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Inability to detect free cylindrospermopsin in spiked aquatic organism extracts plausibly suggests protein binding

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ABSTRACT

Even though the frequency and prevalence of cylindrospermopsin producing cyanobacteria are increasing, several publications have reported the absence of free cylindrospermopsin bioaccumulation in aquatic food chains. Cylindrospermopsin modification by protein binding has been suggested, however, only one publication has investigated this with eukaryotic reticulocyte lysate and concluded that cylindrospermopsin binds non-covalently to soluble proteins larger than 100 kDa associated with eukaryotic translation. With this as the extent of knowledge regarding cylindrospermopsin binding, the present study aimed to determine whether cylindrospermopsin binding also occurred with other proteins. In the present study, proteins from various organisms were extracted, incubated with cylindrospermopsin, and the amount of free cylindrospermopsin was determined by liquid chromatography tandem mass spectroscopy. Additionally, cylindrospermopsin binding to various ammonium sulfate precipitation fractions of *Egeria densa* protein, as well as with selected amino acids was investigated. We find that the percentage of free cylindrospermopsin varied with exposure to various animal and plant proteins as well as with various fractions of proteins but found no binding with single amino acids.

1. Introduction

Cylindrospermopsin (CYN), a zwitterionic and hydrophilic alkaloid toxin consisting of a tricyclic guanidine moiety bridged to a hydroxymethyluracil group, is produced by several genera of cyanobacteria (Chiswell et al., 1999; Harada et al., 1994; Ohtani et al., 1992; Falconer and Humpage, 2005; Preußel et al., 2006). The incidence of CYN detected in aquatic environments has increased in recent years due to an increase in the frequency of bloom events as a result of escalating eutrophication of surface waters and climate change (Kling, 2009). Due to its chemical stability, and therefore slow decomposition (Chiswell et al., 1999), CYN has been reported to be highly persistent in surface waters worldwide (Wörmer et al., 2008) with quantified concentrations as high as 18 μg L^{-1} in European freshwaters (Bogialli et al., 2006; Quesada et al., 2006; Rücker et al., 2007) and 120 μg L^{-1} in subtropical regions of Australia (Shaw et al., 1999; McGregor and Fabbro, 2000). Despite the growing concern of CYN in the environment, the ecotoxicological impact assessment thereof remains neglected.

CYN is one of the least detected cyanotoxin in foodstuffs and bioaccumulation has not been reported. Niedzwiadek et al. (2012) for example, could not detect free CYN in seafood containing several other cyanobacterial toxins. Similarly, other cyanobacteria based products tested negative for the presence of CYN (Liu and Scott, 2011).

Several cyanobacterial toxins have been reported to bind to or interact with substances. For example, microcystins rapidly bind to and inhibit the activity of Ser/Thr protein phosphatases PP1 and PP2A. The inhibition is irreversible and its consequences are well characterized (Ito et al., 2002). Several reports have also suggested that ß-*N*-methyl-amino-L-alanine (BMAA) may be associated with or incorporated into proteins in animals and cultured cells (Andersson et al., 2013; Banack et al., 2006; Dossaji and Bell, 1973; Dunlop et al., 2013; Murch et al., 2004; Xie et al., 2013). Therefore, as with other cyanobacterial toxins, CYN binding has been suggested (Runnegar et al., 1995; Shaw et al., 2000; Norris et al., 2001) and could explain the absence in aquatic food chains where the presence of CYN producing cyanobacteria are often reported. Kinnear (2010) also proposed the simplicity with which CYN may

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bind to tissues based on the structural features of the molecule as described by Duy et al. (2000).

Genotoxicity associated with CYN exposure has been attributed to the covalent binding thereof (or its metabolites) to liver DNA resulting in the formation of single DNA adducts (Shaw et al., 2000; Shen et al., 2002). Norris et al. (2001, 2002) found evidence of CYN/or metabolite(s) binding to proteins in the liver, and high performance liquid chromatography analysis indicated that this tightly bound CYN derivative was in part a hydrophilic metabolite with a different chromatographic elution time. Even though CYN bioaccumulation reports are scarce (Ferrão-Filho and Kozlowsky-Suzukim, 2011), free CYN has previously been detected in aquatic organism (Saker et al., 2004; Saker and Eaglesham, 1999; Nogueira et al., 2006). However, White et al. (2005) explained the lack of free CYN bioconcentration in exposed *Hydrilla verticillata,* conceivably as a result of becoming enzymatically bound and therefore not detectable by the employed analytical technique. They concluded that it is problematic as actual CYN concentrations may therefore be grossly underestimated. Froscio et al. (2008) found that CYN bound non-covalently and concentration dependently to soluble proteins of 100 kDa and larger in reticulocyte lysate associated with eukaryotic translation. However, the exact nature of the associations of CYN with proteins remains poorly tested and understood. With this as the extent of knowledge related to CYN protein binding, the present study aimed at determining whether the binding occurred with other proteins from various species.

2. Materials and methods

2.1. Chemical, reagents and organisms

All chemicals used in the investigation were analytical grade, unless specified otherwise and purchased from Sigma-Aldrich. CYN (purity >95%) was purchased from Enzo Life Sciences (ALX-350-149-C100) and stock solutions were prepared in 100% methanol (MS-grade).

The thirteen organisms used for the investigation were purchased and cultivated as specified below. *Taxiphyllum barbieri* (Java moss), *Cladophora glomerata*, *Ceratophyllum demersum* (Hornwort), *Lemna minor* (common duckweed), and *Egeria densa* (Brazilian waterweed) were purchased from ExtraPlant and cultivated in Provasoli medium (Esterhuizen et al., 2011) under constant illumination (30 mmol m^{-2} s^{-1}) for several weeks prior to the extractions. *Lactuca sativa* (Lettuce), and *Lepidium sativum* (Garden cress) were purchased from a local supermarket. *Daphnia pulex* (water flea), isolated from the Müggelsee (Berlin, Germany), and cultivated in the department for several years, was grown in daphnia medium (Esterhuizen-Londt et al., 2015a). *Lumbriculus variegatus* (blackworm), the department's own culture, was cultivated in Hoagland medium (Hoagland and Arnon, 1950). *Danio rerio* (zebrafish), *Caridina multidentata* (Amano shrimp), *Clea helena* (Bumble bee snail), and *Chironomus* sp. (Mosquito larvae) were purchased from Spree Aquarium (Berlin, Germany). Mouse (CD-1, male) S9 Fractions, and Pooled human liver S9 fractions were purchased from Thermo Fisher Scientific (Darmstadt, Germany).

2.2. Crude lysate preparations

Crude lysates were prepared in quadruplicate from *D. pulex*, *L. variegatus*, *C. demersum* and *E. densa* by collecting approximately 2 g wet weight plant material, 20 worms and 100 daphnids, respectively, snap-freezing the biomass in liquid nitrogen and grinding each replicate independently to a fine powder before suspension in a 20 mM sodium phosphate buffer (pH 7.0). The samples were then centrifuged at 10,000 × g for 10 min at 4 °C to remove the insoluble cell material. All samples were kept on ice for the duration of the extraction. The protein content was determined at 595 nm using Bradford reagent (Bradford, 1976) and all replicates were diluted to 50 μg ml^{-1} protein before exposure to CYN (Section 2.5). The concentration of 50 μg ml^{-1} protein was selected based on the lowest amount of protein extracted from the samples in order to keep the protein concentrations S9 human and mouse protein extracts were diluted to 100 μg ml^{-1} in 20 mM sodium phosphate buffer (pH 7.0) prior to incubation with CYN. Sodium phosphate buffer devoid of protein, was used as a control.

2.3. E. densa protein fractionation

E. densa strands of approximately 2 g wet weight per replicate (n = 4), were ground to a fine powder in liquid nitrogen. The powder was homogenized in 0.1 M sodium phosphate buffer (pH 6.5) containing 20% glycerol, 1 mM ethylenediaminetetraacetic acid (EDTA) and 1.4 mM dithioerythritol (DTE) and left to stir on ice for 20 min to allow all proteins to be released from the lysed cells. To remove the cell debris, the samples were centrifuged at 5000 × g for 10 min at 4 °C (Eppendorf Centrifuge 5417 R, Hamburg, Germany). Proteins in the supernatant were fractionated by ammonium sulfate (AS) precipitation at 10% intervals i.e. 10, 20, 30, 40, 50% etc. to 100% saturation. The salt, added to reach each desired salt saturation percentage (calculated from a standard AS precipitation nomogram), was added slowly over a period of 30 min each and left to stir for an additional 30 min (or for as long as the salt was still visible) before centrifugation at 30,000 × g for 30 min at 4 °C. After each centrifugation, the pellet containing the proteins precipitated at that salt concentration was collected, suspended in 20 mM sodium phosphate buffer (pH 7.0) and desalted by gel filtration using NAP-5 Sephadex columns (Amersham GE Healthcare, Uppsala Sweden). The supernatant was used in subsequent salting steps until 100% saturation was achieved. For all samples, the protein content was determined at 595 nm using Bradford reagent (Bradford, 1976) and exposed to CYN without dilution. Bovine serum albumin (Fraction V), prepared at a concentration of 50 μg ml^{-1} in 20 mM sodium phosphate buffer (pH 7.0) as well as sodium phosphate buffer devoid of protein, were used as controls.

2.4. Protein collection from various organisms

Proteins were extracted according Pflugmacher (2004) with minor modifications, from the following specimens in triplicate: *T. barbieri*, *C. glomerata*, *C. demersum*, *L. sativa*, *L. sativum*, *L. minor*, *E. densa*, *L. variegatus*, *D. pulex*, *D. rerio*, *C. multidentata*, *C. helena*, and *Chironomus* sp. First, the sample material was disrupted mechanically by grinding in liquid nitrogen. Thereafter, the tissue samples were homogenized in 0.1 M sodium phosphate buffer (pH 6.5) containing 20% glycerol, 1 mM EDTA and 1.4 mM DTE and stirred on ice for 20 min to allow all proteins to be released from the lysed cells. After centrifugation at 5000 × g to remove cell debris, the supernatants were subjected to an 80% ammonium sulfate precipitation. The salt was added over a period of 30 min and the samples left to stir for 60 min until all the salt was dissolved. Then, the samples were centrifuged at 30,000 × g for 30 min at 4 °C. The obtained pellets were suspended in 20 mM sodium phosphate buffer (pH 7.0) and desalted by gel filtration using NAP-5 Sephadex columns (Amersham GE Healthcare, Uppsala Sweden). For all samples, the protein content was determined at 595 nm using Bradford reagent (Bradford, 1976) and diluted were applicable to 50 μg ml^{-1} proteins with 20 mM sodium phosphate buffer (pH 7.0) before CYN exposure (based on the lowest amount of protein extracted). Sodium phosphate buffer was used to prepare positive controls conducted in parallel with the exposure.