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Short communication

Electrochemical detection of microcystins, cyanobacterial peptide hepatotoxins, following high-performance liquid chromatography

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Abstract

A novel amperometric HPLC detection method for the cyanobacterial (blue–green algal) peptide toxins microcystin-LR, -YR and -RR was developed. Purified microcystins and cyanobacterial extracts were chromatographed using an internal surface reversed-phase column with acetate- and phosphate-based mobile phase systems. Electrochemical oxidation reactions at 1.20 V vs. Ag/AgCl (glassy carbon working electrode) were shown to originate in arginine and tyrosine residues of microcystins.

Keywords: Cyanobacteria; Detection, LC; Microcystins; Hepatotoxins; Toxins

1. Introduction

Microcystins are cyclic peptide hepatotoxins and tumour promoters produced by freshwater cyanobacteria (blue–green algae). Their general structure is cyclo(-D-Ala-L-**X**-D-*erythro*-β-methylisoAsp-L-**Z**-Adda-D-isoGlu-*N*-methyldehydroAla) where Adda stands for the unusual C_{20} amino acid (2*S*,3*S*,8*S*,9*S*)-3-amino-9-methoxy-2,6,8-trimethyl-10-phenyldeca-4,6-dienoic acid and **X** and **Z** express two variable L-amino acids at positions 2 and 4 [1–4]. The variable L-amino acids are indicated by a two-letter suffix, e.g., the common microcystin-LR contains leucine (L) and arginine (R) at positions 2 and 4. There are more than fifty known analogues of microcystins including also desmethylated microcystins, microcystins with Adda variants and other minor variations [4,5].

The advantages and disadvantages of the different chemical, biochemical and biological analytical methods for microcystins as well as the overall analytical strategy have been discussed recently [6,7]. Chemical analyses of microcystins are usually conducted on reversed-phase high-performance liquid chromatography (HPLC) with UV detection at 238 nm [7]. The UV detection limit for microcystins is a few nanograms in most HPLC systems [8–11]. This paper introduces amperometric electrochemical oxidation as a detection method for microcystins LR, YR and RR. The method is likely to be applicable also to other arginine and tyrosine containing microcystins, as well as to microcystins with other oxidizable residues like tryptophan.

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2. Experimental

2.1. Reagents and toxins

Water was purified to 18.2 MΩ cm on a Milli-Q plus PF apparatus (Millipore, Molsheim, France). HPLC S grade acetonitrile was purchased from Rathburn (Walkerburn, UK). Analytical grade ammonium acetate, potassium dihydrogenphosphate and potassium hydroxide were from Baker (Deventer, Netherlands). L-Arginine (>98% purity) and glycine (>99%) were purchased from Sigma (St. Louis, MO, USA). Peptide toxins microcystin-LR and -RR and their 3-desmethylated analogues were purified from the cyanobacterium *Anabaena* sp. strain 90 grown in our laboratory [12]. The microcystins were identified by their HPLC retention behaviour, UV spectra and electrospray MS masses. Microcystin-YR was purchased from Calbiochem (La Jolla, CA, USA).

2.2. Cyclic voltammetry

A cyclic voltammetric investigation of the electrochemical behaviour of microcystin-LR on a glassy carbon electrode was made in a miniaturized electrochemical cell. The voltammograms were recorded using a laboratory-built potentiostat. Cyclic scans between −0.20 V and 1.50 V (vs. Ag/AgCl, 3 *M* Cl^-) were obtained on a ca. 30 μ*M* solution of microcystin-LR in acetonitrile–0.1 *M* ammonium acetate (15:85).

2.3. HPLC conditions

The HPLC system consisted of a Shimadzu (Kyoto, Japan) LC-7A pump, a Rheodyne (Cotati, CA, USA) 7125 injector equipped with a 20-μl sample loop, a Shimadzu SPD-6A UV detector set at 238 nm, a Bioanalytical Systems (West Lafayette, IN, USA) LC-4B amperometric detector and a Shimadzu CR-5A integrator. The electrochemical detection (ED) system was equipped with a thin-layer flow cell (gasket thickness 0.05 mm) and operated at a potential of 1.20 V. The working electrode was glassy carbon, counter electrode stainless steel and reference electrode Ag/AgCl (3 *M* Cl^-). The column for the analytical work was a 250 mm×4.6 mm I.D. GFF-S5-80 internal surface reversed-phase column (Regis Chemical, Morton Grove, IL, USA) protected by a Rheodyne 7335 (0.5 μm) filter and a GFF pre-column. Two mobile phases were tested. Mobile phase A consisted of acetonitrile–0.1 *M* ammonium acetate (15:85). Mobile phase P consisted of acetonitrile–0.1 *M* potassium dihydrogenphosphate adjusted to pH 6.5 with KOH (15:85). The flow-rate was 1 ml min^{-1}. All separations were carried out at room temperature (23–24°C). To test the electrochemical behaviour of free arginine and glycine in the ED system the free amino acids were dissolved in water in separate vials to give concentrations of 57 nmol per 20 μl (10 μg per 20 μl and 4.3 μg per 20 μl, respectively). The solutions were then injected into the ED system (1 ml min^{-1} of mobile phase A as a carrier, column replaced by capillary tubing) and the detector responses were recorded.

2.4. Preparation of cyanobacterial samples

Samples of the cyanobacterium *Oscillatoria agardhii* taken from 5 m depth in Lake Vargsundet on Åland Islands (SW Finland) were prepared for a comparison between UV and ED in natural samples. A 500-ml volume of lake water was filtered on a Whatman (Maidstone, UK) GF/C filter (diameter 47 mm) and freeze-dried. The freeze-dried filter was extracted twice in a polypropylene Eppendorf tube using 5% acetic acid [9], 1 ml at a time, and 5 min bath sonication in a Branson B2210 (Danbury, CT, USA) ultrasonic bath. The collected extracts were pooled and centrifuged at 10 000 rpm for 10 min in a Sorvall MC 12V centrifuge (DuPont, Newtown, CT, USA). The extracts were concentrated fivefold using a 500-mg Varian (Harbor City, CA, USA) Bond-Elut C_{18} solid-phase extraction cartridge and reconstituted in mobile phase A. The extracts were found to have trace amounts of microcystin-LR and 3-desmethyl-microcystin-RR. The extracts were then spiked (1 volume sample+1 volume spiking solution) with microcystin-LR and 3-desmethyl-microcystin-RR to give concentrations equivalent to 16.4 μg/l and 26.0 μg/l, respectively, in lake water. These toxin levels have been typical for toxic *O. agardhii* blooms [13]. The concentration of 3-desmethyl-microcystin-RR was calculated according to the calibration curve for

microcystin-RR corrected with the molar absorptivity difference reported for a corresponding pair of toxins (microcystin-LR and the 3-desmethyl analogue) 39 800 vs. 31 600 [14].

3. Results

3.1. Cyclic voltammetry

Cyclic scans between −0.20 V and 1.50 V (vs. Ag/AgCl, 3 *M* Cl^-) of microcystin-LR in mobile phase A produced rather featureless voltammograms. An increase in oxidation current could be seen at potentials above ca. 1.2 V. However, no clear oxidation wave or peak could be distinguished. This behaviour is indicative of the slow charge transfer rate of an irreversible oxidation reaction.

3.2. Chromatography of toxins

The phosphate-based mobile phase P gave somewhat better separation of microcystin-LR, -YR and -RR than the acetate-based mobile phase A. This was especially true for the toxin pair microcystin-LR and -YR. Examples of separations with mobile phase A are shown in Fig. 1 (microcystin separations with phosphate-based mobile phases can be found in Refs. [10,15,16]). However, the baseline in ED was more stable with mobile phase A which was therefore chosen for work with cyanobacterial extracts. Peaks on the UV chromatograms were quantified by peak area whereas noise and baseline disturbances necessitated the use of manual peak height measurement in ED. Both UV and ED calibration curves for microcystin-LR and -RR were linear in the test range 13–250 ng. The *r* values for the linear regression

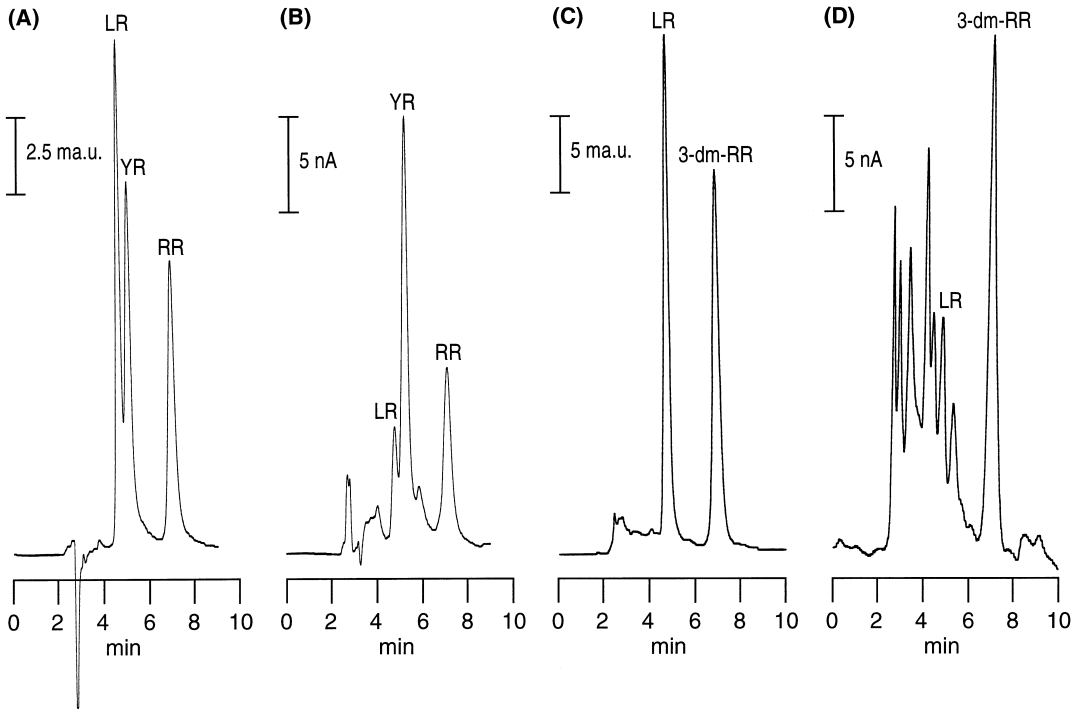

Fig. 1. Chromatograms of microcystin separations with UV detection and ED. Column: Regis ISRP GFF-S5-80 250 mm×4.6 mm I.D. Mobile phase: acetonitrile–0.1 *M* ammonium acetate (15:85) (mobile phase A); flow-rate: 1 ml min^{-1}. UV detection was performed at 238 nm (ma.u.=milliabsorbance units); ED at 1.2 V vs. Ag/AgCl, glassy carbon electrode. Panels A (UV) and B (ED): traces of purified microcystin-LR (93 ng), -YR (91 ng) and -RR (96 ng). Panels C (UV) and D (ED): traces of extracts of *Oscillatoria agardhii* spiked with microcystin-LR and 3-desmethyl-microcystin-RR to give concentrations equivalent to 16.4 μg l^{-1} and 26.0 μg l^{-1}, respectively, in the original water sample.

lines were >0.9994 for all other plots than the ED plot for microcystin-LR in mobile phase P (r= 0.998).

3.3. Electrochemically active residues in microcystins

The molar ED response of microcystin-RR was about 1.8 (height-based calculation, mobile phase A) and 1.5 (height-based calculation, mobile phase P) times stronger than that of microcystin-LR. This leads to the conclusion that the electrochemical reaction occurs in the arginine residue (one arginine in microcystin-LR, two in -RR). The microcystin-RR peaks under the used HPLC conditions are more than 30% broader at half height than those of -LR. If a correction for the resulting peak height difference is applied both ratios become higher than two. This finding indicates that the Arg residue at position 2 is more easily oxidized than the Arg residue at position 4. Further evidence for the participation of the arginine residue in the electrochemical reaction was given by the comparison of the ED signals of Gly and Arg. Equimolar injections of Gly and Arg (57 nmol per 20 μl) showed that the area of the arginine-derived signal was 43-times larger than that of glycine.

The tyrosine-containing microcystin-YR gave the highest ED signal in comparison with the UV signal (Fig. 1A,B). This is likely due to a strong oxidation signal of the tyrosine residue. The height-based ED signal ratio between equimolar amounts of microcystin-YR and -LR was about 3.8 in mobile phase A and 3.3 in mobile phase P.

3.4. Natural sample

The cyanobacterial sample from Lake Vargsundet contained several electrochemically active compounds (Fig. 1D) which eluted mainly before microcystin-LR and 3-desmethyl-microcystin-RR. These electrochemically active compounds are not microcystins as they do not absorb at the characteristic wavelength of 238 nm (Fig. 1C). The height-based ED quantification of microcystin-LR and 3-desmethyl-microcystin-RR was not affected by other electrochemically active substances.

4. Discussion

Electrochemical oxidation of the aromatic amino acids tyrosine, tryptophan and related bioactive compounds is well documented [17–23] but much less has been reported on the electrochemical reaction of arginine. Arginine and other guanidino compounds have been determined by anion-exchange chromatography and ED at 0.45 V vs. Ag/AgCl using a basic aqueous eluent and a nickel(III) oxide working electrode [24]. The authors speculated that other electrode types could improve the electrochemical selectivity for guanidines although this was not tested.

The present ED system can be used e.g. for verification of peak identities, especially in the context of a single-wavelength UV detector. Reliable identification of microcystins calls for a variety of techniques [25]. Thus far the only real alternative detection technique for microcystins has been mass spectrometry [26–28], a technique still too expensive for smaller laboratories. ED should also prove useful in the detection of other microcystins that contain arginine or tyrosine (e.g., microcystin-FR, -YM, -YA, -LY). Furthermore, the reported oxidation conditions for tryptophan (ca. 1.0 V vs. Ag/AgCl, carbon paste or glassy carbon electrode [18,20]) indicate that microcystins with a tryptophan residue could be detected electrochemically in our system. Due to lack of suitable microcystins this could not be tested. Arginine and tyrosine containing microcystins are the most common analogues as illustrated by microcystin lists in Refs. [4,5]. If the ED conditions detect all microcystins containing arginine, tyrosine and tryptophan as we believe, the only major, i.e., common, non-detectable microcystin is microcystin-LA. Nodularins, microcystin-related pentapeptides from brackish and marine sources, have the general structure cyclo(-D-*erythro*-β-methylisoAsp-L-**Z**-Adda-D-isoGlu-*N*-methyldehydrobutyrine) [3,4,29–31]. The majority of the known nodularins have L-Arg as the variable amino acid (**Z**) and should thus be detectable electrochemically.

We see the described HPLC detection system as the first generation in ED of microcystins. Due to the relatively high background activity the applicability of this method is limited when analyzing crude cyanobacterial extracts that have not been purified on

solid-phase extraction cartridges. We are currently investigating more effective ways of sample pretreatment to lower the amounts of impurities. A second objective is to derivatize microcystins with electrochemically active reagents that can be oxidized at a lower potential. At least three moieties will be subjected for derivatization trials: the α,β-unsaturated carbonyl group in the *N*-methyldehydroalanine residue, the carboxyl groups and the conjugated diene system in the Adda residue.

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References

[1] D.P. Botes, A.A. Tuinman, P.L. Wessels, C.C. Viljoen, H. Kruger, D.H. Williams, S. Santikarn, R.J. Smith, 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, D.H. Williams, J. Chem. Soc. Perkin Trans. 1, (1985) 2747.
[3] K.L. Rinehart, K.-i. Harada, M. Namikoshi, C. Chen, C.A. Harvis, M.H.G. Munro, J.W. Blunt, P.E. Mulligan, V.R. Beasley, A.M. Dahlem, W.W. Carmichael, J. Am. Chem. Soc. 110 (1988) 8557.
[4] K.L. Rinehart, M. Namikoshi, B.W. Choi, J. Appl. Phycol. 6 (1994) 159.
[5] K.-i. Harada, in: M.F. Watanabe, K.-i. Harada, W.W. Carmichael, H. Fujiki (Eds.), Toxic *Microcystis*, CRC Press, Boca Raton, FL, 1996, p. 103.
[6] J. Meriluoto, A.-S. Härmälä-Braskén, J. Eriksson, D. Toivola, T. Lindholm, Phycologia 35(Suppl. 6) (1996) 125.
[7] J. Meriluoto, Anal. Chim. Acta 352 (1997) 277.
[8] P.S. Gathercole, P.G. Thiel, J. Chromatogr. 408 (1987) 435.
[9] K.-i. Harada, K. Matsuura, M. Suzuki, H. Oka, M.F. Watanabe, S. Oishi, A.M. Dahlem, V.R. Beasley, W.W. Carmichael, J. Chromatogr. 448 (1988) 275.
[10] J.A.O. Meriluoto, J.E. Eriksson, K.-i. Harada, A.M. Dahlem, K. Sivonen, W.W. Carmichael, J. Chromatogr. 509 (1990) 390.
[11] L.A. Lawton, C. Edwards, G.A. Codd, Analyst (London) 119 (1994) 1525.
[12] J.A.O. Meriluoto, A. Sandström, J.E. Eriksson, G. Remaud, A.G. Craig, J. Chattopadhyaya, Toxicon 27 (1989) 1021.
[13] T. Lindholm, J.A.O. Meriluoto, Can. J. Fish. Aquat. Sci. 48 (1991) 1629.
[14] K.-i. Harada, K. Ogawa, K. Matsuura, H. Nagai, H. Murata, M. Suzuki, Y. Itezono, N. Nakayama, M. Shirai, M. Nakano, Toxicon 29 (1991) 479.
[15] J.A.O. Meriluoto, J.E. Eriksson, J. Chromatogr. 438 (1988) 93.
[16] J.A.O. Meriluoto, K. Isaksson, H. Soini, S.E. Nygård, J.E. Eriksson, Chromatographia 30 (1990) 301.
[17] M.D. Hawley, S.V. Tatawawadi, S. Piekarski, R.N. Adams, J. Am. Chem. Soc. 89 (1967) 447.
[18] D.D. Koch, P.T. Kissinger, J. Chromatogr. 164 (1979) 441.
[19] R. Dawson Jr., J.P. Steves, J.F. Lorden, S. Oparil, Peptides 6 (1985) 1173.
[20] Y. Sagara, Y. Okatani, S. Yamanaka, T. Kiriyama, J. Chromatogr. 431 (1988) 170.
[21] L. Dou, J. Mazzeo, I.S. Krull, BioChromatography 5 (1990) 74.
[22] L. Doorn, E.H.J.M. Jansen, F.X.R. van Leeuwen, J. Chromatogr. 553 (1991) 135.
[23] T. Nagatsu, J. Chromatogr. 566 (1991) 287.
[24] W. Ding, H. Veening, R.M. van Effen, J. Chromatogr. 526 (1990) 355.
[25] R.W. Moollan, B. Rae, A. Verbeek, Analyst (London) 121 (1996) 233.
[26] G.K. Poon, L.J. Griggs, C. Edwards, K.A. Beattie, G.A. Codd, J. Chromatogr. 628 (1993) 215.
[27] C. Edwards, L.A. Lawton, K.A. Beattie, G.A. Codd, S. Pleasance, G.J. Dear, Rapid Commun. Mass Spectrom. 7 (1993) 714.
[28] F. Kondo, Y. Ikai, H. Oka, H. Matsumoto, S. Yamada, N. Ishikawa, K. Tsuji, K.-i. Harada, T. Shimada, M. Oshikata, M. Suzuki, Nat. Toxins 3 (1995) 41.
[29] A. Sandström, C. Glemarec, J.A.O. Meriluoto, J.E. Eriksson, J. Chattopadhyaya, Toxicon 28 (1990) 535.
[30] E.D. de Silva, D.E. Williams, R.J. Andersen, H. Klix, C.F.B. Holmes, T.M. Allen, Tetrahedron Lett. 33 (1992) 1561.
[31] M. Namikoshi, B.W. Choi, R. Sakai, F. Sun, K.L. Rinehart, W.W. Carmichael, W.R. Evans, P. Cruz, M.H.G. Munro, J.W. Blunt, J. Org. Chem. 59 (1994) 2349.