Production and Degradation of Cyanobacterial Toxin in Water Reservoir, Lake Soyang

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Cyanobacterial toxins, microcystins are very potent hepatotoxins and their occurrence has been reported all over the world. They could threaten human health when toxic *Microcystis* occurs in water supply reservoirs. In this study, the effects of several environmental factors on production and degradation of toxins produced by cyanobacteria in Lake Soyang have been studied. A new rapid quantification method of microcystins using fluorescence for a detection signal and a lateral-flow-type immunochromatography as a separation system was used. Culture age, temperature, light intensity, pH, N-nutrient concentration, P-nutrient concentration, iron and zinc concentration were the most importantly examined factors. The toxin content was the highest on 17-18 days and at temperatures between 20 ^{o}C and 25 ^{o}C, and at pH between 8.4 and 8.8.

Key Words : Cyanobacteria, Toxin, Production, Degradation

Introduction

The occurrence of hepatotoxin-producing cyanobacteria is well-documented in freshwaters around the world,[1,2] and they are recognized as a potential threat to human health. Risk may be through acute exposure resulting in hepatic injury, which can in extreme cases prove fatal. One such incident occurred recently that resulted in the death of around 50 dialysis patients due to the use of microcystin-contaminated water in their treatment.[3] Chronic exposure can occur due the presence of microcystins in drinking water and is thought to be a contributing factor in primary liver cancer through the known tumor-promoting activities of these compounds.[4]

Microcystins are a family of cyclic heptapeptides with the generic structure cyclo(*d*-Ala-X-*d*-MeAsp-Z-Adda-*d*-Glu-Mdha) where Adda is an unusual 20 carbon amino acid, Mdha is *N*-methyldehydroalanine, and X and Z are variable amino acids.[5] The structure of microcystin-LR, the subject of this study, is shown in Figure 1. Microcystins are typically produced by planktonic cyanobacteria, which are increasingly found in water bodies at high densities (water blooms) as a result of eutrophication. Many of the water bodies in which microcystin-producing blooms occur are used for drinking water supply, and it is believed that conventional water treatment methods are ineffective in removing these toxins from potable supplies.[6,7]

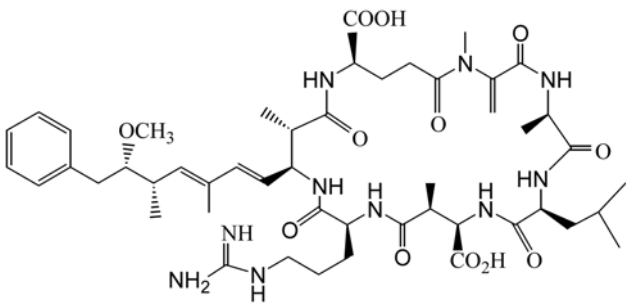

Figure 1. Structure of microcystin LR.

Toxic cyanobacterial blooms have been reported in many countries.[8] Toxic waterblooms cause death of domestic animals and wildlife, and human illness. Cyanobacterial toxins are toxic to zooplankton and fish[9] and can be accumulated in fish and aquatic animals.[10]

The most widely used procedures for isolation of microcystins from cyanobacterial cells are as follows: microcystins are extracted from the lyophilized cyanobacterial cells with organic solvents several times and then the extracts are applied to multi-step column chromatography.[11] In this method, 5% aqueous acetic acid solution was used as an extracting solvent and microcystins are isolated with using ODS column chromatography, Silica gel column chromatography and Gel permeation chromatography. And then, a new method for the fast extraction of microcystin RR, and LR was developed using supercritical fluid extraction (SFE) technique.[12]

As microcystins are proven potent hepatotoxins for humans and animals, the development of sensitive and reliable detection methods becomes of great importance. Since managing surface and drinking water is essential to protect human and animal health, it is very important to develop fast, reliable, and accurate analytical methods to detect microcystins (MCs). Several methods have been developed through the years including microchip based assay,[13] enzyme phosphatase-inhibition assay,[14] and micro-column high performance liquid chromatography (HPLC).[15] The developments of biological methods were first focused on enzyme-linked immunosorbent assays (ELISA), however ELISA need rather long analysis time and require appropriate training.[16] While the other detection methods have their own advantages, they are still inconvenient since they require time-consuming procedures, special safety when handling isotopes, expensive equipment and a qualified expert to administer the method. There has been a great

demand for developing a fast and convenient analytical method to detect microcystins.

In this paper, we developed a rapid quantification method of microcystins using fluorescence as a detection signal and a lateral-flow-type immunochromatography as a separation system. Using this fluorescence immunochromatographic assay, the effects of several environmental factors on production and degradation of toxins produced by cyanobacteria in lake Soyang have been studied. Culture age, temperature, light intensity, pH, N-nutrient concentration, P-nutrient concentration, iron and zinc concentration were the most importantly examined factors.

Experimental Section

Chemicals. Keyhole Limpet Hemocyanin (KLH), and 1-ethyl-3-(3-dimethylaminopropyl) carbadimide (EDAC) were purchased from Sigma (St. Louis, MO). Streptoavidin, Protein G, and 3,3',5,5'-tetramethylbenzidine (TMB) were purchased from Kem-En-Tec (Copenhagen, Denmak). Cyanobacterial cells were collected from Lake Soyang which is the biggest water source in South Korea. Algal cells were freeze-dried for extraction; 100 mg of dried cells, pre-extracted with a ternary mixture (90% CO_2, 9.0% methanol, and 1.0% water) at 40 °C and 250 atmosphere were used. Microcystin-L-leucine-R-arginine (MCLR) was extracted form *Microcystis aeruginosa* in the Analytical Chemistry Laboratory, Kangwon National University.

Production and Purification of Microcystin. Several kinds of *Microcystis aeruginosa* (MA), cyanobacteria known to produce microcystins (MCs) were used. One liter batch cultures of cells were grown in MA medium. Cultures were maintained at 20-25 °C under constant illumination by white fluorescent light incident on the surface of the growth flask. Cells were harvested by centrifugation (9000 × g, 5 min) and were lyophilized before storage at −20 °C. Microcytins were identified by high performance liquid chromatography using Beckman equipment. The equipment included a 116 pump (SYSTEM GOLD Programmable Solvent Module 126), 126 Detector (SYSTEM GOLD Programmable Detector Module 166) and a multi solvent delivery system. Chromatograms were monitored at UV 238 nm. The column was an Ultrasphere 5 μm ODS (Beckman 4.6 mm × 25 cm), Methanol/0.02 M Na_2SO_4 aqueous solution (55:45) was used as a mobile phase at a flow rate of 2 mL min^{-1}.

Production of Monoclonal Antibody against Microcystin-LR. To produce a good quality of mouse monoclonal antibody (mAb), MCLR was conjugated to bovine serum albumin (BSA) or keyhole limpet hemocyanin (KLH) in the presence of 1-ethyl-3,3'-dimethylaminopropyl carbodiimide (EDAC). The immunization, cell fusion and screening of hybridoma cells producing anti-MCLR antibody were conducted according to the standard method.[17] BALB (Bagg Albno)/c mice were immunized with MCLR-KLH. The initial injection used 0.2 mL of the conjugate solution and 0.2 mL of complete Freund's adjuvant. Booster injections used conjugate solutions and incomplete Freund's adjuvant. The mAbMC was produced in BALB/c mice by the hybridoma cell line, SP_2/O-Ag14. Two weeks after fusion, the hybridomas were screened for the production of anti-MCLR antibodies by an indirect fluorescence immunochromatography, in which the MCLR-protein conjugates were coated onto plates. Hybridomas were estimated as positive for MCLR-BSA and MCLR-KLH. The positive hybridomas were cloned several times by a limiting dilution method. Each of the established hybridoma cells producing the antibody was grown in a medium supplemented with HT (Hydroxy Tryptamine). Large quantities of antibodies were prepared from serum-free cultured supernatants of hybridomas by membrane ultra-filtration and ammonium sulfate precipitation, and finally purified using protein G-column.

Fluorescence-Immunochromatographic Strip and Cartridge. In the fluorescence immunochromatographic assay, an assay strip was in-house-fabricated to fit into the holder of a laser fluorescence scanner, which mainly consisted of a nitrocellulose membrane (NC), a sample pad, an absorption pad, and a backing card. The backing polystyrene card is a support that the nitrocellulose membrane, sample, and absorption pad are laid on its adhesive side. The nitrocellulose membrane (Millipore HF 180) is placed where the detection zone is located, and the bottom side of membrane was coated with a plastic thin film. The test and control line on the detection zone was dispensed with anti-MCLR-mAb and streptavidin for detection of MCLR in a

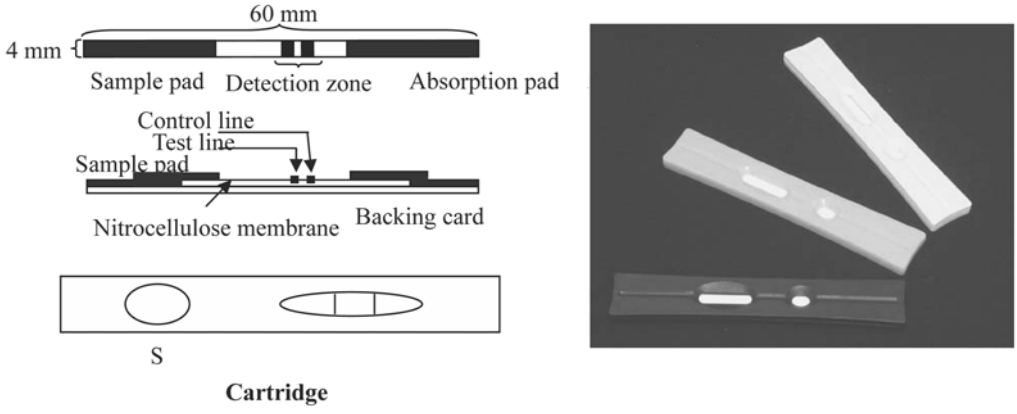

Figure 2. Schematic Diagram of Fluorescence Immunochromatographic Assay Strip.

sample. The control and the test line were located 31 and 33.5 mm down from the sample pad, respectively (Figure 2). Before being placed on the nitrocellulose membrane, the sample pad (S&S 903, 4×25 mm^2) was completely soaked in PBS (Phosphate Buffered Saline) containing 1% BSA and 0.05% Tween-20, and vacuum dried at 50 °C for 1 hr. The absorption pad (S&S 470, 4×20 mm^2) was set up on the nitrocellulose membrane along the detection zone to remove the post reaction solution, which passed by the detection zone. The assembled strip on a polystyrene card was placed into a plastic housing (15×90 mm^2) which was designed to fit into the holder of the laser fluorescence scanner. The oval window of the plastic housing for the scanning of the detection zone was 15 mm, and the diameter of the sample well for holding 100 μL of sample mixture was 5 mm. In the case the test line on nitrocellulose membrane being dispensed with anti-MCLR-mAb, the detection solution was a mixture of MCLR-FL and biotin-FL. The intensity of fluorescence conjugates on the detection zone is scanned by a laser fluorescence scanner and converted to area value. The concentration of MCs was calculated from the equation of standard curve.

Results and Discussion

In the fluorescence immonochromatographic assay system, an unknown sample containing MCs is simply mixed with the detection solution containing fluorophore-conjugated MCs (or fluorophore-conjugated mAb) and fluorescence conjugated biotin as an internal standard. The sample pad was pretreated with the PBS buffer containing 1% BSA and 0.05% Tween 20 to prevent nonspecific binding and to ensure that the tests could be reproduced.

When a water sample is placed on fluorescence immunochromato-graphic strip, two chromatographic lines of fluorescence intensity curves always appear. MCs in the sample and fluorescence conjugated-MCs in the detection solution compete for binding to capture antibodies which are coated at the test line on the detection zone as they flow laterally from the sample pad to absorption pad. The fluorescence conjugated biotin in the sample is captured by the streptavidin that was dispensed at the control line on the detection zone. The fluorescence intensity of the first line (the test line) is inversely proportional to the concentration of microcystin in water sample. Second line of fluorescence intensity curves (control line) is related to the mass transport of the sample and should show a constant value regardless of the concentration of microcystin in the water sample. This phenomena result from the method of making the fluorescence immunochromatographic strip. Anti-MCLR-mAb (350 μg mL^{-1}) and streptavidin (2.5 mg mL^{-1}) were dispensed at the test line and control line of an internal standard in the detection zone, respectively. A sample mixture of 100 μL containing 80 μL of sample and 20 μL of the detection solution was loaded onto the sample pad of the immunochromatographic assay strip. The detector solution contained the MCLR-FL (1.18 μg mL^{-1}) ad the biotin-FL (92 ng mL^{-1}).

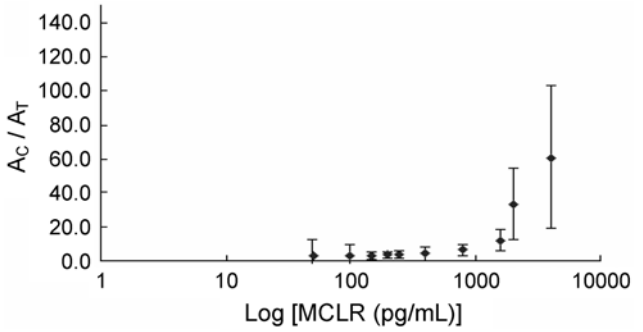

Figure 3. A_T/A_C were plotted against different concentrations of microcystin. Each points on graph represented the mean values and error bars represented standard deviation values of five independent experiments. Ten different concentrations (4000, 2000, 1600, 800, 400, 250, 200, 150, 100, 50 pg/mL) of free microcystin samples were used.

A series of experiments was performed using standard solutions of different concentrations of MCLR to evaluate the performance of the fluorescence immunochromatographic strip. The area value of the fluorescence peak at control line (A_C) was set as constant as possible by applying the same amount of fluorescence labeled biotin. In the mean time, the area value of the fluorescence peak at the test line (A_T) was inversely proportional to the concentration of microcystin in water. Thus, the ratio of A_C/A_T increases as the concentration of microcystin increases in the water sample. The ratio of A_C/A_T were plotted against different concentration of MCLR and was shown in Figure 3. In Figure 3, the data points in the range of 150 pg mL^{-1} to 1600 pg mL^{-1} show small error bars, which means small standard deviation values. It can be concluded that the low and high limits of quantification of the fluorescence immunochromatographic strip assay are 150 and 1600 pg mL^{-1}.

Using the fluorescence immunochormatographic strips, the effect of culture age on production and decomposition of cyanobacterial toxins produced by cyanobacteria (NIER10004) isolated from lake Soyang was investigated (Fig. 4). The 'Ratio' of the Y-axis in Figure 4 means the ratio of the fluorescence peak area at the control line to that at the test

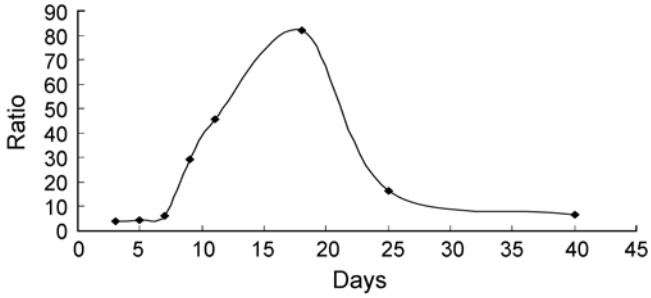

Figure 4. The amount of cyanobacterial toxin produced from NIER10004 as a function of time (NIER10004 is *Microcystis aeruginosa* obtained from lake Soyang by National Institute of Environmental Research) Experimental conditions; 25 °C, 4500 lx, PH=8.6, initial solution: 1.0×10^7 cells mL$^{-1} \times 100$ mL.

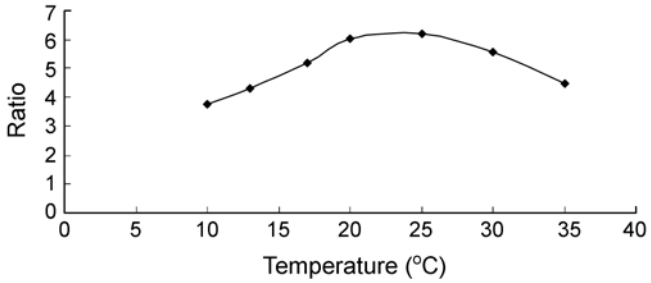

Figure 5. The amount of cyanobacterial toxin produced from NIER10004 as a function of water temperature.

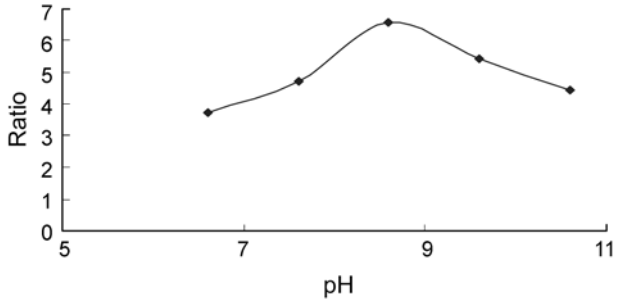

Figure 6. The amount of cyanobacterial toxin produced from NIER10004 as a function of pH of MA medium.

line. This ratio is proportional to the quantity of toxin in sample. In Figure 4, we can observe that the toxin was continuously produced for until 17 or 18 days however, after 17 or 18 days the toxin was decomposed rapidly. The reasons of rapid decrease of the toxin content after 17-18 days could be the death of cyanobacteria due to the lack of nutrients and chemical degradation of the toxin due to the long exposure of the light. In the light condition, microcystins can undergo slow photochemical breakdown and isomerisation.[18]

In order to observe the effect of temperature of water on production of toxin, the temperature was varied from 10 °C to 35 °C (Fig. 5). The toxin content was highest at temperatures between 20 °C and 25 °C, whereas low or very high temperatures decreased the toxin content. In Figure 6, the effect of pH can be observed that the toxin content was the highest at pH 8.6 whereas at lower or higher PH conditions the toxin content decreased. These studies indicate that cyanobacteria produce most toxins under conditions which are most favourable for their growth.

Light intensity is also an important environmental factor on toxin production by cyanobacteria. To test the effect of light intensity, four different intensities of lights (200, 800, 1500, 3000 lux) were used (Fig. 7). Figure 7 indicates that the greater light intensities produces the greater amount of toxin.

The effects of phosphorus- and nitrogen- nutrient concentrations on toxin production were also investigated (Fig. 8). As the concentration of phosphorus-nutrient increases from 0.05 M to 0.2 M, the production of cyanobacterial toxin was rapidly increased but the slope is lowered above the concentration of 0.2 M. However, the production of cyanobacterial toxin was very low up to the 1.5 M of nitrogen-nutrient but it increased more rapidly above that concentration.

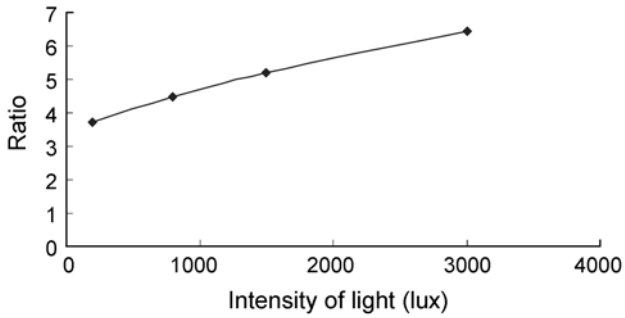

Figure 7. The amount of cyanobacterial toxin produced from NIER10004 as a function of light intensity.

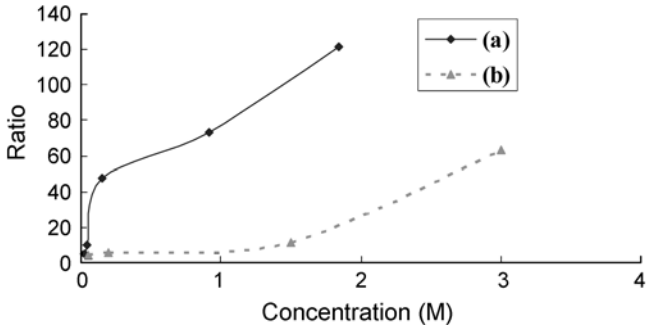

Figure 8. The amount of cyanobacterial toxin produced from NIER10004 as a function of (a) P-nutrient concentration and (b) N-nutrient concentration.

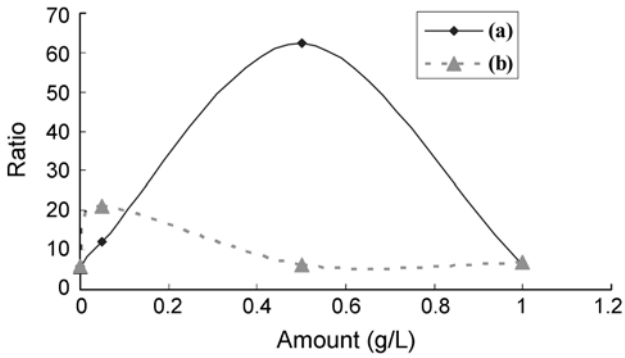

Figure 9. The amount of cyanobacterial toxin produced from NIER10004 as a function of (a) Zn concentration and (b) Fe concentration.

Finally, the effect of metals (iron and zinc) on toxin production were investigated (Fig. 9). Relatively small amount of iron (0.05 g/L) was necessary for the cyanobacterial toxin production, however, in the case of zinc, relatively high concentration of zinc (0.5 g/L) was required for optimal toxin production (Fig. 9).

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