**Lake Erie Protection Fund (SG 472-2014)**

# Final Report

## *Rapid and Sensitive Microcystin Detection*

**Submitted by:**

**Jiyoung Lee**
**Chenlin Hu**

**Division of Environmental Health Sciences**
**College of Public Health**
**The Ohio State University**

**406 Cunz Hall**
**1841 Neil Avenue**
**Columbus, OH 43210**

**November 2015**

**ABSTRACT**

Microcystins belong to a group of cyclic heptapeptides, produced mainly by freshwater cyanobacteria, such as *Microcystis, Anabaena, and Planktothrix*. Microcystins pose a serious human and animal health threat due to their potent hepatotoxicity and tumor-promoting activities. Consequently, there is a great need to develop a cost-effective, rapid, and sensitive method to detect microcystin. With this aim, this study attempted to develop a novel, noncompetitive immunoassay to detect microcystin based on its interaction with ribulose-1, 5-bisphosphate carboxylase/oxygenase (Rubisco) and microcystins. We have expressed and purified the large subunit of Rubisco (RbcL) of *Microcystis aeruginosa* PCC 7806 from *Esherichia coli;* however, the issue of insolubility of the heterologously expressed RbcL limits the development of a novel method. Therefore, we utilized the commercial Rubisco product, purified from spinach, and established a novel, noncompetitive sandwich immunoassay to detect microcystin. Unlike the traditional indirect competitive ELISA, the detection signal in the present method is directly proportional to the concentration of microcystin in the tested samples. In addition, the application of Rubisco significantly reduces the cost of the entire immunoassay. However, the sensitivity of the proposed method (high to 1 μg/mL) needs to be further improved via systematic optimization of reaction conditions between microcystin and RbcL, enabling the detection of low concentrations (~0.1 μg/L) of microcystin in natural water samples. In summary, the method proposed in this study established a first, important step towards developing a convenient, cheap, fast, and reliable immunoassay for microcystin detection.

**INTRODUCTION**

Microcystins (MCs) are a family of cyclic heptapeptides produced mainly by freshwater cyanobacteria such as *Microcystis*, *Anabaena, Plankothrix, and Nostoc* (Sivonen and Jones, 1998). Microcystins exhibit potent hepatotoxicity and tumor-promoting activities (Carmichael et al., 1992; Nishiwaki-Matsushima et al., 1992); therefore, they pose a serious health threat to humans and animals and have received considerable scientific and public attention.

Microcystins are comprised of seven amino acids that form a ring structure. The general structure of microcystins is cyclo[D-alanine$^{1}$-X$^{2}$-D-MeAsp$^{3}$-Z$^{4}$-Adda$^{5}$-D-glutamate$^{6}$-Mdha$^{7}$], in which X and Z are two variable amino acids. Today, more than 80 different variants of microcystin are known, MC-LR is the most common and well-studied. The World Health Organization has established safe guidelines for drinking and recreational waters (1 μg/L and 20 μg/L of MC-LR-equivalents, respectively) (WHO, 1998). The U.S. Environmental Protection Agency (USEPA) also placed MC-LR on its Drinking Water Contaminant Candidate List 3 (CCL 3) for future potential regulation (USEPA, 2009). In this context, it is vitally important to develop a sensitive and convenient quantitative approach to monitor microcystin levels in water samples.

In the past decades, a serial of bioassays, immunoassays, enzymatic and physic-chemical approaches have been developed to analyze microcystin, including enzyme-linked

immunosorbent (ELISA) and protein phosphate inhibition (PPI) assays, high-performance liquid chromatography (HPLC), liquid chromatography coupled with mass spectrometry analysis (LC-MS), capillary electrophoresis (CE), nuclear magnetic resonance (NMR), gas chromatography (GC) and GC with MS detection (GC/MS), surface plasmon resonance (SPR) biosensor, and other various types of immunosensors and immunostrips (Singh et al., 2012; Weller, 2013) were developed. Among these methods, HPLC and LC-MS are able to qualify and quantify microcystin variants and importantly, they generate highly reliable and reproducible analyses; however, they require a complex sample pretreatment, skilled personnel, and relatively expensive facilities, which limits their wide applications. Although ELISA cannot differentiate microcystin variants, it can offer fast, convenient, and sensitive screening. Thus, it has received increasing popularity and is widely being employed in various settings.

ELISA encompasses two basic immunoassay principles: competitive and noncompetitive formats. For example, in the competitive format, the analyte (e.g. antigen) in the test sample and the conjugated antigen in a standard solution compete for binding the same antibody, as a consequence, the final detection signal is reversely proportional to the concentration of analyte. In the noncompetitive format, the analyte (e.g. antigen) is first captured by the immobilized antibody (IA), then, is directly detected by another specific antibody (IIA), eliminating the competition step of the reaction; the final detection signal is thus directly proportional to the concentration of analyte in the test sample. The noncompetitive immunoassay displays obvious superiority in sensitivity (Jackson and Ekins, 1986). However, most of the immunodetection approaches uniformly employ the competitive immunoassay format to detect microcystin (Chu et al., 1989; Nagata et al., 1995; Zeck et al., 2002; Lei et al., 2003; Kim et al., 2003; Campàs et al., 2006; Hu et al., 2008 and 2009;); mainly because the small molecular microcystin (~1000 Da) has only a single eptitope that is recognized by the anti-microcystin antibody. Thus, it hinders the feasibility to develop the noncompetitive immunoassay format for microcystin detection using microcystin-specific antibodies (Nagata et al., 1999). Until now, there has only been limited success in developing a noncompetitive immunoassay for detection of microcystin. Nagata and colleagues (1999) developed a noncompetitive sandwich immunoassay for microcystin using a new monoclonal antibody, specific to the immune complex formed by microcystin and an anti-microcystin monoclonal antibody. It is still a challenge to develop a real noncompetitive, sandwich immunoassay for microcystin. Very recently, there was encouraging evidence that microcystin can primarily react with the protein ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco) large subunit (RbcL) (Zilliges et al., 2009), probably via the addition reaction between the cysteine moieties of RbcL and the N-methyl-dehydroalanine moiety of microcystin (Zilleges, et al., 2009; Kehr et al., 2009).

**PURPOSE AND SCOPE**

This finding prompts us to utilize the Rubisco large subunit to develop a novel, noncompetitive sandwich immunoassay for microcystin. The entire procedure of the proposed method in this study is illustrated in Figure 1. The most significant feature of this proposed method is to utilize

Rubisco protein to capture microcystin in test samples, which is then directly detected by anti-microcystin monoclonal antibody (mAb). Therefore, the analyte is sandwiched by Rubisco and mAb. The current study has two major aims: 1) test the feasibility of developing a noncompetitive immunoassay using RbcL to detect microcystin; and 2) analyze the cost of the proposed method. Considering the convenient operation of the proposed method, as well as the convenient availability of Rubisco that is almost the most abundant protein on earth (Ellis et al., 1979), we envision that the method proposed in this study can represent a first important step towards developing a fast, cheap, and reliable immunoassay for detecting microcystin.

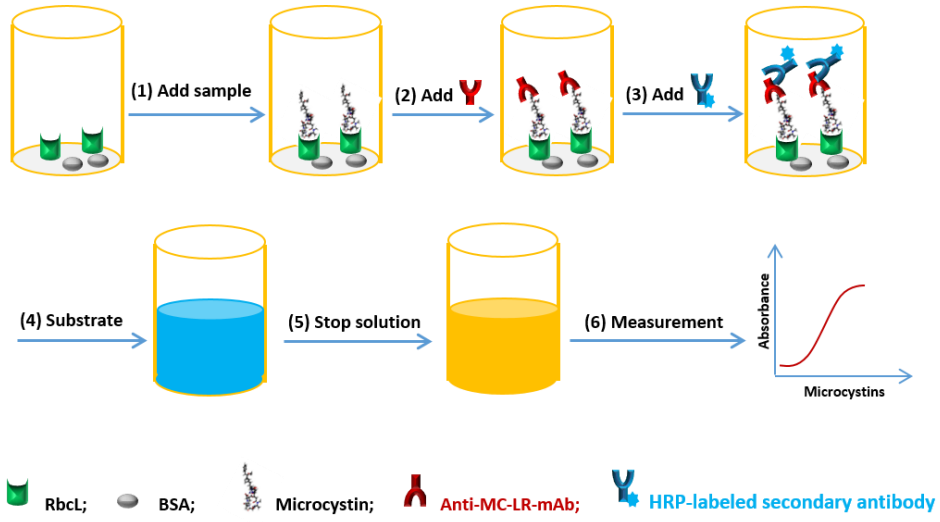


**Figure 1.** The working principle (not to scale) of the novel, RbcL-based noncompetitive immunoassay for detection of microcystin. The entire immunoassay encompasses six major steps: 1) microcystin in test sample was captured by the RbcL immobilized on the ELISA microplate; 2) the captured microcystin was recognized by the specific anti-microcystin monoclonal antibody (mAb); 3) the bound mAb was further recognized by the horseradish perioxidase (HRP)-labeled secondary antibody; 4) HRP-catalyzed color development of the substrate TMB; 5) color development was stopped by the diluted sulfuric acid; and, 6) reading the optical density of the reaction mixture and quantifying the microcystin concentration according to the standard curve.

## METHODS

### *Materials and reagents*

D-Ribulose 1, 5-Diphosphate Carboxylase (Rubisco, 20 mg), partially purified from spinach, was purchased from Sigma-Aldrich. Microcystin standard (MC-LR) (>95% purity, HPLC-grade) was purchased from Beagle (Ohio, USA); mouse monoclonal antibody (mAb, AD4G2) against microcystin was purchased from Enzo Life Sciences Inc. (NY,USA) and this mAb was raised against the unusual amino acid Adda [(2S,3S,8S,9S)-3-amino-9-methoxy-2,6,8-trimethyl-

10-phenyldeca-4E,6E-dienoic acid, which is a common part of microcystins and nodularins (Zeck et al., 2002). The Nunc MaxiSorb ELISA microplate, BlockerTM BSA (10%) in PBS (Lot: QD214812), stabilized peroxidase conjugated goat-anti-mouse (H+L) (10 μg/ml), and 1-Step™ Ultra 3,3', 5,5' Tetramethylbenzidine (TMB) solution were purchase from Thermo Scientific (USA). Primer in this study was synthesized by ABI Company (Life technologies, USA) and FastDigest restriction enzyme was purchased from ThermoFisher Scientific (USA). All the other buffer solutions were prepared with deionized water (18.2 MΩ•cm) from a MilliQ system (Millipore, Bedford, MA, USA).

***Extraction of genomic DNA from Microcystis aeruginosa strain PCC7806***

Genomic DNA in *Microcystis aeruginosa* PCC7806 was extracted using the xanthogenate-sodium dodecyl sulfate (XS)-based DNA extraction protocol with minor modification (Tillet et al., 2001). Briefly, a *Microcystis* culture in exponential growth phase (about 10 mL) was harvested by centrifugation at room temperature; the obtained algal pellet was incubated in XS buffer (1 mL) at 70°C for around 2h, the mixture vortexed for 10s, and placed in ice for 30 min. After centrifugation at 13,000 rpm for 15 min at 4°C, the supernatant was carefully pipetted off and transferred to a new tube and mixed well with an identical volume of 100% isopropanol. Subsequently, the mixture was applied to the Econospin Mini Spin column and centrifuged for one minute at 10,000 rpm. After sequential washing with AW1- and AW2-buffers, DNA in the column was eluted with 100μl of AE buffer and stored at -20°C for further PCR analysis.

***PCR Amplification of rbcL gene***

An 1.4-kb *rbcL* gene was amplified from *Microcystis aeruginosa* PCC7806 genomic DNA using the primers RbcL_BamHI_FW (5-GGCCGGTTGGATCCGATGGTGCAAGCCAAATCC-3) and RbcL_BlpI_RV (5-CAACAAGCTCAGCGAGGGTATCCATAGCCTC-3); the restriction enzyme site (BamHI and BlpI) was separately introduced into the 5' end of the primers (the sequences underlined are the restriction sites). The PCR reaction mixture (20μL) contained: 5× Phusion HF buffer (4μL), dNTP (0.5μL, 10mM), Phusion High-Fidelity DNA polymerases (0.1μL, Thermo Scientific, USA), and DNA template (2μL).The PCR profile was one cycle of an initial denaturation step (95°C for 10 min); 35 cycles of 95°C for 30s, 55°C for 1 min and 72°C for 90s; and one cycle of final extension (72°C for 10 min), which was conducted on the MultiGene Thermal Cycler (TC9600-G, Labnet, USA). The PCR products (10μL) were applied to the agarose gel (1.2%) in TAE buffer (National Diagnostics, USA) for electrophoresis separation at 100V for around 15-20 min, which was conducted in the RunOne™ Electrophoresis Cell (Embi Tec, USA). The PCR product was visualized with ethidum bromide staining on the BioRad UV illuminator. After confirming the right size of PCR fragment, PCR product was purified with the QIAquick PCR Purification Kit purify kit (Qiagen, USA) according to the manufacture's instructions. The purified PCR product was kept at -20°C for further analysis.

***Construction of expression plasmid pET-15b-rbcL***

The purified PCR product was digested with FastDigest restriction enzyme (BlpI and BamHI) to obtain the sticky ends; meanwhile, the commercial vector pET-15b was also linearized with

restriction enzymes (BlpI and BamHI). After purification using the Quick purify kit (Qiagen, USA), the BlpI /BamHI-digested *rbcL* fragment was ligated into the BlpI/BamHI double-digested pET-15b to generate the recombinant expression construct pET-15b-rbcL, which was subsequently transformed into *Escherichia coli* DH5α competent cells via the classic heat shock approach. 500µl of Luria-Bertani (LB) medium was added to recover the growth of the transformed cells at 37˚C for one hour. The tranformants were grown on LB-agar plates (1.5%) with selection by ampicillin at 100µg/ml at 37˚C overnight. The positive clone was screened out via colony PCR with the primers (RbcL_BamHI_FW and RbcL_BlpI_RV) according to the PCR profile described above. The positive clones were picked and inoculated into LB medium with a final concentration of ampicillin at 100µg/ml. After overnight (around 15h) culturing at 37˚C, the plasmid in the positive clone was isolated using the QIAprep Spin Minprep Kit (Qiagen, USA) and was sequenced at The Ohio State University's Plant-Microbe Genomics Facility (Columbus, Ohio, USA). The plasmid with the right nucleotide sequence was ready for downstream experiment.

***Heterologous expression of Rubisco large subunit (RbcL)***

The transformed clone was randomly picked and inoculated into 5ml of LB medium containing 100µg/ml of ampicillin for overnight culturing at 37˚C at a shaking speed of 220 rpm. On the following day, the overnight culture was inoculated at a 1:100 dilution into 50 ml of LB medium containing 100µg/ml of ampicillin and grown at 37˚C at a shaking speed of 220 rpm until the optical density ($OD_{\lambda=600nm}$) of the cell culture reached approximately 0.6. Subsequently, the expression of recombinant RbcL protein was induced by adding the inducer (Isopropyl β-D-1-thiogalactopyranoside, IPTG) at a final concentration of 1mM; the entire induction time persisted was about 3-4h. The cells were harvested by centrifuging at 3,000 rpm for 10 min at 4˚C; the pellets were stored at -80˚C for subsequent protein extraction. In the optimizing experiment for obtaining soluble RbcL protein, varying incubation temperatures (16, 25, 27, 30 and 37˚C) and varying concentrations of the inducer, IPTG (0.1, 0.3, 0.5, 0.7 and 1 mM) were tested.

***Extraction and purification of the recombinant protein RbcL***

The recombinant RbcL protein was purified using the ProBond™ Purification System (Invitrogen, USA). Prior to purification of the recombinant RbcL protein at a large scale, a preliminary test for the solubility or insolubility of RbcL was performed as follows. Briefly, an appropriate amount of harvested cells were resuspended in the native binding buffer supplemented with the protease inhibitor cocktail (1:100 dilution, Sigma, USA). Cells were lysed by freeze-thaw cycles and ultrasonification treatment (60 Sonic Dismebrator, Fisher Scientific). Subsequently, the lysed cell mixture was centrifuged at 4˚C for 10 min. The supernantant fraction containing soluble protein was transferred to a new tube and 5% SDS was added into the pellet to re-dissolve the water-insoluble protein. The soluble and insoluble proteins were applied to SDS – PAGE for analysis. The recombinant RbcL protein proved to be water-insoluble in this study despite numerous optimization trials; therefore, the recombinant RbcL was purified under denaturing conditions according to the manufacture's instructions. Briefly, the cell pellet was

resuspended in Guanidinium Lysis Buffer and then subjected to sonication treatment. After centrifugation, the supernant was transferred to a ready-to-use column that contained resin (2ml) and was already prepared according to instructions. Sufficient binding between protein and resin was facilitated via gentle agitation using a rotating wheel for 30 min. The protein-bound column was sequentially washed using the Denaturing Wash Buffer (pH6.0) and Denaturing Wash buffer (pH. 5.3) that were supplied with the kit. The protein bound to the column was eluted in 1ml fractions using 5ml of Denaturing Elution Buffer. The purity of protein in each fraction was evaluated via subsequent SDS-PAGE analysis.

***SDS-polyacryamide gel electrophoresis (SDS-PAGE)***

Proteins were separated via SDS-PAGE as follows. Briefly, gels consisted of a separating gel and a stacking gel, and SDS (0.1%, w/v). The protein sample was mixed well with 5× loading buffer [Tris-HCl (250 mM pH 6.8); bromophenol blue (0.5%); SDS (10%, w/v); Glycerol (50%, v/v); 2-mercaptoethanol (500 mM)] and heated at 95°C for 10 min. The sample mixture was then applied to gel electrophoresis. The running buffer consisted of Glycine (192mM), Tris-HCl (25mM, pH 8.0) and SDS (0.1%, w/v). The gels were run at a constant current of 25 mA per gel in the Mini PROTEAN® Tetra Cell (Bio-Rad) for around one hour. The gel was stained with GelCode Blue Stain Reagent (Pierce), destained using the buffer overnight, and then photographed.

***Rubisco-based noncompetitive sandwich immunoassay***

Each well in the ELISA microplate was coated with 100µL of commercial Rubisco (3 µg/mL) in 50mM $Na_2CO_3$-$NaHCO_3$ buffer (pH 9.6) at 4°C overnight, and blocked with 200µL of BSA (1%) in PBS (pH 7.4) at 37°C for one hour. After washing the microplate three times with PBST [1×PBS (pH 7.4);Tween-20 (0.05%, v/v)], the test sample (microcystin standard or unknown sample, 99µL) and dithiothreitol (DTT) solution (1M, 1µL) were added to each Rubisco-coated plate and incubated at 30°C for 45 min; after washing three times with PBST, 100µL of anti-ADDA mAb (1:5000 dilution) was added and incubated at 37°C for 45 min. After washing three times with PBST, the stabilized peroxidase conjugated goat-anti-mouse (H+L) (1:200 dilution) was added and incubated at 37°C for 30 min. After washing three times with PBST, the HRP substrate, 1-Step™ Ultra TMB-ELISA Substrate solution (100µL) was added to develop the blue color for 5-10 min, and then the reaction was stopped by adding 50µl of 1M $H_2SO_4$. The absorbance was read at 450nm in a microplate reader (SpectraMax Plus 384 Microplate Reader, Molecular Devices, USA).

**RESULTS AND DISCUSSION**

***Construction of expression plasmid pET-15b-rbcL***

To construct the expression plasmid for the recombinant RbcL of *Microcystis aerguinosa* PCC7806, we introduced the restriction site, BamHI and BlpI, into the 5' end of the forward and

reverse primers. Meanwhile, a high-fidelity polymerase was employed to minimize the potential mutation risk or other amplification errors during the PCR steps. The ligation mixture for constructing the expression plasmid pET-15b-rbcL was transformed into *E.coli* DH5a competent cells, subsequently, four transformants were picked at random and applied to a colony PCR assay for screening. As a result, clones 1 and 2 generated a positive amplification; their PCR product size coincided with the 1.4 kbp of *rbcL* gene in *Microcystis aeruginosa* PCC7806 (Figure 2), indicating that the *rbcL* gene might successfully be cloned into pET-15b. Subsequently, the sequencing analysis verified the right reading frame in pET-15b-rbcL, ensuring that the right amino acids of the recombinant RbcL can be translated as expected. Therefore, the expression plasmid pET-15b-rbcL can be used for a downstream protein expression experiment.

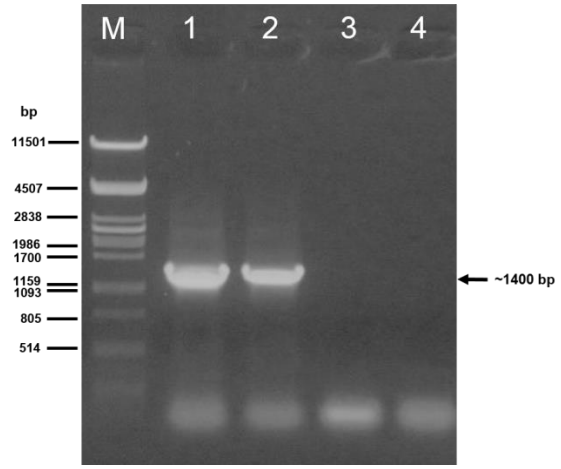


**Figure 2**. Colony PCR to screen transformants for constructing expression plasmid pET-15b-rbcL, PCR product was separated on an electrophoresis gel (1%), M: λ/PstI marker, Clone 1-4 were random picked from the agar plate for screening purposes. Clones 1 and 2 generated positive PCR amplification and clones 3 and 4 generated negative PCR amplification.

***Heterologous expression of the rbcL gene of Microcystis aeruginosa PCC7806***

A preliminary experiment was first conducted to test whether the cloned *rbcL* gene of *Microcystis aeruginosa* PCC7806 can be expressed in *E. coli* BL21 (DE3) under induction of IPTG. SDS-PAGE analysis showed that a ~55 kDa protein was substantially expressed under the IPTG-inducing condition, compared to the control group without the inducer (Figure 3). The molecular weight of the ~55 kDa protein appearing in the SDS-PAGE gel coincided with the anticipated size of the recombinant protein derived from expression plasmid pET-15b-rbcL. Unfortunately, the recombinant protein seemed to be expressed exclusively in the insoluble form under the inducing conditions of 1mM IPTG and 37ºC. This might be due to the high-level expression of the recombinant protein, which may usually result in the miss-folded insoluble protein that was highly aggregated to form inclusion bodies (Kane and Hartley, 1988). The insolubility issue of the recombinant RbcL protein would limit our downstream development of the proposed method. Therefore, to obtain soluble recombinant protein, we conducted a series of optimization experiments using lowering the culturing temperature and the concentration of inducer, IPTG. However, little success was achieved in spite of numerous trials. For example, we

did not detect an obviously increasing proportion of the soluble recombinant protein (Figure 4), when the IPTG inducer (ranging from 0.1 to 1 mM) and lower temperatures (27ºC and 16ºC) were used. (data not shown). We attempted to refold the insoluble recombinant protein, which was purified under denatured conditions, via the dialysis approach. Although we could obtain the highly pure recombinant protein (Figure 5), the recovery efficiency of the refolding protein via dialysis approach was unsatisfactory and we were still unable to obtain the soluble recombinant protein for subsequent experimentation Currently, we cannot explain why the recombinant RbcL in this study could not be expressed into the soluble form. It might be related to the plasmid construct in this study, which lacked the charperin that improved the solubility of recombinant protein (Kyratsous et al., 2009). However, one cannot exclude the possibility of an insolubility nature of the RbcL, and that the solubility of the recombinant RbcL requires its specific chaperon, e.g. RbcX (Saschenbrecker et al., 2007). Previously, multiple studies have demonstrated that the recombinant RbcL accumulates in *E.coli* as the insoluble and catalytically inactive form (Gurevitz et al. 1985, Larimer and Soper, 1993).

As a critically important enzyme involved in both photosynthesis and photorespiration, Rubisco is also the most abundant protein on earth. Structurally, Rubisco is a hexadecamerica, multisubunit complex, consisting of eight large subnits (L8, molecular weight of RbcL:50-55 kDa) and eight small subunits (S8, molecular weight of RbcS: 10-18 kDa) (Spreitzer et al., 2012). Although the amino acid composition of the small subunit of Rubisco can significantly vary among different organisms, there is relatively high conservation in the primary structure of the large subunit of Rubisco. Thus, we wondered whether or not microcystins can also bind with Rubisco from a higher plant; to answer this question, we utilized a commercial Rubisco product purified from spinach.

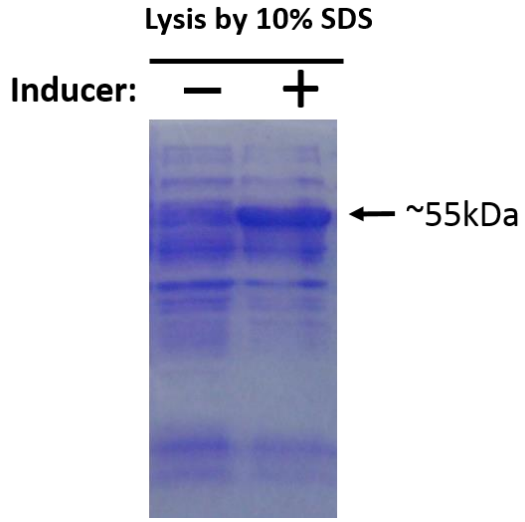


**Figure 3.** SDS-PAGE analysis of the expression of *rbcL* gene in *E. coli* BL21 (DE3) grown in LB medium with (+) and without (-) 1 mM IPTG as the inducer.

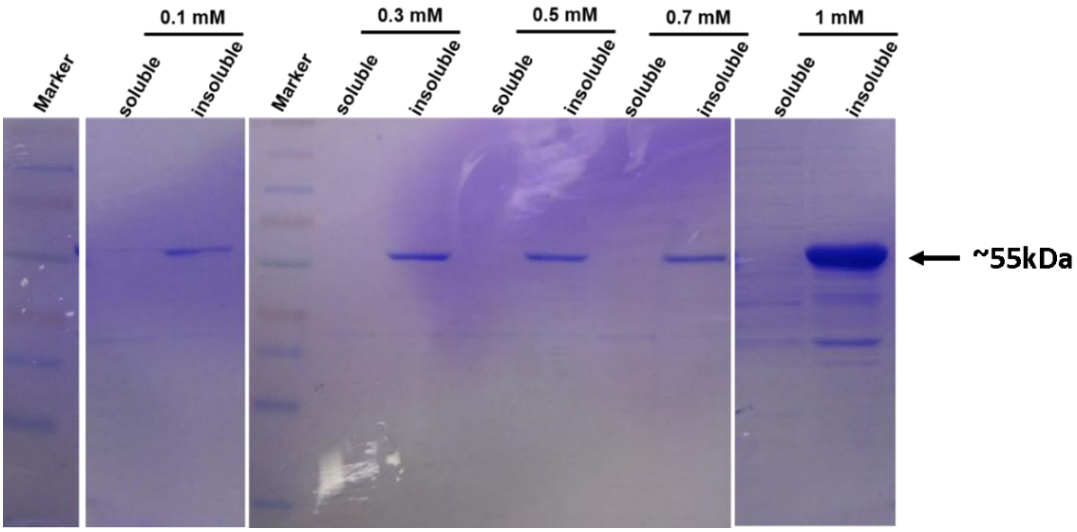


**Figure 4.** Effect of the varying concentrations of inducer (IPTG, 0.1, 0.3, 0.5, 0.7 and 1mM) on the production of soluble recombinant protein RbcL in *Esherichia coli* BL21 (DE3) with the expression plasmid pET-15b-rbcL at 27$^{o}$C.

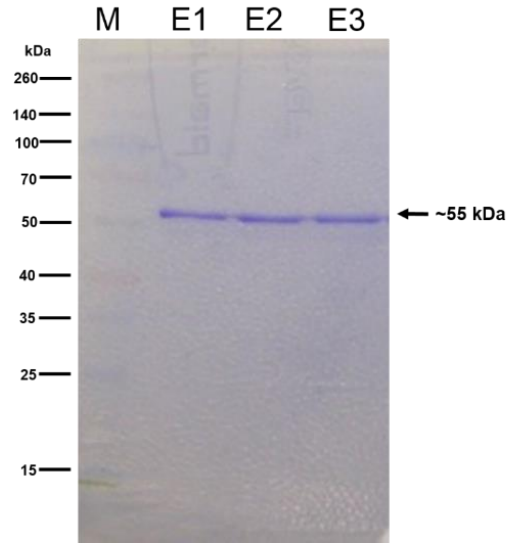


**Figure 5.** SDS-PAGE analysis of the purity of the protein obtained under denaturing conditions. M, marker, E1-3 represent the first, second and third eluted fractions from the column.

***Optimization of coating amount of RbcL protein***

This study employed the noncompetitive immunoassay format to detect microcystin (Figure 1). It is crucially important to obtain an optimal amount of Rubisco coated on the ELISA plate; too high of a level of coated Rubisco might affect the spatial access of microcystin. In the present optimization experiment, serial dilutions of a 1mg/ml Rubisco stock (1:5, 1:10, 1:20, 1:40, 1:80, 1:160, and 1:320) were tested; a blank without microcystin was used as the control. As a result, when the concentration of the coating Rubisco ranged from 6.25 to 200μg/ml, we observed

that the relative response ($B/B_0$) did not change significantly. However, when the concentration of Rubisco coating decreased to around 3μg/ml, the relative response significantly increased by a factor of 2.6 (Figure 6), corroborating the belief that a high level of Rubisco coated on the ELSIA plate did not necessarily capture the corresponding high level of microcystin. In the subsequent experiments, a 3 μg/ml concentration of coating Rubisco was used as the working concentration.

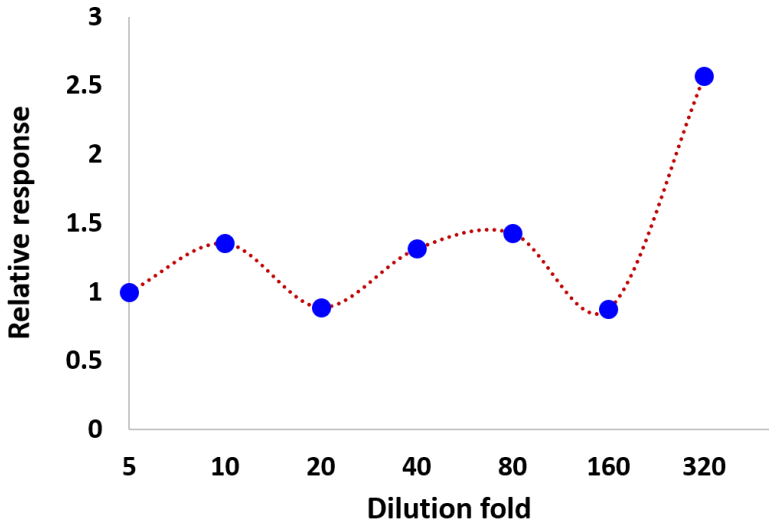


**Figure 6.** Optimization of the concentration of coating Rubisco on ELISA plates in noncompetitive immunoassay format. The stock solution of Rubisco [1mg/ml in in 50mM $Na_2CO_3$-$NaHCO_3$ buffer (pH 9.6)] was serially diluted 5, 10, 20, 40, 80, 160, and 320-fold, respectively.

***Determination of microcystin and standard curve***

According to the procedure of the noncompetitive immunoassay format (Scheme 1), a series of MC-LR standards (0.1, 1, 10, 100, 1000, 10000 μg/L) were added to the Rubisco-coated microwells. Subsequently, anti-ADDA monoclonal antibody and HRP-labeling secondary antibody were sequentially added to detect microcystin bound to the Rubisco on the ELISA plate. We observed that the relative optical density value $[(B-B_0)/B_0]$ went up with an increase in microcystin concentration (Figure 6), which was the typical pattern of an analyte-response commonly observed in the noncompetitive immunoassay format. However, as seen in Figure 6, the proposed method in this study was not as sensitive as the conventional competitive ELISA, other ELISA-like immunoassays, and immunosensors (Lei et al., 2004; Hu et al., 2008 and 2009), which usually display a superior detection sensitivity (0.01-1μg/L). We speculate that there are three major reasons for the poor sensitivity of the method proposed in this study. (1) The incubation condition (pH 9.6 and 30 minutes) used in this study might be not be optimal for the

interaction between Rubisco and microcystin; it is well-known that pH has a significant impact on the reducing capability of DTT. Additionally, incubation time might also affect the binding of microcystin with Rubisco. (2) The unfavorable orientation of the Rubisco coating on the ELISA plate might have significantly reduced the access of the binding site to analyte microcystin; as a consequence, microcystins could not be captured by Rubisco despite a sufficient amount of coated Rubisco on the plate. (3) The reaction of Rubisco and microcystin might be not be as sensitive as that between microcystin and anti-microcystin antibody, which might have limited the binding of microcystin with Rubisco despite the high concentrations of microcystin used. Currently, we are still unclear which of the above constitutes the major factor explaining the poor detection limit of the proposed method. More optimization studies are required to further improve the detection limit of the method proposed in this study. With respect to the analysis time, the entire operation of the new proposed method requires approximately 2.33h including washing steps, similar to that of the conventional competitive ELISA. However, compared to the conventional indirect competitive ELISA kit ($400/kit), the cost of the proposed method for microcystin detection can be reduced significantly by utilizing Rubisco, which is easily purified from spinach. For example, the entire cost of all the reagents used in this proposed method required less than $50, raising the possibility of developing a cost-effective immunoassay for detection of microcystin.

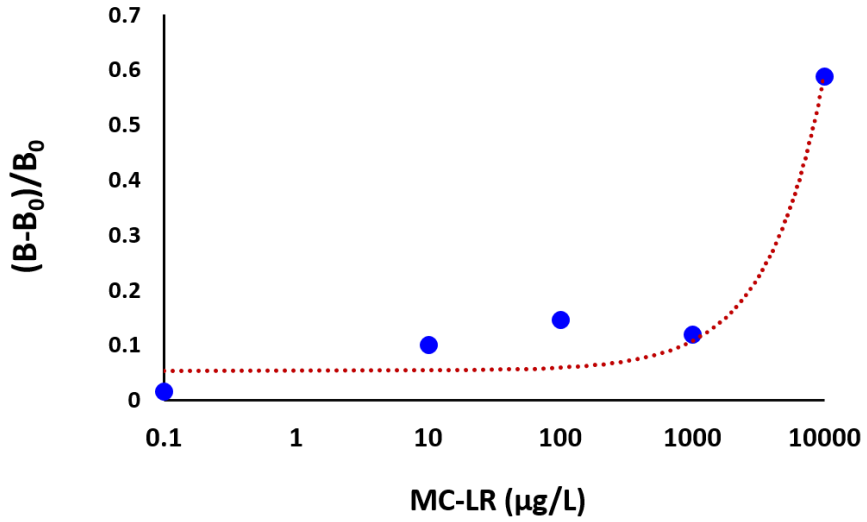


**Figure 7.** Rubisco-based noncompetitive immunoassay detecting free MC-LR (0, 0.1, 1, 10, 100, 1000, 10000 µg/L) in 50mM $Na_2CO_3$-$NaHCO_3$ buffer (pH 9.6)

## SIGNIFICANCE AND OUTLOOK

For the first time, we proposed a novel, noncompetitive immunoassay format for detecting microcystin using the most abundant protein on earth, rubulose-bisphosphate carboxylase

oxygenase (Rubisco). The novelty of this new method is based on the interaction between microcystin and the large subunit of Rubisco (RbcL). Rubisco or RbcL coated on microplates, captured mirocystin in test samples, which was subsequently detected by anti-microcystin monoclonal antibody. Therefore, the final detection signal is directly proportional to the concentration of microcystin in the test sample. To test this hypothesis, we first attempted to obtain pure, soluble, recombinant RbcL from *Microcystis aerguinsoa* PCC7806 through heterologous expression in *E.coli.* However, little success was achieved despite numerous trials with all types of optimization. This study highlights the fact that recombinant RbcL of *Microcystis aerguinsa* PCC7806 might be expressed exclusively in the insoluble form under the present expression system, probably due to a lack of appropriate chaperones. However, by utilizing commercial Rubisco, we succeeded in establishing a noncompetitive sandwich immunoassay to indirectly detect microcystin, suggesting that microcystin can also bind with Rubisco of higher plants (e.g. spinach), expanding our prior view concerning the interaction between microcystin and cyanobacterial Rubisco. Under the present working conditions, the detection limitation of this newly developed method is 1000 µg/L, much higher than 0.1µg/L of the conventional competitive ELISA. However, the sensitivity of the newly developed method can be improved through further optimizing the incubation conditions between microcystin and the Rubisco protein (e.g. pH, temperature, and time) as well as improving the favorable orientation of Rubisco that is coated on the plate. The analysis time of the newly developed method is similar to that of the traditional ELISA. Noteworthy, this newly proposed method can significantly reduce the entire cost of all the reagents to within $50 per kit for analysis of 40 samples, mainly through utilizing Rubisco that is easily available. Overall, the method proposed in this study constitutes an important, first step towards developing a cost-effective, conventional, noncompetitive immunoassay for detecting microcystin.

**ACKNOWLEDGEMENTS**

This study was supported by funding from the Lake Erie Protection Fund. We are grateful for the support by Scott Fletcher of ODNR-Parks. The authors would like to thank Dr. Darryl B. Hood and Dr. Tongwen Zhang for supporting in protein analysis.

**REFERENCES**

Campàs, M. and Marty J.L. (2007) Highly sensitive amperometric immunosensors for microcystin detection in algae. *Biosens Bioelectron* 22:1034-1040.

Chu, F.S., Huang, X., Wei, R.D., Carmichael, W.W. (1989) Production and Characterization of Antibodies against Microcystins. *Appl Environ Microbiol* 55:1928-1933.

Carmichael, W.W. (1992) Cyanobacteria secondary metabolites--the cyanotoxins. *J Appl Bacteriol* 72:445-459.

Ellis, R.J. (1979) The most abundant protein in the world. *Trends in Biochemical Sciences* 4:241-244.

Gurevitz, M., Somerville, C.R., McIntosh, L. (1985) Pathway of assembly of ribulosebisphophate carboxylase/oxgenase from Anabaena 7120 expressed in Escherichia coli. *Proc Natl Acad Sci USA* 82: 6546-6550.

Hu, C.L., Gan, N.Q., Chen, Y.Y., Bi, L.J., Zhang, X.E., Song, L.R. (2009) Detection of microcystins in environmental samples using surface plasmon resonance biosensor. *Talanta* 80:407-410.

Hu, C., Gan, N.Q., He, Z.K., Song, L.R. (2008) A novel chemiluminescent immunoassay for microcystin (MC) detection based on gold nanoparticles label and its application to MC analysis in aquatic environmental samples. *Inter J Environ Anal Chem*. 88: 267-277.

Jackson, T.M. and Ekins, R.P. (1986) Theoretical limitations on immunoassay sensitivity Current practice and potential advantages of fluorescent $Eu^{3+}$chelates as non-radioisotopic tracers. *J Immunol Methods* 87:13-20.

Kane, J.F. and Hartley, D.L. (1988) Formation of recombinant protein inclusion bodies in *Escherichia coli*. *Trends Biotechnol* 6: 95-10.

Kehr, C.J. (2008) Characterisation of the lectin microvirin from Microcystis aeruginosa PCC7806 and new insights into the role of microcystin. PhD. Dissertation.

Kim, Y.M., Oh, S.W., Jeong, S.Y., Pyo, D.J., Choi, E.Y. (2003) Development of an ultrarapid one-step fluorescence immunochromatographic assay system for the quantification of microcystins. *Environ Sci Technol* 37:1899-1904.

Kyratsous, C.A., Silverstein, S.J., DeLong, C.R., Panagiotidis, C.A. (2009) Chaperone-fusion expression plasmid vectors for improved solubility of recombinant proteins in *Escherichia coli*. *Gene* 440:9-15.

Larimer, F.W. and Soper, T.S. (1993) Overproduction of *Anabaena* 7120 ribulose-bisphosphate carboxylase/oxygenase in *Escherichia coli*. Gene 126:85-92.

Lei, L-M., Wu, Y-S., Gan N-Q., Song, L-R., (2004) An ELISA-like time-resolved fluorescence immunoassay for microcystin detection. *Clinica Chimica Acta* 348:177-180.

Nagata, S., Soutome, H., Tsutsumi, T., Hasegawa, A., Sekijima, M., Sugamata, M., Harada, K., Suganuma, M., Yoshio Ueno, Y. (1995) Novel Monoclonal Antibodies Against Microcystin and Their Protective Activity for Hepatotoxicity. *Nat Toxins* 3:78-86.

Nagata, S., Tsutsumi, T., Yoshida, F, Ueno, Y. (1999) A new type sandwich immunoassay for microcystin: production of monoclonal antibodies specific to the immune complex formed by microcystin and an anti-microcystin monoclonal antibody. *Nat Toxins* 7:49-55.

Nishiwaki-Matsushima, R., Ohta. T., Nishiwaki. S., Suganuma. M., Kohyama. K., Ishikawa, T., Carmichael, W.W., Fujiki H. (1992) Liver tumor promotion by the cyanobacterial cyclic peptide toxin microcystin-LR. *J Cancer Res Clin Oncol* 118:420-424.

Saschenbrecker, S., Bracher A., Rao K.V., Rao, B.V., Hartl, F.U., Hayer-Hartl, M. (2007) Structure and function of RbcX, an assembly chaperone for hexadecameric Rubisco. *Cell* 129:1189-200.

Singh, S., Srivastava, A., Oh, H.M., Ahn, C.Y., Choi, G.G., Asthana, R.K. (2012) Recent trends in development of biosensors for detection of microcystin. *Toxicon* 60:878-894.

Sivonen, K. and Jones, G. (1999) Cyanobacterial toxins. In Toxic cyanobacteria in water: A guide to their public health consequences, monitoring and management ed. Chorus, I. and Bartram, J. pp.41-111. London: E&FN Spon.

Spreitzer, R.J. and Salvucci M.E. (2002) Rubisco: structure, regulatory interactions, and possibilities for a better enzyme. *Annu Rev Plant Biol.* 53:449-475.

U.S. Environmental Protection Agency (USEPA). (2009) Fact Sheet: Final Third Drinking Water Contaminant Candidate List 3 (CCL 3).

Weller, M.G. (2013) Immunoassays and Biosensors for the Detection of Cyanobacterial Toxins in Water. *Sensors (Basel)*. 13: 15085-15112.

WHO, (1998) Guidelines for Drinking Water Quality, Addendum to Vol. 1, 2nd ed. World Health Organization, Geneva.

Zilliges Y., Kehr, J-C., Sven Meissner, S., Ishida, K., Mikkat, S., Hagemann, M., Kaplan, A., Börner, T., Dittmann, E. 2009. The Cyanobacterial Hepatotoxin Microcystin Binds to Proteins and Increases the Fitness of *Microcystis* under Oxidative Stress Conditions. *PLoS One*. 2011; 6(3): e17615

Zeck, A., Weller, M.G., Bursill, D., Niessner, R. (2002) Generic microcystin immunoassay based on monoclonal antibodies against Adda. *Analyst* 126:2002-2007.