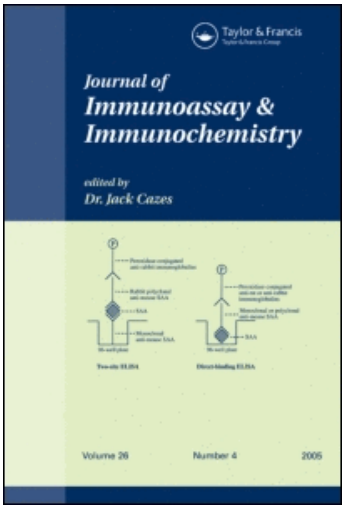


**Journal of Immunoassay and Immunochemistry**
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597271

# Degradation of Cyanobacterial Toxin, Microcystin LR, using Chemical Oxidants

Dongjin Pyo[a]; Jisun Yoo[a]
[a] Department of Chemistry, Kangwon National University, Chunchon, Korea

Online publication date: 05 November 2010

**To cite this Article** Pyo, Dongjin and Yoo, Jisun(2008) 'Degradation of Cyanobacterial Toxin, Microcystin LR, using Chemical Oxidants', Journal of Immunoassay and Immunochemistry, 29: 3, 211 — 219
**To link to this Article: DOI:** 10.1080/15321810802122202
**URL:** http://dx.doi.org/10.1080/15321810802122202

*Journal of Immunoassay and Immunochemistry*, 29: 211–219, 2008

ISSN: 1532-1819 print/1532-4230 online
DOI: 10.1080/15321810802122202



# Degradation of Cyanobacterial Toxin, Microcystin LR, using Chemical Oxidants

**Dongjin Pyo and Jisun Yoo**
Department of Chemistry, Kangwon National University, Chunchon, Korea



**Abstract:** 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. Chlorine, potassium permanganate, and hydrogen peroxide were used as chemical oxidants for the degradation of microcystin LR. When chlorine was used, the efficiency of degradation was the highest. The degradation reaction took 40 minutes.

**Keywords:** Cyanobacteria; Degradation; Microcystin; Toxin

## 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

Correspondence: Dongjin Pyo Department of Chemistry, Kangwon National University, Chunchon 200-701, Korea. E-mail: pyod@kangwon.ac.kr

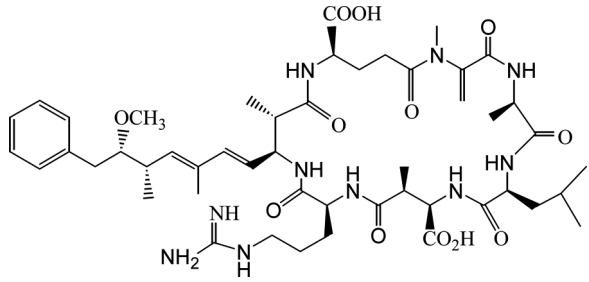


**Figure 1.** Structure of microcystin LR.

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, which 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]

Toxic cyanobacterial blooms have been reported in many countries.[8] Toxic water blooms 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 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 a 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 microcolumn high performance liquid chromatography (HPLC).[15] The developments of biological methods were first focused on enzyme-linked immunosorbent assays (ELISA); however, ELISAs 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 various chemical oxidants on degradation of toxins produced by cyanobacteria have been studied.



## EXPERIMENTAL

### Chemicals

Keyhole Limpet Hemocyanin (KLH), and 1-ethyl-3-(3-dimethylaminopropyl) carbadimide (EDAC) were purchased from Sigma (St. Louis, MO). Streptoavidin, Protein G, and 33′55′,-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 atmospheres were used. Microcystin-L-leucine-R-arginine (MCLR) was extracted form *Microcystis aeruginosa* in the Analytical Chemistry Laboratory, Kangwon National University.

### Preparation of Microcystins

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 (9,000 × g, 5 min)

and were lyophilized before storage at $-20^{\circ}C$. 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 ultrafiltration and ammonium sulfate precipitation, and finally purified using a 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

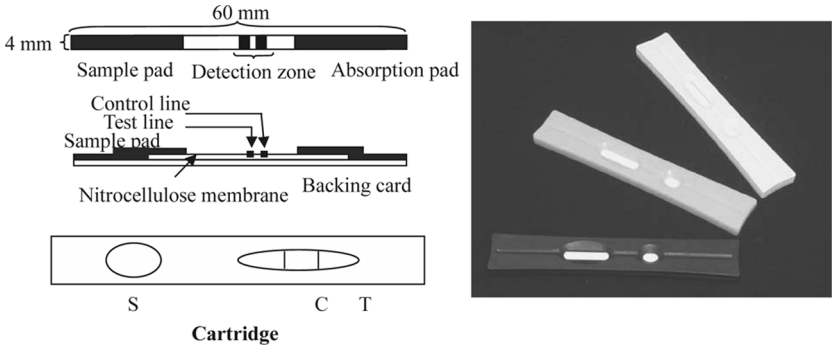


**Figure 2.** Schematic diagram of fluorescence immunochromatographic assay strip.



(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 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, $4 \times 25\,mm^2$) 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, $4 \times 20\,mm^2$) 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 ($15 \times 90\,mm^2$) 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 of 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 an 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 immunochromatographic 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 the 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. The 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 phenomenon results from the method of making the fluorescence immunochromatographic strip. Anti-MCLR-mAb ($350\,\mu g\,mL^{-1}$) 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 $100\,\mu L$ containing $80\,\mu L$ of sample and $20\,\mu L$ of the detection solution was loaded onto the sample pad of the immunochromatographic assay strip. The detector solution contained the MCLR-FL ($1.18\,\mu g\,mL^{-1}$) and the biotin-FL ($92\,ng\,mL^{-1}$). 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 meantime, 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 ratios of $A_C/A_T$ were plotted against different concentrations of MCLR and are shown in Figure 3. In Figure 3, the data points in the range of $150\,pg\,mL^{-1}$ to $1{,}600\,pg\,mL^{-1}$ 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 $1{,}600\,pg\,mL^{-1}$.

First of all, using the fluorescence immunochormatographic strips, the effect of chlorine on degradation of cyanobacterial toxin, microcystin LR produced by cyanobacteria was investigated (Fig. 4). The 'Ratio of Degradation' of the Y-axis in Figure 4 means the ratio of the fluorescence peak area at the test line to that at the control line. This ratio is proportional to the quantity of toxin degraded in the sample. In Figure 4, we can observe that the toxin was continuously degraded for until 12 ppm

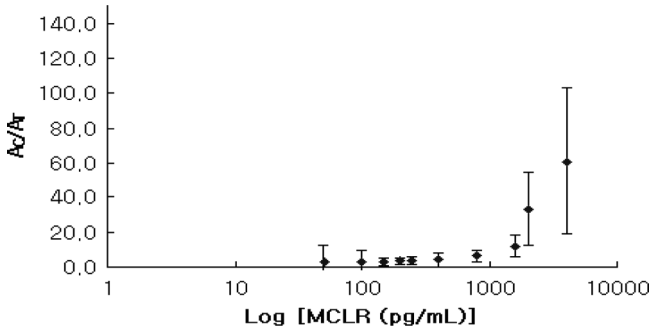


**Figure 3.** $A_T/A_C$ plotted against different concentrations of microcystin. Each point on the graph represents the mean values and the error bars represent standard deviation values of five independent experiments. Ten different concentrations (4,000, 2,000, 1,600, 800, 400, 250, 200, 150, 100, and 50 pg/mL) of free microcystin samples were used.



of chlorine solution; however, after 12 ppm, the degradation ratio was decreased rapidly. The reason of rapid decrease of the degradation of toxin content after 12 ppm could be the fast reaction of microcystin and chlorine. The mechanism of reaction of microcystin and chlorine can be estimated as the following:

When potassium permanganate was used as a chemical oxidant, a small amount of potassium permanganate was sufficient to degrade microcystin LR (Fig. 5). In Figure 5, 82% of the toxin was degraded with

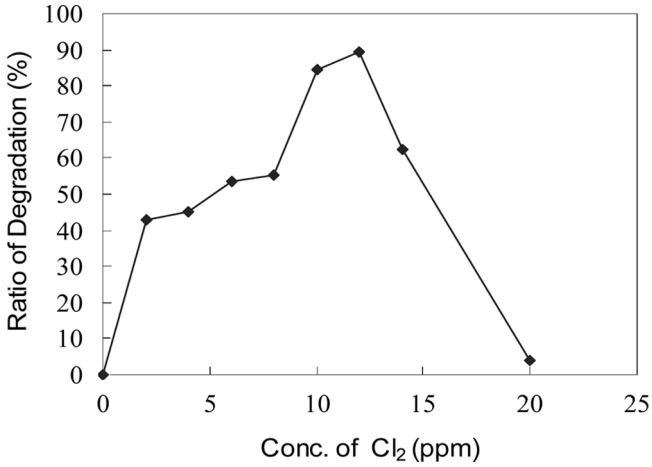


**Figure 4.** The ratio of degradation of microctstin LR using various concentrations of $Cl_2$.Nine different concentrations (20, 14, 12, 10, 8, 6, 4, 2, 0 ppm) of chlorine were used.

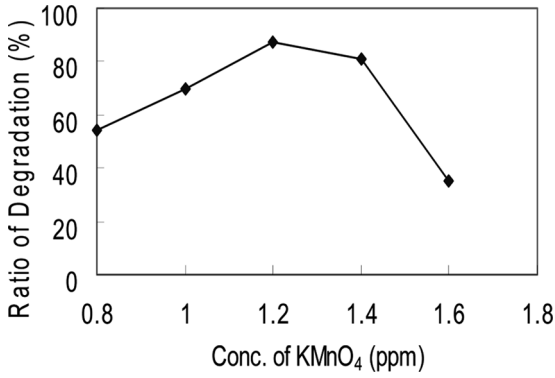


**Figure 5.** The ratio of degradation of microctstin LR using various concentrations of $KMnO_4$. Five different concentrations (1.6, 1.4, 1.2, 1.0, 0.8 ppm) of potassium permanganate were used.



1.2 ppm potassium permanganate solution. In the case of potassium permanganate, after 1.2 ppm, the degradation ratio was decreased relatively slowly. The reason for slow decrease of degradation of toxin could be the relatively slow reaction of microcystin and potassium permanganate, due to the formation of a ring structure. The mechanism of reaction of microcystin and potassium permanganate can be estimated as the following:

Finally, hydrogen peroxide was tried as a chemical oxidant (Fig. 6). In Fig. 6, we could observe that hydrogen peroxide could degrade only 0–14% of micocystin. These studies indicate that chlorine and potassium

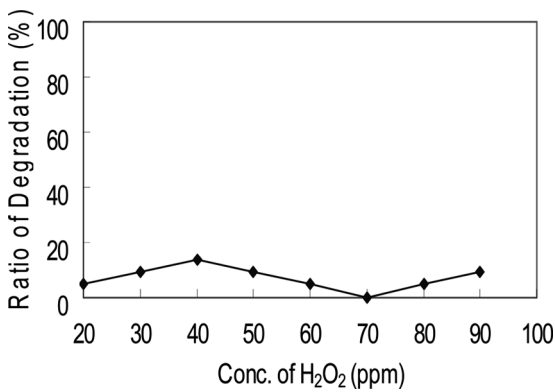


**Figure 6.** The ratio of degradation of microctstin LR using various concentrations of $H_2O_2$. Eight different hyrogen peroxide concentrations (20 to 90 ppm) were used.

permanganate were effective for the degradation of microcystin LR in water; however, hydrogen peroxide was not effective.

## ACKNOWLEDGMENT

This work was supported by the Korea Science and Engineering Foundation (KOSEF) grant funded by the Korea government (MOST) (No. R01-2007-000-20353-0).

## REFERENCES

1. Carmichael, W.W.; Hallegraeff, G.M.; Anderson, D.M.; Cembella, A.D. Scientific and Cultural Organization: Paris, 1995.
2. Sivonen, K. Phycologia **1996**, *35*, 12.
3. Dunn, J.B. Med. J. **1996**, *312*, 1183.
4. Yu, S.-Z.; Steffensen, D.A.; Nichoson, B.C. Austral. Centre Water Qual. Res.: Salisbury, Australia, 1994.
5. Botes, D.P.; Wessels, P.; Kruger, L.H.; Runnegar, MT.C.; Santikarn, S.; Smith, R.J.; Barna C.J.; Williams, D.H. J. Chem. Soc. Perkins Trans. **1985**, *1*, 2747.
6. Lahti, K.; Hiisvirta, L. Water Supply **1989**, *7*, 149.
7. Keijola, A.M.; Himberg, K.; Esala, A.L.; Sivonen, K.; Hiisvirta, L. Toxicol. Assess. **1988**, *3*, 643.
8. Watanabe, M.F.; Harada, K.-I.; Fujiki, H. *Waterbloom of Bluegreen Algae and their Toxins*; Tokyo, University of Tokyo Press: Japan, 1994.
9. Sugaya, Y.; Yasuno M.; Yanai, T. Jpn. J. Limnol. **1990**, *51*, 149.
10. Erikson, J.E.; Meriluoto. J.A.O.; Lindholm, T. Hydrobiologia **1989**, *183*, 211.
11. Harada, K.-L.; Matsuura, K; Sujuki, M.; Oka, H.; Watanabe, M.F; Oishi, S.; Dahlem, A.-M.; Beasley, V.R.; Carmichael, W.W. J. Chromatogr. **1988**, *448*, 275.
12. Pyo, D.; Shin, H. Anal Chem. **1999**, *71* (20), 4772.
13. Pyo, D.; Huang Y.; Kim, Y.; Hahn J.H. Bull. Korean Chem Soc. **2005**, *26*, 939.
14. Oh, H.-M.; Lee, S.-J.; Kim, J.-H.; Yoon, B.-D. Appl. Envir. Mico. **2002**, *67*, 1484.
15. Pyo, D.; Moon D. Bull. Korean Chem Soc. **2005**, *26*, 2089.
16. Pyo, D.; Choi, E.; Cha, G.S.; Lee, J.; Jung, S.; Kim, M.S. Bull. Korean Chem Soc. **2003**, *24*, 126.
17. Ha, S.G.; Park, J.B.; Ko, K.K.; Choi, E.Y. Hybridoma **1999**, *18*, 371.
18. Tsuji, K.; Naito, S.; Kondo, F.; Ishikawa, N.; Watanabe, M.F.; Suzuki, M.; Harada K.-I. Envir. Sci. Technol. **1993**, *28*, 173.

Received February 7, 2008
Accepted February 25, 2008
Manuscript 3277