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First Report of Microcystins in Cyanobacteria *Microcystis* sp. Isolated from Dianchi Lake, China*

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Abstract: *Isolates of* Microcystis *blooms collected from Dianchi Lake of South-western China in different seasons were tested by mouse bioassays. The* LD_{50} *of the* microcystis *isolates corresponding* M. wesenbergii, M.viridis *and* M. aeruginosa *were 221 mg · kg^{-1}, 188 mg · kg^{-1}, and 198 mg · kg^{-1} respectively. The cells of the three samples contained hepatotoxins or microcystins. The HPLC (high performance liquid chromatography) analysis showed that all the three* Microcystis *species contained microcystin LR. In addition,* M. wesenbergii *contained microcystin RR,* M. viridis *contained microcystinYR, M. aeruginosa contained both microcystin RR and YR. The results suggested that in different seasons the toxins which were produced by the dominating* Microcystis *species were fluctuent with regard to the composition and content of the toxins and consenquently were obvious factors that aroused the water quality to decrease and change.*
Keywords: Microcystis, *microcystin, HPLC*

1. Introduction

Blooms of cyanobacteria (blue-green algae) occur in eutrophicated lakes, ponds and rivers all over the world (Carmichael, 1992). A lot of species and strains of blue-green algae (cyanobacteria) forming blooms produce potent hepatotoxins or neurotoxins which are responsible for the death of wild and domestic animals consuming water contaminated with bloom (Schwimmer and Schwimmer, 1968; Tsuji, *et al.*, 1994). *Microcystis, Oscillatoria, Anabaena, Aphanizomenon, Nostoc* and *Nodularia* produced these lethal toxins (Elleman *et al.* 1978; Botes *et al.*, 1982; Watanabe, *et al.*, 1989; Harada, *et al.*, 1991). The colonial species *Microcystis aeruginosa* is the most common hepatotoxic cyanobacterium in eutrophic freshwaters (Carmichael, 1992).

Cyanobacterial heptotoxic cyclic heptapeptides are well known as microcystins possessing the general structure cyclo(-D-Ala-X-D-MeAsp-Z-Adda-D-Glu-Mdha-), where X and Z are variable L-amino acids, D-MeAsp is D-erythro-β-methylasparetic acid, and Mdha is N-methy-

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ldehydroalanine (Carmichael, *et al.*, 1988). More than 50 microcystins have been isolated from cyanobacteria (blue-green algae), such as a coccoid *Microcystis* and filamentous *Anabaena, Nostoc*, and *Oscillatoria* strains (Carmichael, 1988, 1992; Harada, *et al*; 1991, Sivonen, *et al*, 1992 a,b). Species and strains of Microcystis produce more than 40 types of related cyclic peptides called microcystins (Sivonen, *et al.*, 1992). The toxins also inhibit protein phosphatases 1 and 2A in a similar manner to akadaic acid and have a tumor-promoting activity in rat liver (Nishiwaki-Matsushima, *et al.*, 1992). Microcystins as liver tumor promoters would threaten human health.

Eutrophication of Dianchi Lake in South-western China has resulted in massive increases of algae, often dominated and successed by the cyanobacteria *M. wesenbergii*, *M. viridis* and *M. aeruginosa* in different seasons of the year (Zhao Yijun, *et al.*, 1996). This problem becomes intensified due to the lake's environmental characteristics necessary for cyanobacterial growth during the entire year. These include high light intensity, high nutrient concentrations, water temperatures between 15 to 30℃and little water movement.

Several analytical methods have been established for microcystins in cells of cyanobacteria, but high performance liquid chromatography (HPLC) is used most widespreadly (Brooks and Codd, 1986; Dierstein, *et al.*, 1988). We reported the toxic *M. wesenbergii* first found in Taihu Lake of South-eastern China (He Jiawan, *et al.*, 1996). In order to understand the occurrence and toxin production by the cyanobacteria blooms that were found in Dianchi Lake, we used the HPLC to work on the isolation, purification and characterization of microcystins from the *Microcystis* species dominating alternatively in different seasons of the year. In this paper we report the HPLC analytical results of microcystins in three *Microcystis* species including a new Chinese toxic cyanobacterium—*M. viridis* isolated from Dianchi Lake which were found to produce microcystins.

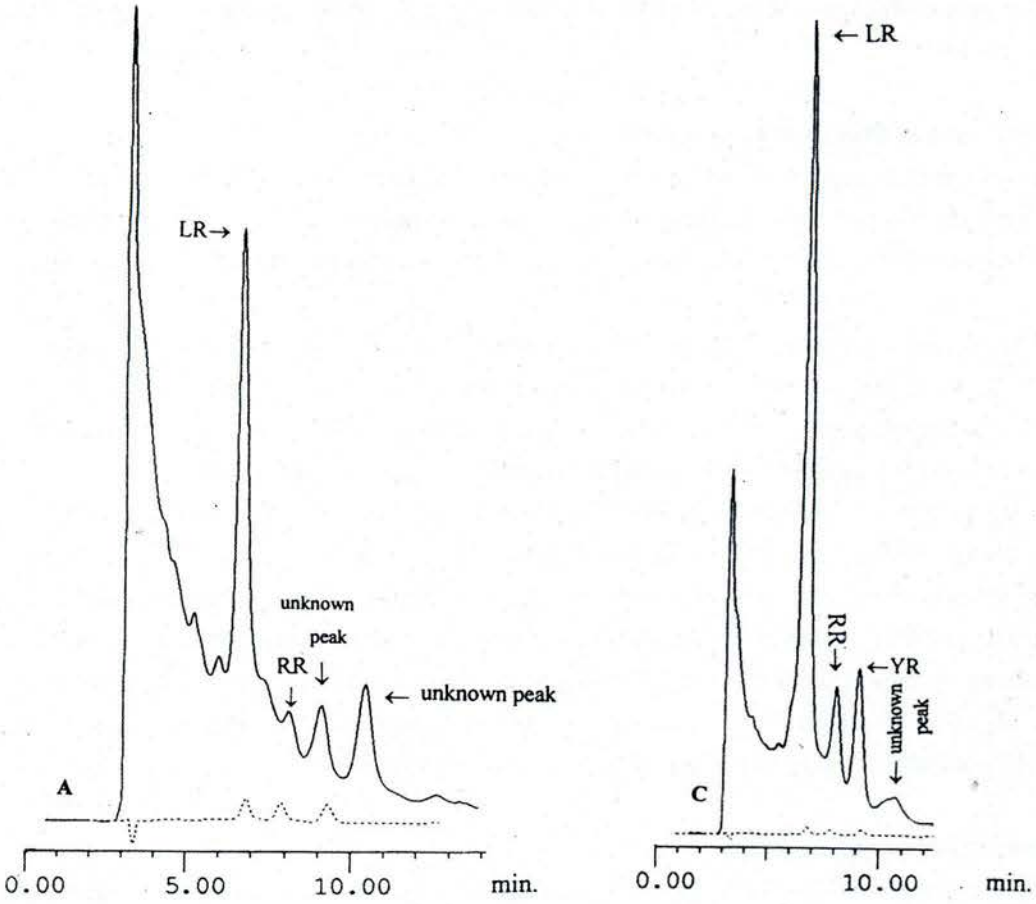
2. Materials and methods

2.1 Cultures

M. wesenbeigii, M. viridis and *M. aeruginosa* were isolated from bloom samples collected in Dianchi Lake in Octorber 1995, January 1996 and June 1996 seperately. The concentrations of *M. wesenbeigii, M. viridis* and *M. aeruginosa* were about 90 %, 65 % and 80 % of the overal cyanobacteria respectively. Cells growing under optimatic conditions were harvested in the late exponential phase, washed, concentrated by centrifugation and then lyophilized for storage, toxicity tests and toxin extraction.

2.2 Bioassays

Mice weighing about 30 g were used to test the toxicity of the algae samples. The lyophilized *Microcystis* purified were first diluted in a small quantity of methnol and diluted with deionizd water as a vesicle. The dilution of 0.2 ml volumes were injected i.p. into each of two mice. The



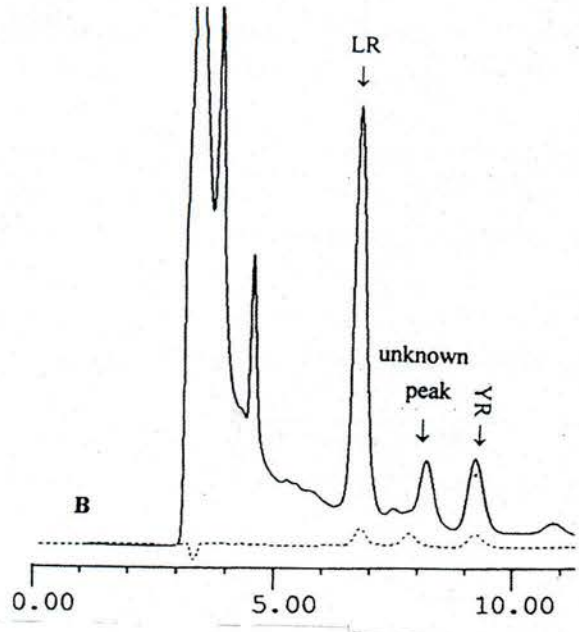


Fig. 1 The HPLC analysis results: The separation was accomplished under isocratic conditions with a mobil phase of 20 mm ammonium acetate, pH 5.0, and acetonitrile (7:3) for 15 min, followed by a gradient of 30 %-50 % acetonitrile in water for 10 min. The volume injected was 1.0 ml with UV detection at 238 nm and a flow raye of about 4.0 ml·min^{-1}.

A: *Microcystis wesenbrergii*

B: *M. viridis*

C: *M. aeruginosa*

——standard; -----sample

concentration of methonol in the dilutions were less than 1 %, which did not have a lethal effect on the animals.

2.3 Toxin extraction and purification

Lyophilized cells (1g) of the algae were extracted three times (1h) with 1-buanol: methanol: water (1:4:15, v/v) and cells debris was removed by centrifugation.The supernatants were combined and air-dried to 30 % of the original volume. This material was passed through C-18 cartridges.

The C-18 cartridges (Bond Elut, USA) were first washed with water then eluted in sequenses with 20 %, 80 % and 100 % methanol. The toxic fraction was eluted with 80 % methanol. This fraction was filtered through a 0.45 μm nylon filter, the methanol removed by evaporation and the toxins seperated and purified by HPLC (high performance liquid chromatography).

The toxins were analyzed using a HPLC system equiped with a Gilson 206 pump and 811C stirring mixer with the analyzing colume (Rpp-250×4.6, Japan). The seperation was accomplished under isocratic conditions with a mobile phase of 20mM ammonium acetate, pH 5.0, and acetonitrile (7:3) for 20 min, followed by a gradient of 30-50 % acetonitrile in water for 15 min. The volume injected was 0.1 ml with UV detection at 238 nm (Gilson 121 type UV detector) and a flow rate of of 1.0 ml · min^{-1}. All peakes were tested for toxicity by bioassay after evaporation of the mobile phase and resolubilization in 1.0 ml of deionied water.

3. Results and discussion

Lyophilized cells of *M. wesenbergii, M. viridis* and *M. aeruginosa* and all peaks of HPLC analysis were tested for hepatotoxicity by the intraperitional mouse assay. The estimated LD_{50} of three lyophilized cell suspensions were 221 mg·kg^{-1}, 188 mg·kg^{-1} and 198 mg·kg^{-1} respectively (Tab.1). Signs of poisoning were identical to those observed with other microcystins-producing toxic cyanobacteria.

The HPLC (high performance liquid chromatography) analysis results showed that all three *Microcystis* species contained microcystin LR. In addition, *M. wesenbergii* contained microcystin RR, *M. viridis* contained microcystinYR, *M. aeruginosa* contained both microcystin RR and YR (Fig.1).

Tab. Composition of microcystins and toxicity of *Microcystis* blooms in different seasons in Dianchi Lake

Sample	Season	*Microcystis* Isolate	LD_{50} (mg·kg^{-1}mouse)	Microcystin-LR	Microcystin-YR	Microcystin-RR
A	Autumn	*Microcystis wesenbergii*	221	+	−	+
B	Winter	*M.viridis*	188	+	+	−
C	Summer	*M.aeruginosa*	198	+	+	+

On preparative HPLC, two unknown toxic peaks were obtained from *M. wesenbergii,* one unknown peak was obtained from both *M. viridis* and *M. aeruginosa* (Fig.1: A, B, C). The first peak from *M. wesenbergii* was detected under isocratic conditions after 9 min and the second was detected under gradient conditions after 10 min (Fig.1: A). Toxic one was further purified by analitical HPLC. Toxin two was sufficiently purified by the preparative HPLC.

The LD_{50} (i.p. mouse) of microcytin LR and RR were reported to be about 50 (Krishnamurthy, *et al.*, 1986) and 600 (Watanabe, *et al.*, 1988) $\mu g \cdot kg^{-1}$ respectively. In our experiment, the LD_{50} of the large toxic peaks (Fig.1) corresponding the standard microcystins LR were between 45 to 70 $\mu g \cdot kg^{-1}$, the microcystin RR were between 88 to 180 $\mu g \cdot kg^{-1}$, and the microcystin YR in our experiment was about 160 $\mu g \cdot kg^{-1}$.

All peaks were isolated and tested by bioassay. Three unknown peaks were found to be toxic to the mouse and the symbols were similar to other microcystins. But the characterizations of the toxins were not determined owing to the shortage of stardard microcystins. The toxins might need to be analyzed by structural determination methods such as amino acid analysis and NMR (nuclear magnetic resonance) techniques (Harada, *et al.*, 1990).

The occurrence of toxic cyanobacteria that produce hepatotoxic microcystins in the Chinese fresh waters is more and more serious in recent years. Dianchi Lake is one of the largest freshwater lakes in south-eastern China which produces at least 100 000 tons biomass (wet weight) of cyanobacteria per year (Zhao Yijun, *et al.*, 1996). However, the toxin pollution in the lake has not aroused the official attention yet though the lake is the main source of drinking water for the 180 million residents of Kunming City. Our research has confirmed the toxic *Microcystis* occurred during the entire year (1995-1996) with the successions of dominating species of *M. wesenbergii, M. vividis* and *M. aeruginosa*. The findings suggest that it is necessary to monitor the occerrence of cyanobacteria blooms and their toxins in Chinese drinking water supplies. This will alert authorities to possible public health hazards from this potent hepatotoxic tumor promoters.

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