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Isolation of activity and partial characterization of large non-proteinaceous lytic allelochemicals produced by the marine dinoflagellate *Alexandrium tamarense*

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ABSTRACT

Certain strains of the toxigenic dinoflagellate *Alexandrium tamarense* produce potent allelochemicals with lytic activity against a wide variety of marine microorganisms. Our efforts to characterize these allelochemicals from a lytic strain focused on the less polar components because of their higher lytic activity. Fractionation and partial purification after solid phase extraction (SPE) were achieved via alternative chromatographic methods, namely HPLC separation on C8 and HILIC phases. Through MALDI-TOF mass spectrometry we compared the mass differences in SPE, C8 HPLC, and HILIC fractions between a lytic and non-lytic strain of *A. tamarense*. Several large species with masses between 7 kDa and 15 kDa were found in the HILIC lytic fraction by MALDI-TOF MS. Tryptic digestion and tryptic digestion-coupled size-exclusion chromatography (SEC) suggested that the lytic compounds are large non-proteinaceous molecules (<23.3 kDa, trypsin). Although there is no direct proof that the large molecules found in the lytic HILIC fraction are responsible for the lytic activity of this fraction, the mass range deduced from SEC strongly supports this hypothesis. Total sugar content analysis showed that the lytic HILIC fraction contained two-fold more sugar than the non-lytic one. Nevertheless, the low percentage of saccharide per dry mass equivalent ($0.18 \pm 0.01\%$) indicates that sugar residues are likely not a major component of the lytic compounds. We concluded that at least one group of lytic allelochemicals produced by *A. tamarense* comprise a suite of large non-proteinaceous and probably non-polysaccharide compounds between 7 kDa and 15 kDa.

1. Introduction

Allelopathy is a biological phenomenon by which an organism produces one or more biochemicals that influence the growth, survival, and/or reproduction of other co-existing organisms (Rice, 1984). Generation and release of allelochemicals therefore constitutes an evolutionary strategy whereby producers overcome competitors for limited resources, such as space, nutrients, and light, or which can serve as a defence mechanism. In marine ecosystems, allelochemicals produced by microalgae may act to deter predators such as heterotrophic protists, tintinnid ciliates and copepods, or to inhibit the growth of co-occurring species (reviewed by Cembella, 2003; Legrand et al., 2003).

In spite of the paucity of knowledge on the structure and function of allelochemicals, allelopathy has long been believed to play a crucial role in phytoplankton bloom formation and succession in both freshwater and marine ecosystems (Pratt, 1966; Keating, 1977; Rice, 1984; Lewis, 1986; Wolfe, 2000; Rengefors and Legrand, 2001; Vardi et al., 2002; Tillmann and John, 2002; Legrand et al., 2003; Fistarol et al., 2004; Suikkanen et al., 2005). In recent years, special attention has been paid to allelopathic interactions involving species responsible for harmful algal blooms (HABs) (Granéli and Hansen, 2006; Tillmann et al., 2008a). This is partly a reflection of the notorious consequences of HAB events on human activities, such as public health, fisheries, aquaculture and tourism, as well as the devastating effects on aquatic ecosystems. Moreover, the potent toxicity of many HAB species, even at rather low cell concentrations, led to the hypothesis that the production of phycotoxins evidenced an allelochemical mechanism. Structurally well characterized phycotoxins with high potency in mammalian systems, e.g. as neurotoxins or protein phosphatase inhibitors, were suspected to also act as allelochemicals in aquatic ecosystems, thereby spawning much further research on these compounds. For example, diarrheic shellfish poisoning (DSP) toxins, okadaic acid

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(OA) and dinophysistoxin-1 (DTX-1), were reported to effectively inhibit the growth of several microalgae (Windust et al., 1996, 1997). However, a later study (Sugg and VanDolah, 1999) found that other compounds must be involved in the toxic effect of the filtrate from the dinoflagellate *Prorocentrum lima*, an OA producer. Brevetoxins produced by the fish-killing dinoflagellate *Karenia brevis* are not apparently responsible for the allelopathic effects against other phytoplankton species, although they slightly inhibit the growth of the diatom *Skeletonema costatum* (Kubanek et al., 2005). Recent research suggested that *K. brevis* produces multiple allelopathic compounds other than brevetoxins that are inhibitory towards the diatom *Asterionellopsis glacialis* (Prince et al., 2010).

Most allelochemicals from HAB species remain unknown, only a few have been characterized by structure and/or biological activity (Granéli et al., 2008). One allelopathic effect is the direct lysis of target species membranes, thus toxins with hemolytic and/or ichthyotoxic capacity were suspected to have an allelopathic effect. The polyoxy-polyene-polyether toxins prymnesin 1 and 2 produced by the prymnesiophyte *Prymnesium parvum* (Igarashi et al., 1998) are believed to perforate cell membranes of other cells, and can even cause fish kills. Similarly, glycosylglycerolipids with ichthyotoxic and hemolytic potential were found in the fish-killing prymnesiophyte *Chrysochromulina polylepis* and the dinoflagellate *Karenia mikimotoi* (Yasumoto et al., 1990). Karlotoxins (KmTxs) with hemolytic activity from the dinflagellate *Karlodinium veneficum* inhibit the growth of several phytoplankton species (Adolf et al., 2006), and the dinoflagellate grazer *Oxyrrhis marina* (Adolf et al., 2007). The allelochemicals of the raphidophyte *Heterosigma akashiwo* that inhibit its diatom competitors *S. costatum* and *Thalassiosira rotula* were recently identified as high-molecular weight polysaccharide–protein complexes (Yamasaki et al., 2009).

Allelopathy is widely found among *Alexandrium* spp. upon other microalgae (Blanco and Campos, 1988; Arzul et al., 1999; Fistarol et al., 2004; Tillmann et al., 2007, 2008b) and towards heterotrophic protists (Hansen, 1989; Hansen et al., 1992; Matsuoka et al., 2000; Tillmann and John, 2002). Details of the molecular structures and exact mode of action of allelochemicals from *Alexandrium* species remain scarce. Previous investigation of the major allelochemicals produced by *A. tamarense* (Ma et al., 2009) indicated that they are large amphipathic compounds with secondary structure, and are clearly unrelated to the known toxins produced by this genus, namely Paralytic Shellfish Poisoning (PSP) toxins (Tillmann and John, 2002) or spirolides (Tillmann et al., 2007). Here we further characterized these allelopathic compounds produced by *A. tamarense* by advanced mass spectrometric techniques. For the identification of candidate masses of compounds related to lytic activity, we compared purified fractions of a lytic and non-lytic strain of *A. tamarense* by alternative chromatographic and mass spectrometric techniques.

2. Materials and methods

2.1. Cell culture

One clonal isolate of the marine dinoflagellate *Alexandrium tamarense* (Alex2) was selected as the source of lytic compounds based on its high lytic activity as quantified comparatively in previous experiments (Alpermann et al., 2009; Tillmann et al., 2009). In some experiments, another isolate Alex5, which does not produce lytic compounds in measurable amounts (Tillmann et al., 2009) and in comparison to Alex2 did not show any allelopathic effects on other algae (Tillmann and Hansen, 2009), was used as a negative control. These clones were selected from a collection of >60 clones of the North American ribotype (Lilly et al., 2007) isolated simultaneously in May, 2004 from the Scottish east coast of the North Sea (56°05′47″N; 1°42′35″W). Dinoflagellate cultures were grown in K-medium (Keller et al., 1987), supplemented with selenite (Dahl et al., 1989) prepared from sterile-filtered (VacuCap 0.2 μm Pall Life Sciences) North Sea seawater (salinity 32 psu) in 1 L Erlenmeyer flasks. Cultures were maintained under controlled conditions at 15 °C under cool-white fluorescent light at a photon flux density (PFD) of 100 μmol photons m^{-2} s^{-1} on a 16 h light:8 h dark photocycle.

The cryptophyte *Rhodomonas salina* (Kalmar culture collection; KAC30) cultured under the same condition as *A. tamarense* described above served as target species to monitor lytic activity at each isolation step, and throughout the various treatments. The bioassay was performed as described before (Tillmann et al., 2008b; Ma et al., 2009).

2.2. Isolation and purification methods

2.2.1. Reversed phase high performance liquid chromatography (HPLC)

Reversed phase solid phase extraction (SPE) fractions were prepared as previously described (Ma et al., 2009). Approximately 1 L *A. tamarense* supernatant, acquired through 15 min centrifugation of cell culture at 3220 × *g* at 15 °C, was passed over a preconditioned C-18 SPE cartridge (500 mg, 6 mL, Sigma–Aldrich, Deisenhofen, Germany). The cartridge was washed with 10 mL deionized water and 10 mL 20% methanol and eluted with 30 mL 50% methanol and finally 30 mL 80% methanol. The 80% methanol fraction was brought to dryness by rotary evaporation, and the residue was re-suspended in 3.5 mL deionized water, and stored at −20 °C before further use. The concentrated lytic SPE fraction was thawed, and spin-filtered (0.45 μm, Durapore, Millipore) in a centrifuge (Eppendorf 5415R) for 2 min at 15,000 × *g* at room temperature.

The HPLC separation procedure on a C8 analytical column was performed as previously described (Ma et al., 2009). Ten runs (100 μL injection volume) of fractions with retention time of 18–19 min were pooled and dried under N_2, and frozen at −20 °C before further use.

2.2.2. Hydrophilic interaction ion-chromatography (HILIC)

Fifty microliter 80% methanol SPE fraction, purified from approximately 600 mL supernatant, were separated on an analytical column (150 mm × 4.6 mm) packed with 5 μm ZIC-HILIC, 200 Å particles (SeQuant, Haltern, Germany) and maintained at 25 °C. A pre-column with the same packing material was also used. The flow rate was 0.7 mL min^{-1} and gradient elution was performed with two eluents. Eluent A was 2 mM formic acid and 5 mM ammonium formate in 20% deionized water and 80% acetonitrile; eluent B was 10 mM formic acid and 10 mM ammonium formate in deionized water. The gradient was as follows: column equilibration with 0% eluent B until 20 min, then linear gradient to 100% B until 35 min, followed by isocratic elution with 100% eluent B until 45 min and finally return to initial 0% eluent until 46 min. Activity in the *Rhodomonas* bioassay was found in the fraction with retention time of 7–9 min. To reduce the sample complexity, the retention time of the collected fraction was narrowed to between 7.5 and 8.5 min. And the dry mass equivalent of the residue was measured.

2.3. Triple quadrupole and orbitrap mass spectrometry

The LC conditions were the same as for the C8 HPLC or HILIC separation procedures. Triple quadrupole experiments were performed on an API 4000 QTrap instrument (Applied Biosystems, Darmstadt, Germany) equipped with a Turbo ion-spray source. The instrument was operated in the full scan mode in the mass range of