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Characterization and quantification of karlotoxins by liquid chromatography–mass spectrometry

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Abstract

Karlodinium veneficum is a cosmopolitan dinoflagellate with a worldwide distribution in mesohaline temperate waters. The toxins from *K. veneficum*, or karlotoxins (KmTxs), which have been implicated in fish kill events, have been purified from monoalgal cultures, and shown to possess hemolytic, cytotoxic and ichthyotoxic activities. Three karlotoxins (KmTx 1–1, KmTx 1–3 and KmTx 2) have been isolated from two different North American strains of *K. veneficum* and characterized using liquid chromatography–mass spectrometry (LC–MS). KmTx 1 karlotoxins have a UV absorption maximum (λ_{max} 225 nm) at lower wavelengths than KmTx 2 karlotoxins (λ_{max} 235 nm). The exact masses and predicted empirical formulae for the karlotoxins (KmTx 1–1, 1308.8210, $C_{67}H_{120}O_{24}$; KmTx 1–3, 1322.8637, and $C_{69}H_{126}O_{23}$; KmTx 2, 1344.7938, $C_{67}H_{121}ClO_{24}$) were determined using high resolution mass spectrometry. Although the individual toxins produce a single peak in reverse phase high performance liquid chromatography (HPLC), MS revealed congeners co-eluting within each peak. These congeners could be separated under normal phase chromatography and revealed a single hydroxylation being responsible for the mass differences. Multistage MS (MS^n) showed that the three KmTxs and their congeners share a large portion of their structures including an identical 907 amu core fragment.

These data were used to develop a quantitative LC–MS assay for karlotoxins from cultures and environmental samples. The sensitivity afforded by MS detection compared to UV absorbance allowed toxin quantification at 0.2 ng when injected on column. Aqueous solutions of karlotoxins were found to quantitatively adsorb to PTFE and nylon membrane filters. Aliquots from whole cultures or environmental samples could be concentrated and desalted by adsorption to PTFE syringe filters and karlotoxins eluted with methanol for analysis by LC–MS. This simplified solid phase cleanup afforded new data indicating that each karlotoxin may also exist as sulfated derivatives and also provided a rapid detection method for karlotoxin from environmental samples and whole cultures.

Keywords: Dinoflagellate; Karlotoxins; Liquid chromatography; Mass spectrometry

1. Introduction

Karlodinium veneficum is a small (10–15 μm diameter), unarmored mixotrophic dinoflagellate implicated in fish kills around the world including Europe (Delgado, 1998), South Africa (Copenhagen, 1953; Braarud, 1957), the Eastern United States seaboard (Kempton et al., 2002; Deeds et al., 2002; Mooney et al., 2007) and Western Australia. While *K. veneficum* is easily identified live, poor fixation, its small size, a lack of distinguishing features and nomenclature confusion has made it difficult to link *K. veneficum* blooms with fish kill events in the past. For example a 1950 fish kill in Walvis Bay, South Africa was attributed to *Gyrodinium galatheanum* (Copenhagen, 1953; Braarud, 1957; Pieterse and Van Der Post, 1967). Laboratory experiments showed this organism causes mortality in juvenile cod and reduced growth in mussels (Nielsen and Strømgen, 1991; Nielsen, 1993). In Alfacs Bay, Spain, *Gyrodinium corsicum* (Paulmier et al., 1995) was implicated in killing of *Sparus aurata* Linnaeus (gilthead seabream) in aquaculture ponds (Delgado, 1998). Numerous fish kills in the Chesapeake Bay, USA, had been attributed to

Abbreviations: LC–MS, liquid chromatography–mass spectrometry; PTFE, polytetrafluoroethylene; SSU, small subunit rRNA; ITS, intervening transcribed sequence; PCR, polymerase chain reaction; DAD, diode array detector; ESI-TOFMS, electrospray-time of flight mass spectrometry; th, thomson a unit of *m/z*, where 1 Th = 1 u/e; SIM, single ion monitoring; TIC, total ion current.

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Gyrodinium estuariale (Goshorn et al., 2004), and *Gymnodinium veneficum* from Plymouth, England produces a toxin that kills mice in 2 min when injected (Abbott and Ballantine, 1957). Based on recent re-examinations of these incidents, all appear likely due to the same *Karlodinium* species (Daugbjerg et al., 2000; Bergholtz et al., 2005). A further complication in several of these cases is that non-toxic and toxic varieties were described in the literature as different species (e.g. Ballantine, 1956).

The toxins associated with *K. veneficum*, which we refer to as karlotoxins or KmTxs, can readily be isolated from natural water samples at fish kill sites as well as from cultures isolated from fish kills (Deeds et al., 2002; Kempton et al., 2002). The purified toxins reproducibly kill fish in a dose dependent manner in laboratory assays (Deeds et al., 2006). Purified toxins, sonicated cultures or culture filtrants from toxic cultures have hemolytic and ichthyotoxic activity. This activity is similar to prymnesin, isolated from *Prymnesium parvum* (Yariv and Hestrin, 1961; Martin and Padilla, 1971) and to amphidinols, a family of hemolytic toxins isolated from the dinoflagellate genus *Amphidnium* (Echigoya et al., 2004). The primary human impact of *K. veneficum* blooms is fish kill events, but it seems likely that KmTxs, like prymnesin may function as anti-grazing or allelopathic compounds (Tillmann, 2003; Adolf et al., 2006, 2007) as well as prey immobilization agents for mixotrophy.

From the United States Atlantic coast two different toxin types have been detected in *K. veneficum*. Toxins observed in Chesapeake Bay cultures and field samples differ from those found in strains south of the Chesapeake based on chromatographic profiles and UV absorption spectra (Deeds et al., 2004). All *K. veneficum* strains from Chesapeake Bay produce KmTx 1 (λ_{max} 225 nm) while all *K. veneficum* strains south of the Chesapeake Bay produce KmTx 2 (λ_{max} 235 nm). The strains reported in this paper have identical plastid SSU rRNA (Tengs et al., 2001) and nuclear ITS sequences (Bachvaroff et al., 2008). Although PCR-based detection assays to monitor *K. veneficum* distributions exist (Tengs et al., 2001), no such high-throughput assay has been developed for karlotoxins.

In this work three karlotoxins (KmTx 1–1, KmTx 1–3 and KmTx 2) were purified and characterized using several mass spectrometric approaches. The empirical formulae of KmTxs 1 and 2 were established using high resolution MS. Multistage MS (MS^n) fragmentation patterns highlighted both structural differences and similarities among the KmTxs and provided structural information on several novel toxin congeners found in both toxin types. This information was used to develop an LC–MS toxin detection method useful for the quantification of different KmTx types found in both environmental and cultured samples.

2. Methods

2.1. Materials

All organic solvents and water used were of HPLC grade or better and were purchased from Burdick & Jackson. Acrodisc syringe (13 mm diameter, 0.2 μm pore size) filters (GHP, PFTE, Nylon, PVDF, PES, HT Tuffryn or polysulfone) were obtained from Pall Life Sciences. Additional PFTE, Nylon (13 mm diameter, 0.2 μm pore size) and GF/F (13 mm diameter, 1 μm pore size) filters were obtained from Whatman.

2.2. Culturing

Karlodinium strains CCMP 1974 and CCMP 2064 were acquired from the Center for the Culture of Marine Phytoplankton (CCMP). The algae, all clonal but not axenic, were cultured autotrophically in 15 psu filtered (0.22 μm) natural seawater combined with f/2 nutrients and vitamins without Si (Andersen et al., 1997) at 100 μEinstein m^{-2} s^{-1} and 20 °C ambient temperature. Cell densities were measured using a Coulter Multisizer II (Beckman-Coulter) counter and cells mL^{-1} determined using Accucomp (Version 2.01) software.

2.3. Toxin isolation

To obtain adequate quantities of toxin from the two strains for structural characterization, two replicates of 1.0×10^8 cells (1–2 L of culture) were grown, filtered onto 125 mm GF/F filters (Whatman), and the toxin from the filtrate was concentrated with a 3 mL packed Sep-Pak tC-18 solid phase extraction cartridge (Waters). After the filtrate was loaded onto the cartridge it was washed with 12 mL of increasing concentrations of methanol/water from 0% methanol (v/v) to 100% methanol in 20% increments. Prior work had shown that greater than 90% of the toxic activity was found in the 80% methanol fraction (Deeds et al., 2002; Kempton et al., 2002). The 80% methanol fraction was collected, dried under a vacuum, resuspended in 1 mL of methanol, and filtered with a GF/B filter prior to HPLC analysis.

2.4. HPLC and LC–MS analysis – reverse phase LC–MS

LC–MS was performed using an Agilent 1100 Series LC-MSD system, comprising binary pump system, autosampler and diode array detector (DAD) with a micro high-pressure flow cell (6 mm pathlength, 1.7 μL volume), fraction collector and quadrupole mass spectrometer (G1956A SL) equipped with an electrospray ionization (ESI) interface. Toxin samples in 30% methanol/H_2O solutions were injected onto a C8 reverse phase (LiChrosphere 125 mm × 4 mm 5 μm bead size RP-8, Agilent) column and subjected to a 1 mL min^{-1} binary methanol/water gradient from 10 to 95% methanol over 25 min. Toxin peaks were detected over the wavelength range 190–950 nm. Based on the UV–vis spectra the absorption at 225 nm was used to detect KmTx 1 while absorption at 235 nm was used to detect KmTx 2. The entire UV–vis spectra were saved for each UV detectable peak. The eluate from the DAD was split (1/3 to 1/6) using a graduated micro-splitter valve (Upchurch Scientific). The major portion of the eluate was collected in multiple fractions while the remaining portion was subjected to MS analysis under the following spray chamber