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A method to extract algae toxin of microcystin-LR

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Abstract: A simple and low-cost method to obtain cyanobacterial toxin microcystin-LR(MC-LR) was developed. A new strain of *Microcystis aeruginosa*, named DC-1, producing microcystin-LR but not microcystin-RR, was separated from the field blooming algae samples of Dianchi Lake, in southwest of China. Following three times' freeze and thaw treatment, the cultivated DC-1 cells were extracted with 40% methanol in water. The extract was centrifuged and the supernatant applied to a Hydrophilic-Lipophilic Balance(HLB) SPE cartridge. Eluted impurities with a certain gradient from 30% to 50% methanol in water, MC-LR was finally eluted from the HLB cartridge with 60% methanol in water, and samples containing 3.85% to 14.8% of MC-LR were obtained. These MC-LR samples may be used in adsorption and biodegradation experiments instead of using expensive standard reagents.

Keywords: purification; extraction; microcystins; HLB cartridge

Introduction

Cyanobacterial blooms are more frequently observed in eutrophic water bodies worldwide. Accordingly, microcystins, a group of bioactive compounds produced by some bloom-forming cyanobacteria, receive more and more attentions for their characteristics of inhibiting protein phosphatases(type 1, 2A and 3), promoting tumors, and even causing deaths(Saito, 2002; Lawton, 2001).

Microcystins have the general structure of cyclo-(D-Ala-X-MeAsp-Z-Adda-D-Glu-Mdha) where X and Z are variable L-amino acids (Fig. 1). MeAsp stands for D-*erythro*-β-methylaspartic acid, Adda is 3-amino-9-methoxy-10-phenyl-2, 6, 8-trimethyladeca-4, 6- dienoic acid, and Mdha, N-methyl-dehydroalanine. LR and RR are two most familiar microcystins that X and Z are replaced by leucine(L) and arginine(R) and both by arginine(Lawton, 2001).

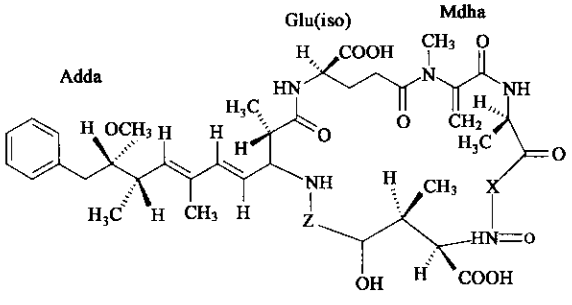

Fig.1 Structure of microcystins

Increasing interests in these compounds, such as investigations into their toxicology, environmental chemistry, treatment, production of analytical standards for environmental analysis and their use as biochemical tools have greatly increased the demand for pure microcystins. Chemical synthesis of these compounds is not successful so far. So it is important to develop methods to obtain microcystins from microcystins-producing algal cells. Until now, many methods have been developed to obtain microcystins from algal cells (Edwards, 1996a; 1996b; Harada, 1988a; 1988b; Lawton, 1994; 1999; 2001; Ramanan, 2000). Solvent extraction is usually used in the first step, followed by the concentration(such as evaporation and solid-phase extraction) and separation steps. In the course of separation, the most crucial step to purify microcystins, many experimental methods have been adopted such as size extraction, ion exchange, thin-layer chromatography, flash/column chromatography, and preparative HPLC. But it was usually difficult to separate different kinds of microcystins from each other, even with the combination of several methods mentioned above. Furthermore, all of them involve expensive equipment, which greatly limit the studies of microcystins.

In this study, we separated a new strain of cyanobacteria that produced only MC-LR but not MC-RR. Using the HLB SPE cartridges, we developed a simple and low-cost procedure for the extraction and purification of MC-LR.

1 Experimental section

1.1 Materials

1.1.1 Chemicals

Microcystin-LR ($C_{49}H_{74}N_{10}O_{12}$, molecular weight: 995.2) and microcystin-RR ($C_{49}H_{75}N_{13}O_{12}$, molecular weight: 1038.2) standards with purities of more than 95% were purchased from Sigma Company, and stored under −18℃. Their purities were more than 95%. Methanol and acetonitrile were HPLC grade, and all the other reagents were analytical grade.

1.1.2 Field algae samples

Crude cell slurry was collected from Dianchi Lake in southwest China during a serious bloom in July 2001. After being concentrated with a 40 μm plankton net, the blooming algae samples were air-dried, screened through 100 meshes, and stored under −18℃ in refrigerator.

1.1.3 Cultured algae samples

A microcystins-producing algae strain was separated from the crude algal sample of Dianchi Lake. The algae strain was identified to be a *M. aeruginosa* species and named DC-

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1 by the Institute of Hydrobiology, the Chinese Academy of Sciences. This toxic strain was incubated in sterilized 250 ml glass flasks containing 200 ml aqueous MA growth medium at 25 ± 1 ℃ under fluorescent light (1000 lx, 12 h light/12 h dark). MA medium consists of $NaNO_3$ 50 mg/L, $Ca(NO_3)_2 \cdot 4H_2O$ 50 mg/L, KNO_3 100 mg/L, Bicine 500 mg/L, β-$C_3H_7O_6PNa_2$ 100 mg/L, Na_2SO_4 40 mg/L, $MgCl_2 \cdot 6H_2O$ 50 mg/L, $MgSO_4 \cdot 7\ H_2O$ Na_2 EDTA 5 mg/L, H_3BO_3 20 mg/L, $FeCl_3 \cdot 6H_2O$ 0.5 mg/L, $MnCl_2 \cdot 4H_2O$ 5 mg/L, $ZnCl_2$ 0.5 mg/L, $CoCl_2 \cdot 6H_2O$ 5 mg/L, $Na_2MoO_4 \cdot 2H_2O$ 0.8 mg/L. The solid MA medium used to separate and purify single algae strain was prepared by adding 1% (w/v) agar into MA medium solution.

1.1.4 SPE cartridge

SPE (solid phase extraction) cartridges were purchased from the Waters Corporation. In the cartridges, a kind of new material, the OASIS® HLB (hydrophilic-lipophilic balance) reversed-phase sorbent was adopted as sorbent, which is a macroporous copolymer made from a balanced ratio of two monomers, the lipophilic divinylbenzene and the hydrophilic N-vinylpyrrolidone. It was found that the OASIS® sorbent has higher capacity and recoveries than that of normal ODS.

1.2 Methods

1.2.1 The separation of single *M. aeruginosa* species DC-1 from the crude algal samples of Dianchi Lake

The crude algal solution was daubed evenly onto the surface of the solid MA medium. Incubated for 15 d (under 1000 lx fluorescent light, 25 ± 1 ℃, 12 h light/12 h dark), some cells from the clone lawn was inoculated into the aqueous MA medium. As the result, the purer culture of a single *M. aeruginosa* species was obtained. At the exponential stage, the purer algal solution was used, and another two similar purification courses were repeated. Finally, the almost pure algal solution of the single *M. aeruginosa* was preserved in aqueous MA medium, and its purity was manifested by microscope.

After 15 d, the single cloned new strains of cyanobacteria were picked from the surface, and inoculated in the MA solution. The character of microcystins-producing was determined through the extract and analytical methods mentioned above.

1.2.2 Extraction of microcystins

For dried field algae samples as described above, certain amount of dry algae powder was added into extracting solution. The algae serosity, including 100 g dry algae per liter extracting solution, was treated by ultrasonic for at least 20 min. After 2 h, the algae serosity was centrifuged at 12000 r/min to separate the residue. Then the solution was filtrated through 0.45 μm and kept in refrigerator. To extract MC from cultured DC-1 algae, algal cells were harvested by centrifugation at the exponential growth phase. The harvested cells were then frozen and thawed in the extracting solution for three times, followed by the treatment as described above.

1.2.3 HPLC analysis

HPLC analysis was carried out using Shimadzu (Kyoto, Japan) instruments consisting of a Model LC-10A solvent-delivery system, a Model LC-10AD constant-flow pump and a Model SPD-DAD photodiode-array detector at 200—300 nm with 1 nm resolution. A Waters μBondapak C_{18} column (300 × 3.9 mm i.d.; Millipore) was used. The eluents were water-acetonitrile-trifluoroacetic acid (TFA) = 60:40:0.05. The pH of 0.05% TFA aqueous solution was about 2.30. The flow-rate was 1.0 ml/min. A volume of 20 μl was injected every time. Before analysis, all samples were filtrated through 0.45 μm microfiber filters.

The UV-detector was set to 239.5 nm in the analysis, which was found to be the optimal absorbance wavelength for both MC-LR and MC-RR. This wavelength also was the same to those most researchers adopted.

2 Results and discussion

2.1 Extraction efficiencies of MC-LR and MC-RR

As reported (Lawton, 2001), extraction efficiencies of MC-LR and MC-RR varied with the concentration of methanol solutions, for their different polarities and dissolving abilities. Here, the highest efficiency was displayed with 40% methanol for MC-LR and MC-RR extracted from the field samples (Fig.2).

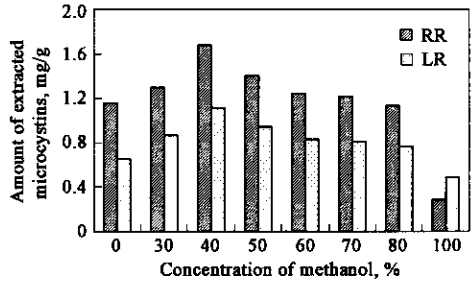

Fig. 2 Extraction efficiencies of MC-LR and MC-RR by different concentration of methanol

When 100 g dry algae were extracted with one liter 40% methanol solution, 1.67 mg RR and 1.11 mg LR per gram dry algae were obtained. All the other methanol solutions showed lower efficiencies, while pure methanol was the lowest. So 40% methanol solution was used to extract LR in following studies.

2.2 A new algal strain producing only MC-LR

With the above method, a new strain of *M. aeruginosa*, named DC-1, was successfully isolated from the field sample of Dianchi Lake. It was found that DC-1 produced only MC-LR but not MC-RR. So it was convenient to obtain MC-LR using this algae sample without adulterating MC-RR.

As mentioned above, all the microcystins shared one structure with very similar characteristics. So it was very difficult to separate several microcystins from each other and finally purify them. Here, the problem was better solved through isolation and purification of algae strain producing MC-LR only. Similarly, one could screen out or design some other strains by gene technique, which usually produced only one specific microcystin. With this strain, purification of microcystins would be greatly simplified.

2.3 Eluting efficiencies of MC-LR from SPE cartridge

LR crude extraction solution was obtained by extracting the DC-1 cells with 40% methanol (100 g dry algae per liter extracting solution), and then diluted to less than 20% methanol before concentrated onto HLB cartridge. The cartridge was eluted by 1 ml solutions involving 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90% and 100%

methanol solutions. From the results of HPLC analysis, it was found that the highest LR eluting efficiency was obtained with 60% or 70% methanol. Solutions involving methanol less than 60% showed very low efficiency, but eluted most of impurities (such as substances A and B in Fig.3).

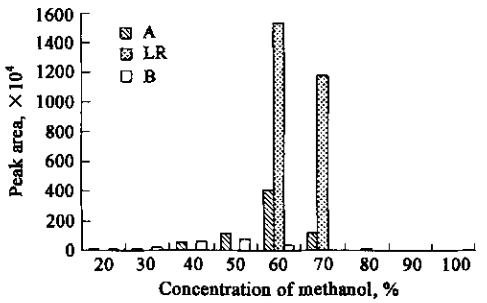

Fig.3 Eluting efficiencies of MC-LR from HLB cartridge by different concentration of methanol

2.4 Purification of LR

As seen in Fig.3, one should use 30%, 40% and 50% of methanol to remove most of impurities first, then 60% methanol solution to elute LR from HLB cartridge. Each eluent in the process was collected and analyzed respectively by HPLC. Their HPLC chromatograms are shown in Fig.4. There were several impurities such as A and B in crude extracted samples. Eluted in the order of 30%, 40% and 50% methanol, B and A were discharged adequately from the HLB cartridge but not losing any MC-LR (from their scanning chromatograms, it was known that B and A were not microcystins). Finally, the eluent of 60% methanol containing most MC-LR (more than 90% of total peak area in HPLC chromatogram) and a little impurity B were obtained. After removing unwanted methanol from eluent by N_2 blow-off or rotary evaporimeter, MC-LR sample with certain purity was obtained.

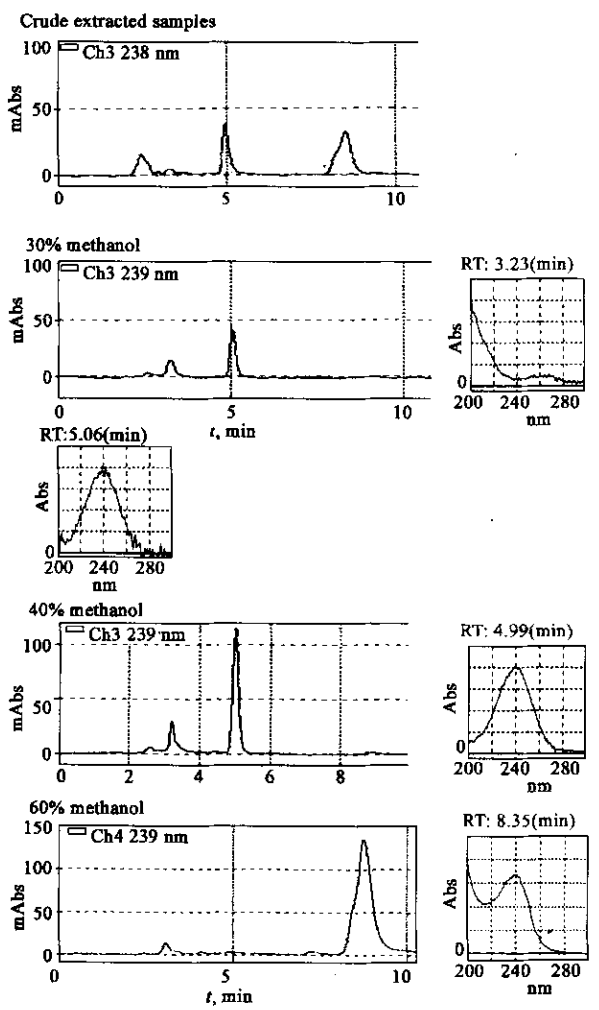

Fig.4 HPLC chromatograms of eluents from the HLB cartridge during the purification of MC-LR

The whole extraction and purification course shown in Fig.5 was repeated several times. It was found that the purities were varied from 3.85% to 14.8% considering the concentration of TOC of LR in purified samples.

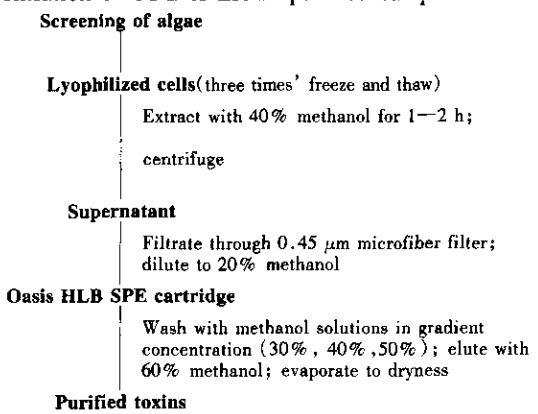

Fig.5 Procedure for extraction and purification of MC-LR

3 Conclusions

A new strain of *Microcystis aeruginosa*, named DC-1, producing mainly MC-LR, was successfully separated from the field blooming algae samples.

With simple extraction and gradient eluting from HLB SPE cartridge, MC-LR was obtained from the DC-1 sample. Its purity (3.85%—14.8%) was not so high, but enough for some studies, especially in some developing and polluted badly areas.

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