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Note

Purification procedure for peptide toxins from the cyanobacterium *Microcystis aeruginosa* involving high-performance thin-layer chromatography

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Several species of the cyanobacteria (blue-green algae), which dominate nutrient-rich fresh and brackish waters, produce toxins which are responsible for poisonings of wild and domestic animals, birds and fish throughout the world[1,2]. The toxins have also been implicated in human health problems[1–4] and are thus of interest to the water industry. *Microcystis aeruginosa* has been the most often encountered cyanobacterium in poisoning episodes. The symptoms of intoxication of animals and fish by this organism include extensive liver damage and haemorrhages[5–9]. The *Microcystis* toxins responsible are low-molecular-weight (*ca.* 1000 a.m.u.) cyclic hexapeptides[10]. On acid hydrolysis, they have a characteristic fragmentation pattern, usually forming valine, leucine, glutamic acid, methyl aspartic acid and an unusual amino acid containing a substituted phenyldecadienoic acid[11].

Methods for the purification of *Microcystis* peptide toxins have long been available[12] and have been refined recently[13,14]. The usual modern protocol involves solvent extraction of freeze-dried cells, followed by adsorption from the extract onto C_{18} Sep-Pak cartridges, gel filtration column chromatography and reversed-phase high-performance liquid chromatography (HPLC). These procedures have revealed one or two toxic peptides in some cases (*e.g.* refs. 13–15) although the presence of up to seven toxins has been indicated in some *M. aeruginosa* isolates[16]. We wished to develop a simpler and more rapid method for peptide toxin extraction and purification and this paper describes how this can be achieved by high-performance thin-layer chromatography (HPTLC).

EXPERIMENTAL

Organisms

Three toxic strains of *Microcystis aeruginosa* were used: *Microcystis* PCC 7820 and PCC 7813 were isolated from toxic blooms in Loch Balgavies near Dundee and

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are deposited at the Institut Pasteur, Paris. *Microcystis* AIT was isolated from a toxic *Microcystis* bloom from Thailand, kindly provided by Dr. M. J. Phillips, University of Stirling. Organisms were grown in BG-11 medium containing nitrate in photoautotrophic batch culture as detailed earlier[17].

Materials

HPLC grade acetonitrile, water and ammonium acetate were purchased from Fisons (Loughborough, U.K.). Elution solvents for HPLC were filtered through a 0.22-μm Nylon 66 membrane (Alltech, Carnforth, U.K.) and degassed before use. The C_{18} Sep-Pak cartridges were supplied by Waters Assoc. (Northwich, U.K.). HPTLC plates precoated with silica gel 60 (10 × 10 cm), and preparative plates precoated with RP-C_{18} (20 × 20 cm) were manufactured by Merck (Darmstadt, F.R.G.) and Whatman (Maidstone, U.K.), respectively.

Toxin extraction

Lyophilized late log-phase cells (1 g dry weight) were extracted three times in 1-butanol–methanol–water (5:20:80, v/v; 160 ml) for 3 h at 4°C. The mixture was centrifuged at 23 000 *g* for 40 min. The supernatants were combined and evaporated *in vacuo* at 40°C until *ca.* 100 ml remained. The samples were passed through two C_{18} Sep-Pak cartridges connected in series and the toxic material was eluted with about 20 ml methanol. The eluates were dried *in vacuo* at 40°C.

HPTLC analysis

The extracted samples were suspended in 0.5 ml methanol and were delivered to the preparative RP-C_{18} plates as a single streak 3 cm from the bottom of the plate. The tank was lined with Whatman No. 1 filter paper and equilibrated by the addition of solvent 10 min before inserting the plates. The developing solvent was varied according to each sample (Table I). After the solvent had reached the top of the plates, they were examined by placing on a UV transilluminator. The appropriate spots were scraped into 10 ml methanol, vortexed and spun in a bench centrifuge at 20 000 *g* for 5 min. The supernatant was concentrated under reduced pressure and rechromatographed on silica gel 60 plates to confirm purity (Table I).

TABLE I

SOLVENT SYSTEMS FOR HPTLC SEPARATION OF TOXIC PEPTIDES FROM *MICROCYSTIS AERUGINOSA* STRAINS

Microcystis strain	*Solvent system*	
	Silica gel 60	*RP-C_{18}*
PCC 7813	Dichloromethane–methanol (2:3)	Chloroform–methanol (1:1)
PCC 7820	Chloroform–methanol (1:1)	Chloroform–methanol (1:1)
AIT	Chloroform–methanol (1:3)	Chloroform–methanol (2:1)

Toxicity testing

The purified material was tested by mouse bioassay. The concentrated HPTLC eluates were diluted in water and injected intraperitoneally (1 ml) into Balb *c* mice of 20–25 g body weight as detailed previously[18].

UV characterization of the toxins

All of the HPTLC spots which were toxic to mice were scanned by a UV–VIS spectrophotometer (LKB Ultrospec 4050) to determine the maximum UV absorbance of each compound.

HPLC analysis

A Gilson liquid chromatograph equipped with two solvent delivery pumps (Model 303) and a holochrome (Model HM) with a single channel UV–VIS absorbance detector were used. The chromatograms were recorded on a Shimadzu Chromatopac C-RIB recorder. The column was a 25 cm × 0.4 cm I.D. Hypersil 5 ODS column (HPLC Technology, Wilmslow, U.K.). The operating solvent was acetonitrile–10 m*M* ammonium acetate (26:74). The flow-rate was 0.8 ml min^{-1} and the UV absorbance was monitored at 240 nm for all samples. Each sample was filtered through a 0.45-μm membrane filter before injection into the HPLC system.

RESULTS AND DISCUSSION

Typical profiles of the toxins extracted from three strains of *Microcystis aeruginosa* and separated by HPTLC on silica gel 60 plates are shown in Fig. 1. The toxins appeared as discrete pale blue-white spots under UV and were well separated

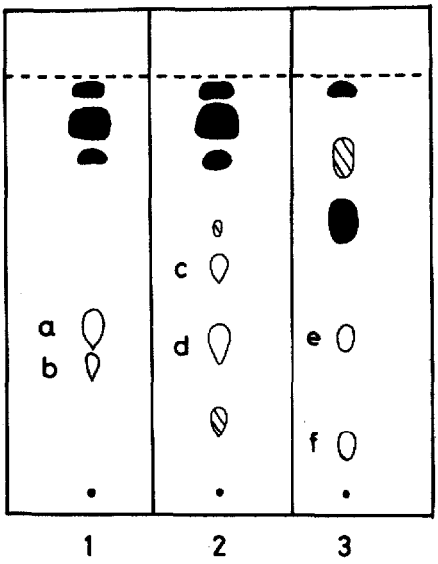

Fig. 1. Typical separations of peptide toxins by HPTLC from strains of *Microcystis aeruginosa*. For respective elution conditions see Table I. (1) *Microcystis* PCC 7813; (2) *Microcystis* PCC 7820; (3) *Microcystis* AIT. Spots a–f, peptide toxins. Hatched spots, non-toxic compounds; filled spots, photosynthetic pigments.

TABLE II

R_F VALUES OF *MICROCYSTIS* PEPTIDE TOXINS SEPARATED BY HPTLC

Microcystis strains	*Toxin compound**	R_F *values*	
		Silica gel 60	*RP-C_{18}*
PCC 7813	a	0.41	0.48
	b	0.32	0.30
PCC 7820	c	0.56	0.67
	d	0.38	0.44
AIT	e	0.38	0.26
	f	0.12	0.15

* Letters a–f refer to toxic spots shown in Fig. 1.

from the green and yellow/orange photosynthetic pigments also resolved by the procedure. Two toxic compounds, according to mouse bioassay, were obtained from *Microcystis* strains PCC 7820, PCC 7813 and AIT (Fig. 1). The R_F values of the individual toxins are given in Table II for the HPTLC silica gel 60 and RP-C_{18} systems used. R_F values for toxins from individual strains were distinguishable from one another.

HPLC analysis of individual eluted *Microcystis* peptide toxins produced chromatograms containing a single peak, indicating that the compounds obtained were

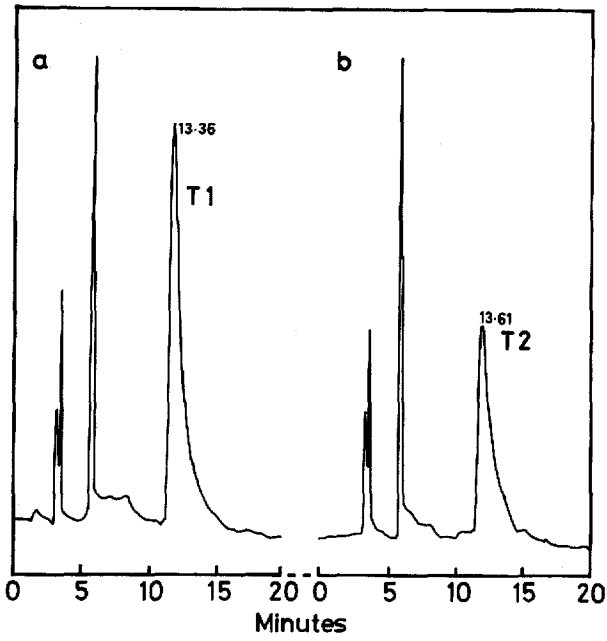

Fig. 2. Typical HPLC profiles of toxins eluted from HPTLC plates. a and b were obtained from the respective spots a and b from *Microcystis* PCC 7813 shown in Fig. 1. T1, T2, toxic peptides shown with retention times (min). Earlier peaks were due to water.

pure (*e.g.* Fig. 2). Comparative studies are in progress on the composition and structure of individual toxic peptides purified by HPTLC from the *Microcystis* strains.

The toxin purification procedure described here is easy to perform. It eliminates the need for gel filtration column chromatography and HPLC[12–15]. Our method involving Sep-Paks and HPTLC does not require expensive equipment and should find wide applications.

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