiety comprising 3-amino-9-methoxy-10-phenyl-2,3,8-trimethyldeca-4,6-dienoic acid (Adda), N-methyldehydroalanine (Mdha), D-alanine, β-linked D-erythro-β-methylaspartic acid and γ-linked -D-glutamic acid, plus two L-amino acids as variants.[7,8] The most extensively studied form is microcystin-LR which contains L-leucine and L-arginine in the two main variant positions.



Mdha : N- methldehydroalanine
Dha : dehydroalanine
Adda : (2S,3S,8S,9S)-3-amino-9-methoxy -10-phenyl-2,6,8-trimethyl -deca -4,6- dienoic acid

	R_1	R_2	R_3	R_4	MW
Microcystin - LR	Leu	Arg	CH_3	CH_3	994
Microcystin - YR	Tyr	Arg	CH_3	CH_3	1044
Microcystin - RR	Arg	Arg	CH_3	CH_3	1037

Figure 1. Structure of microcystins. A characteristic of microcystins and related cyanobacterial toxins is the hydrophobic amino acid, Adda, which contains, in position 5, two conjugated double bonds. Numbers represent the positions of the corresponding amino acids.

Toxic cyanobacterial blooms have been reported in many countries.[9] Toxic waterblooms cause death of domestic animals and wildlife, and human illness. Cyanobacterial toxins are toxic to zooplankton and fish[10] and can be accumulated in fish and aquatic animals.[11]

The structures and function of toxin are classified into three groups: neurotoxin, hepatotoxin, and lipopolysaccharide. Microcystis aeruginosa, which is the most common toxin-producing cyanobacteria found worldwide, produces microcystins.

Many studies showed that these microcystins and nodularin inhibit in vitro activity of protein phosphatase in a cytosolic fraction of mouse liver.[12,13] Liver is reported as the target organ that shows the greatest degree of histopathological change when animals are poisoned by these cyclic peptides. The cause of death in mice is at least partially known and is concluded to be hypovolemic shock caused by interstitial hemorrhage.[14]

The analysis of microcystins in laboratories has mainly been based on reversed-phase high performance liquid chromatographic methods[15] which require expensive equipment and highly qualified personnel due to the high variability of toxin structures. HPLC also involves lengthy analysis time and methods to concentrate or clean the samples, since the detection level is relatively high. Therefore, the method is not optimal for rapid detection of low toxin concentrations which is needed in the trace analysis of cyanobacterial hepatotoxins, microcystins in water.

It is well known that an enzyme-linked immunosorbent assay (ELISA) is highly specific, sensitive, and is a quick method.[16,17] Therefore, for the trace analysis of microcystins in water, various ELISA systems using either polyclonal[18,19] or monoclonal[20] antibodies were introduced. Using polyclonal antibodies, the detection limit was first ng/mL,[18] and, later on, it was 95 pg/mL.[19] The use of a monoclonal antibodies that selectively recognize microcystin can enhance the detection limit in the trace analysis of free microcystin in water samples. The aim of this study is to evaluate the performance of a newly developed ELISA system in terms of the sensitivity and the linear responses of monoclonal antibodies with different concentrations of microcystin LR.

EXPERIMENTAL

Keyhole limpet hemocyanin (KLH), 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDAC), and microcystine-leucine-alanine (MCLR from Microcystis aeruginosa) were extracted in the laboratory and also purchased from Sigma (St. Louis, MO). 3,3′,5,5′-tetramethyl benzidine (TMB) was purchased from Kem-En-Tec (Copenhagen, Denmark). Cyanobacterial cells were collected in the summer from Korean lakes

and rivers. Cells were collected with plankton net or surface scums were collected by a dip net when dense surface scum was floating. Algal cells were freeze-dried for extraction. 100 mg of dried cells, pre-extracted with a ternary mixture (90% CO_2, 9.0% methanol, 1.0% water) at 40°C and 250 atm, were used.

KLH-conjugated MCLR was used as an immunogen for the production of mouse monoclonal antibody against microcystins (Mabmc). The immunization, cell fusion, and screening of hybridoma cells producing anti-MCLR antibody were conducted according to a standard method.[21] Six to eight week-old BALB/c mice were immunized with 100 μg of a MCLR-KLH conjugate mixed with complete Freund's adjuvant. The first injection was followed by three or four booster injections of the same amount of immunogen mixed with incomplete Freund's adjuvant at 3–4 week intervals. Serum was taken from the tail of the mouse and tested for antibody titer usually after the third injection. For the fusion, spleen cells from immunized mice were combined with SP2/0-Ag-14 myeloma cells, and 1 mL of 50% poly-(ethylene glycol) (PEG) 1,500 in DME medium was added drop by drop for over 60 s. The fused cells were selected in a hypoxantine-aminopterin-thymidine medium for 2 weeks. Next, the culture supernatants from the hybridoma cells were then collected and screened with BSA-conjugated MCLR by ELISA. The positive clones were frozen first and selected further by two successive limiting dilutions after thawing. Ascitic fluids were generated for a large-scale production of Mabmcs by injecting 1×10^7 hybridoma cells into the peritoneal cavity of a mouse. We purified the Mabmc from the supernatant of the hybridoma cell or ascites fluids through a series of purification steps: membrane ultrafiltration, ammonium sulfate precipitation, and protein-G column chromatography. The eluted Mabmc from the column with 100 mM glycine-HCl (pH 2.5) was neutralized by adding 0.1 vol. of 1 M Tris (pH 8.0) and stored at −70°C until use.

Polystyrene beads were attached with MCLR (antigen) and introduced into a microchip chamber and selected as the reaction solid phase. To avoid the nonspecific absorbance, the beads were treated with bovine serum albumin (BSA) beforehand. Different concentrations of MCLR sample were mixed with the antibody mAb-HRP for the competitive reaction. After the conjugation, the mixture was injected into the chamber and the remaining free mAb-HRP would have the reaction with the MCLR antigens on the surface of the polystyrene beads. Following the washing step, HPR bound to the bead surface via the antigen-antibody complex would catalyze the introduced substrate-TMB, and the color change of TMB was detected by a wave-guide.

To maintain the reaction solid phase, i.e., the polystyrene beads in the microchip, a chamber and a frit were fabricated. The chamber is shown in Figure 2, and the circle in its center was designed for promoting

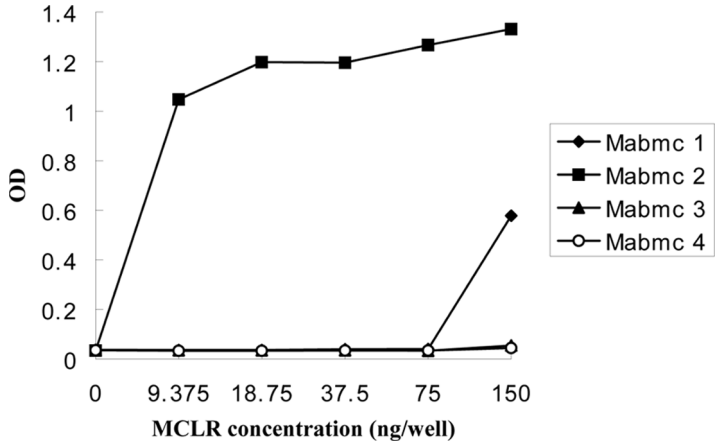


Figure 2. Reactivities of monoclonal antibodies to MCLR-conjugate. Each Mab at an appropriate concentration, mixed with indicated concentrations of MCLR, was added to MCLR-KLH-coated wells of ELISA microtiter plates followed by the addition of HRP-labeled anti-mouse Ig G antibody. Mabmc 2 clones show the highest reactivity to MC-KLH complex.

the full filling of solution. The frit was made by filling a 1.5 cm length of capillary (OD: 366 μm, ID: 248 μm) with about 0.5 mm length of GF/B filter, which can retain the microparticles down to 0.1 μm. Then, the frit was inserted into the microchannel between the chamber and the channel of detection chip, so that the 56 μm size of polystyrene beads would stay in the chamber.

RESULTS AND DISCUSSION

Reactivity of Monoclonal Antibodies to MCLR-Conjugate

To prepare a large quanty of antigen (microcytin LR, MCLR), a supercritical fluid extraction method was used. MCLR included in this study is sparsely soluble in neat CO_2. For example, when neat CO_2 was used as the extraction fluid at 40°C and 250 atm, no microcystin LR could be extracted from freeze-dried cyanobacterial cells. To solve this problem, we used a mixed solvent supercritical fluid extraction method[22,23] developed by our laboratory. Using mixed solvent supercritical carbon dioxide as a supercritical fluid, the microcystins could be extracted from cyanobacterium. The microcystin LR was successfully extracted with an extraction efficiency of 94% using a ternary mixture (90% carbon dioxide, 9.0% methanol, 1.0% water). This supercritical fluid extraction method has

several advantages over solid-phase extraction sample preparation for the analysis of microcystins. Sample handling steps are minimized, thus reducing possible losses of analytes and saving analysis time. No organic solvent extractions are involved in this method and no clean-up steps are employed.

KLH-conjugated microcystin LR was used as an immunogen for the production of mouse monoclonal antibody. Immunization, cell fusion, and screening of hybridoma cells producing anti-microcystin LR antibody were conducted.

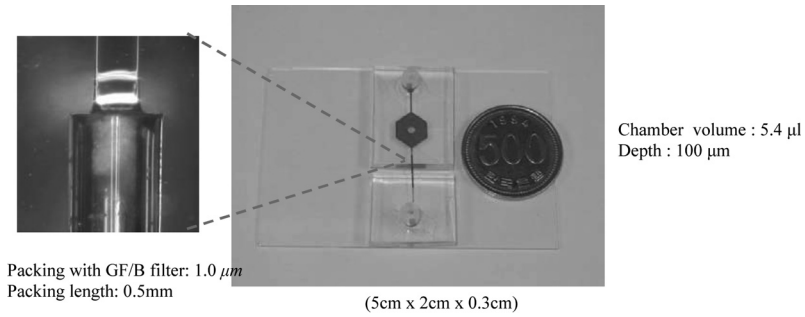
Two fusion experiments yielded a total four hybridoma secreting Mabmcs to MCs. They reacted with MCLR-KLH. The binding of Mabmcs to the MCLR-KLH coated to the plates was inhibited by free MCLR, and the concentrations required for complete competition were varied (Fig. 2). Mabmc 2 clones show the highest reactivity to MC-KLH complex.

Trace Analysis of Microcystins using Microchip Based Enzyme-Linked Immunosorbent Assay (ELISA)

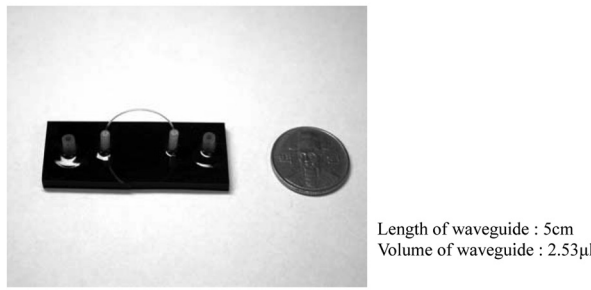
For the trace analysis of microcystins using microchip based enzyme-linked immunosorbent assay, the microchip system was composed of two chips, i.e., reaction chip and detection chip (Fig. 3). In the construction of the microcystin detection chip, the light source is a red LED (650 nm); the irradiation source is a UV pen lamp (254 nm, 90%, Oriel Instrument). A photomultiplier tube, H5784-04 (Hamamatsu, Japan), is used as the sensitive light detector. All the solutions are pumped into the system by 4-channel peristaltic pump (Ismatec, Switzerland). The PMT data acquisition system is Autochro Data Module (Younglin Instrument, Korea).

The chip was washed with PBS buffer before use to maintain a stable pH value. 25 μL of MCLR was mixed with 5 μL of mAb-HRP for 5 min reaction. At the same time, 9 μL of polystyrene beads were injected into the chamber by syringe, and then 9 μL of the mixture was also introduced into the chamber. After 5 min reaction, all of the solutions were washed away with PBS buffer. In the following detection, the continuous flow of PBS buffer was regarded as the detection background, and then, 9 μL of TMB was pushed by the peristaltic pump to obtain the absorbance signal.

To evaluate the performance of our microchip based ELISA system, in terms of the linear responses of monoclonal antibodies with different concentrations of microcystin LR, we conducted an experiment of competition ELISA assay with free MCLR, anti-MCLR-Mab, and MCLR-BSA conjugate. As shown in Figure 4, MCLR-BSA conjugate coated on beads competes with free MCLR in binding to anti-MCLR-Mab.



(a)



(b)

Figure 3. PDMS microchip for enzyme-linked immunosorbent assay. (a) Reaction Chip (transparent PDMS); (b) Detection Chip (black PDMS).

The binding interaction between anti-MCLR-Mab and MCLR-BSA conjugate was well inhibited by free MCLR in the water samples when five different concentrations (0.3,0.2,0.1,0.05,0.025 ng/mL) of aqueous MCLR samples were tested. From Figure 4, we could observe that the minimum detection level of our microchip based ELISA system would be about 0.025 ng/mL and the linear responses of monoclonal antibodies with different concentrations of microcystin LR could be well established between 0.3 and 0.025 ng/mL.

In the conventional ELISA, because of performing on the plate, the precious reagent consumption is a big problem. For the microcystin detection, since algal growth depends on environmental factors, particularly on temperature and sunshine, and at the same time, it is not easy to extract the MCLR from the variants of microcystin, small sample consumption is necessary and economical. In fact, in our experiment, we at first mixed 25 μL MCLR sample with 5 μL HRP-mAb and, then, extracted only 9 μL of them; this meant that we only needed 7.5 μL of

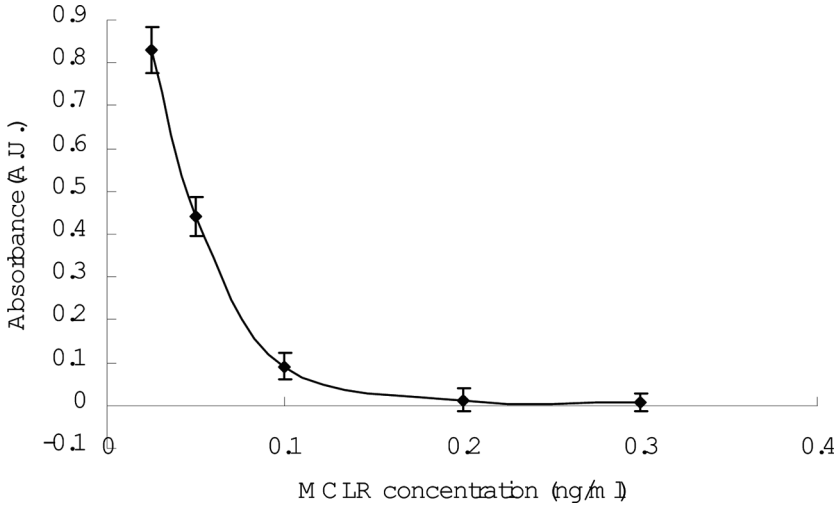


Figure 4. Calibration curve for MCLR with our PDMS microchip-based ELISA. Each point on the graph represented the mean values and error bars represent standard deviation values of three independent experiments.

the MCLR sample. The use of a small amount of sample is a great advantage in the environmental analysis.

CONCLUSION

The aim of this study was an application of the microchip based ELISA system with our monoclonal antibodies that is sensitive enough for monitoring of microcystins in water. The performance of the microchip based ELISA system with our monoclonal antibodies was satisfactory. We could also observe that the linear responses of monoclonal antibodies with different concentrations of microcystin LR were well established between 0.3 and 0.025 ng/mL.

Since microchip based ELISA assay is easy to perform and its quantitative range is within microcystin concentrations in natural waters, it shows potential for routine use in monitoring of microcystins in water.

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