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A new simple screening method for the detection of cytotoxic substances produced by harmful red tide phytoplankton

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Abstract

A highly sensitive, but simple and quantitative, cytotoxic assay method for the detection of toxic substances produced by red tide phytoplankton was developed by utilizing Vero cells which were the most resistant to seawater among the six cell lines tested. *Heterocapsa circularisquama*, which is known to be highly toxic to shellfish, showed cytotoxicity to Vero cells in a cell-density dependent manner when Vero cells were directly exposed to the cell suspension of *H. circularisquama* in seawater-based plankton culture medium, whereas *Heterocapsa triquetra*, which is morphologically similar to *H. circularisquama* but non-toxic to shellfish, showed no cytotoxic effect. Since the potent cytotoxicity was also detected in the cell-free culture supernatant of *H. circularisquama*, it was suggested that a certain cytotoxic substance is extracellularly secreted by *H. circularisquama*. Furthermore, by this direct exposure method, we found that *Alexandrium fraterculus*, *Alexandrium tamiyavanichii*, *Alexandrium tamarense*, and *Alexandrium affine* but not *Alexandrium taylorii* and *Alexandrium catenella* cause toxic effect on Vero cells with different extent depending on species. By gel-filtration and subsequent two cytotoxicity assays using Vero and mouse neuroblastoma cell line (Neuro-2a), we found that high molecular weight cytotoxic substance distinct from paralytic shellfish poisoning toxins is present in the aqueous extract of *A. tamarense*. These results suggest that our 96-well microplate cytotoxicity assay using Vero cells is useful not only as a primary screening assay for the detection of potential toxic activity of harmful phytoplankton but also as a quantitative routine toxicity assay for following the active substances during the extraction and purification processes.

Keywords: Cytotoxicity; Screening assay; *Alexandrium tamarense*; Vero cells; Harmful dinoflagellate

1. Introduction

Harmful algal blooms (HABs) often cause mass mortality of natural and aquacultured fish or shellfish, which not only results in serious economic loss but also contributes to pollution of the coastal areas. In addition to the direct effects of HABs on various marine organisms, HABs can also become a potential threat to human health resulting from the consumption of phycotoxins accumulated in shellfish and other marine organisms. Therefore, the detection and quantification of the causative toxic substances produced by algae is a crucial subject of HABs study. To investigate the potential toxic effects of algae, direct exposure experiments using fish or shellfish to algae are generally employed as a primary investigation. However, these procedures are tedious and time-consuming, and often require large scale equipments, facility, and certain amounts of algal culture. Furthermore, such bioassay is

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recognized to have poor reproducibility and high variability, and it seems to be difficult to obtain quantitative results (Jellett, 1993; Suzuki et al., 1996).

In the case of phycotoxins such as paralytic shellfish poisoning (PSP) toxins, mouse bioassay (Yasumoto et al., 1984), which is sanctioned by the Association of Official Analytical Chemists as an official method, is routinely used world wide to detect PSP toxins in acid shellfish extracts (Williams, 1984). In this assay, toxin levels in shellfish extracts are estimated after intraperitoneal injection of the extracts into mice and subsequent monitoring the survival rate of the animals. However, there is increasing opposition from the general public to the use of live animals for routine toxicity assay, and this assay may not be suitable for detail quantitative analysis of the potentially toxic substances which might contain still unknown toxins. Several methods have been proposed as alternatives to mouse bioassay such as high performance liquid chromatography (HPLC) (Sullivan and Wekell, 1984) and enzyme-linked immunosorbent assay (ELISA) (Chu et al., 1996). The HPLC analysis is sensitive and reliable, but this method requires expensive equipments and sophisticated technical skills, and analytical standards for the each toxin to identify the toxin type are prerequisite. The ELISA is also highly sensitive and specific method as long as specific antibodies are available, but such available antibodies are limited. Thus, these chemical methods are fundamentally inappropriate for the detection and investigation of novel toxins.

In addition to producing PSP toxins, direct detrimental effects of some species of *Alexandrium* on several marine living organisms have been reported. Involvement of *Alexandrium tamarense* in a fish kill of farmed salmon and rainbow trout has been pointed out (Mortensen, 1985). Lush et al. (1997) have reported that juvenile greenback flounder (*Rhombosolea taparina*) exposed to *Alexandrium minutum* whole cell suspension showed gill damage characterized by severe epithelial swelling. *A. tamarense* has been reported to produce lethal effects on surf smelt (*Hypomesus pretiosus japonicus*) (Ogata and Kodama, 1986). It has been reported that *A. minutum* showed potent toxic effects on brine shrimp (*Artemia salina*) (Lush and Hallegraeff, 1996) and harpacticoid copepod (*Euterpina acutifrons*) (Bagøien et al., 1996). It seems that these harmful effects of *Alexandrium* spp. may be mediated by the secretion of toxic substances. However, in the most cases described above, the biochemical features of such toxic substances are still unclear. For the detail analysis of such toxic substances, establishment of suitable assay method that allows quantitative measurement of toxicity with small scale equipment is required.

As one of such assay methods, in vitro cell-culture-based bioassay has been described for the detection of some phycotoxins. For instance, tetrodotoxin, saxitoxin and related toxins, which are sodium channel blockers, can be detected by examining their effects on mouse neuroblastoma (Neuro-2a) which were pretreated with veratridine and ouabain (Jellett et al., 1992). The cooperative action of veratridine and ouabain on neuroblastoma cells induces sodium ion influx and eventual cytotoxicity which can be prevented or reduced by sodium channel blockers in a concentration-dependent manner. From the extent of the protective effect, the concentrations of tetrodotoxin, saxitoxin or related toxins can be estimated. Cytotoxicity assays for the diarrheic shellfish poisons (DSPs), which are based on the effects of the toxins on cell viability or cellular morphological changes in appropriate cell lines, have also been reported (Flanagan et al., 2001). In general, cytotoxicity assays using established cell lines are simple and inexpensive, but sensitive and quantitative, and simultaneous detection of a number of toxins with small scale experiments such as microplate assay is possible.

In this study, we developed a microplate cytotoxicity assay using Vero cells for screening and detection of algal-derived toxic substances. Since Vero cells were capable to live in seawater for a few hours, even direct exposure experiment to phytoplankton cell suspension was feasible. Potential usefulness of the assay method was also examined in several dinoflagellates. By this exposure experiment, we found that *Heterocapsa circularisquama*, which is known to be toxic to bivalves, showed cytotoxicity to Vero cells, whereas *Heterocapsa triquetra*, a harmless species, had no toxic effect. Furthermore, the cytotoxicity assay also revealed that *Alexandrium* spp. caused toxic effect on Vero cells with different extent depending on the species. In the gel-filtration of the extract prepared from *A. tamarense*, the cytotoxic activity was detected in relatively high molecular weight fractions which were clearly distinct from the fractions of PSP like activity.

2. Materials and methods

2.1. Plankton culture

Clonal cultures of *Heterocapsa circularisquama* and *H. triquetra* were isolated in Ago Bay and Hiroshima Bay, Japan, respectively. *Alexandrium taylorii*, *Alexandrium fraterculus* and *Alexandrium affine* were