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Application of reversed-phase medium-pressure liquid chromatography to the isolation, separation and amino acid analysis of two closely related peptide toxins of the cyanobacterium *Microcystis aeruginosa* strain PCC 7806

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ABSTRACT

A mixture of two compounds, related to cyanoginosin-LR, was isolated from the cyanobacterium *Microcystis aeruginosa* strain PCC 7806. Using an optimized reversed-phase fast protein liquid chromatography (RP-FPLC) method, up to 1.5 mg of this toxin mixture could be separated into two distinct compounds by a single preparative scale run. To perform an amino acid analysis of the two components, the hydrolysates derivatized with phenylisothiocyanate were analysed separately by RP-FPLC. A method was developed which could be used for the automatic sequential analysis of up to fourteen samples. The amino acid analysis by RP-FPLC was linear in the range 1–5 nmol. On the basis of this analysis, the two compounds differ by only a methylated *versus* a demethylated aspartate. Only six of the seven amino acids known for cyanoginosin-LR could be reliably detected.

INTRODUCTION

Microcystis sp., which produce highly active hepatotoxins, are algal organisms, most frequently isolated from toxic algal blooms in water [11]. The organisms occur in supplies of drinking water, and, as a result of their toxicity and high stability, are a threat for drinking water hygiene [2–4].

The most important toxic substances of *Microcystis* have been identified as cyclic heptapeptides with a molecular weight of about 1000. These are composed of three D-amino acids, one dehydroamino acid, one atypical β-amino acid and two strain-dependent variable L-amino acids. *Microcystis* toxins are generally termed cyanoginosin-XY. The variables X,Y describe the two variable L-amino acids [5].

It is assumed that the toxin produced by *Microcystis aeruginosa* strain PCC 7806 and cyanoginosin-LR are identical [6], with a structure of cyclo-(D-Ala-L-Leu-D-*erythro*-β-methylaspartate-L-Arg-Adda-D-Glu-N-methyldehydro-Ala-). The atypical amino acid Adda is a 3-amino-9-methoxy-10-phenyl-2,6,8-trimethyldeca-4,6-dienoic acid [7].

Reversed-phase high-performance liquid chromatographic (RP-HPLC) studies imply that the toxic fraction of PCC 7806 is not only one substance [6,8]. This is in accordance with previous findings using thin-layer chromatography (TLC) (unpublished data). It has been shown that this *Microcystis* strain produces two very similar components which have the properties of the cyanoginosins. Considering the possible implications for further toxicological studies, it was intended to isolate, separate and analyse these two substances on a preparative scale. Until now, the cyanoginosins have been purified and analysed by HPLC [8–11]. In this work the preparative and analytical capacity of the fast protein liquid chromatography (FPLC) technique was investigated. The chromatographic conditions were optimized by systematic variation and methods were developed to perform amino acid analysis by FPLC.

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EXPERIMENTAL

Algal strain and cultivation

Microcystis aeruginosa strain PCC 7806 was kindly supplied by Professor Weckesser, Institute for Microbiology of the University of Freiburg, Germany. Cyanobacteria were harvested from 10 l of fermentation cultures, continuously supplied with BG-11 medium [12] and illuminated with 2000-lux fluorescent light.

Isolation of the toxin

Lyophilized cyanobacteria (5 g) were extracted in 500 ml of doubly distilled water by exposure to ultrasound and centrifugation at 30 000 *g* for 30 min. For solid-phase extraction the supernatant was applied to a column packed with 5 g of C_{18} (J. T. Baker, Gross-Gerau, Germany), previously activated by flushing with 50 ml of 100% methanol (Uvasol Merck, Darmstadt, Germany) and subsequently with 50 ml of water. The toxic fraction was desorbed by elution with 100% methanol. After evaporation to dryness, the residue was dissolved in 200 ml of methanol–water (30:70, v/v). The precipitated lipids were removed by centrifugation at 30 000 *g* and subsequent passage of the supernatant through a 0.22-μm filter (Acrodisc, Gelman Sciences, Frankfurt, Germany).

To perform anion-exchange chromatography, an XK 16/10 column (Pharmacia, Freiburg, Germany), packed with 5 g of QMA (Waters Millipore, Eschborn, Germany) was used [13]. Prior to loading of the C_{18} eluate (200 ml), the QMA column was equilibrated with 100 ml of 0.1 *M* ammonium hydrogencarbonate in methanol–water (30:70, v/v) and washed by rinsing with 100 ml of the salt-free solvent. The toxic fraction was almost selectively detached from the matrix by elution with 0.02 *M* ammonium hydrogencarbonate (methanol–water, 30:70, v/v; flow-rate 2 ml/min; monitored at 240 nm). Finally, the toxic QMA eluate was evaporated to dryness and redissolved in methanol–water (30:70, v/v) and photometrically adjusted to the desired concentration using a standard calibration graph (Cyanoginosin-LR, molecular weight 994, Medor, Hersching, Germany).

Preparative RP-FPLC

The components contained in the QMA eluate were separated on a preparative scale using an FPLC system (Pharmacia) with the following configuration: LCC-500 plus gradient controller, two P-500 pumps, MV7 motor injection valve, 500-μl sample loop, VWM 2141 variable-wavelength monitor and FRAC-100 fraction collector. FPLC software (FPLC Manager, Pharmacia) facilitated the operation, data processing and documentation. A preparative PepRPC HR 16/10 column (pre-packed with 15-μm silica particles with covalently bonded C_2/C_{18}, average pore size 100 Å) was used as the stationary phase. The solvents were acetonitrile (LiChrosolv, Merck) and Milli-Q water (Millipore). For the optimum separation the application of very shallow gradients was necessary and therefore the eluents were premixed: solvent A, acetonitrile–water (24:76, v/v); solvent B, acetonitrile–water (50:50, v/v). Depending on the experiment trifluoroacetic acid (TFA) or pentafluoropropanate (PFPA, Merck) were added in different amounts to both solvents. The solutions were freshly prepared and degassed for 15 min before use. After separation the toxin fractions were desalted by the HR 16/10 column. For evaluation, the resolution (R_s) of the separated compounds was calculated as follows [14]:

$$\alpha = r_2/r_1 \quad (1)$$

$$N = (r_1/w_{0.5})^2 \quad (2)$$

$$k' = (r_1 + r_2)/2 \quad (3)$$

$$R_s = 0.25\,[(\alpha - 1)/\alpha]\,N^{1/2}[k'/(1 + k')] \quad (4)$$

where r_1 = retention time of peak 1, r_2 = retention time of peak 2, $w_{0.5}$ = peak width at half height, α = selectivity, N = efficiency and k' = capacity factor. A baseline separation is achieved, if R_s exceeds 1.5.

UV spectroscopy

The UV spectra of toxin samples were recorded on a MQ-3 spectrophotometer (Zeiss, Oberkochen, Germany). The samples were evaporated, dissolved in methanol–water (30:70, v/v) and adjusted to 0.6 absorption units at 240 nm ($A.U._{240\ nm}$).

TLC

UV_{254nm}-C_{18} RP-HPTLC plates, 10 × 20 cm (Merck) were used. The tank was equilibrated with

100 ml of methanol–water–acetic acid (70:28:2, v/v/v) 1 h before inserting the plates. Sample volumes of 10–20 μl dissolved in methanol–water (50:50, v/v) were spotted on the plates. After developing, bands were visualized by UV light (254 nm).

Amino acid analysis

Automatic FPLC amino acid analyser. The FPLC system could be programmed and automated for amino acid analysis. For automatic sample application, two MV8 valves (MV8-2 and MV8-3), each with eight inlets and one outlet, and the peristaltic pump C were connected in series between the solvent reservoir A and the motor injection valve MV7. As position .1 of the MV8 valves is reserved for wash functions, the valve positions .2 to .8 are available for sample loading. Programming methods for the LCC-500 gradient controller, sample loading by defined MV8 settings and the sample injection, gradient control and washing of the sample application system could be performed automatically (Table I). Fourteen LCC-500 methods (MV8.1 to MV8.14) were provided for the different valve positions and the FPLC Manager program was used for control.The program automatically analysed each of the fourteen samples successively by activating the LCC-500 methods in addition to recording, evaluating and printing the corresponding chromatograms.

Sample preparation. Standard L-amino acids (Asp, Glu, Ser, Gly, Thr, Ala, Arg, Tyr, Met, Leu) were purchased from Merck. D-Glu, D-Ala, DL-*threo*-β-methylaspartate and methylamine were obtained from Sigma (Deisenhofen, Germany). Defined aliquots of standards and purified toxin samples were suspended in 4 *M* hydrochloric acid (Titrisol, Merck) and placed in glass tubes. After evacuating and flushing with nitrogen, the tubes were sealed and incubated at 110°C for 18 h. To eliminate residues of hydrochloric acid, the samples were evaporated to dryness by vacuum centrifugation. The samples were then dissolved in 10 μl of an ethanol–water–triethylamine solution (2:2:1, v/v/v) (Pierce, Oud Beijerland, Netherlands). After redry-

TABLE I

FUNCTIONS OF THE LCC-500 METHODS MV8.%1 CONTROLLING THE MV8- VALVE SETTING AND THE GRADIENT

Programmed methods are MV8.1 to MV8.7 for valve MV8-2 and MV8.8 to MV8.14 for valve MV8-3. The valve position is controlled by the the FPLC Manager program calling up the appropriate method by the value for the variable %1, corresponding to the numeral behind the point in the method name.

Time (min)	Processing of		Setting	Description
	Sample	Gradient		
0		Conc. %B	0	
0		ml/min	1	Adjust flow-rate
0	VALVE.POS		2.X or 3.X[a]	Set valve to sample
0	FLOW C		0.5	Load sample (pump C)
1		cm/min	0.5	Activate recorder
1	FLOW C		0	Loop filled
1	VALVE.POS		1.2	Inject (valve 1 = MV7)
2	VALVE.POS		1.1	Stop injection
2	VALVE.POS		2.1 or 3.1	Reset active valve
2	FLOW C		1	Wash loop
3		Conc. %B	0	Start gradient
5	FLOW C		0	Stop washing
34		Conc. %B	32	Start column washing
49		Conc. %B	100	and reequilibrate to
53		Conc. %B	100	initial conditions
55		Conc. %B	0	
60		Conc. %B	0	END METHOD

[a] To determine the valve position depending on the method activated, fixed X-values from 2 to 8 are programmed for the methods MV8.1 to MV8.7 and MV8 to MV8.14.

ing for 1 h, 20 μl of the coupling solution [ethanol–water–triethylamine–phenylisothiocyanate (PITC), 70:19:10:1, v/v/v/v] were added, followed by mixing and incubation for 20 min at room temperature. By reaction with free amino acids, phenylthiocarbamyl (PTC) derivatives are obtained. Any excess PITC was removed by vacuum centrifugation. The samples were dissolved in eluent A prior to chromatographic separation.

Column chromatography. For eluent A, 0.9 l of water (HPLC grade, Merck) plus 0.8 ml of phosphoric acid (85%) were adjusted to pH 6.4 with sodium hydroxide solution 30%. The volume was adjusted to 1 l with HPLC-grade water and 5 ml of acetonitrile were added. Eluent B consisted of 300 ml of eluent A and 700 ml of acetonitrile. Before use, the freshly prepared solutions were degassed for 15 min. An HR 5/5 column (prepacked with C_2/C_8 silica, average particle diameter 5 μm, pore size 100 Å) was used as the stationary phase. The separation was carried out with 0% B for the first 2 min and then a linear gradient of 1% B per min for 32 min. Washing and re-equilibration was controlled by the program (Table I).

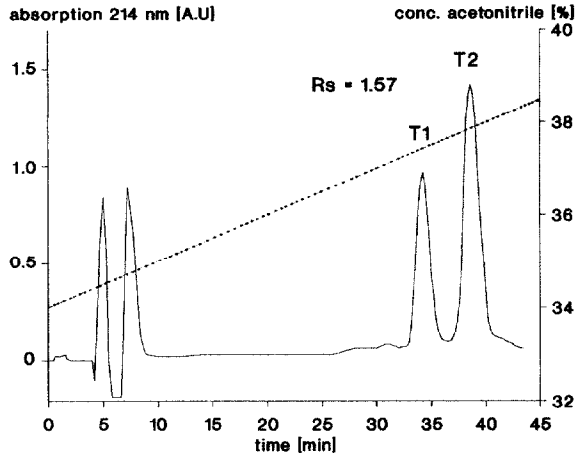

Fig. 1. Separation of the cyanoginosin homologues T1 and T2, isolated from strain PCC 7806, on the PepRPC HR16/10 column. The resolution R_s was calculated by eqn. 4. Chromatographic conditions: gradient start, 34% acetonitrile; slope, 0.1% min; additive, 0.3% PFPA; flow-rate, 3 ml/min; temperature, 4.3°C; sample, 1.4 mg per 500 μl (methanol–water, 30:70, v/v). The first signals (< 10 min) are solvent peaks.

RESULTS

Toxin preparation

Characteristic UV absorption spectra and the typical double-banded spot on HPTLC indicate the presence of cyanoginosin in the QMA eluate. The isolation procedure to the stage of the QMA eluate seems to give an almost pure toxin preparation; the RP-FPLC elution profiles of the QMA eluate recorded at 214 nm (Fig. 1), TLC (Fig. 2) and UV absorption spectra (Fig. 3) displayed no significant contamination.

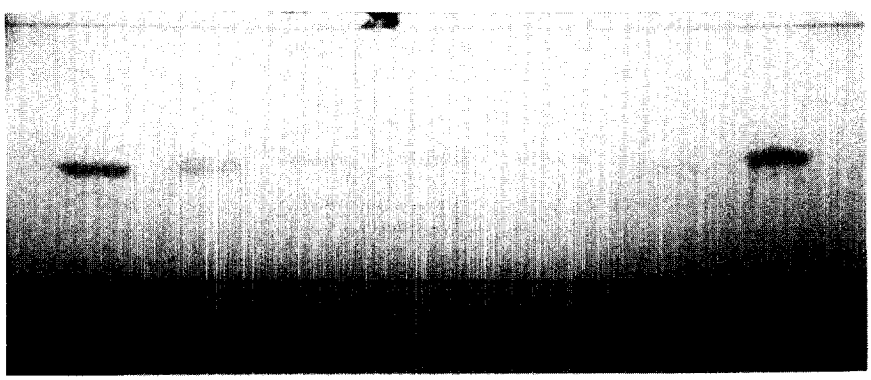

Fig. 2. HPTLC separation (chromatographic conditions as described in the text). The lanes are numbered from left to right. The arrangement of the samples was: lane 2, mixture of T1 and T2 (QMA eluate) prior to RP-FPLC; lanes 1 and 6, T1 after separation; lanes 3, 5 and 7, T2 after separation; lane 4, cyanoginosin-LR standard (Medor). The amounts spotted were about 20 μg. Lanes 1 and 7 were overloaded to visualize possible contaminations.

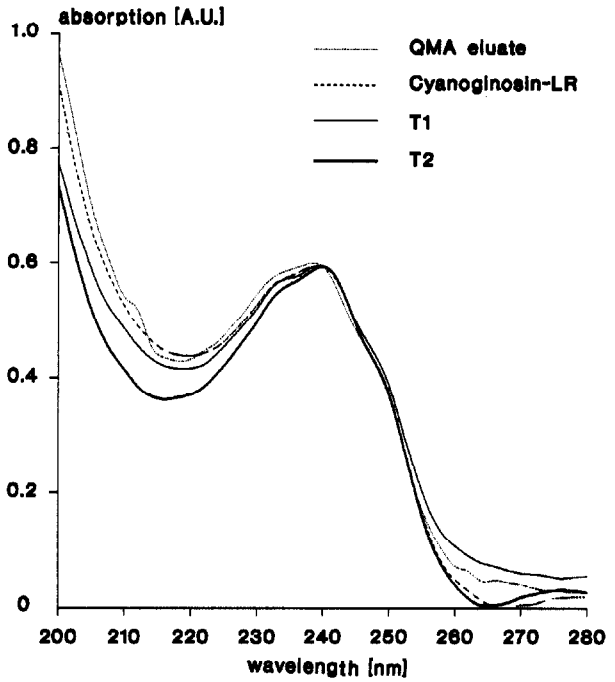

Fig. 3. UV absorption spectra of QMA eluate, T1, T2 and cyanoginosin-LR standard. Solvent was methanol–water (30:70, v/v).

Optimization of separation of the peptides by FPLC

The organic solvent concentration at which the desired components were eluted from the HR 16/10 PepRPC was in the range 35–42% acetonitrile. First 0.1% TFA was used as the ion-pairing agent, but under these conditions the resolution was not satisfactory. Therefore the more hydrophic PFPA was introduced. By increasing the retention time and selectivity by the addition of at least 0.3% PFPA, the separation was significantly improved. For further optimization the influence of different gradient slopes and different flow-rates was investigated. Starting at a concentration of 32% acetonitrile, the gradient slope was decreased stepwise from 0.32 to 0.08% per min and the flow-rate from 8 to 2 ml/min. The resolution was increased by lowering the flow-rate and gradient slope at the expense of time taken for the analysis and peak sharpness, which affected the efficiency (Table II). Isocratic elution in the concentration range 32–38% acetonitrile was not of advantage.

The following conditions provided an acceptable separation within 60 min: gradient start, 34% acetonitrile; gradient slope, 0.1% per min; flow-rate, 3 ml/min.

The influence of temperature in the range 0–30°C was investigated. Below 10°C almost baseline separation could be achieved (Fig. 1). Mathematically, the two components (T1 and T2) are separated completely. As a result of the slightly raised baseline, the fraction between the peaks T1 and T2 below the threshold of 0.15 A.U. was ignored to ensure physical separation. Up to 1.5 mg of the T1–T2 mixture can be separated by this method.

Analysis of separated peaks T1 and T2

The separation results were verified by repeating the chromatographic separation and by HPTLC (Fig. 2). The separation of T1 and T2 was complete and the absorption at 214, 239 and 254 nm suggests that the purity of the substances approaches 100%. The R_F values of the toxin standard (Cyanoginosin-

TABLE II

INFLUENCE OF GRADIENT SLOPE AND FLOW-RATE ON THE RESOLUTION AND RETENTION TIME

Conditions: temperature, 20°C, 0.3% PFPA to solvent A and B.

Gradient slope (% min)	Flow-rate (ml/min)					
	8		4		2	
	R_s	Time (min)	R_s	Time (min)	R_s	Time (min)
0.33	0.88	18.60	1.10	28.00	0.83	40.91
0.17	0.93	23.53	1.15	37.06	1.20	55.79
0.08	0.96	28.04	1.14	47.03	1.26	74.19

LR, molecular weight 994, Medor) was 0.48, whereas the components T1 and T2 had R_F values of 0.44 and 0.48, respectively.

A further indication of the structural relationship of the isolates to cyanoginosin-LR was obtained by the UV absorption spectra from 200 to 280 nm (Fig. 3). Apart from slight differences, the QMA eluate, T1 and T2 showed similar UV absorption behaviour to the cyanoginosin-LR standard with an absorption maximum at 240 nm.

Amino acid analysis by RP-FPLC

Standards were chosen according to known amino acid composition of cyanoginosin-LR. The sequence of the standards eluted from the HR 5/5 PepRpc column could be determined reproducibly. Comparing the standard chromatograms from different series ($n \leqslant 5$), each performed with fresh eluent preparations, the maximum standard deviation of the retention times was about 0.6 min. As the cyanoginosins contain D-Glu and D-Ala, the corresponding D- and L-stereoisomers were tested. Significant differences in retention times were not detectable (Table III).

Almost idential chromatograms were recorded for the PITC-derivatized T1 and T2 hydrolysates. The only difference was the exchange of one amino acid (Fig. 4A, peak 1; Fig. 4B, peak 2). Asp, Glu, Ala, Arg and Leu could be identified directly by the standards (Tables III and IV; for discrepancies of the retention times, see Discussion). The cyanoginosin component N-methyldehydroalanine (N-Medha) and the atypical amino acid Adda are destroyed by the hydrolysis procedure [6,15,16], but N-Medha is known to liberate methylamine (MeAmine) during hydrolysis. The presence of MeAmine was proved by a PTC derivative of a MeAmine standard (Fig. 4C, Tables III and IV). The commercially available standard for β-MeAsp is a *threo*-diastereomer, which co-eluted with Glu. On the basis that the racemization of the β-MeAsp standard to an equimolar mixture of *threo* and *erythro* configurations can be effected by exposure to acid and heat (see Discussion) [15,17], this standard was also submitted to the hydrolysis procedure (4 *M* hydrochloric acid, 110°C, 18 h) and then derivatized with PITC (Fig. 4D). Only after this treatment the analysis of the β-MeAsp standard did reveal an additional peak at 6 min. A fraction of

TABLE III

ELUTION SEQUENCE OF SINGLE PTC STANDARD AMINO ACIDS ON THE HR 5/5 PepRPC COLUMN

See also Fig. 4C for Asp, Glu, *threo*-β-MeAsp, Ala, Arg, MeAmine and Leu.

PTC derivative[a]	Time (min)	Standard deviation (min)	*n*
L-Asp	3.05	0,.37	4
DL-*threo*-β-MeAsp	3.76	0.08	5
D-Glu	3.89	0.18	2
L-Glu	3.93	0.15	5
L-Ser	7.77	0.41	2
Gly	8.57	–	1
L-Thr	11.03	0.44	2
D-Ala	11.73	0.31	2
L-Ala	11.89	0.55	5
L-Arg	13.84	0.62	3
MeAmine	16.78	0.38	3
L-Tyr	20.59	–	1
L-Met	21.43	–	1
L-Leu	24.98	0.29	3

[a] Known components of cyanoginosin-LR are printed in bold.

15–20% of *threo*-β-MeAsp was converted to another substance, which is assumed to be *erythro*-β-MeAsp (quantification by peak-area distribution). This compound had exactly the same retention time as the second peak of the T2 hydrolysate (Fig. 4B, Table IV).

The elution pattern of T1 and T2 hydrolysates could be qualitatively reproduced by a mixture of amino acids which are assumed to be components of the cyanoginosin-LR (Fig. 4C). This standard mixture was also exposed to the acid hydrolysis conditions (for discrepancies of the retention times, see Discussion).

The characteristic elution behaviour of the T1- and T2-derived PTC amino acids could be confirmed by repetitive determinations of the retention times and amounts of amino acids. To test the reproducibility, toxin samples from different preparations were hydrolysed, derivatized separately and chromatographed in one series. Approximately equimolar amounts of the detected amino acid components were calculated for T1 and T2 (Table V).

Determining the range for the analysis of PITC-derivatized cyanoginosin hydrolysates by RP-FPLC, the quantification of amino acids is linear in

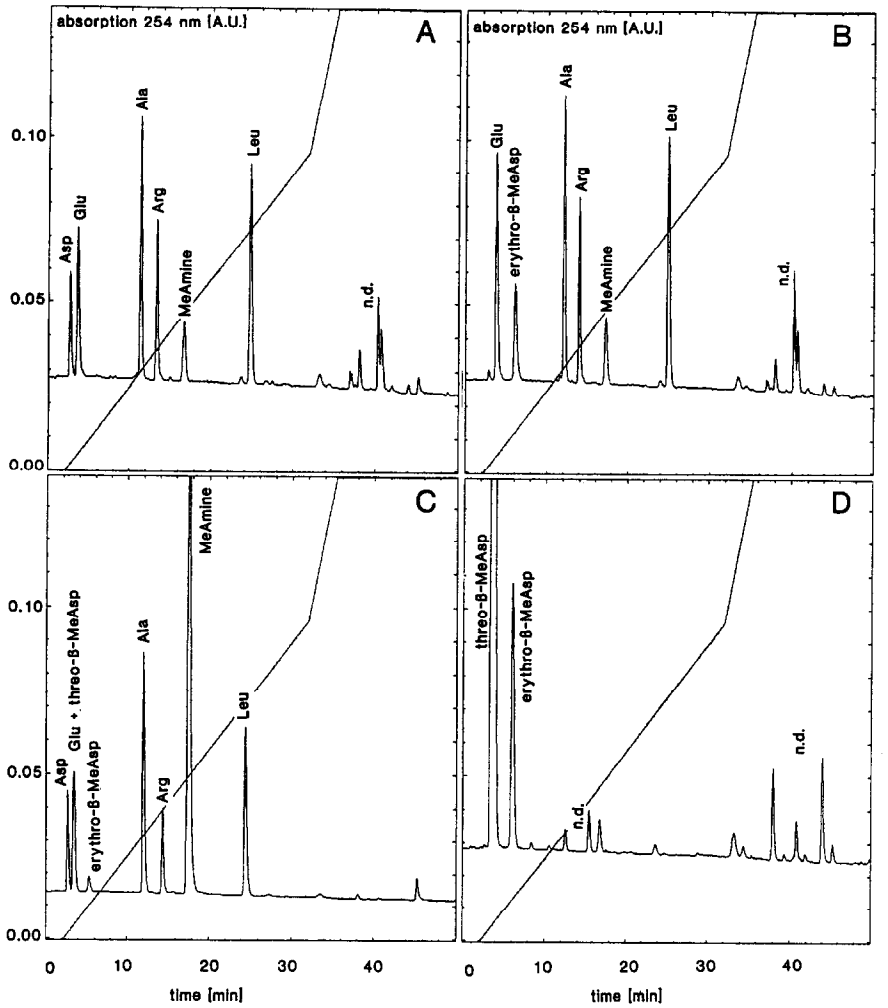

Fig. 4. (A) Amino acid analysis of 3 nmol PITC-derivatized T1 hyrolysate; (B) 3 nmol T2 hydrolysate; (C) Elution sequence of PTC standard amino acids exposed to acid hydrolysis prior to the derivatization (concentration range about 1–3 nmol of each amino acid and about 10 nmol MeAmine). Glu and *threo*-β-MeAsp are not resolved; (D) Chromatogram of *threo*-β-MeAsp standard (34 nmol) exposed to acid hydrolysis prior to the derivatization. About 4.8 nmol were converted to *erythro*-β-MeAsp (for discrepancies, comparing the retention times of C to A, B and D, see Table IV and the comments in the discussion). MeAmine = methylamine, a hydrolysis product of N-methyldehydroalanine; n.d. = unidentified PITC derivatives produced during sample derivatization.

the range 1–5 nmol. The detection limit was less than 1 nmol.

DISCUSSION

RP-HPLC is a powerful tool for separating different peptide toxins of *Microcystis aeruginosa* sp. at the analytical and semi-preparative scales [8–11]. The data presented here verify that RP-FPLC offers a practical alternative. The method allows the separation of 1.5 mg of a T1–T2 mixture, two very similar cyanoginosin-LR homologues produced by *Microcystis aeruginosa* PCC 7806, in a single run within 45 min. Although the similar UV absorption spectra and HPTLC analysis confirm purity of the two isolates, non-UV-absorbing impurities might still be present [18]. However, the amino acid analysis detected no significant contamination.

TABLE IV

ELUTION BEHAVIOUR OF THE TOXIN-DERIVED PTC-AMINO ACIDS COMPARED WITH THE PTC DERIVATIVES OF A STANDARD MIXTURE AND *threo*-β-METHYL-ASPARATE ON THE HR 5/5 PepRPC COLUMN

All standards were submitted to the hydrolysis procedure prior to derivatization. The data correspond to the chromatograms in Fig. 4. Glu and Ala in the mixture were of L configuration.

PTC derivative	Hydrolysate		Standard mixture	*Threo*-β-MeAsp
	T1	T2		
L-Asp	2.65	–	2.62	–
DL-β-MeAsp	–	–	3.41[a]	3.27[b]
D-Glu/L-Glu	3.58	3.71	3.41[a]	–
DL-*erythro*-β-MeAsp	–	6.03	5.32	6.03[b]
D-Ala/L-Ala	11.37	12.07	11.97	–
L-Arg	13.38	13.98	14.42	
MeAmine	16.69	17.27	17.56	–
L-Leu	24.70	24.91	24.42	–

[a] *Threo*-β-MeAsp and Glu are not resolved by the PepRPC column.
[b] *Threo*-β-MeAsp is chromatographed in two peaks only when exposed to acid hydrolysis; it is assumed that a conformational change from the *threo* to the *erythro* diastereomer occurs (see also Discussion).

The close relationship of the isolates to cyanoginosin-LR is illustrated by comparing the QMA eluate, T1 and T2 to the standard cyanoginosin-LR by UV absorption spectra and HPTLC. Almost equivalent UV spectra with absorption maxima at 240 nm, which are typical for the cyanoginosins [8], were recorded. By TLC the T2 compound had the same R_F value as the standard; T1 displayed only a slightly lower mobility. For further chemical characterization an RP-FPLC method for the analysis of amino acids in the isolates was developed.

Compared with the commonly used RP-HPLC methods for amino acid analysis, the analytical capacity of FPLC, which is predominantly used at a preparative scale, may have some technical limitations. Factors influencing the reproducibility and

TABLE V

COMPARISON OF THE RETENTION TIMES AND THE MOLAR RATIOS OF THE T1- AND T2-DERIVED PTC-AMINO ACIDS

Compound	T1 ($n=6$)			T2 ($n=6$)		
	Time (mean ± S.D.) (min)	Q[a] (nmol	M.R.[a]	Time (mean ± S.D.) (min)	Q (nmol)	M.R.
Asp	2.71 ± 0.10	3.82	1.12(1)	–	–	–
Glu	3.68 ± 0.12	3.45	1.01(1)	3.68 ± 0.15	3.20	1.18(1)
β-MeAsp[b]				6.06 ± 0.27	1.78	0.66(1)
Ala	11.47 ± 0.16	3.41	1.00(1)	11.71 ± 0.33	2.70	1.00(1)
Arg	13.36 ± 0.12	3.04	0.89(1)	13.55 ± 0.33	2.97	1.10(1)
MeAmine[c]	16.68 ± 0.11	1.91	0.56(1)	16.86 ± 0.30	1.93	0.71(1)
Leu	24.81 ± 0.37	2.54	0.74(1)	24.90 ± 0.17	2.46	0.91(1)

[a] Q = quantity and M.R. = molar ratio of the amino acids present in the peptides with respect to molarity of Ala. The value in brackets is the rounded M.R. value.
[b] The distinct lower molarity of *erythro*-β-MeAsp might be due to racemization to *threo*-β-MeAsp which co-elutes with Glu.
[c] It is unknown whether methyldehydroalanine liberates MeAmine quantitatively.

sensitivity are, for example, higher dead volumes, slight fluctuations in gradient steering, flow-rate and pressure, and column parameters. To account for the reported discrepancies in retention times, it should be noted that FPLC requires 1 h for the chromatographic separation of one sample. As an autosampler was not available and sampling had to be performed by two FPLC MV-8 valves, only fourteen samples could be analysed in one series. Therefore some deviations may be due to variations in preparing fresh eluents for new analytical series. For instance, the slightly different retention times of the *erythro*-β-MeAsp in the standard mixture (Fig. 4C, 5.32 min) to the hydrolysate (Fig. 4B, 6.03 min) and the single standard (Fig. 4D, 6.03 min) is most probably due to variations in the eluents, because chromatogram C was recorded within a different series to A, B and D. The maximum standard deviation of the retention times obtained from different series was 0.6 min. Despite this relatively high scattering, in most instances a definite identification of single peaks is still possible if the wide spread elution profile and the selectivity of the peaks are considered. However, signals of PITC-derived toxin components and standard amino acids could be measured with sufficient reproducibility to less than 1 nmol (Tables III and V). Thus the analytical capability of this method is sufficient for a realiable amino acid analysis.

The major problem occurred in identifying unusual amino acids (Adda, N-methyldehydroalanine, *erythro*-β-methylaspartate) in the toxin hydrolysates. It is known that not all amino acid components of the cyanoginosins can be detected by the commonly used derivatization procedures. A reference substance for *erythro*-β-MeAsp is not available. The atypical β-amino acids Adda and N-Medha are destroyed by the hydrolysis procedure [6,15,16]. However, N-Medha could be detected by its hydrolysis product, methylamine, which reacts with PITC [10,11] (peak 5 in Fig. 4A and B). Another difficulty was identifying peak 2 of the T2 hydrolysate by a standard substance. With respect to the basic structure of cyanoginosin-LR, the most likely amino acid responsible for this peak is the *erythro*-β-methylaspartate. Against this, the commercially available β-MeAsp standard has to be of almost 100% D,L-*threo* configuration (an *erythro* standard does not exist). The standard *threo*-β-MeAsp was co-eluted with glutamate (Tables III and IV; Fig. 4C). For this reason a definite assignment of this peak was not possible. Nevertheless, *erythro*-β-MeAsp is one of the five D-amino acids of cyanoginosin-LR (molecular weight 994) [5]. This has been confirmed electrophoretically and by stereospecific gas chromatography [15,19]. On HPTLC the T2 had the same R_F value as the standard cyanoginosin-LR (Fig. 2), for which a molecular weight of 994 is documented. This indicates chemical identity and consequently the presence of *erythro*-MeAsp in the T2 hydrolysate. The solution of this problem was racemization of the *threo*-β-MeAsp to the *erythro* diastereomer to produce an "*erythro*-β-MeAsp standard". A significant peak at 6 min appeared only if the standard β-MeAsp was exposed to acid hydrolysis (4 *M* hydrochloric-acid for 18 h). This behaviour is probably a result of a configuration change (Table IV, Fig. 4C and D). The molar ratio of the *threo* to *erythro* diastereomer after 18 h of acid hydrolysis was about 6:1. After 72 h of hydrolysis in 5.7 *M* hydrochloric acid, the racemization proceeds to equimolar amounts of the diastereomers [15,17]. These findings agree with other RP-HPLC studies in which the PTC-derivatives of *threo*- and *erythro*-β-MeAsp were chromatographed to give two separate peaks with a time difference of 4 min [10]. Additionally, the HPLC elution patterns of PTC derivatives of cyanoginosin-LR, originated by other cyanobacteria [11], are very similar to those obtained by FPLC; even the elution behaviour of *erythro*-β-MeAsp is comparable. In consideration of these findings, peak 2 of the T2 hydrolysate is assigned to *erythro*-β-MeAsp. Obviously, the configuration considerably infuences the polarity of the PTC-β-MeAsp derivatives.

Amino acid analysis in association with the UV absorption spectra and TLC relate the T1 and T2 isolates very closely to cyanoginosin-LR. According to the chromatographic separation of the PTC derivatives, the molar ratio of the T1 and T2 amino acids was equal to 1. As D- and L-configurations cannot be separated, no further specification of the compounds is possible. The atypical amino Adda cannot be detected by amino acid analysis. However, considering that the dien conformation in Adda is regarded to be responsible for the characteristic UV spectra with an absorption maximum at 240 nm [10,16], it should be a component of T1 and T2.

It is concluded that the presented data give some evidence that *Microcystis aeruginosa* PCC 7806 produces cyanoginosin-LR and a demethylated homologue. The two peptides were separated on a preparative scale and a partial amino acid analysis was performed by RP-FPLC. Of the seven amino acids known for cyanoginosin-LR, six could be reliably detected.

The possibility of separating both cyanoginosin homologues may have some relevance for further toxicological studies as the demethylation is accompanied by a distinct loss of toxicity [11].

RP-FPLC is a valuable supplement to the current chromatographic techniques for the isolation and analysis of small amphiphilic peptides. In particular, the possibility of connecting analytical and preparative applications is of advantage. Even complex problems such as amino acid analysis can be resolved in a reasonable time using automation.

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