Toxicology in Vitro 22 (2008) 1714–1718

Contents lists available at ScienceDirect

Toxicology in Vitro

journal homepage: www.elsevier.com/locate/toxinvit

Exploring the interaction of microcystin-LR with proteins and DNA

L. Vela [a,1], E. Sevilla [a,1], C. Gonzalez [b], M.T. Bes [a], M.F. Fillat [a], M.L. Peleato [a,*]

[a] Departamento de Bioquimica y Biologia Molecular y Celular, Facultad de Ciencias, and BIFI, Universidad de Zaragoza, Pedro Cerbuna 12, 50009-Zaragoza, Spain
[b] Instituto de Quimica-Fisica Rocasolano (CSIC), Madrid, Spain

ARTICLE INFO

Article history:
Received 1 April 2008
Accepted 7 July 2008
Available online 15 July 2008

Keywords:
Microcystin-LR
Cyanotoxin
Hepatotoxin

ABSTRACT

The physiological role of microcystin-LR is still under discussion, and since binding of microcystin-LR to proteins different from their main cellular targets was described, we have performed experiments in order to explore this interaction. A non-specific interaction of microcystin-LR with a variety of soluble proteins *in vitro* is disrupted when using organic solvents such as methanol. The isoelectric point of proteins is not affected by their interaction with microcystin-LR, even though the presence of microcystin-LR alters the pool of peptides obtained by tryptic digestions. Under the conditions tested, microcystin-LR does not exhibit affinity for DNA. Although it is unlikely that the non-specific binding of microcystin-LR to proteins has a physiological meaning, one must be aware of the fact that determinations of the toxin extracted from any biological sample may be affected by the presence of proteins in the extracts. Consequently, we strongly recommend use organic solvents and to lyophilise the tissue samples to guarantee the accessibility of these organic solvents to microcystin-LR when performing experiments with tissue or cell extracts.

1. Introduction

Microcystins are cyclic nonribosomal heptapeptides produced by some cyanobacterial strains that entail serious ecological problems and a health risk to humans and livestock for their toxicity. Microcystins are the most prevalent of cyanobacterial toxins and specifically inhibit eukaryotic protein phosphatases 1 and 2A (Runnegar et al., 1993). As consequence of phosphorylation/dephosphorylation disfunction, one of the predominant effects of microcystins on eukaryotic organisms is the alteration of the cytoskeleton, which is involved in key functions such as cell shape determination, cell movement, cell-to-cell recognition and cell division (Zurawell et al., 2005). Despite the large amount of published literature concerning such toxins, very little is known about the role of microcystins. These toxins are considered as intracellular secondary metabolites and must exhibit some sort of function for their structural complexity and the energetic cost involved in their biosynthesis. Microcystins reach high concentrations inside the cyanobacteria, up to mM (Wiedner et al., 2003), and Juttner and Luthi (2008) proposed that such high concentration of microcystin is only possible when bound to proteins.

Immunogold localization of microcystins in the producing cells showed that microcystin-LR was mostly associated with thylakoids, and to a lesser extend located in the nucleoplasmic region (Young et al., 2005). In contrast, other authors found that microcystin-LR was hardly detected within the thylakoid membranes of *Microcystis aeruginosa*, where the photosystems I and II are located and the photosynthetic activity takes place, while polyphosphate bodies and carboxysomes were found to be microcystin-rich regions (Young et al., 2005; Gerbersdorf, 2006). Since polyphosphate bodies have been reported to trap metals such as zinc (Andrade et al., 2004), Young et al. (2005) proposed that the presence of microcystin-LR may well be involved in a metal detoxification mechanism inside the cell. In fact, Utkilen and Gjolme (1995) suggested a putative role for microcystin-LR as an intracellular chelating molecule.

Also, microcystins have been shown to induce DNA damage *in vitro* and *in vivo*, and its genotoxicity seems to be mediated by reactive oxygen species (Zegura et al., 2004; Gaudin et al., 2008). Several host defense peptides, such as indolicidin, forms covalent links with DNA (Marchand et al., 2006), but no information is available concerning microcystins and a possible direct interaction with the nucleic acid.

Binding of microcystin-LR to proteins different from their main cellular target was described (Dittmann et al., 2006; Juttner and Luthi, 2008), opening a new perspective in the study of the physiological status of the toxin. In this paper, we present a set of experiments designed to clarify the nature and significance of the interaction of microcystin-LR with proteins.

* Corresponding author. Tel.: +34 976 762479; fax: +34 976 762123.
E-mail address: mpeleato@unizar.es (M.L. Peleato).
[1] These authors contributed equally to this work.

0887-2333/$ - see front matter © 2008 Elsevier Ltd. All rights reserved.
doi:10.1016/j.tiv.2008.07.008

2. Materials and methods

2.1. Growth conditions

The axenic strains *M. aeruginosa* PCC7806 and *Nostoc* sp PCC7120 were provided by the Pasteur Culture Collection (Paris, France) and grown in BG11 medium. In the case of *M. aeruginosa*, the medium contained 2 mM $NaNO_3$ and 10 mM $NaHCO_3$ (Rippka et al., 1979) as suggested by the Pasteur Institute. Cells were grown in batch conditions with continuous aeration at 25 °C.

2.2. Analytical methods

Crude extracts were prepared by sonication of *M. aeruginosa*. PCC7806 cells followed by centrifugation to remove cell debris. Total protein was quantified using the bicinchoninic acid assay (BCA™ Protein Assay Reagent Kit, Pierce, USA). Microcystin-LR in cells was determined by HPLC according to the procedure described by Lawton and Edwards (2001). Isoelectric focusing was carried out on Phast System from Pharmacia following the directions given by the manufacturer. SDS–PAGE was performed using 17% (w/w) polyacrylamide gels. Immunoblotting was carried out using monoclonal antibodies against microcystin-LR (Alexis Biochemical, Germany). Samples were electrophoresed and electroblotted to a Hybond™-C Extra Nitrocellulose membrane from Amersham Bioscience® (United Kingdom) at 20 V for 40 min. Immunodetection was performed according to Towbin et al. (1979). Ferredoxin-$NADP^+$ reductase (FNR) was purified from *Nostoc* PCC7120 as previously described (Razquin et al., 1996).

2.3. Tryptic digestion and peptide separation

Horse myoglobin (0.15 mg) was incubated with microcystin-LR (Alexis Biochemical, Germany) in the presence of 1% (w/w) DPCC-treated trypsin from bovine pancreas (Sigma–Aldrich, Germany). Incubation was performed in 50 mM Tris–HCl buffer (pH 8), with 20 mM $CaCl_2$ and 0.1% (w/v) SDS at 25 °C in a final sample volume of 90 μl. Trypsin addition to the samples was repeated after 1 h and 4 h of incubation. After 24 h, samples were frozen in order to stop the digestion. Peptides were separated by HPLC using a C18 reverse phase column (Symmetry 300™ C18 5 μm, from Waters, USA) running a linear gradient of water/acetonitrile containing 0.1% TFA at a flow rate of 1 ml/min (40 min). Absorbance of the separated fractions was recorded at 220 nm.

2.4. DNA assays

Electrophoretic mobility shift assays (EMSA) were performed as described previously (Martin-Luna et al., 2006).

NMR spectra were acquired on a Bruker Advance (Bruker, Spain) spectrometer operating at 600 MHz and equipped with a cryoprobe. Water suppression was achieved through a WATERGATE module (Piotto et al., 1992) in the pulse sequence prior to acquisition. ^1H NMR spectra of microcystin-LR (1 μg/ml) were acquired using increasing amounts of a saturated solution of calf thymus DNA. Spectra were recorded at 25 °C.

3. Results

3.1. Microcystin-LR binds to proteins in a non-specific manner

In order to study the possible association of microcystin-LR with proteins described previously by Dittmann et al. (2006) and Juttner and Luthi (2008), we analysed crude extracts of the toxic cyanobacterium *M. aeruginosa* PCC7806 by Western blotting

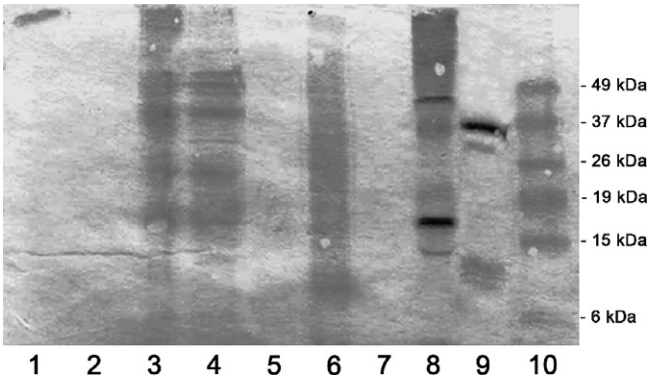

Fig. 1. Western blot of a 17% SDS–PAGE gel, immunodetected with anti-microcystin-LR antibodies. Lane 1: positive control: bovine IgG-microcystin-LR conjugate. Lane 2: negative control: bovine IgG. Lane 3: crude extract from *M. aeruginosa* PCC7808 (25 μg total protein). Lane 4: crude extract from *M. aeruginosa* PCC7808 (25 μg total protein), with 45 mM DTT. Lane 5: crude extract from *Nostoc* sp PCC7120 (50 μg total protein). Lane 6: crude extract from *Nostoc* sp PCC7120 (50 μg total protein), with 3 nmol of microcystin-LR. Lane 7: protein mixture: 0.11 nmol BSA, 0.7 nmol lysozyme, 0.45 nmol *Nostoc* flavodoxin, 0.3 nmol horse myoglobin. Lane 8: protein mixture with 3 nmol microcystin-LR. Lane 9: 0.10 nmol FNR with 3 nmol microcystin-LR. Lane 10: prestained molecular weight markers.

(Fig. 1). The blotted membrane showed that many proteins were immunodetected using anti-microcystin-LR antibodies (Fig. 1, lane 3), suggesting some kind of binding of microcystin-LR to proteins. These protein–microcystin-LR complexes remained even after boiling the samples in the presence of SDS. The binding was not affected by the addition of a reducing agent such as DTT (Fig. 1, lane 4), thus the attachment of microcystin-LR to the proteins was not affected by the redox status of the cysteine residues (Fig. 1, lanes 3 and 4), an interesting data since microcystin-LR binds covalently to a cysteine residue of its target, the phosphatase 1 (MacKintosh et al., 1995). Crude extracts of *Nostoc* PCC7120, a non-microcystin-producing strain, were also loaded on the gel, in the absence (Fig. 1, lane 5) and presence of microcystin-LR (Fig. 1, lane 6). As observed, the protein profile showed microcystin-LR bound to the *Nostoc* proteins. A mixture of several proteins, namely bovine seroalbumin, lysozyme, flavodoxin, myoglobin and ferredoxin-$NADP^+$ reductase, was also studied in the presence or absence of microcystin-LR. The results revealed that microcystin-LR binds to all of these proteins (Fig. 1, lanes 8 and 9). In order to clarify if the binding to these species was an artefact as a result of the SDS treatment, proteins were separated on native-PAGE gels. The results were identical to those obtained under denaturing con-

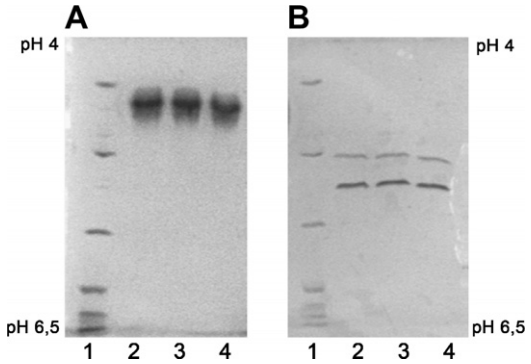

Fig. 2. Isoelectric focusing gels (pI range 4–6.5) of two proteins in presence of microcystin-LR. Gel A. Lane 1: pI protein standards. Lane 2: 3.7 μg BSA. Lane 3: 3.7 μg BSA with 0.8 μg microcystin-LR (1 mol protein: 3 mol toxin). Lane 4: 3 μg BSA with 1.3 μg microcystin-LR (1 mol protein: 6 mol toxin). Gel B: similar to A, but using 6 μg FNR as protein, and 0.6 and 1 μg of microcystin-LR.