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Seven microcystins* from *Microcystis* waterbloom in Lake Dalai, China

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Abstract—— Seven types of microcystins, isolated from Microcystis waterbloom in Lake Dalai, were characterized. The major toxins; MCYST-LR, MCYST-RR, [D-Asp^3] MCYST-LR and [Dha^7] MCYST-LR were identified by high performance liquid chromatography (HPLC), as compared with the authentic microcystins. The minor toxins ; MCYST FR, [L-Mser^7] MCYST-LR and an unknown MCYST which was most likely to be MCYST-(H_4) YR were identified with frit fast atom bombardment liquid chromatography/mass spectrometry (Frit-FAB LC/MS) and amino acid analysis. The toxigenic diversity in blue-green algae (cyanobacteria) was discussed.

Keywords: microcystin; cyanobacteria; waterbloom; identification; Frit-FAB LC/MS.

* Abbreviation: CH_3CN, acetonoitrile; NH_4OAc, ammonium acetate; Adda, 3-amino-9-methoxy-2,6, 8-trimethyl-10-phenyldeca-4, 6-dienoic acid; Dha, dehydroalanine; Masp, D-erythro-β-methylaspartic acid; Mdha, N-methyldehydroalanine; Mamine, *n*-methylamine.

1 Introduction

Lake Dalai, also known as the Hulun Lake, is situated in Inner Mongolia Autonomous Region, with an area of about 2300km^2. It is a semi-salty lake with high alkalinity. The water body has been polluted by nutrients and industrial wastes, and became mesotrophic-eutrophic(Jin, 1990). A heavy waterbloom occurred in the summer of recent years. The dominant species of the waterbloom in Lake Dalai has been *Microcystis aeruginosa*, and it is toxic to sheep and cattle. A report indicated that more than four hundred sheep and one hundred cattle died due to water bloom poisoning in the past thirty years(Qiao, 1994).

It had been known that the MCYSTs are hepatotoxin. It not only impacts on wildlife (including aquatic invertabrate) and domestic animals, but also threats the health of humans. Laboratory studies, using subacute levels of the toxins, have shown that they are potent promoters of liver tumors. So it is necessary to appraise the effects of MCYSTs on aquatic environment.

For studying the toxicogenic organisms and the toxins of the waterbloom in Lake Dalai, we collected the waterbloom samples in 1991 and 1992, isolated and determined their toxic compounds using HPLC, Frit-FAB LC/MS and amino acid analysis. It was found that there were seven types of microcystins in the waterbloom.

So far, about 50 types of microcystins have been reported in the scientific literature. The general structure of MCYST is characterized as cyclo(D-Ala^1-X^2-D-Masp^3-Z^4-Adda^5-D-Glu^6-Mdha^7-), in which X and Z are variable L amino acids. The amino acid X has been most commonly found to be leucine(L), arginine (R) or tyrosine(Y); and Z is arginine (R) or alanine(A). Common variations also include demethylation of D-Masp or Mdha. L-Serine is also found in place of Mdha.

We have studied the variation of MCYST present in different ecological environment (He, 1988; 1993; Carmichael, 1988; Harada, 1988). In this paper, we report the results of analysis and determination of seven microcystins isolated from the waterbloom in Lake Dalai, and discuss the toxigenic diversity in the cyanobacteria.

2 Materials and methods

2.1 Collection of cyanobacteria material

All materials of waterblooms were collected from northwest coast of Lake Dalai in the summer of 1991 and 1992. The dominant species (over 95%) were *microcystis aeruginosa*. They are all toxic waterbloom with a toxicity of LD_{50}70 mg/kg(intraperitoneal injection of mouse).

2.2 Toxicity testing

Toxicity testing was done using bioassay method. The experimental animals (Kunming strain mice, 17—20g) were injected intraperitoneally (i. p.) with various amounts toxins (He, 1989).

2.3 Extraction and purification of toxins

Dried materials (60g) were extracted three times with methanol: butanol: H_2O=25:5:70 solution for 1h (in ice bath). The extract was centrifuged at 10000 r/min for 35 min, and the supernatant was loaded into a Bond Elut C18 cartridge. The cartridge was rinsed first with water and then 20% methanol in water, finally 100% methanol was used to elute the toxins and a crude toxin extract was obtained. The crude toxin extract was evaporated to dryness. The residue was dissolved in 5% methanol in water solution for running gel filtration.

Gel filtration conditions: column (25 × 700 mm) filled with Sephadex G-25, eluted with 5% methanol in water. Toxic fraction (detected at 238 nm) was collected and evaporated to dryness. Proper volume of solution (HPLC mobile phase) was added to residue to make up crude toxin.

2.4 HPLC purification and analysis

The crude toxin obtained from gel filtration was further purified with HPLC. Analysis was accomplished under reversed phase conditions with an ODS column (semi-preparative column 10×250mm, Alltech Appl. Sci. Labs, or Cosmosil 5C 18-AR 4.6×150 mm). Mobile phase: CH_3CN: 0.025mol/L NH_4OAc=26:74, flow rate: 3.0ml/min or CH_3CN: 0.1 mol/L NH_4OAc=25:75, flow rate: 1.0 ml/min. UV and photodiode array detector at wave length of 238 nm were used.

2.5 Frit-FAB LC/MS analysis

A HPLC equipped with a constant pump was used under the following chromatographic conditions: column:Develosil ODS-HG-5 (150x0.3mm); mobile phase:MeOH: 0.05% TFA =58:42; flow rate:4μl/min. The solvent flow was split between the HPLC pump and the injector using restriction column, so that 4μl/min was introduced into the mass spectrometer.

A mass spectrometer (JMS-AX 505W, JEOL Co. Ltd.) was used. The FAB mass spectra were obtained in positive-ion mode by scanning from m/z 40—1500 at a cycle time of 6.5 seconds. The fast atom beam was operated at 3kV with xenon gas, and the spectrometer was operated at a 5kV accelerating potential.

3 Results and discussion

The toxins present in waterbloom collected from Lake Dalai through isolation and purification by Bond Elut C18 Cartridge, Sephadex-G25 gel filter and semi-preparative HPLC, have been purified near one thousand times. There were five toxic components (five peaks, named as PI, PII, PIII, PIV and PV) in the waterbloom , as shown in the HPLC profile (Fig. 1).

The amino acid composition of all five toxic components are shown in Table 1. Their toxicity and toxin content in the algal cells are also listed in Table 1.

Table 1 The amino acid composition, toxicity and toxin content of toxic components (microcystins) from Microcystis waterbloom in Lake Dalai

Toxic components (Microcystins)	Amino acid composition	Toxin content, %	Toxicity in LD_{50}, μg/kg
PI Unknown	β-Masp, Glu, Ala, Ser, Phe Tyr, Arg, Mamine, Leu	2.18	190
PII MCYST-LR	β-Masp, Glu, Arg, Ala, Mamine, Leu	73.61	42
P III[Dha^7] MCYST-LR	β-Masp, Glu, Arg, Ala, Mamine,Leu	0.67	40
PIV [D-Asp^3] MCYSY-LR	Asp, Glu, Arg, Ala, Mamine,Leu	3.36	200
PV MCYST-RR	β-Masp, Glu, Arg, Ala, Mamine	20.17	129

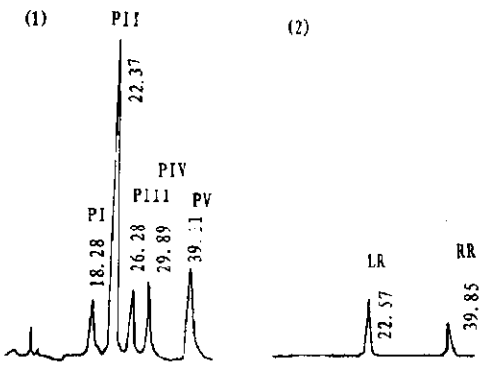

Fig. 1 HPLC profile of waterbloom toxins from Lake Dalai (1) Toxic components of waterbloom from Lake Dalai; (2) Standard toxins. HPLC conditions: column: nucleosis C18 semi-preparative column. 10x250 mm; mobile phase: CH_3CN 26% + 0.025mol/L ammonium acetate 74%; flow rate: 2.5 ml/min

The toxic components PII and PV in Fig. 1 were determined to be microcystin-LR and microcystin-RR, as compared with authentic microcystins. Microcystin-LR and microcystin-RR were the major toxins in the *Microcystis* waterbloom. They consisted of 73.61% and 20.17% of total toxin (Table 1) respectively.

PIII and PIV were also two pure microcystins. They had the same molecular ion peak at m/z 981 in the profile of analysis FABMS (Fig. 2). But they had the different retention time in HPLC (Fig. 3). And their amino acid composition (Table 2) were also different. According to congruence of their retention time with standard microcystins (Fig. 2), the PIII must be [Dha^7] MCYST-LR, and PIV must be [D-Asp^3] MCYST-LR.

Concerning the toxic component PI, it essentially contained different types of toxins but could not be separated by semi-preparative HPLC analysis. We used Frit-FAB LC/MS analysis procedure to find out that PI consisted of four types of toxins: PI-1, PI-2, PI-3, and PI 4(MCYST-LR) (Fig. 4). As shown in the mass spectra, four peaks all possess the characteristic peaks at m/z 135, which are the fragment ion peaks of [$phCH_2CH(OH_3)$, m/z 135], a constituent of Adda. So they all were heptapeptide microcystins.

Peak PI-1 provided a $[M+H]^+$ at m/z 1029(Fig. 4(b)), and contained the amino acid of phenylalanine (Table 1). The most possibility is the microcystin MCYST-FR.

Peak PI-2 contributed the $[M+H]^+$ at m/z 1049 corresponding to the microcystin-(H_4) YR. In which four hydrogen atoms added to tyrosine and formed the 1, 2, 3, 4-tetrahydrotyrosine. Microcystin-(H_4) YR had a molecular weight of 1048 with molecular formular

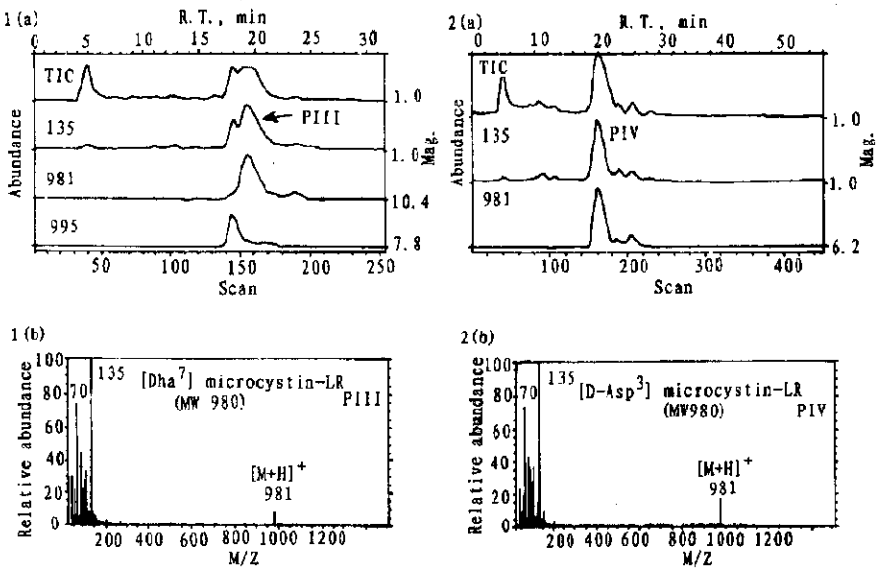

Fig. 2 Frit-FAB LC/MC analysis of toxic components PIII[1(a) and 1(b)] and PIV[2(a) and 2(b)]; 1(a) and 2(a) total ion chromatogram and mass chromatogram monitored at m/z 135, 981 and 995. 1(b) and 2(b), frit-FAB LC/MS mass spectra of major peak (Analysis conditions are the same as described above)

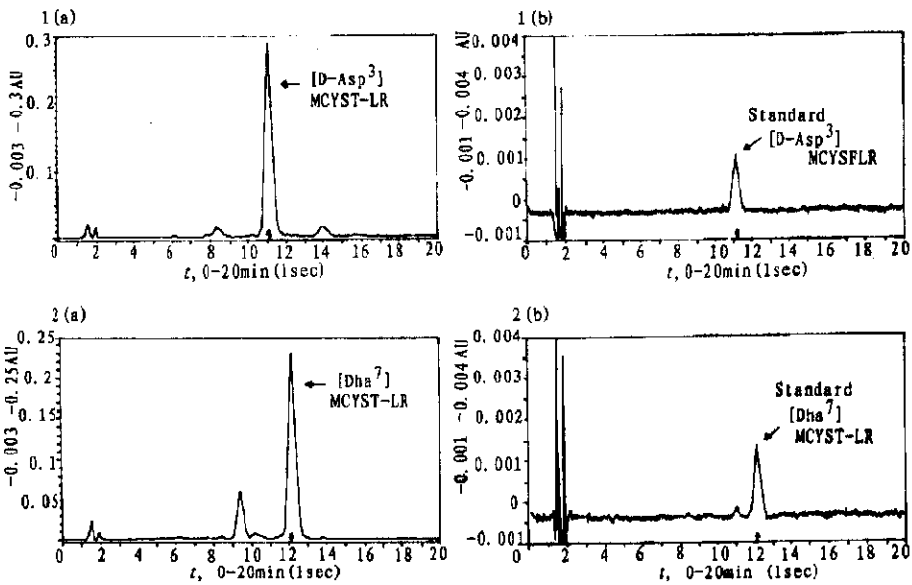

Fig. 3 HPLC chromatogram comparison between toxic components PIII, PIV and the standard microcystins. 1(a) toxic component PIII; 1(b), standard [D-Asp^3] MCYST-LR; 2(a), toxic component PIV; 2(b), standard [Dha^7] MCYST-LR; HPLC conditions: column: cosmosil 5C18-AR, 150 x4.6 mm; mobile phase: CH_3CN: 0.1mol/L NH_4OAc=25:75. Flow rate: 1.0ml/min; monitoring at 238 nm

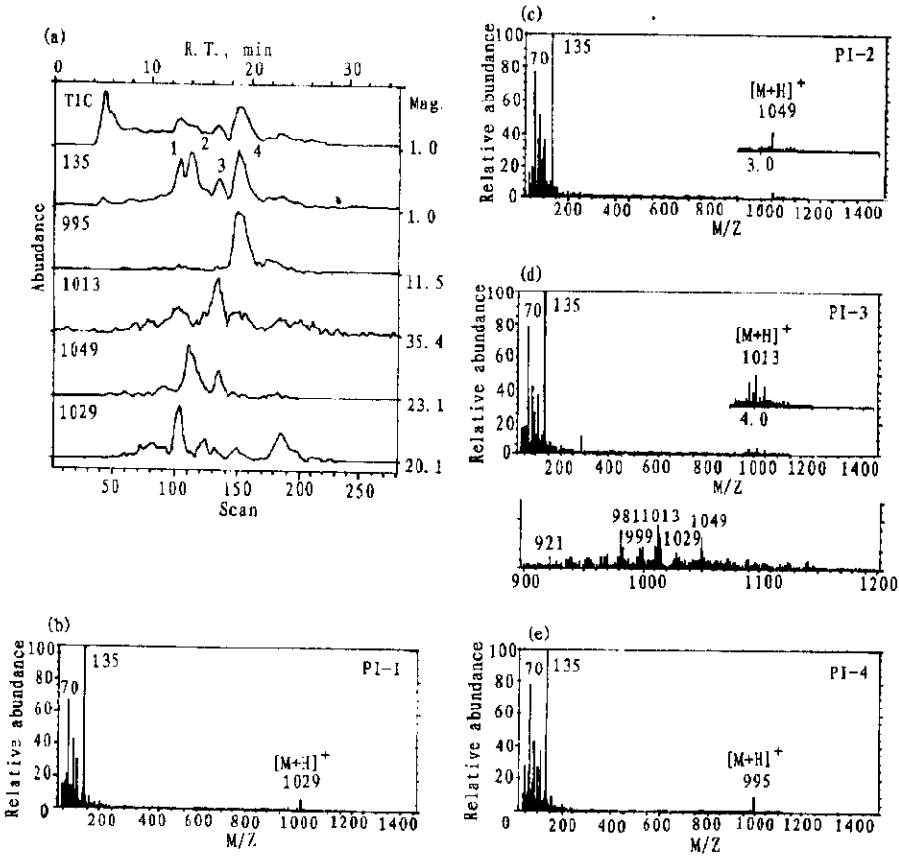

Fig. 4 Frit-FAB LC/MS analysis of PI. (a) Total ion chromatogram and mass chromatograms monitored at m/z 135, 995, 1013, 1049 and 1029. (b), (c), (d) and (e): mass spectra of individual major peaks of PI-1(b), PI-2(c), PI-3(d) and PI-4(e)

$C_{52}H_{76}N_{10}O_{13}$.

Peak PI-3 had a molecule ion peak at m/z 1013 [Fig. 4(d)] corresponding to microcystin [L-Mser^7] MCYST-LR. In the same figure we can find another ion peak at m/z 999. It is derived from demethylation of [L-Mser^7] MCYST-LR. Moreover, we had found the amine acid residue serine included in its profile of amino acid analysis (Table 1), so we obtained the peak PI-3 to be [L-Mser^7] MCYST-LR.

To sum up, the toxin composition of *Microcystis* waterbloom from Lake Dalai is presented in Table 2.

We used a procedure combining semi-preparative analysis with Frit-FAB LC/MS determination to find out seven types of microcystins, which existed in *Microcystis* waterbloom from Lake Dalai. The analysis procedure is a reliable sensitive method for determination of individual microcystin in complicated matrices (Kondo, 1992; 1995).

From the previous studies (Carmichael, 1992; Rinehart, 1994), all seven types of microcystins from Lake Dalai could be found in *Microcystis* as well as in *Anabaena* and *Oscillato-*

Table 2 Total MCYST contained in *Microcystis* waterbloom from Lake Dalai

No.	Type of MCYST	Retention time, min	Mol. wt.	Mol. formula
1	MCYST-RR	10.20	1037	$C_{49}H_{75}N_{13}O_{12}$
2	MCYST-FR	11.42	1028	$C_{52}H_{72}N_{10}O_{12}$
3	MCYST-(H_4)YR	12.85	1048	$C_{52}H_{76}N_{10}O_{13}$
4	[L-Mser^7]MCYST-LR	15.71	1012	$C_{49}H_{76}N_{10}O_{13}$
5	MCYST-LR	17.86	994	$C_{49}H_{74}N_{10}O_{12}$
6	[Dha^7]MCYST-LR	18.57	980	$C_{48}H_{72}N_{10}O_{12}$
7	[D-Asp^3]MCYST-LR	19.28	980	$C_{48}H_{72}N_{10}O_{12}$

ria. It seems that these toxins are common in various toxigenic cyanobacteria. Up to now, a total of 47 types of microcystis have been found. They nearly contained all kinds of amino acids in living cells. It is obvious that the cyanobacteria markedly manifest their toxigenic diversity. Although a proposed biosynthetic pathway of microcystin have been reported(Rinehart, 1994), perhaps the biosynthelic pathway is also diversity.

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