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ANALYSIS AND PURIFICATION OF TOXIC PEPTIDES FROM CYANOBACTERIA BY REVERSED-PHASE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

A simple, rapid and reliable chemical analysis method for microcystins (cyanoginosins) has been studied. Three different mobile phases for high-performance liquid chromatography were selected and optimized. Also the adsorptive powers of three commercially available C_{18} cartridges were compared and the results successfully applied to the clean up of three of the toxins. Finally a total system for the analysis and isolation of microcystins was established.

INTRODUCTION

Low-molecular-weight peptide toxins which affect the liver have been the predominant toxins involved in cases of animal poisonings due to cyanobacterial toxins. They are mainly produced by *Microcystis aeruginosa* and are called microcystins or cyanoginosins[1]. Several microcystins have been isolated and their structures determined to be cyclic heptapeptides[2,3] (Fig. 1). Although mouse bioassay provides a general assessment of the toxicity[4], it is necessary to determine the concentrations of the toxins more accurately by an appropriate chemical assay. The establishment of a suitable chemical assay method would also facilitate studies on toxin production and toxicokinetics.

Some chemical assay methods for microcystins using high-performance liquid chromatography (HPLC)[5–7] and thin-layer chromatography (TLC)[8] have been reported. However, these methods have been used mainly for the separation of

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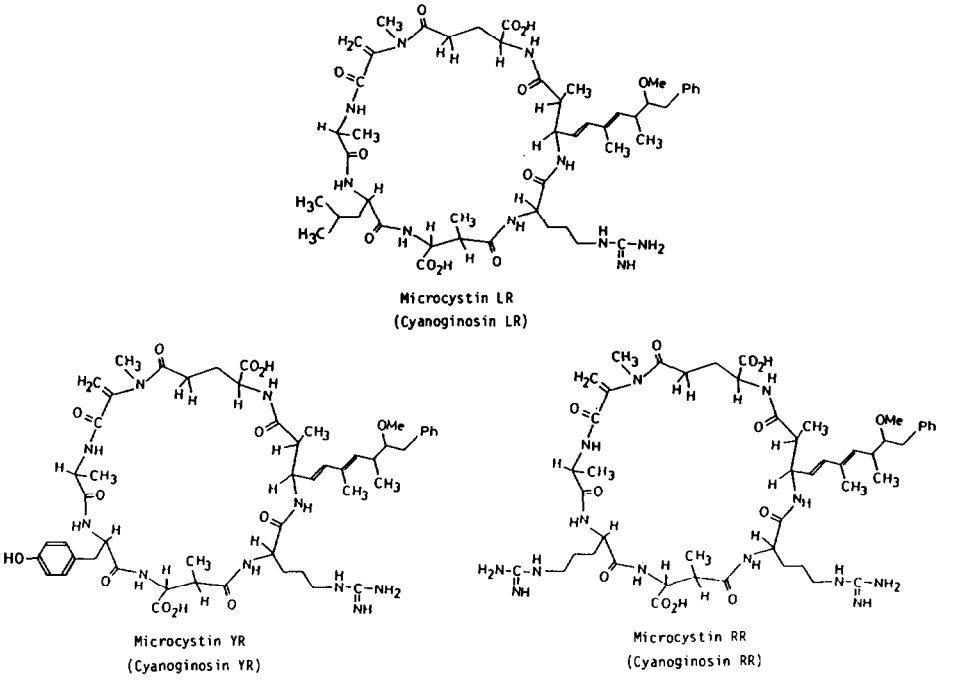


Fig. 1. Structures of microcystins LR, YR and RR (cyanoginosins LR, YR and RR).

microcystins LR (cyanoginosin LR). We have recently proposed an efficient purification method which consists mainly of extraction on ODS (octadecylsilanized)-silica gel, separation on silica gel and HPLC with ODS-silica gel, and which has been successfully applied to some *Microcystis aeruginosa* strains[9].

In this paper we describe a chemical analysis method using reversed-phase HPLC with modifications based on the above purification method. The method can now be used in the detection and determination of all microcystins we have investigated to date and was applied successfully to the analysis of the toxins from several *Microcystis* species. This work establishes a total system for both analysis and isolation of microcystins.

EXPERIMENTAL

Materials

Three *Microcystis* cell materials were used. The first was from culture strain *Microcystis aeruginosa*, M-228[10], the second from culture strain *Microcystis viridis*, TAC-44[11] and the third from a naturally occurring surface bloom, *Microcystis aeruginosa*, Monroe strain[12].

Baker 10 C_{18}, Bond Elut C_{18} and Sep-Pak C_{18} were obtained from J. T. Baker (Phillipsburgh, NJ, U.S.A.), Analytichem International (Harbour City, CA, U.S.A.) and Waters Assoc. (Milford, MA, U.S.A.), respectively. The HPLC packing materials were Nucleosil $3C_{18}$ and $5C_{18}$ (Chemco Scientific, Osaka, Japan) having particle sizes of 3 and 5 μm, respectively.

Methanol, chloroform, ethyl acetate, isopropanol, trifluoroacetic acid (TFA), acetic acid, sodium sulphate, disodium hydrogenphosphate, potassium dihydrogenphosphate and phosphoric acid were analytical grade.

Toxins

Microcystins LR, YR and RR (cyanoginosins LR, YR and RR) were isolated and purified from M-228 and TAC-44 according to the previously reported method[9].

Thin-layer chromatography

Samples applied to plates (Kieselgel $60F_{254}$; E. Merck, Darmstadt, F.R.G.) were developed with the following solvent systems; ethyl acetate–isopropanol–water (4:3:7, upper layer) and chloroform–methanol–water (65:35:10, lower layer). The developed plate was heated at 105°C for 5 min to evaporate remaining solvents. The plate was visualized under short-wavelength UV light and then placed in a chamber with iodine vapour, and after 5 min the positions of the toxins were evaluated. Toxin concentrations as low as 100 ng can be detected with this detection system.

High-performance liquid chromatography

An high-performance liquid chromatograph equipped with a constant-flow pump (LC-5A; Shimadzu, Kyoto, Japan) was used with a variable-wavelength UV detector (Shimadzu SPD-2AM) operated at 238 nm. The separation was performed on Nucleosil $3C_{18}$ (3 μm, 75 mm × 4.6 mm I.D.) and $5C_{18}$ (5 μm, 150 mm × 4.6 mm I.D.) columns with the following mobile phases; (A) methanol–0.05% (v/v) TFA (6:4), (B) methanol–0.05 *M* phosphate buffer (pH 3.0, 6:4) and (C) methanol–0.05 *M* sodium sulphate (1:1).

Analytical procedure

Lyophilized cells (500 mg) were extracted three times with 50 ml of 5% (v/v) acetic acid for 30 min while stirring. The extract was centrifuged at 9300 *g* and then the supernatant was applied to a C_{18} cartridge. The cartridge which contained microcystins was rinsed with 20 ml of water, followed by 20 ml of 10–25% methanol in water (10% for Sep-Pak C_{18}, 20% for Bond Elut C_{18} and 25% for Baker 10 C_{18}, respectively). Microcystins were finally eluted from the C_{18} cartridge with 20 ml of methanol. The eluate was evaporated under reduced pressure and then the residue was dissolved in 0.5 ml of methanol. The solution was subjected to TLC or HPLC analysis.

RESULTS AND DISCUSSION

In our previous study[9] we found that: (1) extraction with 5% aqueous acetic acid was very effective because excellent toxin recovery was obtained and pigment extraction was limited; (2) clean-up using ODS-silica gel efficiently eliminated inorganic materials and polar contaminants; (3) TLC with silica gel gave good separation and (4) HPLC with methanol–0.05% TFA as a mobile phase provided excellent resolution between microcystins LR and YR.

Separation of microcystins by HPLC

In several previous reports on HPLC methods for separation of microcystins,

acetonitrile–10 m*M* ammonium acetate (26:74) has often been used as a mobile phase on C_{18} columns[5–8]. We attempted to separate microcystins LR and YR under these HPLC conditions but they were coeluted. We were previously able to separate microcystins LR and YR using a mobile phase containing TFA[9]. However, more than five microcystins exist in this group[2,3], so the identification of the toxins should be achieved by the complementary usage of plural HPLC solvent systems. In addition, preparative HPLC is a suitable technique to obtain pure microcystins. Therefore three different HPLC conditions were compared for separation between microcystins LR and YR in view of the analysis and preparation and the results are discussed below.

As shown in Fig. 2, the three different mobile phases [(A) methanol–0.05% TFA (6:4), (B) methanol–0.05 *M* phosphate buffer (pH 3.0) (6:4) and (C) methanol–0.05 *M* sodium sulphate (1:1)] gave good separation between both toxins on a short column (Nucleosil $3C_{18}$, 3 μm, 75 mm × 4.6 mm I.D.). However, since microcystin LR is frequently accompanied by a small peak (arrow) in liquid chromatography, the separation between these compounds was also examined. The compound corresponding to the small peak has the same molecular weight as microcystin LR and its isolation and characterization will be discussed elsewhere[13]. With the use of mobile phase A, both toxins were separated within 5 min and this mobile phase was applicable to preparative HPLC because it is very easy to evaporate the mobile phase. Despite these advantages the separation between microcystin LR and the small peak was not sufficient. Although mobile phase B was not suitable for preparative HPLC because of

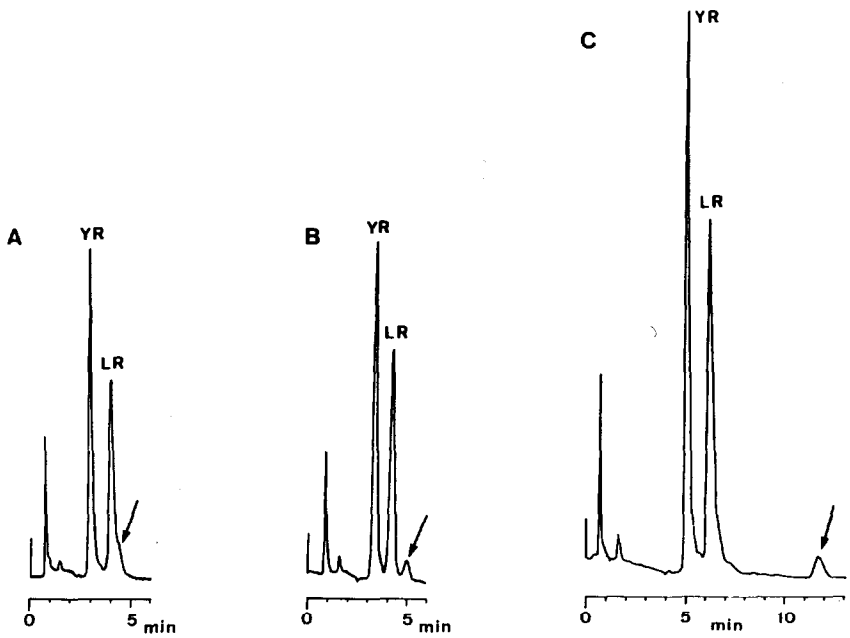


Fig. 2. HPLC separation between microcystins LR and YR. Column: Nucleosil $3C_{18}$ (3 μm, 75 mm × 4.6 mm I.D.). Mobile phases: (A) methanol–0.05% TFA (6:4); (B) methanol–0.05 *M* phosphate buffer (pH 3.0) (6:4); (C) methanol–0.05 *M* sodium sulphate (1:1). Flow-rate: 1 ml/min. Detection: 238 nm. Arrows indicate the small peak.

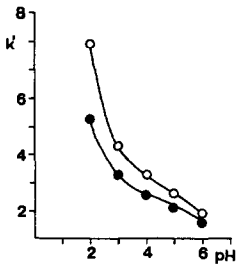


Fig. 3. Influence of the pH on the capacity factor, k', using phosphate buffer in the mobile phase for separation of microcystins LR (○) and YR (●).

the presence of non-volatile salts, all compounds including the small peak were separated within 5 min. Fig. 3 shows the influence of the pH of the phosphate buffer in the mobile phase on the capacity factors, k' (ref. 14), of both toxins. The k' of both toxins are strongly dependent upon the pH. When phosphate buffer with a pH of 3.0 was used, the most suitable k' values of these toxins were obtained. Therefore this mobile phase was adopted subsequently. With mobile phase C, it took 12 min to separate the above three compounds. This mobile phase is the most suitable for preparative HPLC because it was able to separate completely microcystin LR from the small peak and sodium sulphate is inert. We examined the effect of the concentration of sodium sulphate in the mobile phase on k' values of both toxins using methanol–aqueous sodium sulphate (1:1) solutions. The resolution was improved with increasing salt concentration, so that good resolution was obtained above 0.01 *M*. The most suitable retention time was obtained using 0.05 *M* sodium sulphate.

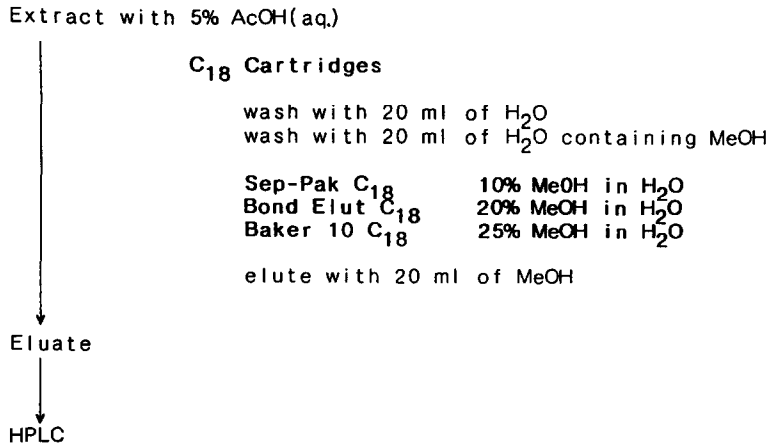
At this time we recommend the following mobile phases for identification and purification of microcystins: A, methanol–0.05% TFA (6:4), B, methanol–0.05 *M* phosphate buffer (pH 3.0) (6:4) and C, methanol–0.05 *M* sodium sulphate (1:1). Their complementary use would enable a ready identification of any microcystins investigated to date, and mobile phase C is most effective for preparative HPLC of the toxins.

Purification of microcystins LR with C_{18} cartridges

Recently, Poon *et al.*[8] and Brooks and Codd[5] extracted microcystins using Sep-Pak C_{18} cartridges. However the adsorbing power of the cartridge is relatively weak, so they were compelled to use two cartridges connected in series. Although Krishnamurthy *et al.*[6] efficiently used Bond Elut C_{18} cartridges, it has not always been possible to optimize the clean-up conditions. We have already shown the differences in the adsorption behaviour of cartridges from different suppliers[15]. Consequently, the clean-up conditions for microcystins were investigated using three commercially available cartridges (Baker 10 C_{18}, Bond Elut C_{18} and Sep-Pak C_{18}).

A lyophilized *Microcystins aeruginosa* (Monroe strain, dry weight 500 mg), which contained only microcystin LR, was extracted three times with 50 ml of 5% aqueous acetic acid solution and then the extract was passed through a C_{18} cartridge. The cartridge was washed with 20 ml of water and then with 20 ml of 5–40% methanol in water. Each eluate passed through the cartridge was subjected to HPLC to test for the elution of microcystin LR from the cartridge. In the case of the Baker 10 C_{18}

cartridge the toxin was completely retained using less than 25% methanol in water as a washing agent. When the cartridge was washed with 30% methanol in water, the toxin began to leak from the cartridge. It is desirable to wash the sample-containing cartridge with 20 ml of 25% methanol in water when the Baker 10 C_{18} cartridge is used at the clean-up step to separate impurities from the toxin. Using Bond Elut and Sep-Pak C_{18} cartridges, and respectively 25 and 15% methanol in water as the washing agents, the toxin began to elute. Twenty and ten percent methanol in water are suitable for Bond Elut and Sep-Pak C_{18} cartridges, respectively because no toxin elution can be detected. These results indicate the adsorptive power for microcystin LR is increasing in the order: Baker 10, Bond Elut and Sep-Pak C_{18}. The clean-up procedures for the toxin using the three different cartridges are shown in Scheme 1. The chromatograms before and after the clean up show the effect of eliminating the more polar contaminants (Fig. 4). The results should be applicable to microcystins other than microcystin LR.



Scheme 1. Clean-up procedure for microcystin LR with C_{18} cartridges. AcOH = Acetic acid; MeOH = methanol.

Finally, in order further to ensure the applicability of the methods mentioned above, mobile phase B and Baker 10 C_{18} were used for the analysis of microcystins from a *Microcystis viridis* (TAC 44) which was collected in Lake Kasumigaura in Japan[11]. In this case a normal type column (Nucleosil $5C_{18}$, 5 μm, 150 mm × 4.6 mm I.D.) and a slightly modified mobile phase [methanol–0.05 *M* phosphate buffer (58:42)] were employed. Fig. 5 shows the high-performance liquid chromatogram after the clean up, and the three toxins are successfully detected. Two of the three peaks were readily identified as microcystins LR and YR by comparison with standard samples. The remaining large peak which has a molecular weight of 1037 is thought to be microcystin RR containing two argines as the L amino acid variants. The toxin is also considered to be the same compound as cyanoviridin RR and cyanogenosin RR recently reported by Kusumi *et al.*[16] and Painuly *et al.*[17], respectively. The toxins from *Microcystins viridis* will be reported in detail elsewhere[18].

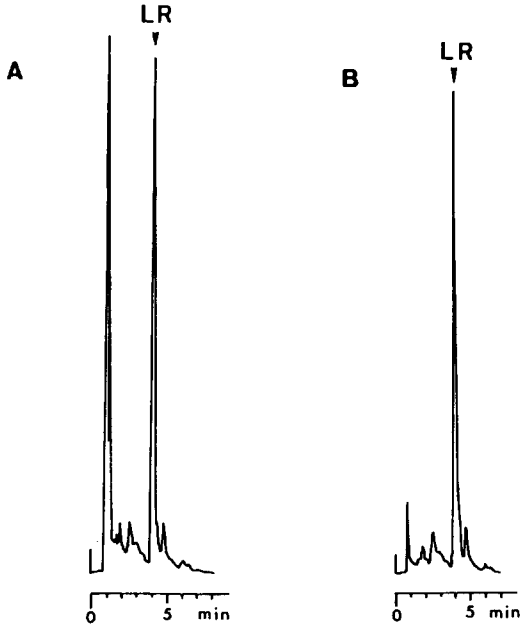


Fig. 4. High-performance liquid chromatograms of the methanol fraction from Monroe cells. (A) Before the clean up using Baker 10 C_{18}; (B) after the clean up using Baker 10 C_{18}. Conditions as in Fig. 2B.

A total system for analysis and isolation of microcystins

Herein we have established analytical methods for microcystins using three HPLC solvent systems and three commercially available C_{18} cartridges. In addition we have also described an isolation procedure for microcystins by using TLC, HPLC and silica gel and C_{18}-silica gel chromatographies[9]. A combination of these methods can therefore provide an isolation and analytical system for the toxins as shown in Scheme 2. Namely, after extraction with 5% aqueous acetic acid the sample is purified with

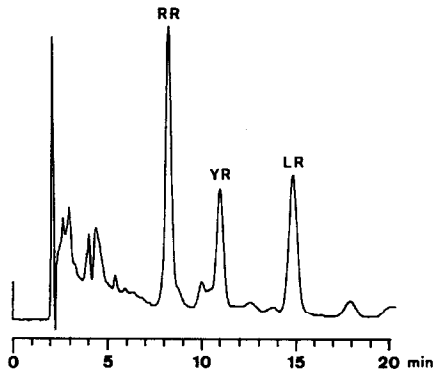
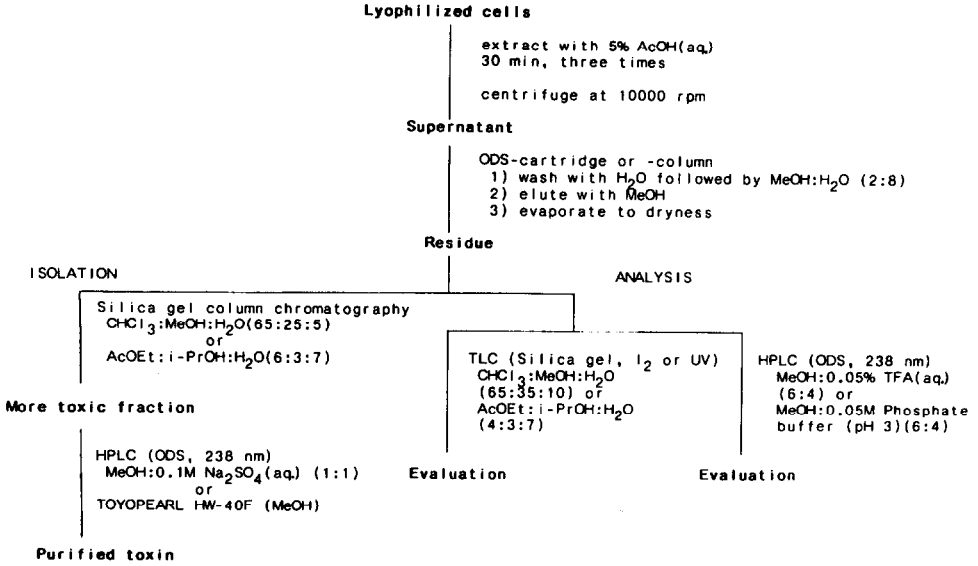


Fig. 5. High-performance liquid chromatogram of the methanol fraction from TAC-44. Column: Nucleosil $5C_{18}$ (5 μm, 150 mm × 4.6 mm I.D.). Mobile phase: methanol–0.05 *M* phosphate buffer (pH 3.0) (58:42). Flow-rate: 1 ml/min. Detection: 238 nm.



Scheme 2. Total system for analysis and isolation of microcystins. MeOH = Methanol; AcOH = acetic acid; AcOEt = ethyl acetate; i-PrOH = isopropanol.

a C_{18} cartridge and then the toxins are detected by HPLC with mobile phase A or B, and TLC using iodine and UV as detection systems. In this HPLC system, 1 ng of the toxin can be precisely detected, while the TLC method enables a ready detection of not only the toxins but also contaminants which are not detected by UV alone. When the toxins are detected at the analysis stage, the toxic fraction from a large amount of cells is applied successively to C_{18}-silica gel and silica gel column chromatographies. Finally the toxins are purified by preparative HPLC (mobile phase C) or Toyopearl HW-40 (methanol) and are checked by HPLC and TLC again. The systems developed are simple, rapid and reliable, and should contribute to progress in various studies on the microcystins.

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