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Detection of cyanobacterial toxins (microcystins) in cell extracts by micellar electrokinetic chromatography

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Abstract

A micellar electrokinetic chromatography (MEKC) method with UV detection is described for the rapid and efficient separation of three microcystins: microcystin-YR (MCYST-YR), microcystin-LR (MCYST-LR) and microcystin-RR (MCYST-RR). A detection limit of 7.5 pg for each toxin was achieved. The UV intensities of these toxins measured at 200 nm showed good linearity in the range 7.5–150 pg. The production of MCYST-LR in three cultured strains of cyanobacteria, namely *Microcystis aeruginosa* strain IP7806, *Microcystis aeruginosa* strain IP7813 and *Oscillatoria agardhii* strain IP7805, was evaluated.

Keywords: Microcystins

1. Introduction

Waterblooms of toxic cyanobacteria (blue-green–algae) have been detected in freshwater lakes and reservoirs for drinking water all over the world [1,2]. These blooms have caused death to domestic animals after drinking affected water [3,4] and may also be a health hazard for humans [5,6]. Cyanobacteria of the genera *Microcystis* and *Oscillatoria* produce a range of cyclic heptapeptide toxins, known as microcystins [7]. Approximately fifty variants of microcystin have been identified [8–11]. Their toxicities vary; LD_{50} values for microcystins (intraperitoneal injection to mice) range from 50 μg/kg for the most toxic (microcystin-LR) to >1 mg/kg for the least toxic [7,12]. Recent research has shown that microcystins may inhibit protein phosphatases and promote tumours in nanomolar concentrations [7,13,14]. These findings suggest that a method for determining microcystins is urgently needed to monitor drinking water. These highly polar toxins are, however, difficult to analyze. A number of approaches for their determination have been evaluated. Immunological-based assays such as ELISA [15] cannot be used for precise quantitative analysis because the microcystin-LR antibody produced has different specificities against each microcystin variant. Chemical assay methods based on high-performance liquid chromatography (HPLC) and gas chromatography (GC) have been reported [16–18]. Other research has shown the application of capillary zone electrophoresis (CZE) with laser fluorescence detection for the determination of microcystin-LR [19]. Recently, a combined liquid chromatographic mass spectrophotometric method was reported for analysis of microcystins [20].

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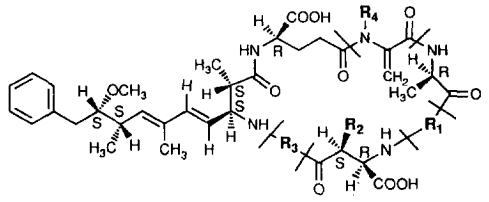

MCYST-YR; R_1 = Tyr, R_2 = CH_3; R_3 = Arg; R_4 = CH_3 (MW = 1044)

MCYST-LR; R_1 = Leu; R_2 = CH_3; R_3 = Arg; R_4 = CH_3 (MW = 994)

MCYST-RR; R_1 = Arg; R_2 = CH_3; R_3 = Arg; R_4 = CH_3 (MW = 1037)

Fig. 1. Structures of cyanobacterial toxins: microcystin-YR (MCYST-YR), microcystin-LR (MCYST-LR) and microcystin-RR (MCYST-RR).

In this work we report on the application of micellar electrokinetic chromatography (MEKC) for the rapid, efficient and sensitive analysis of three microcystins (Fig. 1): microcystin-YR (MCYST-YR), microcystin-LR (MCYST-LR) and microcystin-RR (MCYST-RR).

2. Experimental

2.1. Equipment

A HP 3D-CE system (Hewlett-Packard, Waldbronn, Germany) equipped with diode array detection was used for the MEKC experiments. An untreated fused-silica capillary (Composite Metal Services, London, UK) 64.5 cm (56 cm to the detector)×50 μm I.D., was used in all separations. Sample introduction was performed using hydrodynamic injection at 5 kPa for 5 s. The injection volume was in the order of 3 nl. The micellar phase consisted of 40 m*M* phosphate buffer at pH 6.8 and 10 m*M* sodium dodecyl sulfate (SDS). The applied voltage was +20 kV and UV detection was performed at 200 nm.

2.2. Materials

Three cyanobacteria cell materials were used. The first was from culture strain *Microcystis aeruginosa*, IP7806; the second from culture strain *Microcystis aeruginosa*, IP7813; and the third from culture strain *Oscillatoria agardhii*, IP7805. The strains were obtained from the Institut Pasteur (Paris, France). Microcystins -LR and -RR were purchased from Sigma (Paris, France) and microcystin-YR was purchased from Calbiochem (CA, USA). Methanol, chloroform and water were HPLC grade. The 40 m*M* phosphate buffer (pH 6.8) was prepared by the addition of 268 mg of $Na_2HPO_4 \cdot 7H_2O$ and 156 mg of $NaH_2PO_4 \cdot 2H_2O$ in 50 ml HPLC-grade water.

2.3. Cyanobacteria cultures

Cultures of the three strains of cyanobacteria were maintained in sterilized 250-ml Erlenmeyer flasks containing 100 ml BG-11 growth medium [21] at 22°C under white light (1070 lux, 12 h light–12 h dark). Cells were harvested by filtration at the end of the exponential growth phase, and then dried at 40°C for 4 h.

2.4. Cyanobacteria sample preparation

Dried cells of each strain of cultured cyanobacteria were sonicated in methanol for 5 min. After centrifuging the homogenate at 12 000 *g* for 10 min, the supernatant was evaporated to dryness under reduced pressure. The residue was then distributed two times with methanol–chloroform–water (2:3:10, v/v). After discarding the organic layers, the aqueous layers were then pooled and applied to a C_{18} cartridge (Sep-Pak C_{18}). Microcystins were eluted from the C_{18} cartridge with methanol. The eluate was evaporated under reduced pressure and the residue was dissolved in 25 μl methanol for analysis.

3. Results and discussion

Because of the charged character of the solutes under different pH values, initial experiments were performed in capillary zone electrophoresis (CZE) in the phosphate buffer alone. The performance was poor on the one hand due to the incomplete separation of the three microcystins and on the other hand due to the large band broadening observed i.e. low efficiency, which is attributed to capillary wall interaction. By adding 10 m*M* SDS to the buffer the resolution is improved dramatically (Fig. 2). More-

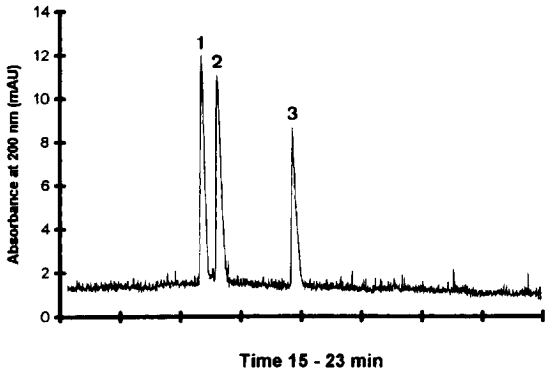

Fig. 2. MEKC separation of a standard solution containing three microcystins: (1) MCYST-YR; (2) MCYST-LR and (3) MCYST-RR. The electropherogram represents an injection of 50 pg of each toxin. For conditions see Section 2.

over good linearity was observed in the concentration range 2.5–50 μg/ml corresponding to 7.5–150 pg absolute amounts.

Calibration graphs for MCYST-YR, MCYST-LR and MCYST-RR are presented in Fig. 3. The graphs show good linear response with correlation coefficients of 0.991, 0.989 and 0.983 respectively. The mass detection limit is in the order of a 7.5 pg injection for each toxin. This detection limit is approximately 600 times lower than that reported with HPLC. The limit of detection for pure standards (MCYST-RR, MCYST-LR, MCYST-LY, MCYST-LW, and MCYST-LF) with HPLC giving a consistently reproducible peak area was found to be 5 ng [17]. The insert in Fig. 3 shows the MEKC analysis achieved at this detection limit for MCYST-YR, MCYST-LR and MCYST-RR with a signal-to-noise ratio of 2, 2 and 1.5 respectively. Reproducibility of migration times was sufficient to ensure proper peak assignment and extremes of migration time varied by no more than 5% on a daily basis.

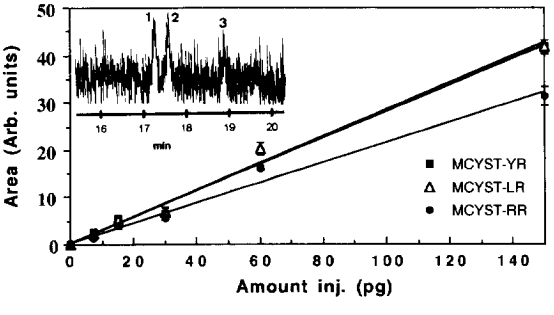

Fig. 3. Calibration curve (wavelength of detection 200 nm) for MCYST-YR, MCYST-LR and MCYST-RR in the concentration range 2.5–50 μg/ml. Bars represent S.E. (n=3). For conditions see Section 2.

The application of this MEKC method was evaluated for the determination of microcystins in the crude extracts of three cultured cyanobacteria. The extracts from the *Microcystis aeruginosa* IP7806, *Microcystis aeruginosa* IP7813 and *Oscillatoria agardhii* IP7805 cultured in our laboratory contain only microcystin-LR [Fig. 4(A), Fig. 5(A) and Fig. 6(A)]. The peak 2 observed in these figures corresponded to the MCYST-LR standard as can be

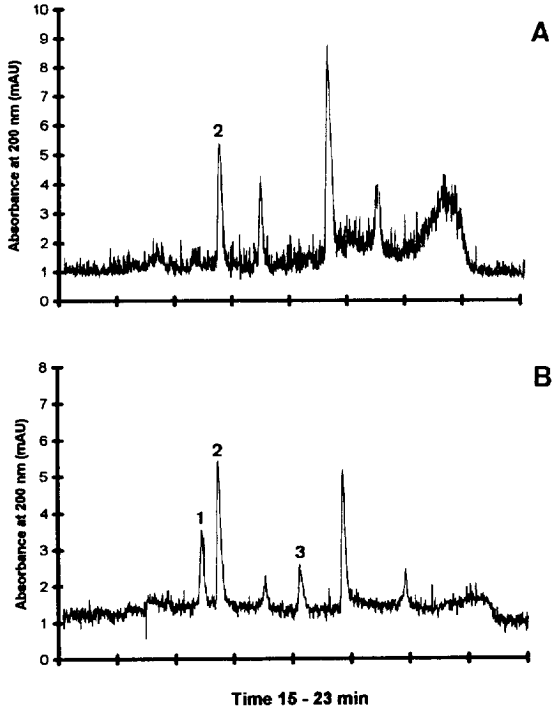

Fig. 4. Electropherograms of (A) extract of cultured cyanobacteria *Microcystis aeruginosa* IP7806 and (B) extract of cultured cyanobacteria *Microcystis aeruginosa* IP7806 spiked with the standard solution containing the three microcystins: (1) MCYST-YR; (2) MCYST-LR and (3) MCYST-RR. For conditions see Section 2.

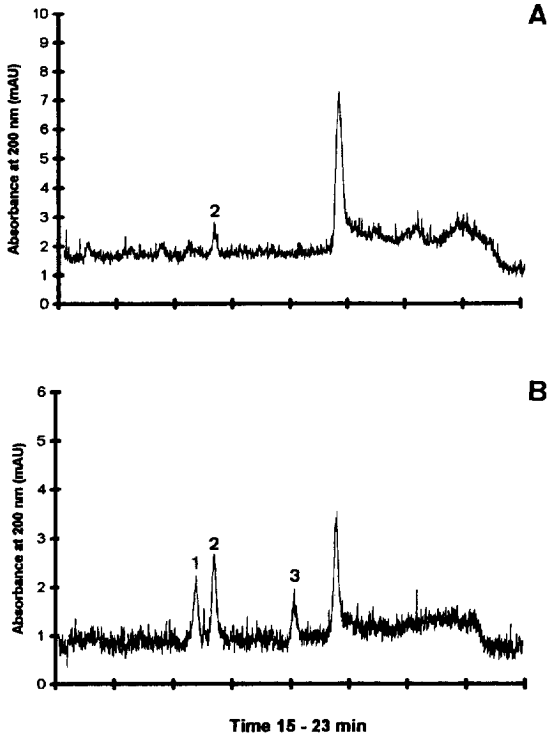

Fig. 5. Electropherograms of (A) extract of cultured cyanobacteria *Microcystis aeruginosa* IP7813 and (B) extract of cultured cyanobacteria *Microcystis aeruginosa* IP7813 spiked with the standard solution containing the three microcystins: (1) MCYST-YR; (2) MCYST-LR and (3) MCYST-RR. For conditions see Section 2.

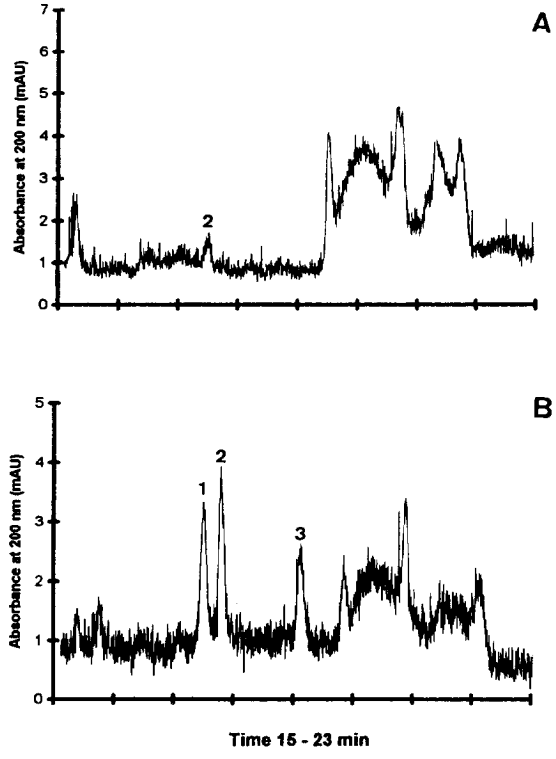

Fig. 6. Electropherograms of (A) extract of cultured cyanobacteria *Oscillatoria agardhii* IP7805 and (B) extract of cultured cyanobacteria *Oscillatoria agardhii* IP7805 spiked with the standard solution containing the three microcystins: (1) MCYST-YR; (2) MCYST-LR and (3) MCYST-RR. For conditions see Section 2.

deduced from Fig. 4(B), Fig. 5(B) and Fig. 6(B) showing the electropherograms with the spiked standards (Fig. 2).

The production of MCYST-LR is different in the cells of the three strains cultured in the same conditions. The yield of MCYST-LR in dried cyanobacteria *Microcystis aeruginosa* IP7806, *Microcystis aeruginosa* IP7813, and *Oscillatoria agardhii* IP7805 is 70, 20 and 6 μg/g dried weight respectively.

Many other peaks with electrophoretic mobilities in the range of those of the microcystin standards are observed in the extracts of each cyanobacterium. All these peaks have a UV absorbance at 238 nm, a characteristic wavelength of many microcystins. Work is in progress to determine the identification of these peaks.

4. Conclusions

MEKC allows the rapid and efficient separation of microcystins. The limit of detection was found to be in the order of femtomoles (7.5 pg). The production of microcystin-LR by three different microorganisms could be evaluated.

In comparison with existing techniques such as liquid chromatography (HPLC), the determination of microcystins by MEKC-UV holds promise for the routine screening of these toxins in natural extracts. However, solid-phase extraction (SPE) appears to be a necessary technique for the extraction of these toxins. This is especially important for microcystin concentrations which are normally present in low concentrations (ng/l) in drinking water.

Particular attractions of this technique are ease of

operation, low cost, small sample consumption, speed of analysis, high efficiency and automation.

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