Effects of Microcin SF608 and Microcystin-LR, Two Cyanotobacterial Compounds Produced by Microcystis sp., on Aquatic Organisms

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ABSTRACT: Effects of two cyanobacterial compounds, microcin SF608 and microcystin-LR, were investigated on different physiological parameters of two organisms, the water moss, Vesicularia dubyana, and the waterflea, Daphnia magna. Both compounds are produced by Microcystis species. Microcystin-LR is a potent inhibitor of protein phosphatases 1 and 2A, and microcin SF608 inhibits serine proteases. Other effects of microcystin-LR are well documented in the literature, but adverse effects of microcin SF608 have not been investigated as yet. This study compared the effects of both compounds on detoxication enzymes, microsomal and soluble glutathione-S-transferase (m-, sGST); oxygen stress enzymes, glutathione peroxidase (GP-X), and peroxidase (POD); photosynthetic oxygen production and chlorophyll a:chlorophyll b ratio. mGST was inhibited by both compounds in both organisms, significantly by microcin SF608, possibly indirectly by inhibition of that serine protease transforming the mGST to its active form. The sGST of D. magna was inhibited by microcin SF608, but elevated by microcystin-LR, and elevated by both compounds in V. dubyana. The GP-X in D. magna was not altered by microcin SF608, but elevated parallel to the sGST, whereas the POD in V. dubyana was decreased by both. Photosynthetic oxygen production as well as the chlorophyll a/b ratio showed typical stress reactions, a decrease of oxygen production, and an increase of chlorophyll b, caused both by microcin SF608 and by microcystin-LR. Microcin SF608 was not likely to be detoxified via conjugation to glutathione. The effects of microcin SF608 and microcystin-LR demonstrate that the impact of cyanobacteria on other organisms may not only be directly related to the presently known toxins.

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INTRODUCTION

The capability of cyanobacteria to produce a range of toxins led to the recognition that they are an increasing problem in
many freshwater ecosystems. The main group of toxins produced by cyanobacteria are the microcystins with more than 60 structure variants, of which the microcystin-LR congener is one of the most toxic (Carmichael, 1994; Watanabe et al., 1996; Codd et al., 1997). Microcystins are cyclic heptapeptides containing d-amino acids and the unusual amino acid Adda. Two of the amino acids are highly variable, and two are less altered, leading to this structural variety. The main biochemical mechanism of their toxicity is the binding and inhibiting of protein phosphatases 1 and 2A, a central control enzyme for metabolism, e.g., the cytoskeleton (Mackintosh et al., 1990).

Because of the high expression of a carrier mechanism in the vertebrate liver, the OATP (unspecific organic anion transporter, bile acid carrier; e.g., Runnegar et al., 1991), these toxins accumulate there, leading to changing liver enzymes, liver necrosis, and sometimes deadly intrahepatic bleeding (Falcoir et al., 1983; Erikson et al., 1990; Råberg et al., 1991). Intoxication has been described since the end of the 19th century, ranging from death of cattle after drinking from lakes contaminated with cyanobacteria (Francis, 1878) to a disastrous accident in a dialysis station, where more than 60 patients died (Pouira, 1998).

Up to a certain concentration, the microcystins are metabolizable by organisms via the glutathione S-transferases (Kondo, 1996; Pflugmacher et al., 1998, 2001; Takenaka, 2001). Glutathione S-transferase (GST) is member of the potential II detoxication enzymes, conjugating electrophile substances to glutathione (GSH), thereby increasing their water solubility to support their excretion. A broad substrate specificity is attained by several soluble GST isoenzymes and by a microsomal enzyme. Microcystins are conjugated to GSH through the terminal methylene of N-methyldehydroalanine (Mdha; Kondo, 1992), which is the moiety that binds to the Cys 273 in the active center of PPA1 (Mackintosh, 1995). In addition to the transformation of the microcystins to a more excretable form, the microcystin-SG conjugate is not able to bind covalently to the PPA. Despite this detoxication pathway the microcystins, particularly the LR congener, express their toxicity and cyanobacterial crude extracts are even more potent than the pure microcystins themselves (e.g., Oberemm et al., 1997; Pietsch et al., 2001). Either the detoxication capacities of the organisms are not sufficient, or other compounds that accompany the microcystins in the cyanobacteria are contributing to the toxicity by their own toxicity or by interference with the detoxication process.

During an ongoing project to characterize metabolites of bloom-forming cyanobacteria, three serine-protease inhibitors were isolated and their structure determined by NMR and MS techniques (Banker and Carmeli, 1999). One of the compounds, microcin SF608, was chosen for the study described below. Microcin SF608 is a linear peptide consisting of two amino acids (L-phenylalanine, 2-carboxy-6-hydroxyoctahydroindole); an α-hydroxy acid, p-hydroxyphenyl-lactic acid; and an agmatine (decarboxylated arginine). It inhibits trypsin and has an IC50 of 0.5 μg/mL (Banker and Carmeli, 1999). Via this mechanism, it could indirectly interfere with several other cell processes, for example, activation of proenzymes by the proteolytic processes of serine proteases. It also is not known if other, more general functions are affected by microcin SF608, especially, if it acts against plants. The aim of this study was to compare the effects of two compounds produced by Microcystis sp., the well-investigated hepatotoxin microcystin-LR and the recently characterized serine-protease inhibitor microcin SF608, on different physiological parameters of two organisms of the aquatic ecosystem. For the study Vesicularia dubyana, a water moss, and the invertebrate Daphnia magna were chosen as test organisms. The parameters microsomal and soluble glutathione S-transferases (m-, sGST) as indicators of the activity of the detoxication system were measured in both organisms. As enzymes dealing with oxidative stress, activity of glutathione-peroxidase (GP-X) was determined in D. magna and of peroxidase (POD) in V. dubyana. In the plant photosynthetic oxygen production as well as chlorophyll a and b content also were quantified as more general metabolic functions that could be altered by this toxic stress.

**MATERIALS AND METHODS**

**Cyanobacterial Compounds**

Microcin SF608 was isolated by Banker and Carmeli (1999) from a Microcystis sp. bloom by HPLC and characterized by FAB MS and 1-D and 2-D NMR. For the exposures Microcin SF608 was used in concentrations of 0.5, 5.0, 50.0, and 500.0 μg/L. Microcystin-LR was obtained from Professor G. A. Codd (University of Dundee, Scotland, UK). The toxin was produced by the nonaxenic culture of Microcystis aeruginosa (PCC 7813) and purified by HPLC according to the procedure explained in Lawton et al. (1995). To compare dose effects of both compounds, microcystin-LR was used in concentrations of 0.5, 5.0, and 50.0 μg/L.

**Rearing of the Test Organisms, Daphnia magna and Vesicularia Dubyana**

Both organisms were maintained in room conditions of a temperature of 20°C and a light/dark regime of 14 h light to 10 h dark. Tank water was reverse osmosis water containing NaHCO3 (0.103 g/L), CaCl2 (0.20 g/L), and sea salt (0.10 g/L). Daphnia magna were kept in culture for several weeks prior to exposure in 20-L tanks. They were fed with Microcell® algae powder, and 50% of the water was exchanged every 3–5 days. Vesicularia dubyana were cultivated for several months, with nutrients supplied by Povasoli’s medium (ESLI 15 mL/L) and an exchange of 50% of the water weekly.
Exposure of the Organisms

The organisms were exposed for 24 h without feeding or nutrient supply. Samples for the enzyme measurement were immediately frozen in liquid nitrogen, and stored at \(-80^\circ C\).

Enzyme Preparation and Measurement

Enzymes were extracted from the daphnids according to Wiegand et al. (2000) and from the water moss according to Pflugmacher et al. (1999a). Briefly, the animals were homogenized in sodium phosphate buffer (0.1 M, pH of 6.5, 20% glycerol, 1.4 mM of DTE, 1 mM of EDTA), and the plants ground in liquid nitrogen to a fine powder before suspending in the same buffer. Cell debris was removed by centrifugation. Microsomal and cytosolic enzymes were separated by centrifugation at 106 000 g followed by ammonium sulfate precipitation and desalting. Enzymes were stored in a sodium phosphate buffer (20 mM, pH of 7.0, 1.4 mM of DTE) at \(-80^\circ C\) until the measurement of activities.

Enzyme activities were measured colorimetrically: the mGST and sGST according to Habig et al. (1974), using CDNB (1-chloro-2,4-dinitrobenzene) as the photometer substrate; the GP-X according to Drotar et al. (1985), using glutathione reductase for the indirect measurement and NADPH and H\(_2\)O\(_2\) as substrate and cosubstrate; and the POD according to Bergmeyer (1983, 1986), using guajacol and H\(_2\)O\(_2\) as substrate and cosubstrate. Protein content of the samples was quantified according to Bradford (1974).

Measurement of Photosynthetic Oxygen Production

After exposure, plants were rinsed twice with water and from 5 replicates of 0.5 g of plant material from each toxin exposure, photosynthesis were measured according to Pflugmacher et al. (1999b), using a Phosy-Mess 400X (Innoconcept GmbH, Berlin, Germany). Oxygen content was determined by a Clark electrode (WTW EO 196-1.5) and calculated in micromoles of O\(_2\)/h/g FW. Measurement conditions were 2000 lx, with a dark/light/dark cycle of 10/12/10 min and a constant temperature of 20°C.

Analysis of Chlorophyll a and b

Chlorophyll a and chlorophyll b were extracted in DMSO for 48 h at 8°C according to Inskeep and Bloom (1985). Measurement of the pigments were done colorimetrically at 645 nm and 664.5 nm against DMSO.

RESULTS

Activities of Detoxication Enzymes—Glutathione S-Transferases

In D. magna, the sGST was not affected by a microcin SF608 concentration of 0.5 \(\mu g/L\), but every higher concentration caused a significant inhibition [Fig. 1(a)]. Contrastingly microcystin-LR caused an elevation of activities up to a concentration of 5.0 \(\mu g/L\); only the highest concentration, 50.0 \(\mu g/L\), gave no significant change. The mGST was inhibited by microcin SF608 proportional to increasing concentrations, whereas the reaction to microcystin-LR was equivocal [Fig. 2(a)]. The sGST of V. dubyana was increased by both cyanobacterial compounds in the concentrations used, significantly with microcin SF608 [Fig. 1(b)], whereas mGST was slightly, but not significantly inhibited [Fig. 2(b)].
Oxidative Stress Enzymes Peroxidase and Glutathione Peroxidase

The GP-X of *D. magna* showed no definite reaction toward microcin SF608, but exposure to microcystin-LR caused a clear dose-dependent elevation of activities up to 5 μg/L, and a decrease of activity at 50 μg/L [Fig. 3(a)]. The POD of *V. dubyana* was strongly inhibited by microcystin-LR at 50 μg/L, but not by microcin SF608 at the same concentration. The high concentration of microcin SF608 (500 μg/L) caused a nearly total inhibition [Fig. 3(b)].

Photosynthetic Oxygen Production and Pigment Pattern

Photosynthetic oxygen production was strongly affected by both cyanobacterial compounds with a decrease of more than 50% by both at 50 μg/L and a decrease of more than 75% by microcin SF608 at 500 μg/L [Fig. 4(a)].

The pigment pattern showed the typical stress reaction of plants: chlorophyll *b* values increased by almost a factor of 4 in both compounds in the concentrations used. In addition, the chlorophyll *a* content was nonsignificantly decreased by microcystin-LR and significantly decreased by microcin SF608 [Fig. 4(b)].

**DISCUSSION**

Both cyanobacterial compounds adversely affected the tested physiological parameters of the organisms. The soluble GSTs of both organisms were increased by microcystin-LR, whereas the microsomal GSTs did not respond so
clearly. This different reactions of the GSTs toward microcystins was also shown for other organisms, such as the zebrafish, *Danio rerio* (Wiegand, 1999, 2001), the coon tail, *Ceratophyllum demersum* (Pflugmacher et al., 1999c), and the zebra mussel, *Dreissena polymorpha* (Schwalbe and Pflugmacher, personal communication). It is possible that the different GST specificities led to the different reactions or that the microcystins in the cells were distributed more toward the cytosol than toward the membranes.

Microcin SF608 affected the mGST more potently than the sGST—it inhibited the mGST of both organisms but only the sGST of the daphnids. It is possible that this was a result of an indirect mechanism. One major in vivo mechanism for activating already synthesized mGST is via limited proteolysis (Morgenstern and Depierre, 1988). In studies by Banker and Carmeli (1999), microcin SF608 showed inhibitory capability toward serin-protease trypsin. An increase of mGST activity is impossible through this mechanism, even if an induction of the enzyme occurred, because the enzyme stays in the inactive form.

sGST consists of several isoenzymes. In *Daphnia magna* seven sGST isoenzymes were isolated (LeBlanc et al., 1988). At a microcin SF608 concentration of 5–500 µg/L, inhibition was detectable, but it was not concentration dependent. Presumably, only distinct isoenzymes were inhibited by this compound, whereas the others were still active. A small amount of microcin SF608 (0.5 µg/L) was not sufficient for inhibition. In the water moss, *Vesicularia dubyana*, activation of sGST was not inhibited by microcin SF608, as activity of sGST actually increased.

Prevention of the transformation of the GST into its active form limits the capacity of the detoxication pathway via the conjugation to glutathione. By this mechanism possible toxicity of microcin SF608 might be explained by the inhibition of the serin proteases, which then cause the inhibition of an important detoxication system in daphnids. From our results it seems very unlikely that microcin SF608 was detoxified via conjugation to glutathione.

The GP-X and POD belong to the group of enzymes dealing with oxidative stress caused by oxygen itself or by reactive oxygen species (ROS). They prevent oxidative damage to the cell, such as lipid peroxidation and DNA or enzyme oxidation. Microcin SF608 did not provoke reactions from GP-X in the daphnids. Contrasted to that, elevation of GP-X by microcystin-LR was concentration dependent, similar to sGST. A parallel elevation of GP-X showed zebra fish embryos after exposure to microcystin-LR (Wiegand et al., 1999). At a microcystin-LR concentration of 50 µg/L the capacity of the enzyme was depleted. There is no evidence about whether the microcystin-LR molecule itself represents or generates reactive oxygen species. The increase in enzyme activity may be caused by simultaneously induction of both GP-X and sGST. The sGST is induced via the DRE (dioxin responsible element) as well as via the ARE (antioxidance responsible element); the latter also induces GP-X (Fernandes et al., 1996). Both compounds caused a decrease in activity of POD in *V. dubyana* but only at high and rarely relevant doses: 500 µL microcin SF608 and 50 µg/L microcystin-LR. Further studies are needed to show whether direct binding of the molecules to the enzyme causes this inhibition.

Photosynthesis in *Vesicularia dubyana* was inhibited by both microcin SF608 and microcystin-LR. It has been shown that microcystins inhibit photosynthesis in various plants (Pflugmacher et al., 2001b). Other cyanobacterial compounds such as fisherellin also caused a decrease in the photosynthetic capacity (Srivatava et al., 1998).

\[ \text{Fig. 4. (a) Photosynthetic oxygen production and (b) chlorophyll a and b contents of Vesicularia dubyana after exposure to microcin SF608 and microcystin-LR. *Significant changes compared with control values by Newmanns–Keuls test at } p = 0.05. \]
gest inhibition was caused by a cyanobacterial crude extract (Pietsch et al., 2001). Increase of the chlorophyll b content with a concurrent decrease in chlorophyll a follows a disturbed photosynthetic apparatus. Decrease of the chlorophyll a to b ratio occurs when the collected light energy cannot be used adequately. This stress reaction of the plant can also be provoked by xenobiotics. Our results, using microcin SF608 and microcystin-LR separately, showed that both were affecting the central energy production mechanism of the plant. The question still remains open whether cyanobacterial secondary metabolites are produced to create an ecologically competitive benefit for the cyanobacteria themselves. That is, as allelochemicals.

It has been shown in several studies that cyanobacterial crude extracts cause different effects than purified toxins or even the equivalent toxin content of the particular crude extract (Fastner et al., 1995; Bury et al., 1996; Oberemm et al., 1997; Pietsch et al., 2001). These results for microcin SF608 and microcystin-LR demonstrate that the effects of cyanobacteria are not related only to the toxins known to date. In the ongoing process of purification and characterization of cyanobacterial compounds, it is likely that more biologically active substances will be revealed. Investigations from different starting points are needed in the ecotoxicological field as well as in the microbial arena to elucidate step by step the natural function of these substances.

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