Available online at www.sciencedirect.com
ScienceDirect
Harmful Algae 7 (2008) 639–645
HARMFUL ALGAE
www.elsevier.com/locate/hal

Effects of *Protoceratium reticulatum* yessotoxin on feeding rates of *Acartia hudsonica*: A bioassay using artificial particles coated with purified toxin

Wataru Makino [a,*], Kotaro Ito [b], Yasukatsu Oshima [b], Jotaro Urabe [a]

[a] *Laboratory of Community and Ecosystem Ecology, Graduate School of Life Sciences, Tohoku University, Aramaki Aza Aoba, Sendai, Miyagi 980-8578, Japan*
[b] *Laboratory of Bioorganic Chemistry, Graduate School of Life Sciences, Tohoku University, Tsutsumidori-Amamiya, Sendai, Miyagi 981-8555, Japan*

Received 10 November 2007; received in revised form 30 January 2008; accepted 30 January 2008

Abstract

Paralytic shellfish toxins produced by dinoflagellates are known to deter copepod grazing. Dinoflagellate species, including *Protoceratium reticulatum*, also produce disulfated polyether yessotoxins that were previously referred to as diarrheic shellfish toxins. However, the role of yessotoxins in predator–prey relationships is not yet clear. In the present study, the effects of purified yessotoxin (YTX) on feeding activities of *Acartia hudsonica* (Copepoda, Calanoida) were experimentally investigated. Polystyrene fluorescent microspheres (10 μm in diameter) colored bright blue or yellow-green were coated with cell extracts of *P. reticulatum* that do not produce yessotoxins. The bright blue microspheres were further coated with YTX, and the yellow-green microspheres were used as the reference. The microspheres were then given to the copepods separately or in combination to measure clearance rates and feeding selectivity. *A. hudsonica* was found to feed on the yellow-green microspheres without YTX at twice the rate of the bright blue microspheres with YTX. We also confirmed that microsphere color *per se* did not affect the feeding rates. The bright blue microspheres adsorbed 1.8–43.3 pg of YTX per microsphere, which is similar to the cell-specific yessotoxin content of toxic *P. reticulatum* found in natural environments. These results suggest that production of yessotoxin is advantageous for *P. reticulatum* by deterring predation by copepods.

Keywords: Yessotoxin; *Protoceratium reticulatum*; *Acartia hudsonica*; Selective feeding; Copepoda

1. Introduction

Dinoflagellates are one of the major primary producers in marine ecosystems. They are preyed upon by primary consumers such as copepods and filter-feeding shellfish. Some marine dinoflagellates, however, produce toxins. Such toxins may be transferred to organisms at higher trophic levels through the foodweb. In this way, bioaccumulation of the toxins can occur, and in some cases, such bioaccumulation has been blamed for mass die-offs of marine mammals and seabirds (Landsberg, 2002). Humans also sometimes suffer from poisoning by toxins in aquacultured shellfish. Paralytic shellfish toxins from some dinoflagellate species are especially dangerous. Several studies have shown that dinoflagellate species producing paralytic shellfish toxins are less vulnerable to copepod predation (Shaw et al., 1997; Teegarden, 1999; Colin and Dam, 2003), suggesting that the production of paralytic shellfish toxins is an antipredator strategy for these dinoflagellates (Cembella, 2003; also see Selander et al., 2006).

Paralytic shellfish toxins are not the sole type of toxin produced by dinoflagellates. Among others, the disulfated polyether yessotoxin and derivatives of yessotoxin are produced by *Protoceratium reticulatum* (Satake et al., 1997, 1999; Eiki et al., 2005), *Lingulodinium polyedrum* (Draisci et al., 1999), and *Gonyaulax spinifera* (Rhodes et al., 2006). Yessotoxin was first isolated from scallops in northern Japan (Murata et al., 1987) and are now detected worldwide (see citations above). Yessotoxin was once regarded as diarrheic shellfish poisons. However, it is now known that yessotoxin and its derivatives are nearly non-toxic when ingested orally,

* Corresponding author. Tel.: +81 22 795 6683; fax: +81 22 795 3683.
E-mail address: wmakino@mail.tains.tohoku.ac.jp (W. Makino).

1568-9883/$ – see front matter © 2008 Elsevier B.V. All rights reserved.
doi:10.1016/j.hal.2008.01.002

although they are highly toxic in mice via intraperitoneal injection (e.g., Tubaro et al., 2003). While there is detailed information on the degrees of toxicity and the chemical structures of yessotoxin, the understanding of the role of yessotoxin in foodweb interactions is still poor.

Uye and Takamatsu (1990) showed that one copepod species, *Acartia omorii*, maintained a high egg production rate when fed *P. reticulatum*, suggesting that this alga is not toxic to that copepod species. However, Huntley et al. (1986) found that the survivorship of *Calanus pacificus* was much lower when they were exposed to *P. reticulatum* at 500 $\mu g\ l^{-1}$, a bloom concentration, compared with individuals exposed to other algae in equal amounts. Furthermore, not only *P. reticulatum* cells, but also the filtrate of *P. reticulatum* culture suspension, suppressed the feeding activity of *C. pacificus*. These results showed that *P. reticulatum* adversely affects at least some copepod species. However, although this alga produces yessotoxin, it is not clear whether the adverse effects on copepod feeding are due to yessotoxin.

In the present study, therefore, we examined the effects of *purified* yessotoxin (hereafter called YTX) on the feeding activities of copepods. We artificially coated fluorescent microspheres with extracts from non-toxic *P. reticulatum* and with YTX isolated from toxic *P. reticulatum*. We then examined feeding preferences of *Acartia hudsonica* Pinhey on the microspheres. In northeastern Japan, toxic *P. reticulatum* has been observed at various sites, including Yamada Bay (39°28′N, 141°59′E) (Satake et al., 1999) and Mutsu Bay (41°09′N, 140°56′E) (Eiki et al., 2005). Although conditions promoting high concentrations of toxins have not been determined, it has been suggested that *P. reticulatum* is a prime factor in determining yessotoxin concentrations in scallops in Okkirai Bay (39°05′N, 141°52′E) (Koike et al., 2006). *A. hudsonica,* formerly *A. clausi* (see Ueda, 1986, 1997), is a dominant copepod in inlet waters there (e.g. Uye, 1982).

2. Materials and methods

We used a non-toxic strain of *P. reticulatum* (isolated from Harima Nada, the Seto Inland Sea of Japan), which was maintained in the laboratory according to the methods of Satake et al. (1997). The non-toxic nature of this strain has been confirmed (see Satake et al., 1999). *A. hudsonica* specimens were collected at a dock in Shiogama Bay (36°20′N, 141°03′E). Live individuals, mostly late copepodites and adults, were transferred to a laboratory where they were fed non-toxic *P. reticulatum* (200 cells ml^{-1}) at 20 °C for one night before the experiments. Seawater was also collected at the dock. Before use, the seawater was filtered through Whatman GF/F glass fiber filters and was autoclaved (hereafter referred to as ‘‘filtered and autoclaved seawater,’’ FASW).

For the preparation of YTX, we used a toxic *P. reticulatum* strain that was isolated from Marlborough Sounds, New Zealand (Satake et al., 1997) and was maintained in the laboratory according to the method of Satake et al. (1997). Detailed purification processes are described in Ito (2007). In short, the culture medium of the toxic *P. reticulatum* strain was passed though the GA-100 glass fiber filter and loaded into an Amberlite XAD2 column that was equilibrated with distilled water. This column was washed with pure methanol (MeOH) and then with MeOH–H_2O (3:7). The yessotoxin eluted in the MeOH–H_2O fraction was evaporated, and the residue dissolved in MeOH–H_2O (3:7) was loaded into a Sep-Pak C18 cartridge column equilibrated with the same solvent. The column was washed stepwise with MeOH–H_2O (3:7) and MeOH in this order. The toxin eluted in the MeOH fraction was evaporated, and the residue dissolved in the mixture of MeOH, MeCN, and distilled water (2:1:2, with 1 mM $AcONH_4$) was given to the first HPLC purification on a Cosmosil 5C18 AR II column (4.6 mm × 200 mm) at the flow rate of 1 ml min^{-1}. A fraction from retention times between 16.6 and 22.3 min was then given to the second HPLC purification, which led to the isolation of YTX (retention time, 4–7 min). The conditions of the second HPLC were identical to those in the first HPLC purification except that the mobile phase lacked $AcONH_4$. The purity of YTX was certified by comparing its MS and NMR spectrums with those of well-characterized reference yessotoxin in the previous structural study (Satake et al., 1996).

As artificial food items in which we manipulated the YTX concentrations, we used Fluoresbrite® bright blue and yellow-green carboxylate microspheres with diameters of 10 μm (Polysciences Inc.) as tracer food particles, and we performed the following experiments.

2.1. Experiment 1

Since we used bright blue microspheres and yellow-green microspheres, we first examined whether the microsphere color *per se* affected the feeding rates of *A. hudsonica* by impregnating bright blue microspheres and yellow-green microspheres with the same extract of non-toxic *P. reticulatum* cells. The impregnation procedure was as follows. Non-toxic *P. reticulatum* was concentrated to a concentration of 10^7 to 10^8 cells ml^{-1} by centrifugation (3000 rpm, 5 min), and the cell solution was sonicated on ice for two 1-min intervals. The sonicated cell solution was filtered through a 0.2-μm PVDV membrane filter (Millipore) and was transferred to a 1.7-ml Eppendorf tube (ca. 1 ml). Then, a drop of the raw microsphere solution (bright blue or yellow-green) was added to the tubes. After gently shaking overnight at 4 °C in the dark, the tubes were centrifuged at 1500 rpm for 5 min, the supernatant was removed, and the microspheres were resuspended in 1 ml of FASW. The concentration of microspheres in FASW was determined by direct count using fluorescence microscopy. We called this procedure coating Method A.

For a feeding run, 30–50 individuals of *A. hudsonica* that were swimming actively were transferred to polycarbonate bottles containing 250 ml of FASW with 200 cells ml^{-1} of non-toxic *P. reticulatum*. One hour later, the coated microspheres (bright blue or yellow-green) were added to the bottles so that the concentration reached 1000 microspheres ml^{-1} (Table 1). Non-toxic *P. reticulatum* that had average (±S.D.) longer and shorter diameters of 29.5 ± 2.1 and 24.0 ± 2.2 μm, respectively ($N = 5$), were left in the bottle since copepods often fail to