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Short Communication

Separation of methylated and non-methylated cyanoginosin-LR homologues of the cyanobacterium *Microcystis aeruginosa* strain PCC 7806 by reversed-phase medium-pressure liquid chromatography

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ABSTRACT

Cyanoginosins produced by *Microcystis aeruginosa* sp. are toxic cyclic heptapeptides with molecular weights of about 1000 dalton. *Microcystis aeruginosa* strain PCC 7806 proved to synthesize a methylated and non-methylated cyanoginosin-LR. A reversed-phase medium-pressure liquid chromatographic method was developed to separate the two cyanoginosin homologues on a preparative scale. The influence of chromatographic conditions on the separation such as solvent system, gradient parameters, flow-rate and temperature was investigated systematically. After optimization, up to 1.5 mg of the two compounds could be separated completely within 40 min.

INTRODUCTION

The formation of toxic cyanobacterial water-blooms in freshwater ecosystems and in drinking-water supplies is a well documented problem [1–4]. The most important organism in this context is the species *Microcystis aeruginosa*, which can produce highly stable hepatotoxins, termed cyanoginosins. The cyanoginosins are cyclic peptides with molecular weights of about 1000 dalton. Regularly these peptides are composed of five constant amino acids (*i.e.*, D-Glu, β-methyl-Asp, D-Ala, N-methyldehydro-Ala and ADDA, an atypical β-amino acid which is a 3-amino-9-methoxy-10-phenyl-2,6,8-trimethyldeca-4,6-dienoic acid) and two strain-dependent variable L-amino acids [5]. Recently it became obvious that the hitherto constant part of the molecule can also exhibit some structural variations that influence toxicity [6,7].

The object of our investigations, *M. aeruginosa* strain PCC 7806, proved to synthesize cyanoginosin-LR (L = L-Leu, R = L-Arg) in two forms. Amino acid analysis confirmed that their difference consists only in methylation or non-methyla-

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tion of the D-Asp residue [8]. For toxicological and immunological studies we tried to separate these two homologues by preparative reversed-phase medium-pressure liquid chromatography (MPLC; maximum pressure = 40 bar). Results of an optimization procedure for the reversed-phase MPLC are presented, establishing that this method is a valuable supplement to separation techniques for small amphiphilic peptides.

EXPERIMENTAL

Cyanobacteria

M. Aeruginosa strain PCC 7806 was grown in continuous cultures supplied with BG-11 medium [9] and illuminated with 2000-lux fluorescent light.

Toxin isolation

Algal dry mass was obtained from the cultures by centrifugation (3000 *g*, 30 min) and lyophilization. After suspension in doubly distilled water, the cyanobacterial cells (5 g dry mass per 500 ml) were extracted by exposure to ultrasonics and centrifugation at 30 000 *g* for 30 min. For the solid-phase extraction, the supernatant was applied to a column (7.5 × 1.6 cm I.D.) containing 10 g of C_{18} silica gel (Baker Bond C_{18}; J. T. Baker, Gross-Gerau, Germany). Hydrophilic components were washed out with 100 ml of water. After elution with methanol, the toxic fraction was evaporated to dryness and the residue was dissolved in 200 ml of methanol–water (30:70, v/v). Precipitated lipids were removed by centrifugation (30 000 *g*, 30 min) and filtration (0.22 μm). At a flow-rate of 2 ml/min, the toxin solution was then pumped through a column (4.8 × 1.6 cm I.D.) containing 5 g of quaternary methylamine anion-exchange resin (QMA; Waters–Millipore, Eschborn, Germany) activated with 0.1 *M* ammonium hydrogencarbonate [in methanol–water (30:70, v/v)]. The loaded column was washed with 100 ml of methanol–water (30:70, v/v) and subsequently the cyanoginosin was eluted with 100 ml 0.02 *M* ammonium hydrogencarbonate [in methanol–water (30:70, v/v)] [10]. After drying, the toxin was dissolved in methanol–water (30:70, v/v) and photometrically adjusted to a concentration of 3 mg/ml with the aid of a standard calibration graph (cyanoginosin-LR; Medor, Herrsching, Germany).

Preparative reversed-phase MPLC

The separation studies of the two cyanoginosin homologues were performed on a fast protein liquid chromatography (FPLC) system (Pharmacia-LKB, Freiburg, Germany) of the following configuration: LCC-500 plus gradient controller connected with FPLC Manager software, two P-500 Model pumps, MV-7 motor injection valve, 500-μl sample loop, VWM 2141 variable-wavelength detector and FRAC-100 fraction collector. As stationary phase a HR 16/10 PepRPC column (10 × 1.6 cm I.D.), packed with 15-μm silica particles with covalently bonded C_2/C_{18}, average pore size 100 Å (Pharmacia–LKB), was used. Solvents for the gradient formation (solvents A and B) were different mixtures of acetonitrile (LiChrosolv; Merck, Darmstadt, Germany) and water purified with a Milli-Q system (Millipore, Eschborn, Germany). Depending on the experiment, trifluoroacetic acid (TFA) or pentafluoropropionic acid (PFPA) (Merck) were added to solvents A and B as ion-pairing agents.

TABLE I

INFLUENCE OF GRADIENT START, GRADIENT SLOPE AND FLOW-RATE ON THE MEAN RETENTION TIME (t_R) AND THE RESOLUTION (R_s)

Conditions	Gradient[a]		Flow-rate (ml/min)											
	Start (%)	Slope (% min)	8		4		2		5		3		1	
			R_s	t_R (min)	R_s	t_R (min)	R_s	t_R (min)	R_s	t_R (min)	R_s	t_R (min)	R_s	t_R (min)
(a)	32	0.32	0.88	19	1.10	28	0.83	41						
	32	0.16	0.93	24	1.15	37	1.20	56						
	32	0.08	0.96	28	1.14	47	1.26	74						
(b)	40	0.00							0.84	11	0.97	19	1.15	55
	36	0.00							1.07	21	1.14	35		
	32	0.00							1.05	60	1.99	99		
(c)	36	0.10							1.06	20	1.08	29	1.12	69
	35	0.10							1.00	22	1.08	34		
	34	0.10							1.13	26	1.20	39		

[a] The percentage values refer to the acetonitrile concentration.

TABLE II

INFLUENCE OF TEMPERATURE ON RETENTION TIME AND RESOLUTION

Conditions: gradient start, 34% acetonitrile; slope, 0.1% min; flow-rate, 3 ml/min.

Temperature (°C)	Retention time (min)		Peak width at half peak height (min)	R_s
	t_{R1}	t_{R2}		
24.8	36.2	39.4	1.42	1.18
13.3	35.6	39.4	1.40	1.42
9.3	35.0	39.2	1.42	1.51
4.3	34.2	38.6	1.42	1.57

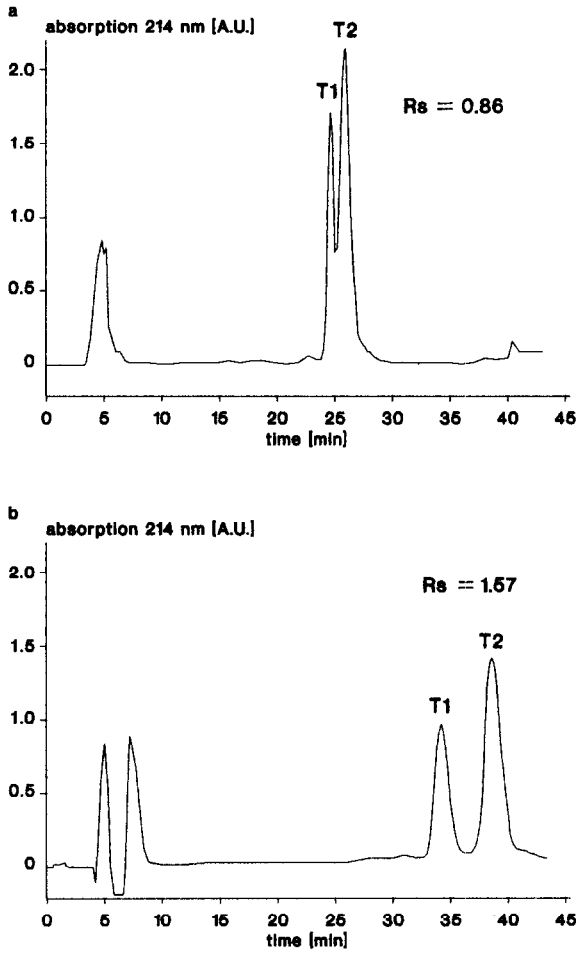

Fig. 1. Separation of the non-methylated (T1) and methylated (T2) cyanoginosin-LR homologues on an HR 16/10 PepRPC MPLC column. Sample, 1.5 mg of peptide mixture in 500 μl of methanol–water (30:70, v/v). (a) Starting conditions: gradient start, 30% acetonitrile; slope, 0.33%/min; flow-rate, 4 ml/min; temperature, 20°C; solvent A, Milli-Q-purified water containing 0.1% of TFA; solvent B, acetonitrile containing 0.1% of TFA. (b) Optimized method: gradient start, 34% acetonitrile; slope, 0.10%/min; flow-rate, 3 ml/min; temperature, 4.3°C, solvent A, acetonitrile–Milli-Q-purified water (24:76, v/v) containing 0.3% of PFPA; solvent B, acetonitrile–Milli-Q-purified water (50:50, v/v) containing 0.3% of PFPA.

RESULTS AND DISCUSSION

Apart from non-UV-absorbing contaminants, a *ca.* 90% pure cyanoginosin preparation was isolated by QMA-anion exchange chromatography. C_{18} thin-layer chromatography clearly demonstrated that the cyanoginosin fraction isolated from *M. aeruginosa* PCC 7806 is made up of two very similar compounds (data not shown). With the aim of isolating these two compounds by preparative reversed-

phase MPLC methods, the chromatographic conditions were investigated systematically and the success was verified by calculation of the resolution (R_s) [11].

In the first step, the concentration of the organic phase in which the two compounds were eluted from the column was determined in the range between 30 and 40% acetonitrile (Fig. 1a). Simply by changing the ion-pairing agent from 0.1% TFA to 0.3% PFPA, the resolution was increased successively from 0.86 to 1.11. This was mainly due to an extended retention time and increased selectivity (data not shown).

The influence of the gradient parameters and the flow-rates on the separation is presented in Table I. The resolution was increased to 1.26 by decreasing the flow-rate and running very shallow acetonitrile gradients [Table I (a)]. Isocratic runs in this range delivered the highest resolution (R_s = 1.99) but in a very long time [99 min, Table I (b)]. In consideration of these data, the influence of different gradient start points and flow-rates at a constant low gradient slope of 0.1%/min on the separation was investigated; as a result, a resolution of 1.20 could be achieved within 40 min [Table I (c)].

The decisive step for the optimization of the separation conditions was the variation of the temperature (Table II). By decreasing the temperature from 24.8 to 4.3°C, the R_s value increased from 1.18 to 1.57. This effect is caused by shortening the retention of the first peak without influencing the peak width, thus strongly increasing the selectivity. A typical chromatogram recorded under these optimized conditions is presented in Fig. 1b. The R_s value of 1.57 indicates that the two compounds are baseline separated according to the mathematical definition of resolution [11].

The results show that reversed-phase MPLC is a powerful method for the preparative separation of cyanoginosin homologues. The excellent performance of the preparative MPLC column, which exhibits a high loading capacity, a high selectivity but a low flow resistance, permits the complete separation of up to 1.5 mg of the peptides, which only differ in one methyl group, within 40 min. This may be an important result for further toxicological and immunological studies.

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