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Aquatic Toxicology 78 (2006) 32–41

AQUATIC TOXICOLOGY

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The presence of microcystins and other cyanobacterial bioactive peptides in aquatic fauna collected from Greek freshwaters

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Received 12 October 2005; received in revised form 25 January 2006; accepted 4 February 2006

Abstract

Toxic bloom-forming cyanobacteria can cause animal death and adversely affect human health. Blooms may contain microcystins (MCs), cyanobacterial heptapeptide hepatotoxins and other peptides such as anabaenopeptins and anabaenopeptilides. MCs have been shown to occur in various aquatic organisms including mussels, water snails, crustaceans and fish. Muscle and viscera samples from eight species of fish (*Acipenser gueldenstaedtii*, *Carassius auratus*, *Carassius gibelio*, *Cyprinus carpio*, *Perca fluviatilis*, *Rutilus rubilio*, *Silurus aristotelis* and *Silurus glanis*), a frog (*Rana eperotica*), a mussel (*Anodonta* sp.) and a water snail (*Viviparus contectus*) were analyzed by high-performance liquid chromatography (HPLC), protein phosphatase 1 (PP1) inhibition assay (PP1IA) and ELISA. MC(s) was detected in all fish, frog, mussel and water snail samples tested by PP1IA and ELISA, including the frog *R. eperotica* and the freshwater snail *V. contectus*, in which the occurrence of MCs was not previously known. MC concentration ranged from 20 to 1500 ng g^{-1} dw and from 25 to 5400 ng g^{-1} dw in muscle and visceral tissue of fishes and frogs, respectively. In mussel and water snail tissue MC concentration ranged from 1650 to 3495 ng g^{-1} dw. HPLC analysis revealed peaks having the same UV spectrum as anabaenopeptin- or anabaenopeptilide-like compounds, not previously known to occur in aquatic fauna tissue. The concentrations of the compounds detected ranged from 1.5 to 230 μg g^{-1} dw. Comparison of the PP1IA and ELISA showed that values obtained with PP1IA where higher than those obtained with ELISA. Anabaenopeptins and/or anabaenopeptilides occurring in faunal tissue may account for the higher PP1IA values as we found that PP1 activity was inhibited by the purified anabaenopeptins A (45–60% inhibition) and B (5–75% inhibition). Purified anabaenopeptilides 90A and 90B exhibited weaker PP1 inhibition activity (5–35 and 5–23% inhibition, respectively). This is the first report of MC occurrence in aquatic animals collected from freshwaters of southern Europe.

Keywords: Microcystins; Anabaenopeptins; Anabaenopeptilides; Aquatic fauna; Protein phosphatase 1 inhibition

1. Introduction

Mass occurrences of cyanobacteria are found worldwide in eutrophic lakes and drinking-water reservoirs. Toxic bloom-forming cyanobacteria can cause animal death and adversely affect human health (Kuiper-Goodman et al., 1999; Carmichael et al., 2001; Codd et al., 2005). Blooms may contain microcystins (MCs), cyanobacterial heptapeptide hepatotoxins produced by cyanobacteria such as *Microcystis*, *Nostoc*, *Oscillatoria/Planktothrix*, *Anabaena* and *Anabaenopsis* (for a review see Sivonen and Jones, 1999). The structure of the more than 60 structural microcystin variants known (Sivonen and Jones, 1999) is *cyclo* (-D-Ala[1]-X[2]-D-MeAsp[3]-Z[4]-Adda[5]-D-Glu[6]-Mdha[7]-), where X and Z indicate variable L-amino acids, D-MeAsp is D-erythro-β-methylaspartic acid and Mdha is *N*-methyldehydroalanine (Carmichael et al., 1988). Adda, (2S, 3S, 8S, 9S)-3-amino-9-methoxy-2,6,8-trimethyl-10-phenyldeca-4,6-dienoic acid, is a unique structural feature of these toxins which confers toxicity (Namikoshi et al., 1989).

MCs have been shown to occur in various aquatic organisms including mussels (Eriksson et al., 1989; Vasconcelos, 1995; Prepas et al., 1997; Williams et al., 1997c; Amorim and Vasconcelos, 1999), water snails (Zurawell et al., 1999; Ozawa et al., 2003), crustaceans (Vasconcelos et al., 2001; Magalhães et al., 2003; Ibelings et al., 2005) and fish (Williams et al., 1997a,b; Malbrouck et al., 2003, 2004; Magalhães et al., 2003;

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0166-445X/$ – see front matter © 2006 Elsevier B.V. All rights reserved.
doi:10.1016/j.aquatox.2006.02.001

Li et al., 2004; Ibelings et al., 2005). Most MCs accumulate in the liver because of the first-pass effect; however, MCs can pass via the liver to other organs including muscle, kidney and brain in detectable amounts (Williams et al., 1997a; Fischer and Dietrich, 2000). MC intoxication kills fish by hepatic necrosis (Li et al., 2004), rather than by the massive hepatic haemorrhage that is frequently reported in mammals (Falconer et al., 1994). After oral uptake followed by intestinal absorption (Fischer and Dietrich, 2000), the toxin is taken up by hepatocytes via a carrier-mediated transport system (Dawson, 1998), and it then binds to the serine/threonine protein phosphatases 1 and 2A and inhibits their activity (MacKintosh et al., 1990, 1995; Goldberg et al., 1995). This inhibition disturbs the cellular phosphorylation balance and cause hyperphosphorylation of a variety of functional proteins, which leads to apoptosis and/or necrosis of hepatocytes (Dawson, 1998).

MCs have been shown to be metabolized in mussels (Karlsson et al., 2005), fish (Wiegand et al., 1999), rodents (Kondo et al., 1996) and plants (Pflugmacher et al., 1999, 2001) by detoxification enzymes, namely soluble glutathione *S*-transferases (sGSTs) (Pflugmacher et al., 1998). sGSTs catalyze the addition of MCs to the thiol group of the tripeptide glutathione (GSH). This can neutralize the electrophilic sites of MCs and increase water solubility and thus enhance excretion of degradation products, like GSH conjugates of MC-LR (Pflugmacher et al., 1998; Wiegand et al., 1999). Conjugation of GSH and cysteine to MC-LR has been observed (Kondo et al., 1992, 1996; Wiegand et al., 1999).

Cyanobacteria may produce other peptides in addition to the hepatotoxic MCs such as cyclic depsipeptides (e.g. anabaenopeptilides and cyanopeptolides), depsipeptides having a tricyclic ring system (e.g. microviridins), linear peptides (e.g. aeruginosins and microginins) and two groups of cyclic peptides possessing a ureido linkage (e.g. anabaenopeptins) or a β-amino acid (nostophycins) (Namikoshi and Rinehart, 1996; Fujii et al., 2000). Anabaenopeptins are unique cyclic peptides that have the common cyclic peptide moiety linked with Tyr, Arg, Lys and Phe, via a ureido bond (Fujii et al., 1996, 2002). Anabaenopeptilides are 19-membered cyclic depsipeptides containing a unique residue, 3-amino-6-hydroxy-2-piperidone (Ahp) (Fujii et al., 1996, 2002). These peptides exhibit diverse bioactivities such as serine protease inhibition (Namikoshi and Rinehart, 1996). Analyses of several toxic and non-toxic strains of cyanobacteria have shown that cyanobacteria may produce MCs and other peptides. Non-toxic cyanobacterial strains may contain bioactive peptides other than MCs (e.g. Fujii et al., 2000). Therefore, a comprehensive understanding of the possible functions of bioactive peptides and their ecological benefits requires studying bioactive peptides as a group rather than focusing on MCs.

Toxic cyanobacterial blooms are widespread phenomena in several freshwaters in Greece (Gkelis et al., 2005) indicating a significant threat to public health, livestock and aquatic fauna. The purpose of this study was to assess the occurrence of MCs in aquatic fauna of freshwaters in Greece and to investigate the possibility that other cyanobacterial peptides, apart from MCs, occur in tissues of aquatic fauna exposed to cyanobacterial blooms.

2. Materials and methods

2.1. Sample collection

Eight species of fish, *Acipenser gueldenstaedtii* (3 specimens), *Carassius auratus* (6 specimens), *Carassius gibelio* (9 specimens), *Cyprinus carpio* (18 specimens), *Perca fluviatilis* (3 specimens), *Rutilus rubilio* (3 specimens), *Silurus aristotelis* (6 specimens), *Silurus glanis* (3 specimens), one frog, *Rana eperotica* (6 specimens), one mussel, *Anodonta* sp. (1 specimen) and one water snail, *Viviparus contectus* (10 specimens) were collected from three freshwaters (Lake Kastoria, Lake Pamvotis and Kerkini Reservoir; see Table 1). One species of fish, *C. carpio* (three specimens) and one of frog, *Rana ridibunda* (three specimens) were collected from two freshwaters (Lake Yliki and the Gallikos River, respectively) where cyanobacterial blooms have not been reported, to be used as controls. Fishes from all freshwaters and the frogs from Lake Pamvotis (consumed by the local people) were bought from local fishermen on the day of fishing. The invertebrate species were collected in situ from each freshwater.

Species were identified by Prof. A. Sinis (fish) and Prof. M. Lazaridou (invertebrates) (Department of Zoology, Aristotle University of Thessaloniki).

2.2. Sample preparation and toxin extraction

Muscle tissues were dissected, separated and lyophilized separately from visceral tissues within 5 h of collection. The whole invertebrate tissue was lyophilized. One specimen of the mussel species, three specimens per fish or frog species and five specimens of the water snail species were combined to form one sample of mussel, fish or frog and water snail, respectively. A total of 34 different samples (Table 1) were prepared out of 74 specimens. Peptides (1 g lyophilized tissue per sample) were extracted by 20 mL of 75% (v/v) methanol in glass tubes, immersed in ice and sonicated for 15 min (Labsonic-U; Braun, Melsungen, Germany). After sonication, the mixture was stirred for 1 h at room temperature, centrifuged for 10 min at 13,000 × *g* and the supernatant was collected. The pellet was resuspended in 20 mL of 75% (v/v) methanol and re-extracted. The procedure was repeated twice. The three supernatants (methanol extracts; 60 mL) were pooled together in a separating funnel and an equal volume of hexane was added. Hexane layers were discarded and the procedure was repeated twice. After methanol evaporation the extract (15 mL) was concentrated using OASIS HLB extraction cartridges (Waters, Milford, MA, USA) and eluted in 4 mL 100% (v/v) methanol. The methanol fraction was dried in an air stream and the residue was redissolved in 1 mL Milli-Q water. 0.5 mL of the aqueous extract was added to 0.5 mL 100% (v/v) methanol and formed a 50% (v/v) methanol solution used for high-performance liquid chromatography (HPLC). The other 0.5 mL was used for ELISA and protein phosphatase 1 inhibition assay (PP1IA).