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Possible mechanism for the foodweb transfer of covalently bound microcystins

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ABSTRACT

Microcystins (MCs) are cyanobacterial toxins that inhibit protein phosphatases 1 and 2A (PP1, PP2A) within an animal through both reversible and covalent interactions. Only MCs that have accumulated in animal tissue in reversible interactions are currently considered when estimating risk to higher trophic levels and humans through food web exposure. However, the majority of MCs is likely covalently bound to target proteins in tissues and these MCs are not quantified or included in these assessments. These covalently bound MCs may be made bioavailable in the digestive system of a consumer through the digestion of their attached protein phosphatase. Three common digestive enzymes, pepsin, chymotrypsin, and trypsin, did not digest cyclic MC-LR and MC-LY, but were very active against a control peptide with typical linkages and standard amino acids in "L" conformation, supporting the possibility for MC-peptide formation during gut passage. To test if digestion products could be biologically active in the consumer, four predicted MC-peptides were synthesized and assayed for activity against PP1 by the protein phosphatase inhibition assay (PPIA). All four MC-peptides were active against PP1 and comparably half (58%) as inhibitory as the parent toxin. This *in vitro* study demonstrated that MCs covalently bound to proteins may represent a reservoir of potential toxicity for consumers.

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1. Introduction

Microcystins (MCs), a class of endotoxins produced by multiple genera of freshwater cyanobacteria, target the liver or hepatopancreas and exposure has led to the death of aquatic and terrestrial animals, including humans (Tencalla et al., 1994; Mez et al., 1997; Carmichael et al., 2001; Zimba et al., 2001). Once transported to the liver or hepatopancreas, MCs (that contain both the Adda (3-amino-9-methoxy-2,6,8-trimethyl-10-phenyldeca-4,6-dienoic acid) and Mdha (*N*-methyldehydroalanine) residues undergo a two-step reaction with protein phosphatases 1 and 2A (PP1, PP2A). First, PP1 or PP2A are reversibly inhibited by hydrophobic reactions between the Adda moiety of the toxin and the catalytic subunit of the protein phosphatase (e.g., PP1c) and hydrogen bonding using the toxin's carboxylic groups (Fig. 1). This step is followed by the formation of a covalent linkage between the toxin's Mdha residue and a cysteine residue within the protein phosphatase active site (MacKintosh et al., 1995; Craig et al., 1996), irreversibly attaching microcystin to form "bound-MC".

The World Health Organization set a maximum guideline for microcystin-LR (MC-LR) in drinking water at 1 μg/L and a tolerable daily intake (TDI) of MC-LR in the diet at 0.04 μg/kg of body weight/d (Falconer et al., 1999). With the exception of a few studies (Williams et al., 1997b, 1997c; Dionisio Pires et al., 2004; Ibelings et al., 2005; Ott and Carmichael, 2006), MCs are extracted from animal tissue using organic solvents (e.g., methanol). Extraction with organic solvents only releases free MCs (i.e., MCs that are dissolved or in reversible interactions with protein phosphatases within the organism); however, the covalently bound portion of MCs can be significant, representing up to 38–99% of the total MCs contained within in the organism's tissues (Williams et al., 1997a, 1997b, 1997c; Ibelings et al., 2005). Nonetheless, free MCs have been generally accepted to represent the total risk to organisms and humans due to the untested assumption that the covalently bound portion was not bioavailable to food web transfer.

Microcystins accumulate in multiple levels of the food web, from zooplankton and mollusks to herbivorous and carnivorous fish species (Smith et al., 2008, and references therein) in natural and aquaculture systems (Vasconcelos, 1999; Magalhães et al.,

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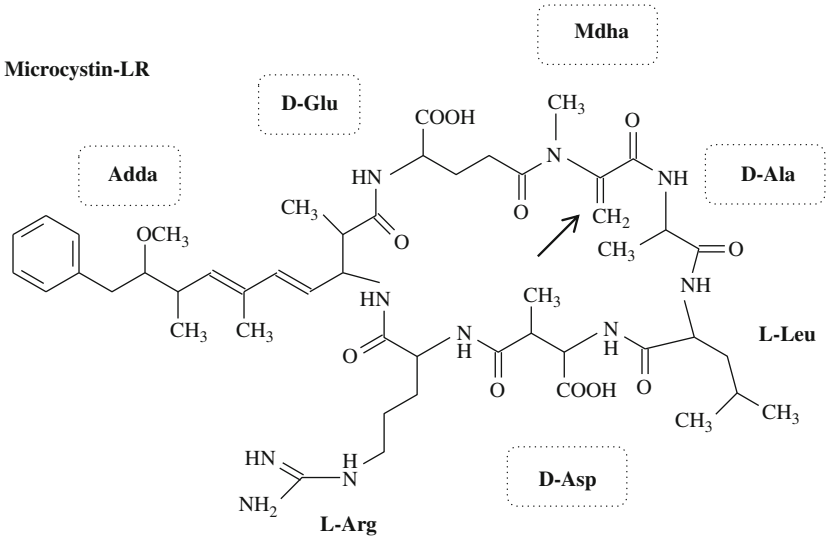

Fig. 1. Structure of microcystin-LR, including the Adda (3-amino-9-methoxy-2,6,8-trimethyl-10-phenyldeca-4,6-dienoic acid) and Mdha (N-methyldehydroalanine) moieties that are largely responsible for the toxin's interaction with protein phosphatases. Dashed boxes indicate residues with non-standard peptide linkages: the linkage is not centered on the chiral carbon, the amino acid is in non-traditional "D" conformation , or the amino acid itself is not standard. Arrow indicates the site for covalent linkage to protein phosphatases 1 and 2A.

2001; Zimba et al., 2001; Mohamed et al., 2003). MCs can be transferred to higher trophic levels, such as carnivorous fish, through the consumption of toxin-laden prey (Smith et al., 2006; Williams et al., 1997b); however, the bioavailability of the covalently bound portion is unknown.

If covalently bound MCs are released during gut passage in an active form, they may represent a significant risk to consumers and should be considered when evaluating the impact of microcystins on the food web. After the consumption of toxin-laden prey, proteolytic enzymes in the gut of a consumer may preferentially digest protein phosphatases covalently bound to the toxin, releasing the MC. MCs, on the other hand, may be resistant to proteolytic digestion as a result of the toxin's non-standard peptide structure: modified amino acids (e.g., Mdha and Adda), "D" amino acids, unique linkages, and cyclical structure (Fig. 1). Released toxin may be available for absorption, transport, and toxic activity in the consumer or toxicity in the environment following excretion.

It is important to point out that we do not expect the covalent linkage between the protein phosphatases and the Mdha residue of MCs to be broken during digestion, but instead, predict that cleavage at a nearby amino acid residue on the protein phosphatases will release MCs with a small residual peptide attached at Mdha. Additionally, we ask if the released MC with a small attached peptide could inhibit protein phosphatases in the consumer (assuming similar transport as MCs across membranes). Inhibition could possibly occur through non-covalent, hydrophobic interactions between the MC-peptide's Adda residue and protein phosphatases in the consumer.

Here we utilized *in vitro* techniques to show that MCs are resistant to digestion by typical digestive enzymes and that the predicted MC-peptides resulting from the digestion of a PP1-MC complex retain a significant fraction of the parent toxin's inhibitory activity.

2. Methods and materials

2.1. Overview

First, MCs and a control peptide were tested for their resistance to common proteolytic enzymes to determine if the protein phosphatase of a PP1-MC complex could be preferentially digested in the gut. If MCs were digested at a slower rate than the control peptide, we would expect the formation of MC-peptides from food in the consumer. Secondly, we wanted to test if MC-peptides retained any inhibitory properties that could be active against the consumer. To do this we purchased peptides that we predicted would result from the digestion of PP1 based on the known amino acid sequence and cleavage sites of common digestive enzymes. These peptides were conjugated to MC to form the predicted MC-peptides and then assayed for inhibitory activity.

2.2. Proteolytic digestion

To determine if microcystins were resistant to digestion, three proteolytic enzymes (pepsin, chymotrypsin, and trypsin) were incubated with MC-LR, MC-LY (Axxora, San Diego, CA) or the linear peptide angiotensin II (Sigma-Aldrich, St. Louis, MO). Angiotensin II (angII, amino acid sequence of DRVYVHPF) was digested by each protease as a positive control for enzyme activity as it contains both arginine (R) and tyrosine (Y) residues for cleavage by trypsin or chymotrypsin and pepsin, respectively. AngII represents a more typical protein or peptide due to its linear conformation, peptide linkages, and standard amino acids in "L" configuration. MC-LR was incubated with trypsin due to its R residue, and MC-LY was incubated with chymotrypsin or pepsin because it contained Y. Negative controls containing buffer and peptide, but no protease, were analyzed to determine any degradation due to the experimental conditions alone.

Chymotrypsin was hydrated in nanopure water for 1 h at 4 °C prior to incubation. Before use, all proteases were washed three times with either 20 mM sodium acetate, pH 2.5 (pepsin) or 5 mM imidazole, 10 mM $CaCl_2$, pH 8.0 (chymotrypsin, trypsin) to remove stabilizers as per manufacturers' instructions. MC-LR (1 μmol) or angII (1 μmol) was incubated with immobilized bovine pancreatic trypsin (10 μl, TPCK treated, >200 TAME units/ml immobilized gel, Pierce, Rockford, IL) and MC-LY (1 μmol) or angII (1 μmol) was incubated with porcine stomach pepsin (50 μl, >6000 units/ml immobilized gel, Pierce) or bovine pancreatic lyophilized chymotrypsin (0.63 mg, ≥2000 units ATEE/mg solid agarose, Sigma-Aldrich). Digestion mixtures containing the protease and peptide were taken to 1 ml with the appropriate buffer, as described above, and incubated for up to 6 h (300 rpm, 37 °C). Subsampling (0.3 ml) occurred at 0, 2, and 6 h; centrifugation (1 min at 7000 × *g*) and syringe filtration (0.45 μm, cellulose acetate, Nalgene) removed immobilized proteases (i.e., enzymes attached to aragose beads). Chymotrypsin and trypsin digests were acidified (to 0.3% formic acid final concentration) prior to HPLC analysis.

Each sample was analyzed using HPLC (Ace C_{18} column, gradient of 5–85% acetonitrile +0.02% TFA over 45 min, 0.5 ml/min, 50 μl injection) coupled with UV photodiode array detection (PDA, 239 nm) and mass spectrometry (ZQ4000, Waters) in positive mode using electrospray ionization (ESI) and a full scan between *m/z* 400 and 2500. Digestion was monitored through the loss of the original peptides, measured using their individual extracted ion currents (EICs): MC-LR [*m/z* 995.5], MC-LY [*m/z* 1002.5], and Angiotensin II [*m/z* 1032.5]. Probable digestion products were monitored to confirm proteolysis: hydrolyzed MC-LR [*m/z* 1013.5], hydrolyzed MC-LY [*m/z* 1020.5], and Angiotensin II fragments [*m/z* 662.1 by pepsin, *m/z* 761.0 by trypsin, *m/z* 552.1 by chymotrypsin].

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