DETERMINATION OF MICROCYSTIN LR FROM BLUE-GREEN ALGAE (*Microcystis aeruginosa* Kutz.) USING REVERSED-PHASE HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

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ABSTRACT

Microcystin-LR in *Microcystis aeruginosa* Kutz. collected from Sri Sakhett, Thailand was extracted and quantitatively analyzed using reversed-phase HPLC with UV detection at 238 nm. The HPLC analysis employed a C-18 column (4.6x125 mm., 5 micron), with methanol-water (60 : 40) at flow rate of 1.0 ml/min as the mobile phase. A linear calibration curve was obtained out using five concentrations in the range from 1 to 5 ppm (r^2=0.9919). A triplicate analysis of 0.5 g of sample yielded $2.93 \times 10^{-4} \pm 3.68 \times 10^{-5}$ g of microcystin-LR ($5.85 \times 10^{-2} \pm 7.35 \times 10^{-3}$ % yield). NMR spectroscopy was also used to confirm the structure of the microcystin-LR standard.

KEYWORDS: Microcystis aeruginosa Kutz, reversed-phase HPLC, Microcystin LR, NMR spectroscopic technique.

1. INTRODUCTION

The development of the planktonic cyanobacteria in lakes and reservoirs under some environmental conditions, such as high temperature, pH, and nutrient, results in the formation of surface bloom. This may accumulate as scum that results in a significant water quality problem since certain species of cyanobacteria are capable of producing toxins. Cyanotoxin, the secondary metabolites produced by cyanobacteria, are very diverse in their chemical structure and toxicity [1]-[3]. For example, microcrystins are hexpatotoxic cyclic compounds that are responsible for periodic poisonings of human and livestock fresh drinking water wherever cyanobacteria are endemic. The presence of microcystins contaminated food and water causes the illness worldwide[4]-[5]. Crop plants that are watered with microcystin contaminated water may suffer hindered growth and development effects, in addition to accumulating the toxins and thereby posing potential risk of toxin transference to human through the food chain. Microcystins are potent cancer promoters in laboratory animals. Chronic exposure to low concentrations of microcystin in drinking water might contribute to the promotion of cancer in humans. Microcystin-LR, one of the most toxic cyanotoxin, is a selective inhibitor of protein phosphatase-2A (PP-2A) (IC_{50}= 0.04 nM) and will completely inhibit this enzyme at a concentration of 0.5 nM without affecting protein phosphatase-1 (PP-1, IC_{50}~1.7 nM)[6]. Microcystins are at least 10 times more potent as serine/threonine PP inhibitors than okadaic acid. Figure 1 shows the structure of microcysting-LR.Microcystins are known to be produced by species belonging to the genera *Anabaena*, *Microcystis*, *Nostoc* and *Anabaenopsis*.[1] Several methods have been described for the separation of Microcystins by HPLC with fixed wavelength UV [7] and diode-array detection [8]-[10]. More accurate identification of microcystins can be obtained using HPLC with mass spectrometry (MS) detection [10]-[14]. The objective of the present study is to investigate the amount of Microcystin-LR from *Microcystis aeruginosa* Kutz. obtained from Sri Sakhett, Thailand using HPLC with UV detector. A simple mobile phase without any buffer was also employed in this study.

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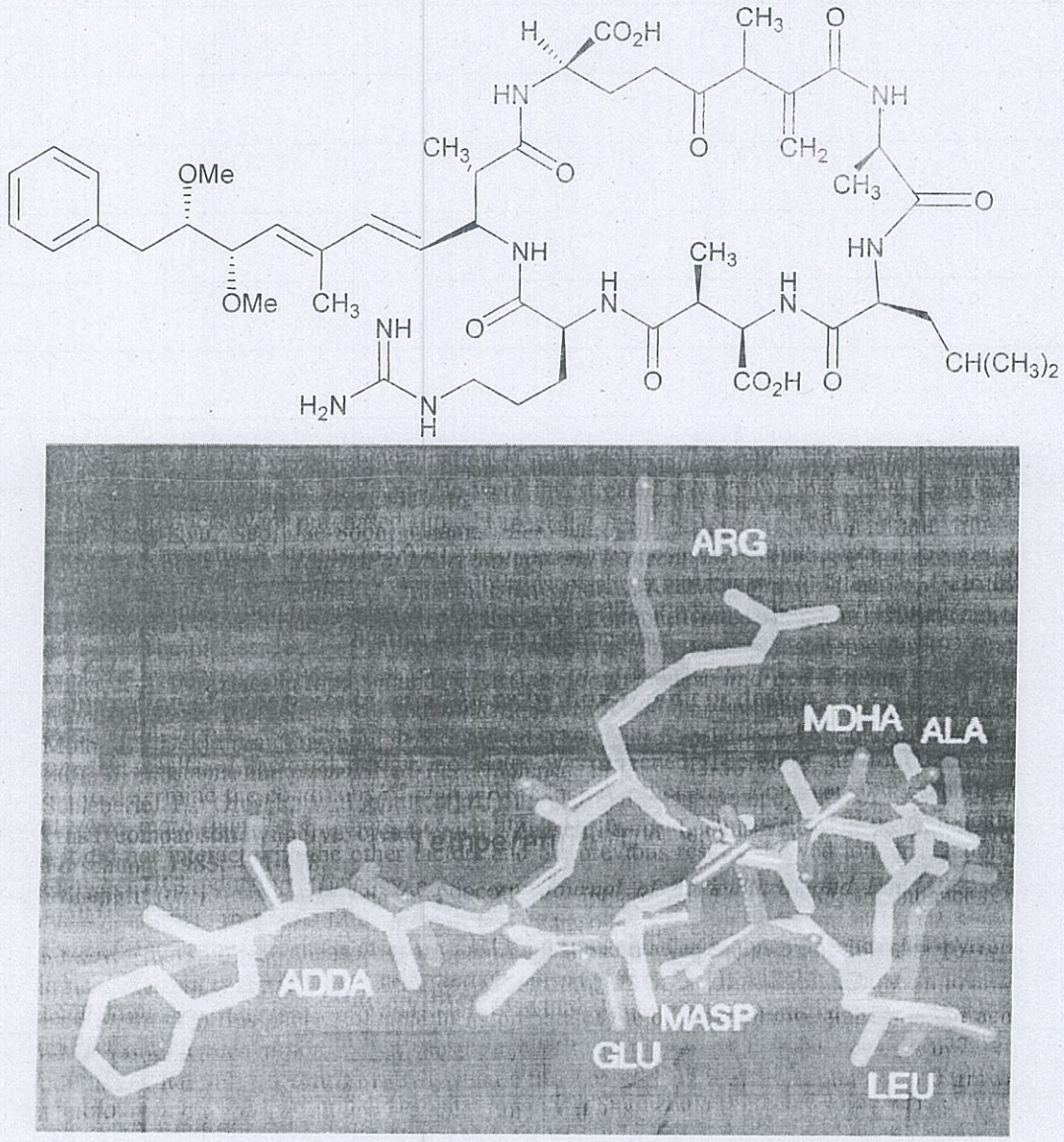

Figure. 1. Structure of Microcystin-LR [15]

2. MATERIALS AND METHODS

Chemicals

Microcystin LR was obtained from the Laboratory of Prof. K. Kaya, NIST, Japan and the exact structure and type of LR was confirmed using 500 MHz NMR (JEOL, Japan). All reagents were analytical grade. Acetic acid was purchased from Merck. Methanol was obtained from BDH. High-purity water with a resistance of 18.2 MΩ. cm^{-1} was obtained from Milli-Q system (Millipore Corporation, Bedford, MA, USA). A microcystin-LR stock solution was prepared at the concentration of 520 ppm in methanol and stored in 10 ml volumetric flask at 4 °C before use.

Instruments

A HP 1100 Series HPLC system (Hewlett-Packard, CA, U.S.A.) consisting of a vacuum degasser, binary pump, manual injector, and variable wavelength detector was used. The equipment,

data collection and manipulation were controlled by the Chemstation software. A UV/Visible Spectrophotometer (Thermo Spectronic, U.S.A.) and a rotary vacuum evaporator (Buchii, Japan) were used.

The study of optimal conditions for HPLC

An investigation of the optimal wavelength was carried using the 520 ppm microcystin -LR stock solution to obtain the spectrum of the compound employing the UV/Visible Spectrophotometer. The extracted sample was used to find the mobile phase composition for good separation (methanol:water, v/v 60:40) with the optimal flow rate of 1.0 ml/min.

The study of linearity of analysis and calibration curve

The stock 520 ppm solution of microcystin-LR was diluted to 1, 2, 3, 4 and 5 ppm, respectively. Each of the standard solutions was filtered through 0.45 micron Nylon membranes and stored in separate 4 ml vial. Then 1 microliter of each concentration was injected into the HPLC, employing conditions as given in Section 2.3. Finally, calibration curve was obtained by plotting peak area against concentration (ppm) of microcystin-LR standard solution.

Sample preparation

Microcystis aeruginosa Kutz. was isolated from a bloom in a channel named Jeeneo, in Sri Sakhett province, Thailand and mass-cultured in Dr. Aparat Mahakran's laboratory at the Thailand Research Council using the condition of Okino [16]. The freeze-dried algae was weighed (0.5g) in Erlenmeyer flask, dissolved with 40 ml of 80 % methanol, stirred for 1-2 hr, filtered (filter paper No.1), and the filtrate collected. The residue was dissolved again and the second filtrate collected. Both filtrates were added together and stored in refrigerator. Then 5 % acetic acid was added, stirred for 3-4 hr, and the final volume of sample was measured. This procedure was repeated in triplicate. All samples were stored in refrigerator prior to HPLC separation.

Sample analyses

Each replicate of sample was filtered through 0.45 micron Nylon membrane and stored in 4 ml vial separately. One microliter of filtered sample was injected into HPLC in triplicate. The peak area of each sample was compared with calibration curve to determine the amount of microcystin-LR.

3. RESULTS AND DISCUSSION

The standard microcystin-LR was obtained from the laboratory of Prof. K. Kaya, NIST, Japan and the exact structure and type of LR was confirmed using a 500 MHz NMR. The spectrum was identical to a structure published in the literature[17]. Chromatographic separation was carried out using a C18 column (4.6x125 mm, 5 micron, Hewlett-Packard) with methanol-water (v/v, 60 : 40). After scanning to find out the optimal wavelength using UV/Visible Spectrophotometer, 238 nm was found to be the maxima and was used in the detection of microcystin-LR. This value also corresponded to that used in a previous study [13]. By plotting peak area against concentration of Microcystin LR, it was found that the linearity range was 1-5 ppm., slope=1.1552 , intercept=4.4611, and correlation coefficient=0.9919. From the HPLC results, the retention time of Microcystin LR was about 2.10 minute as shown in Fig. 3. A typical chromatogram of the microcystins containing cyanobacterial extract is shown in Fig. 4.

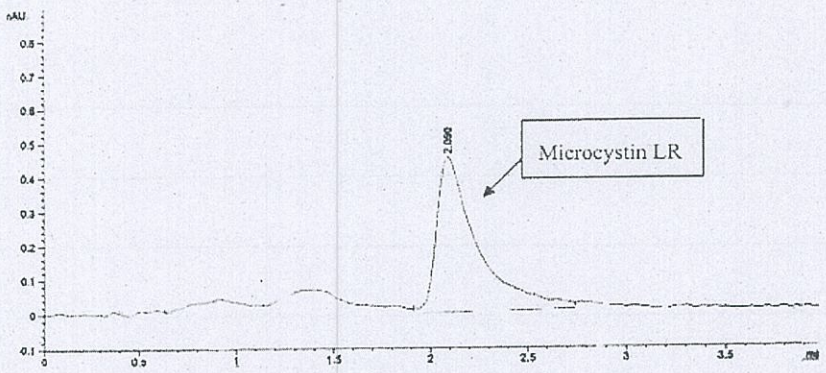

Figure.2 HPLC Chromatogram of Microcystin-LR.

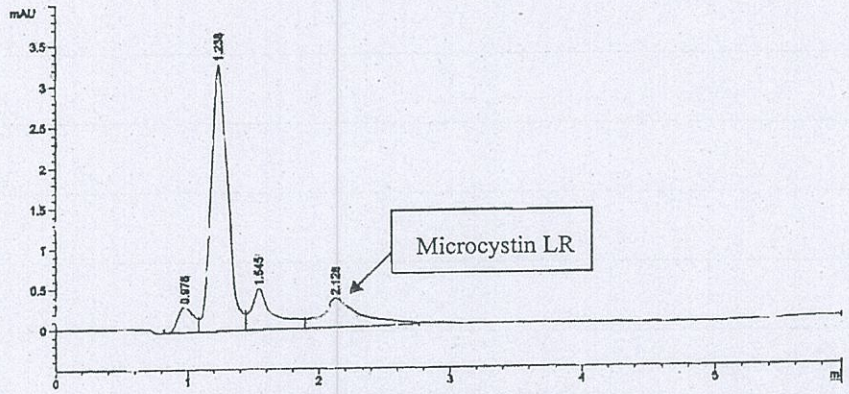

Figure.3 HPLC Chromatogram of sample.

As seen from Fig.2 and 3 , the microcysin LR peaks are broad and the peak intensitie in Fig. 3 is low, which may be a result from the differences of concentration between solvent and sample and the dissolvability of sample in mobile phase. In general, the reversed-phase chromatography, the sample should be dissolved in a solvent. A calibration curve was carried out using five concentrations in the range from 1 to 5 ppm. The regression line is $Y=1.1552X+4.4611$ ($r^2=0.9919$), slope=1.1552 and intercept=4.4611, where Y is the response area, X the microcystin-LR concentration (ppm), and r^2 the correlation coefficient. The amount and percentage of microcystin-LR found in the dried crude cyanobacteria was calculated and shown in Table 1.

Table 1. Amount and percentage of Microcystin LR from Microcystis *aeruginosa* Kutz

Sample injection	Microcystin LR (g)	Percentage of microcystin LR in sample (%)
1	$3.37x10^{-4}$	$6.74x10^{-2}$
2	$2.94x10^{-4}$	$5.88x10^{-2}$
3	$2.47x10^{-4}$	$4.94x10^{-2}$
Mean	$2.93x10^{-4} \pm 3.68 \times 10^{-5}$	$5.85x10^{-2} \pm 7.35 \times 10^{-3}$
S.D.	$3.68X10^{-5}$	$7.35X10^{-3}$

In order to confirm that the peak is that of microcystin LR , pure standard of microcystin LR was spiked in sample and it was found out that the absorbance at the same retention time was increased , thereby the presence of Microcystin LR was confirmed as shown in Fig.5.

4. CONCLOSION

HPLC is an efficient method to determine the amount of microcystin-LR in crude dried *Microcystis aeruginosa* Kutz. sample collected in the Jeeneo channel, Sri Sakhett province, Thailand. The results in Table 1 show that 0.5 g samples contained a mean of 2.93×10^{-4} g ± 3.68×10^{-5} microcystin-LR ($5.85 \times 10^{-2} \pm 7.35 \times 10^{-3}$ % yield).

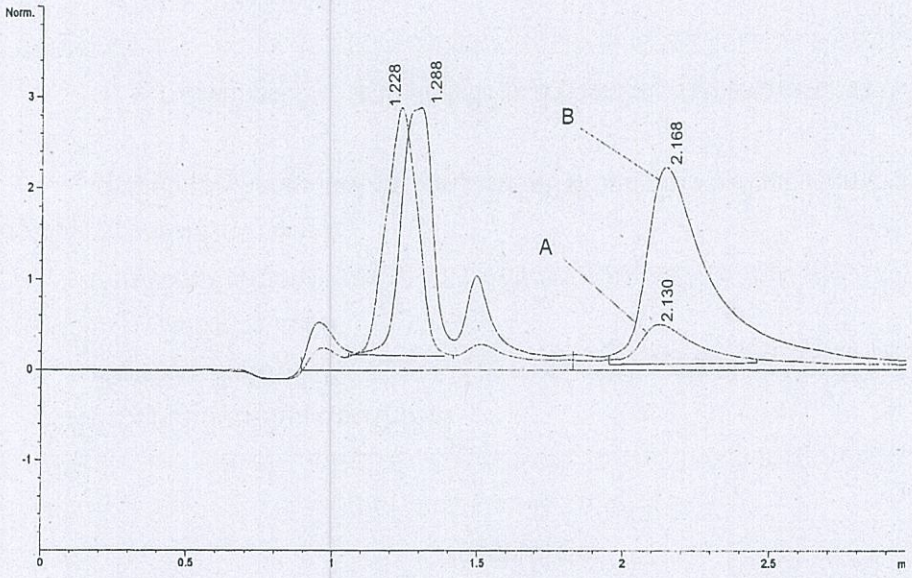

Figure.5 HPLC overlaid chromatograms of (A) sample and (B) sample spiked with microcystin LR pure standard.

5. ACKNOWLEDGEMENT

This work was partly supported by a departmental grant to Senior student Research.

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