Journal of Chromatography, 509 (1990) 390–395
Elsevier Science Publishers B.V., Amsterdam

CHROM. 22 445

Note

Internal surface reversed-phase high-performance liquid chromatographic separation of the cyanobacterial peptide toxins microcystin-LA, -LR, -YR, -RR and nodularin

JUSSI A. O. MERILUOTO*
Department of Biochemistry and Pharmacy, Åbo Akademi University, Porthaninkatu 3, SF-20500 Turku (Finland)
JOHN E. ERIKSSON
Department of Biology, Åbo Akademi University, Porthaninkatu 3, SF-20500 Turku (Finland)
KEN-ICHI HARADA
Faculty of Pharmacy, Meijo University, Tempaku, Nagoya 468 (Japan)
ANDREW M. DAHLEM
Department of Drug Metabolism, Merrell Dow Research Institute, 2110 East Galbraith Road, Cincinnati, OH 45215-6300 (U.S.A.)
KAARINA SIVONEN
Department of Microbiology, University of Helsinki, SF-00710 Helsinki (Finland)
and
WAYNE W. CARMICHAEL
Department of Biological Sciences, Wright State University, Dayton, OH 45435 (U.S.A.)
(First received January 31st, 1990; revised manuscript received March 14th, 1990)

Several species of freshwater cyanobacteria (blue-green algae), *e.g.*, *Microcystis aeruginosa*, *Oscillatoria agardhii* and *Anabaena flos-aquae*, produce a family of low-molecular-weight peptide liver toxins known as microcystins (MCYST). The general structure of these heptapeptide toxins is cyclo(-D-Ala–L-X–D-*erythro*-β-methyl-Asp–L-Y–ADDA–D-Glu–N-methyldehydro-Ala), where X and Y denote the variable amino acids and ADDA represents β-amino acid 3-amino-9-methoxy-2,6,8-trimethyl-10-phenyldeca-4,6-dienoic acid[1–3]. Further, the residues D-*erythro*-β-methyl-Asp and N-methyldehydro-Ala can be desmethylated[4]. One brackish water cyanobacterial species, *Nodularia spumigena*, produces a pentapeptide toxin, nodularin (NODLN), with the structure cyclo(-D-*erythro*-β-methyl-Asp–L-Arg–ADDA–D-Glu–N-methyldehydroaminobutyric acid)[5]. Only one type of nodularin has been reported so far[5–8].

Cyanobacterial toxins have been detected and analysed by the use of mouse bioassay, high-performance liquid chromatography (HPLC) and immunoassay. These methods have their advantages and disadvantages. The mouse bioassay is rapid and does detect degree of toxicity, but it does not detect multiple hepatotoxins in a

0021-9673/90/$03.50

sample and it is difficult to detect hepatotoxins in a sample when the faster acting neurotoxins are also present. In addition, there is more variability in reporting toxicity between different laboratories than is desired for accurate toxicity level reporting. Finally, there is the need to minimize the use of laboratory animals in toxicity testing, if other equally accurate or more accurate clinical detection methods are available.

Immunoassay is a rapid, accurate method of detection for determining the presence and amount of the peptide toxins in water, algal cells and animal tissue[9]; however, only total toxin content is determined, not the amounts or types of the individual peptide toxins. It is important to be able to distinguish between the different microcystins because their toxicities vary.

HPLC has been the method of choice for the separation and analysis of the various cyanobacterial peptide toxins[10–12]. Although optimization of the stationary phase and the mobile phase composition has been used successfully to separate the various toxins, there is always a need for more rapid separations of multiple toxin samples from crude field extracts and laboratory-cultured strains. This paper reports the successful use of internal surface reversed-phase (ISRP) HPLC columns to overcome some of the problems with multiple toxins and crude cell extracts.

ISRP-HPLC was originally developed to simplify drug analysis in protein-rich serum samples[13–15]. It combines size-exclusion sample purification and reversed-phase separation. Although the stationary phase, Gly-L-Phe–L-Phe, is basically a reversed phase, the free carboxyl group in the terminal phenylalanine gives the phase additional functionality as a weak cation exchanger. The stationary phase is located on the inner walls of porous silica material. Only small analytes (molecular weight <5000) penetrate the pores and are retained and separated. Macromolecules are unretained and eluted rapidly.

In a previous study[16] we used ISRP-HPLC for the analysis of three (structurally uncharacterized) cyanobacterial peptide toxins. In this work we used the ISRP approach for the separation of four microcystin analogues and nodularin in an isocratic run.

EXPERIMENTAL

Purification of toxins

The peptide toxins were purified from the following sources as described previously: MCYST-LR (Leu and Arg as the variable residues) from *Microcystis aeruginosa* collected from a natural bloom in lake Akersvatn, Norway[17], MCYST-RR (Arg, Arg) from the *M. viridis* culture TAC44[11], MCYST-YR (Tyr, Arg) from the *M. aeruginosa* culture M-228[18], MCYST-LA (Leu, Ala) from a toxic water bloom in Eau Claire, WI, U.S.A.[9] and NODLN from the *Nodularia spumigena* culture L575[6].

Preparation of a field sample

Lyophilized *M. aeruginosa* material from Akersvatn was used to test the extraction efficiency. A 10-mg amount of cyanobacteria in a 1.5-ml polypropylene Eppendorf tube (Treff, Degersheim, Switzerland) was extracted for 5 min in a bath sonicator using 100 μl/mg of acetonitrile–0.1 *M* potassium dihydrogenphosphate (15:85; pH 6.8, adjusted with 10 *M* potassium hydroxide after addition of acetonitrile; chemicals from Merck, Darmstadt, F.R.G.). The sample was centrifuged at about

10 000 *g* for 10 min, after which the supernatant was collected and the pellet was re-extracted once or twice (four samples per treatment). The combined supernatants were spun once more and the sample was ready to be injected in the HPLC system.

Extracts of *M. aeruginosa* material were also used to check how well the toxins were separated from macromolecules and pigments in a natural cyanobacterial sample. The material was extracted twice in the manner described above.

Internal surface reversed-phase HPLC

The HPLC system consisted of a Shimadzu (Kyoto, Japan) LC-7A pump coupled to an SPD-6A detector set at 238 nm and a CR-5A integrator. The 250 mm × 4.6 mm I.D. GFF-S5-80 ISRP column (serial number 21102) was manufactured by Regis Chemical (Morton Grove, IL, U.S.A.). The column was protected by a Rheodyne (Cotati, CA, U.S.A.) 0.45-μm on-line filter and ISRP precolumn. The mobile phase was acetonitrile–0.1 *M* potassium dihydrogenphosphate (pH 6.8, adjusted with 10 *M* potassium hydroxide after the addition of acetonitrile) (15:85) at a flow-rate of 1 ml/min. Samples of 20 μl of purified toxins or extract were injected with a Rheodyne 7125 injector.

The toxins were first run separately and then as a mixture at concentrations of MCYST-LA 8.5, NODLN 9.5, MCYST-LR 15.5, MCYST-YR 18.0 and MCYST-RR 22.0 μg/ml. The *M. aeruginosa* extracts were run either separately or spiked (1:1, v/v) with the toxin mixture (MCYST-LA, -LR, -YR, -RR and NODLN). The MCYST-LR concentration in the extracts was 16.8 μg/ml, corresponding to 3360 μg/g cyanobacteria.

RESULTS

The third extraction did not improve the toxin yield significantly. More than 97% of MCYST-LR in the *M. aeruginosa* material was extracted after two cycles and the extraction was thus considered to be complete after two cycles.

The four microcystins and nodularin were well separated and had good peak shapes. The retention times were MCYST-LA 3.45, NODLN 4.30, MCYST-LR 5.08, MCYST-YR 5.84 and MCYST-RR 7.45 min (Fig. 1, left panel). The retention times in spiked field sample were MCYST-LA 3.44, NODLN 4.29, MCYST-LR 5.06, MCYST-YR 5.81 and MCYST-RR 7.43 min. The fraction of macromolecules and polar molecules in the beginning of the chromatogram did not interfere with the identification of the toxins (Fig. 1, right panel).

DISCUSSION

Our results together with earlier work[19] show that ISRP-HPLC can be used in applications beyond its original scope, the analysis of drugs in serum samples. Separation of certain peptides seems to be a new area for the ISRP columns. Especially those applications requiring extensive sample pretreatment could benefit from the concept of ISRP to separate macromolecules from analytes.

Analyte selectivity of the ISRP column

A problem in the chemical analysis of cyanobacterial toxins is the number of

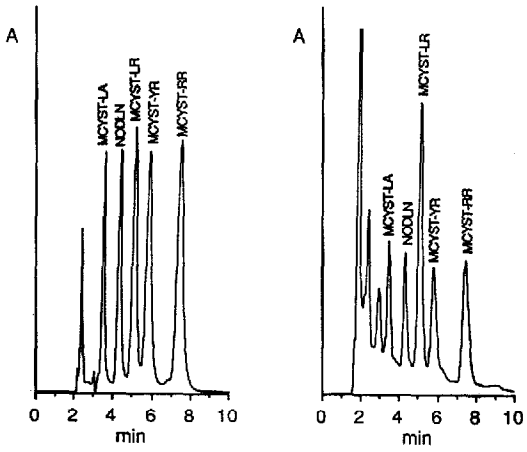

Fig. 1. ISRP-HPLC separation of cyanobacterial peptide toxins. Column, Regis GFF-S5-80 ISRP (250 mm × 4.6 mm I.D.) with an ISRP precolumn; mobile phase, acetonitrile–0.1 *M* potassium dihydrogenphosphate, pH 6.8 (15:85); flow-rate, 1 ml/min; sample size, 20 μl; detection at 238 nm. (Left panel) Separation of the purified toxins MCYST-LA (170 ng), NODLN (190 ng), MCYST-LR (310 ng), MCYST-YR (360 ng) and MCYST-RR (440 ng). The MCYST-LR signal was 41.8 milli-absorbance units. (Right panel), Separation of *M. aeruginosa* extracts spiked (1:1, v/v) with the purified toxins MCYST-LA, -LR, -YR, -RR and NODLN. The amounts of the toxins were MCYST-LA 85, NODLN 95, MCYST-LR 330, MCYST-YR 180 and MCYST-RR 220 ng. The MCYST-LR signal together with the underlying tailing part of the chromatogram was 45.6 milli-absorbance units.

analogues. The ISRP column resolves at least five toxins in a 10-min run. The resolving power of the ISRP column is not surprising as ISRP analyses combine reversed-phased chromatography with cation exchange and size exclusion.

The primary aim of the size-exclusion mode is on-line purification and separation of macromolecules from analytes. Ordinary reversed-phase columns are prone to protein denaturation and column clogging. This is a real problem in the cyanobacterial context because cyanobacteria can contain more than 15% protein (dry weight). Protein binding at neutral pH and small amounts of acetonitrile is not a problem with the ISRP column and the lifetime of the ISRP columns is longer was expected; we used a 15-cm ISRP column with more than 2000 extract and toxin samples without any significant loss of resolution[20].

Reversed-phase and cation-exchange mechanisms in the ISRP phase resolve analytes. Reversed-phase separation is thought to be the primary mode in ISRP-HPLC[13,15,21]. The ISRP material is selective for aromatic and other ring structures. The retention power of ISRP was found to resemble that of a phenyl phase when phenytoin (5,5-diphenyl-2,4-imidazolidinedione) was run on ISRP, phenyl, C_8 and C_{18} columns[13]. In a later study the capacity factors of 36 drugs and related compounds were measured using 0.1 M phosphate–isopropanol–tetrahydrofuran (84:10:6)[15]. In general, single-ring aromatic and aliphatic compounds yielded capacity factors (k') ranging from 0 to 2. Substances with two aromatic rings had $k' = 2$–12 and compounds with three fused rings or diaryl compounds with aliphatic side-chains gave $k' = 14$–26. The obvious selectivity for ring structures is due to the phenylalanine residues in the stationary phase.

However, the apolar residue ADDA, which has a lipophilic side-chain ending in a phenyl ring, seems to play a relatively minor role in the total retention under the conditions used. ADDA is present in all the toxins studied in this work. For example, for MCYST-LA $k' \approx 0.9$ (for bovine serum albumin $k' = 0$). This could indicate that the phenyl ring of ADDA is not available to the ISRP phase. This hypothesis is supported by conformational simulations with the molecular modelling program Chem-X (Chemical Design, Oxford, U.K.). The studies showed that the lipophilic side-chain of the ADDA residue is folded and turned inwards towards the centre of the toxin molecules[22].

Comparison of the separated toxins and work by Pinkerton and Koeplinger[19] showed that cation exchange plays a crucial role in analyte selectivity. The arginine residues are positively charged at pH 6.8 and MCYST-RR is the most cationic of the toxins separated here. It is also the most polar molecule (as indicated by a low capacity factor on a C_{18} phase and a low R_F value on silica gel thin-layer chromatographic plates[11]). However, it is the last to elute ($k' \approx 3.1$). Further evidence of the importance of the cation-exchange mechanism is given by the fact that the tyrosine-containing toxin MCYST-YR is eluted before MCYST-RR.

There are several more microcystins in addition to those studied here. Although speculation, the good separation results obtained in this work suggest that ISRP-HPLC can separate more microcystins. Changes in pH, organic modifier and buffer concentration should provide new separation possibilities. Preliminary studies on toxin separation at lower pH showed that, in general, the retention times become longer and the column has even more resolving power at lower pH. Low-pH separations were suggested by Pinkerton *et al.*[15].

Practical aspects

The retention times in the spiked field sample were slightly shorter than those in the sample with the purified toxins. This difference is probably caused by interfering compounds that block the stationary phase pores so that analytes cannot penetrate all the pores. In most instances the difference is negligible and should not jeopardize identification. However, in critical work the use of a diode-array detector and spectral analysis is justified for identification of peaks.

A diode-array detector can also be used to identify novel toxins and toxins not mentioned in this paper. All the microcystins and nodularin that we isolated had a local maximum at 238–240 nm in their UV spectrum (due to the conjugated double bonds in ADDA).

Although cyanobacterial material can be injected directly without off-line purification, there are samples that require concentration. The detection limit (signal-to-noise ratio = 3) for pure toxin samples is less than 1 ng of toxin per injection in the HPLC system used here. In field samples with interfering contaminants the limit of detection may be about 10 ng per injection. Samples with low toxin levels can be concentrated using C_{18} solid-phase extraction cartridges[11].

Further studies on the ISRP-HPLC of cyanobacterial toxins will be focused on the separation of more analogues and the simultaneous analysis of peptide and alkaloid toxins in cyanobacteria. We are also investigating the ISRP retention mechanisms in detail.

ACKNOWLEDGEMENTS

We thank the Maj and Tor Nessling Foundation, the Academy of Finland (Research Council for Environmental Sciences) and the Oskar Öflund Foundation for their valued financial support. Toxin purification was supported in part by USAMRDC contract DAMD-17-87-C-7019 to W.W.C. We also thank Olav Skulberg, Norwegian Institute for Water Research, for the Akersvatn bloom material.

REFERENCES

1 D. P. Botes, A. A. Tuinman, P. L. Wessels, C. C. Viljoen, H. Kruger, D. H. Williams, S. Santikarn, R. J. Smith and S. J. Hammond, *J. Chem. Soc., Perkin Trans. 1*, (1984) 2311.
2 D. P. Botes, P. L. Wessels, H. Kruger, M. T. C. Runnegar, S. Santikarn, R. J. Smith, J. C. J. Barna and D. H. Williams, *J. Chem. Soc., Perkin Trans. 1*, (1985) 2747.
3 W. W. Carmichael, V. Beasley, D. L. Bunner, J. N. Eloff, I. Falconer, P. Gorham, K. Harada, T. Krishnamurthy, Y. Min-Juan, R. E. Moore, K. Rinehart, M. Runnegar, O. M. Skulberg and M. Watanabe, *Toxicon*, 26 (1988) 971.
4 T. Krishnamurthy, L. Szafraniec, D. F. Hunt, J. Shabanowitz, J. R. Yates, III, C. R. Hauer, W. W. Carmichael, O. Skulberg, G. A. Codd and S. Missler, *Proc. Natl. Acad. Sci. U.S.A.*, 86 (1989) 770.
5 K. L. Rinehart, K. Harada, M. Namikoshi, C. Chen, C. A. Harvis, M. H. G. Munro, J. W. Blunt, P. E. Mulligan, V. R. Beasley, A. M. Dahlem and W. W. Carmichael, *J. Am. Chem. Soc.*, 110 (1988) 8557.
6 W. W. Carmichael, J. T. Eschedor, G. M. L. Patterson and R. E. Moore, *Appl. Environ. Microbiol.*, 54 (1988) 2257.
7 K. Sivonen, K. Kononen, W. W. Carmichael, A. M. Dahlem, K. L. Rinehart, J. Kiviranta and S. I. Niemelä, *Appl. Environ. Microbiol.*, 55 (1989) 1990.
8 A. Sandström, C. Glemarec, J. A. O. Meriluoto, J. E. Eriksson and J. Chattopadhyaya, *Toxicon*, 28 (1990) 535.
9 F. S. Chu, X. Huang, R. D. Wei and W. W. Carmichael, *Appl. Environ. Microbiol.*, 55 (1989) 1928.
10 H. W. Siegelman, W. H. Adams, R. D. Stoner and D. N. Slatkin, in E. P. Ragelis (Editor), *Seafood Toxins*, (*ACS Symposium Series*, No. 262), American Chemical Society, Washington, DC, 1984, p. 407.
11 K.-I. Harada, K. Matsuura, M. Suzuki, H. Oka, M. F. Watanabe, S. Oishi, A. M. Dahlem, V. R. Beasley and W. W. Carmichael, *J. Chromatogr.*, 448 (1988) 275.
12 P. S. Gathercole and P. G. Thiel, *J. Chromatogr.*, 408 (1987) 435.
13 I. H. Hagestam and T. C. Pinkerton, *Anal. Chem.*, 57 (1985) 1757.
14 S. E. Cook and T. C. Pinkerton, *J. Chromatogr.*, 368 (1986) 233.
15 T. C. Pinkerton, T. D. Miller, S. E. Cook, J. A. Perry, J. D. Rateike and T. J. Szczerba, *Biochromatography*, 1 (1986) 96.
16 J. A. O. Meriluoto and J. E. Eriksson, *J. Chromatogr.*, 438 (1988) 93.
17 J. A. O. Meriluoto, A. Sandström, J. E. Eriksson, G. Remaud, A. G. Craig and J. Chattopadhyaya, *Toxicon*, 27 (1989) 1021.
18 K.-I. Harada, M. Suzuki, A. M. Dahlem, V. R. Beasley, W. W. Carmichael and K. L. Rinehart Jr., *Toxicon*, 26 (1988) 433.
19 T. C. Pinkerton and K. A. Koeplinger, *J. Chromatogr.*, 458 (1988) 129.
20 J. Meriluoto and H. Björklund, *LC · GC*, 7 (1989) 738.
21 T. C. Pinkerton, J. A. Perry and J. D. Rateike, *J. Chromatogr.*, 367 (1986) 412.
22 T. Lanaras, Thessaloniki University, unpublished results.