Isolation of Two Toxins from the Blue–green Alga

Microcystis aeruginosa

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(Accepted March 18, 1987)

Attempts were made to isolate toxins from the blue–green alga Microcystis aeruginosa which has been reported to form a toxic water-bloom in freshwater lakes and ponds in various countries. A unialgal strain (M228) from Lake Suwa, Nagano Prefecture, was cultured, and the cells were harvested and freeze-dried. The water extract was subjected to high performance liquid chromatography (HPLC) on a DEAE-5PW column, resulting in appearance of two toxic peaks. HPLC using an ODS-80TM reversed phase column made it possible to isolate two toxins, P-1 and P-2, with yields of 100 μg and 700 μg/g freeze-dried cells, respectively. The specific toxicity of the main toxin, P-2, was 70 μg/kg mice (i.p.).

Microcystis aeruginosa is a freshwater, unicellular blue–green alga. It often forms a toxic water-bloom, which is sometimes lethal to domestic or wild animals when drunk.1) According to Gorham,2) Louw carried out a pioneering work on the responsible Microcystis toxin using an African strain as the material. Since then, the studies on this toxin were spread to various countries: Canada,3,4) Australia,5) etc., and the toxin was assumed to be a peptide. Recently, Botes et al.6,7) revealed that the African Microcystis toxin was composed of several cyclic peptides, each consisting of seven amino acid residues. In Japan, Watanabe et al.8) surveyed the distribution of toxic strains of this alga in various lakes and ponds, finding a highly toxic strain (designated M228) in Lake Suwa, Nagano Pref.9) The present series of investigation was undertaken to characterize the responsible toxin. This paper deals with the isolation of two Microcystis toxins from the strain M228 of M. aeruginosa.

Material and Methods

Material

A unialgal strain M228 of M. aeruginosa, a blue–green alga which was isolated from Lake Suwa, Nagano Pref. in 1973,9) was used. The strain was cultured at 25°C in 40 or 200 l of MA medium10) for 7–10 days by standing, under a continuous light whose intensity was 30.1 μE/m²·s.9) After culture, cells were harvested by centrifugation at 7,000 × g for 30 min, freeze-dried and kept at −20°C until use for isolation of Microcystis toxin and other purposes.

Mouse assay method for Microcystis toxin

First of all, dose–death time curve for this toxin was provided. The freeze-dried cells (1 g) were homogenized with 20 ml of water for 4 min, and centrifuged at 3,000 × g for 20 min. A series of dilutions were prepared from the extract. One ml of each dilution was injected i.p. into a group of three mice. When more than two mice were killed, the solution was judged to be toxic, and the dose was calculated from their death times and the dose–death time curve for Microcystis toxin.

Microcystis toxin was assayed, by use of the curve. A test solution was injected i.p. into a group of three mice. When more than two mice were killed, the solution was judged to be toxic, and the dose was calculated from their death times and the dose–death time curve.

Isolation of Microcystis toxin

Microcystis toxin was purified and isolated from...
the cells according to the scheme shown in Fig. 2.

Ten grams of freeze-dried cells were homogenized with 400 ml of water for 5 min and stirred overnight at 4°C. The homogenate was centrifuged at 3,000 × g for 20 min. The supernatant obtained was freeze-dried, suspended in 40 ml of water, adjusted to pH 4 with 10% acetic acid, and to it was added an equal volume of 1-butanol. The resultant suspension was stirred for 1 h, and centrifuged at 5,000 × g for 30 min. The upper layer was concentrated to dryness under reduced pressure. The crude toxin thus obtained was further purified by the following procedures.

The crude *Microcystis* toxin was dissolved in 10 ml of water, adjusted to pH 8.5 with aqueous ammonia, and applied to a DEAE-5PW (TSK gel) ion-exchange column (0.75 × 7.5 cm). The column was developed with a linear gradient from 5–90 mM NH₄HCO₃. The flow rate was 1 ml/min, and the pressure 10 kg/cm². Two-ml fractions were collected and monitored by UV absorption at 220 nm. Each peak was checked for toxicity by the mouse assay method. Two peaks were toxic and the responsible toxins designated P-1 and P-2 in the order of elution. Fractions of each peak were combined, lyophilized, and subjected to high performance liquid chromatography (HPLC) on an ODS-80TM (TSK gel) reversed phase column (0.5 × 30 cm) with 70% methanol containing 0.1% trifluoroacetic acid for P-1 or 78% methanol containing 0.1% trifluoroacetic acid for P-2 toxin. Column conditions were essentially the same as those in DEAE-5PW column chromatography, except that the pressure applied was changed from 10 to 90 kg/cm².

**Thin-layer chromatography**

TLC was performed on 5 × 20 cm silica gel precoated plates (Merck) with two solvent systems: (1) 1-butanol/acetic acid/water (4:1:2) and (B) chloroform/methanol/water (6:4:1).

Toxins were detected as dark brown spots under UV light at 365 nm, or as light brown spots after spraying with 10% H₂SO₄, followed by a 5 min-heating at 110°C, ninhydrin spraying and another 5 min-heating at 110°C.

**Results and Discussion**

The dose-death time curve for *Microcystis* toxin is shown in Fig. 1. From the curve, one mouse unit (MU) of *Microcystis* toxin was defined as the amount of toxin killing a mouse in 3 h after intraperitoneal administration. From this and the dilution factors of injected solution, lethal dose at 99% (LD₉₀) of the freeze-dried alga in mice was estimated to be 10–15 mg/kg mice. Autopsy of the killed mice found congested and enlarged livers, supporting the claim that *Microcystis* toxin is a hepatotoxin.¹¹

This curve was used to assay for *Microcystis* toxin, as described above.

*Microcystis* toxin was isolated from the freeze-dried cells according to the scheme shown in Fig. 2. Fig. 3 shows an elution profile from the DEAE-5PW column of the butanol extract. Two toxin peaks, P-1 and P-2, appeared at retention times of around 28 and 50 min, respectively. In this con-
Some lots of cultured algal cells gave rise to an elution profile in which two toxin peaks, along with P-1 and P-2, appeared, suggesting that toxin pattern is somewhat variable depending upon culture conditions.

Both toxins thus separated were further purified by reversed phase ODS-80TM column chromatography.

As Figs. 4 and 5 show, P-1 appeared at a retention time of 41 min, whereas P-2 at 40 min. Yields of P-1 and P-2 were 100 and 700 μg/g dried cells, respectively, on an average.

P-2 was determined for LD₉₀ by the mouse assay method, affording 70 μg/kg mice. The LD₉₀ of P-1 was not determined, due to scarcity of the
Both P-1 and P-2 exhibited a single spot in TLC (Fig. 6), Rf values being 0.48 and 0.43 with solvent system A, and 0.65 and 0.51 with solvent B, respectively.

Both toxins were ninhydrin-negative, but turned positive after spraying with 10% H₂SO₄, indicating that they had a blocked N-terminus or were cyclic peptides.¹²,¹³

Further characterization of both toxins are now in progress, and will soon be published elsewhere.

Acknowledgements

The authors express their sincere thanks to Dr. H. Tokuda, Faculty of Agriculture, Univ. of Tokyo, for kind cooperation in the mass culture of alga. This work was supported in part by a Grant-in-Aid for Scientific Research from the Ministry of Education, Science and Culture, Japan.

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