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Identification of okadaic acid production in the marine dinoflagellate *Prorocentrum rathymum* from Florida Bay

Tianying An^{a,b}, Jamie Winshell^{a,b}, Gloria Scorzetti^{b,c}, Jack W. Fell^{b,c}, and Kathleen S. Rein^{a,b}*

^a Department of Chemistry and Biochemistry, Florida International University, 11200 SW 8th St., Miami, FL 33199, USA

^b Center for Oceans and Human Health, University of Miami, 4600 Rickenbacker Cswy, Miami, FL 33149, USA

^c Division of Marine Biology and Fisheries, Rosenstiel School of Marine and Atmospheric Science, University of Miami, 4600 Rickenbacker Cswy, Miami, FL 33149, USA

Abstract

Extracts of fifty-seven newly isolated strains of dinoflagellates and raphidophytes were screened for protein phosphatase (PP2A) inhibition. Five strains, identified by rDNA sequence analysis as *Prorocentrum rathymum*, tested positive and the presence of okadaic acid was confirmed in one strain by HPLC-MS/MS and by HPLC with fluorescence detection and HPLC-MS of the okadaic acid ADAM derivative. Quantitation of the ADAM derivative indicated that the concentration of okadaic acid in the culture medium is 0.153 µg/L.

Keywords

Prorocentrum rathymum; okadaic acid; diarrhetic shellfish poisoning (DSP) toxins; LC-MS; ADAM derivative; HPLC-FLD

The suite of marine toxins that includes okadaic (OA) acid and the dinophysistoxins (DTX) is collectively known as DSP (diarrhetic shellfish poisoning) toxins. The acute symptoms of DSP include diarrhea, nausea, vomiting and abdominal pain. Outbreaks have been documented worldwide and are associated with the consumption of mussels, scallops, or clams tainted with OA, its analogs or derivatives. (Gestal-Otero, 2000). OA has been isolated from several dinoflagellates in the genera *Prorocentrum* and *Dinophysis*, including the species *P. lima* (Yasumoto et al., 1987), *P. hoffmanianum* (Murakami et al., 1982), *P. concavum* (Dickey et al., 1990), *P. maculosum* (Zhou and Fritz, 1993), *P. belizeanum* (Morton et al., 1998), *P. faustiae* (Morton 1998), *P. arenarium* (Ten-Hage et al., 2000), *D. acuta* (Draisci et al., 1998) and *D. fortii* (Murata et al., 1982). In addition to OA, several related polyethers were isolated from these dinoflagellates, including the dinophysis toxins, DTX-1 and DTX-2 which differ in the location and number of methyl substituents (Murata et al., 1982; Yasumoto, et al., 1985; Hu et al., 1993). OA, DTX-1 and DTX-2 are inhibitors of protein phosphatases PP1 and PP2A (Dounay and Forsyth, 2002).

*Corresponding author fax: +1 305 348-3772. reink@fiu.edu.

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We recently reported the isolation of over fifty strains of dinoflagellates by viable high speed single-cell sorting (Sinigalliano et al., 2009). Five of these strains (6–9 and 25) matched most closely with *P. rhathymum* in a BLAST (Altschul et al., 1990) analysis of their large-subunit ribosomal genes (Scorzetti et al., 2009) and tested positive for protein phosphatase (PP2A) inhibition in preliminary screening. The presence of OA was confirmed in one strain by HPLC with fluorescent detection of the ADAM (9-anthryldiazomethane) derivative (Quilliam et al., 1998) and by HPLC-MS and MS/MS experiments.

Fifty-seven field strains were sequenced in the D1/D2 region of the LSU rDNA and compared with GenBank sequence data (Scorzetti et al., 2009). Dinoflagellate and raphidophyte genera identified included *Akashiwo*, *Amphidinium*, *Heterocapsa*, *Cachonina*, *Chatonella*, *Coolia*, *Fragilidinium*, *Heterosigma*, *Karlodinium*, *Karenia*, *Protoceratium*, *Scipscsiella* and *Prorocentrum*. Field strains 6, 7, 8, 9, 23, 25 and identified strains CCMP687 (*Prorocentrum mexicanum*) and CCMP1591 (*Prorocentrum micans*) clustered within a clade of the genus *Prorocentrum* (Fig. 1). The sequences for strains 6, 7, 8 and 9 were identical to each other and matched with two sequences (AY259166 and AY259167) of *P. rhathymum* (Pearce and Hallegraeff unpublished), which are the only two D1/D2 sequences of that species in GenBank. Strain 6 was deposited in the Provasoli-Guillard National Center for culture of Marine Phytoplankton as strain CCCMP2933 and sequenced in the ITS regions (Ferrel and Beaton unpublished). The ITS sequence (EU927561) showed only 2 mismatches from other ITS sequences present in the GenBank database: EU244464 (Rial et al., unpublished) and FJ155840 (Caillaud et al., 2009), thus confirming the identification as *P. rhathymum*. Base pair differences at two locations, particularly in the highly variable ITS region, are considered to be insignificant and may be due to technical difficulties in comparing data from different laboratories (D'Onofrio et al 1999). Strain 23 was more than 20 bp different from its closest match, AF260377, *P. micans*. The sequence of the identified strain of *P. mexicanum* agreed with the GenBank strain AF260378 (Daugbjerg et al., 2000). After its initial description in 1979 by Loeblich, the *P. rhathymum* taxonomy was dissolved and reclassified as *P. mexicanum* (Faust, 1990; Steidinger, 1983). Later the species was reinstated as a unique organism (Cortes-Altamirano, 2003). Our sequence data agrees with the concept that *P. mexicanum* and *P. rhathymum* are separate species. Strain 25 clustered with *P. rhathymum* and differed from that species by 6 bp in the D2 region. It has a distinct position in the clade and demonstrates significant microscopic and macroscopic differences from *P. rhathymum*, which indicates that strain 25 may represent a new taxon or species within the genus. Other members of the clade include *P. micans*, *P. gracile* and strain 23, which may also represent a new species in the genus. Studies among micro-eukaryotes (D'Onofrio et al, 1999; Scorzetti et al, 2002; Montresor et al 2003) have recognized that strains with identical sequences are members of a single species and that sequence differences at the magnitude exhibited by strains 23 and 25 indicate phylogenetically distinct genotypes.

Solid phase C18 extracts of the culture medium of fifty-seven strains of dinoflagellates and raphidophytes were screened for PP2A inhibition (Simon and Vernoux 1994). Pan *et al.* demonstrated that OA, DTX-1 and DTX-2 are more abundant in the culture medium than in the cells (Pan et al., 1999). The large scale isolation of okadaic acid from filtered seawater was recently described by Rundberget et al. (2007). We therefore reasoned that testing of the culture medium would be a simple and efficient way to perform preliminary screens for OA production. C18 extracts of strains 6–9, 25 and 40 (*Prorocentrum hoffmanianum*) were positive for PP2A inhibition. All fifty-one of the remaining field strains, including six strains (21, 22, 23, 27, 54, 55) whose D1/D2 sequence matched most closely with other *Prorocentrum* species (*micans*, *minimum*, *donghaiense* and *dentatum*) (Scorzetti et al., 2009) tested negative for PP2A inhibition.

To confirm the presence of OA by LC-MS, two hundred liters of culture medium from *P. rhathymum*, strain 8 was extracted. LC-MS of the crude extract was performed in negative ion mode with the full scan range of m/z 500–820. Both the OA standard and the crude extract of *P. rhathymum* revealed a [M-1] peak of m/z 803.5 at a retention time of 14.4 min (Fig. 2A and B). Only a single peak, with an m/z of 803.5 was observed, nor did we observe a peak with m/z 817, suggesting that only OA was present and not DTX-1 or DTX-2. LC-MS/MS analyses of standard OA and *P. rhathymum* extract were performed in negative ion mode for the fragmentation of m/z 803.5. The MS/MS spectrum shows four major product ions at m/z 785, 563 and 255 and two minor product ions at m/z 767, 785 and 321 (Fig. 2C) identical to those produced from OA (Torgersen et al., 2008).

Standard OA and *P. rhathymum* extract were derivatized with ADAM. Fig. 2D shows the HPLC-FLD analysis of the OA-ADAM reaction. The peak at a retention time of 12.49 min was absent from the blank and corresponds to ADAM esterified OA. Fig. 2E is the HPLC-FLD analysis of culture extract derivatized with ADAM. A peak was observed with the retention time of 12.54 minutes. The same sample that gave the chromatogram in Fig. 2E was subsequently analyzed by LC-MS and the results are presented in Fig. 2F. The total ion current chromatogram (TIC) showed one major peak, whose relative retention time was 26 min. The mass spectrum recorded in Fig. 2F confirmed this to be the ADAM esterified OA. An [M +NH₄]⁺ ion was observed at m/z 1012.11 and fragment ions due to sequential losses of water were observed at m/z 977.6 and 959.6. Thus the unambiguous identification of OA from *P. rhathymum* was made based on the retention time, molecular mass and fragment ion information. Quantitative analysis of the OA-ADAM derivative indicated that the concentration of OA in the culture medium of *P. rhathymum* was 0.153 $\mu\text{g/L}$.

We demonstrated the production of okadaic acid by *P. rhathymum* isolated from Florida Bay. While the presence of OA was confirmed by LC-MS or HPLC-FLD for strain 6 only, strains 7–9 and 25 were positive for PP2A inhibition. As we know of no other PP2A inhibitor produced by a dinoflagellate, we consider this indicative of OA production as well. Recently, OA has been identified in another strain of *P. rhathymum* from Malaysia, further supporting our findings (Caillaud et al., 2009). In our work, okadaic acid was isolated from the culture medium and not, as is traditionally done, from the cells. We are not aware of any other report of screening for OA in this way. The concentration of OA in the medium of our cultures of *P. rhathymum* was as much as two to three orders of magnitude lower (0.153 $\mu\text{g/L}$) than other known OA producers such as *P. lima* or *P. hoffmanianum* when the culture medium was analyzed in the same way (Perez et al., 2008). However, OA and DTX-1/2 are stored in the dinoflagellate cells as esters, while the free acids are released into the medium. This suggests that the total OA in the cultures could be much higher than our analysis indicates. At the time of sampling, strains 6–9 were the most abundant protists and may impact seafood safety in Florida Bay. In addition, future research should consider the pectenotoxins, which are often associated with OA producing organisms.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Appendix. Supplementary information

Supplementary data associated with this article including experimental details may be found at XXXXXXXX

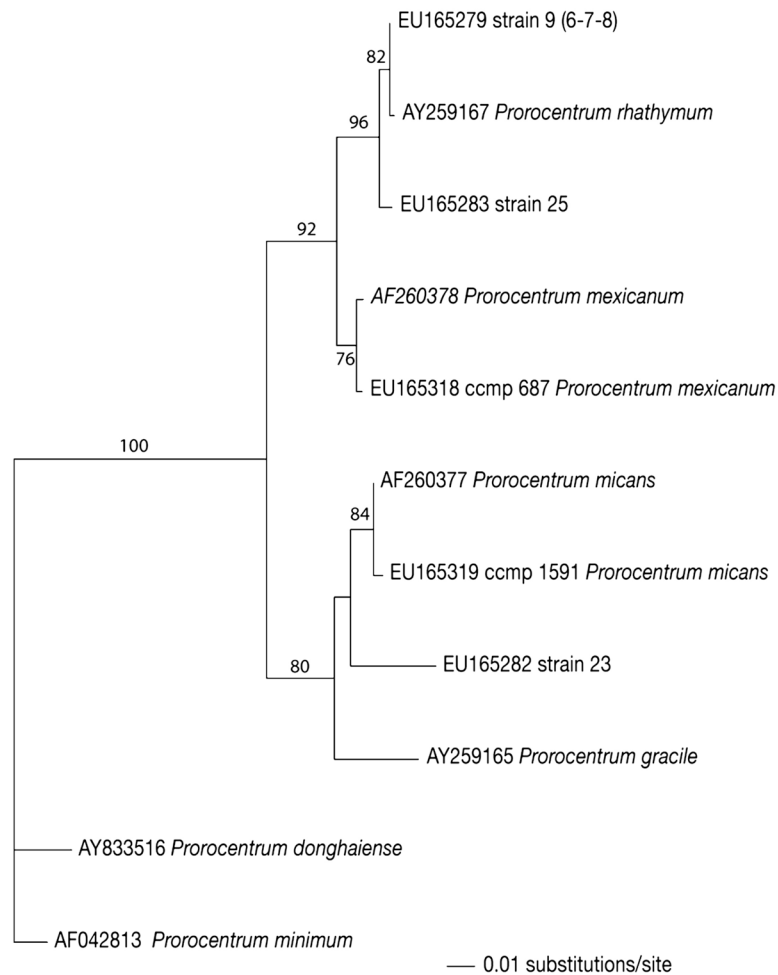


Fig. 1. LSU D1D2 rRNA phylogenetic tree comprising studied strains and related GenBank sequences. The tree was constructed with likelihood analysis in heuristic search (stepwise-addition option with 1000 replicates). Bootstrap values were reported on branches when higher than 50%. Sequences for *Prorocentrum minimum* and *Prorocentrum donghaiae* were used as outgroup.

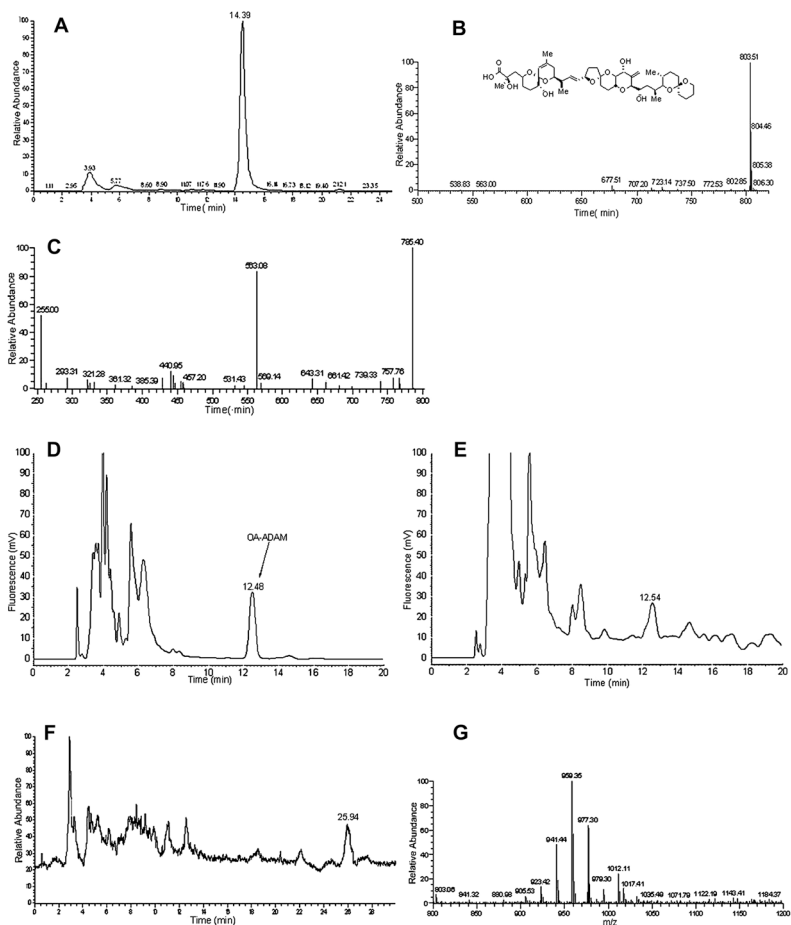


Fig. 2.

A. The chromatogram of *P. rhathymum* extract monitored for m/z 803.5. **B.** MS spectrum of 14.39 min. peak from **Fig. 2A**. **C.** The LC-MS/MS of the m/z 803.5 ion as the precursor. **D.** HPLC-FLD analysis for reaction of "in situ" ADAM reagent system with 8.65 ng of an okadaic acid standard. **E.** HPLC-FLD analysis for reaction of "in situ" ADAM reagent system with *P. rhathymum* supernatant extract. **F.** Total ion chromatogram (TIC) of the "in situ" ADAM reaction of *P. rhathymum* supernatant extract. **G.** MS spectrum of 25.94 min. peak from **Fig. 2F**.