Harmful Algae 16 (2012) 58–62

Contents lists available at SciVerse ScienceDirect

Harmful Algae

journal homepage: www.elsevier.com/locate/hal

Development of theca specific antisera for the profiling of cell surface proteins in the marine toxic dinoflagellate genus *Alexandrium* Halim

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ARTICLE INFO

Article history:
Received 9 June 2011
Received in revised form 26 August 2011
Accepted 23 January 2012
Available online 31 January 2012

Keywords:
Alexandrium affine
A. tamarense
Cell surface protein (CSP)
Paralytic shellfish toxin (PST)

ABSTRACT

Revealing the profile of dinoflagellate cell surface proteins (CSPs) is a crucial step for developing molecular probes for effective identification, separation and enumeration of toxic and non-toxic dinoflagellates. This study aimed to develop theca-specific antibodies against the dinoflagellates *Alexandrium affine* (non-toxic) and *A. tamarense* (toxic) to distinguish the two species, and verify if these antibodies would enable the analysis of multiple CSPs for probing phytoplankton's nutrient physiology, and facilitating rapid detection and enumeration of these harmful algal species. Using 2-DE immunoblots, we evaluated the specificity and effectiveness of the theca-specific polyclonal antisera against two types of antigens generated from fresh or fixed whole cell and insoluble cellular fractions, respectively. Our results showed that, of the four cell surface antigens, paraformaldehyde fixed whole cell antigen derived antiserum specifically recognized weakly bound theca-associated CSPs in toxic *Alexandrium* strain. Using the optimized theca-specific antisera, about 187 and 110 cell surface associated antigenic spots were identified on the 2-DE immunoblots of *A. affine* and *A. tamarense*, respectively. This immunoproteomic approach is proven to be very useful for phytoplankton CSP studies, permitting a more in-depth elucidation of the relationship among nutrient condition, bloom dynamic and toxin production of the harmful algae in the marine environment.

1. Introduction

Harmful algal blooms (HABs, commonly called "red tides"), which are characterized by the proliferation and occasional dominance of particular species of toxic or harmful algae, have spread worldwide and become a global problem causing enormous economic loss and serious impacts on human health (Anderson, 2009). HABs show great diversity due to the range of causative organisms involved, but dinoflagellates are the most important group of marine phytoplankton producing HABs (Anderson et al., 1989). Some dinoflagellates also carry toxins. For instance, dinoflagellates in the genus *Alexandrium* can produce paralytic shellfish poisoning (PSP) toxins (Hamasaki et al., 2001). During blooms of these toxic dinoflagellates, bivalves can accumulate the toxins by ingesting the dinoflagellates. Toxins can then be transferred to, and accumulated in, human beings by consuming these toxic bivalves, and thus causing serious food borne PSP poisoning.

Traditionally, *Alexandrium* cells are identified based primarily on microscopic examination of morphological features, particularly plate arrangement and apical pore complex. However, because of small sizes, these fine structures are extremely difficult to identify, requiring multiple light microscope observations. Meanwhile, near real-time monitoring of *Alexandrium* species has not been achieved because of difficulties resulting from the highly similar morphologies of the species. In particular, difficulties arise from the so-called tamarensis species complex. For example, *A. tamarense* and *A. exavatum*, as well as *A. tamiyavanichii*, share characteristics with *A. affine* and *A. fraterculus* that are related to chain formation and occurrence in natural seawater columns, this overlapping of morphological features confounds their identification using conventional microscopic examination. Cell surface proteins are the most common molecular targets, which employ antibody and immunofluoresecence-based detection (Vrieling and Anderson, 1996).

A. tamarense AT-Polar was identified and isolated from the Drake Passage in Antarctic Ocean by Prof. K. C. HO (The Open University of Hong Kong) in a research cruise from Novermber to December 2001. It produced mainly C-2 toxins but very little

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doi:10.1016/j.hal.2012.01.004

saxitoxin and gonyautoxin. Its bloom coincided with a massive death of penguins in the Falkland Island in December 2002 to January 2003. Experiments showed that this strain of *A. tamarense* has a high tolerance to temperature variations and could survive at temperatures ranging from 5 to 26 °C (Ho et al., 2003). This study aimed to develop theca-specific antibodies against the antigens generated from toxic and non-toxic dinoflagellate *Alexandrium* species, and evaluate if these antibodies would enable the analysis of multiple CSPs for probing phytoplankton's nutrient physiology, and facilitating rapid detection and enumeration of this harmful algal species. In brief, theca-specific antisera were produced against four types of cell surface antigens from *Alexandrium* species: (A) two types being the whole cell antigen (WCA) and insoluble cellular fractions (ICF) (presumably containing fragments of cellular membranes and amphiesma); and (B) two types being from different initial preparation procedures involving fresh antigens without fixation and antigens fixed with paraformaldehyde (PFA). The four antigen types include, fresh-WCA, fresh-ICF, PFA-fixed WCA and PFA-fixed ICF. Our results showed that PFA-fixed WCA-derived antiserum specifically recognized weakly bound CSPs in all extracts. Using the optimized theca-specific antisera, 187 and 110 cell surface associated antigenic spots were identified on the 2-DE immunoblots of *A. affine* and *A. tamarense*.

2. Materials and methods

2.1. Cultures and growth condition

Cells of *Alexandrium affine* AF-HKJB (non-toxic) were isolated from Junk Bay in Hong Kong, while *A. tamarense* AT-Polar (toxic) was isolated from the Southern Ocean by Prof. K.C. Ho (The Open University of Hong Kong). All isolates were batch cultured in K medium (Keller et al., 1987) in a 12:12 h light:dark cycle at 20 °C in a Conviron growth chamber S10H (Conviron Controlled Environments, Winnipeg, Canada) (150 μmol m^{-2} s^{-1} photo density from fluorescent lamps), for 14 days to attain the mid-exponential growth phase.

2.2. Production of antisera

2.2.1. Preparation of antigens

Four different antigens were prepared for antisera production: two types of antigens, the WCA and the ICF, were produced using two different antigen preparation procedures, that were fresh antigens without fixation and antigens fixed with PFA.

To prepare the fresh WCA, approximately 1×10^6 cells were harvested using centrifugation and then washed three times with either phosphate buffer solution (PBS) (0.02 M phosphate, 0.15 M NaCl, pH 7.5) or filtered and sterilized seawater. The washed cells were collected as the WCA. Two-dimensional electrophoresis (2-DE) (Chan et al., 2002) was used to monitor the proteins that might have been lost from the two washes. Sterilized seawater wash was found to be more efficient than PBS and, during the subsequent antigen preparation procedure, it was therefore used instead of PBS.

The ICF was prepared as described by Lin et al. (2003) with slight modifications. Briefly, a cell pellet containing about 1×10^7 cells was frozen at −80 °C, homogenized with a Tissue Grinder Kit (Fisher Scientific, Pittsburgh, Pennsylvania, USA) to thaw completely, and then passed through three frozen-homogenization cycles. Cell slurry was resuspended in a small volume of pre-cooled filtered sterilized seawater, and centrifuged at 12,000 × *g* for 15 min at 4 °C. The resulting pellet was collected as ICF, containing most of cellular membranes and amphiesma associated proteins.

For the PFA fixed antigen preparation procedure, both the WCA and ICF were preserved in PBS containing 0.5% PFA at 4 °C. Before the immunization, antigens were washed three times with PBS, and then re-suspended in 1 ml PBS.

2.2.2. Production of theca-specific polyclonal antisera

Two female Wister rats were immunized with each antigen. For the initial inoculation, 1 ml of antigen was mixed with an equal volume of Freund's Complete Adjuvant and injected into multiple sites in the back region. Subsequent boosts were made using Freund's Incomplete Adjuvant at monthly intervals. Final bleeds were collected from the tail vein on day 14, clotted overnight at room temperature and then centrifuged for antisera collection.

2.3. Test specificity of antisera produced using different protocols

2.3.1. Preparation of subcellular fractions for immunoblotting

A combination of WCA and ICF extraction protocols were applied and yielded four subcellular fractions: a highly enriched cell surface fraction (WCA), an insoluble cellular fraction (ICF), a pure cytosolic fraction (WCA-CS), and an intracellular fraction (ICF-CS). For the WCA fractions, individual cells of *A. affine* AF-HKJB and *A. tamarense* AT-Polar (approximately 1×10^6 cells) were collected by centrifugation, washed twice with pre-cooled filtered and sterilized seawater, and then had 1 ml of buffer I (0.2 M $CaCl_2$, 2 mM dithiothreitol (DTT), 1 M NaCl and 50 mM 1,2-cyclohexanediaminetetraacetic acid (CDTA) in 50 mM sodium acetate (pH 6.5)) and 0.5 ml of buffer II (0.2 M borate (pH 7.5)), added sequentially with vortexing for 30 min at 4 °C after each addition. Extracts from the two procedures were collected and pooled as the WCA. The resulting pelleted cells were resuspended in 0.5 ml of 40 mM Tris–HCl buffer (pH 8.7) and disrupted with a Microtip-probe sonicator 250 (Branson Ultrasonics, Danbury, Connecticut, USA). The supernatant, representing the WCA-CS, was obtained by ultrafiltration through an Amicon YM-3 membrane (Millipore, Bedford, Massachusetts, USA). For the ICF, approximately 1×10^6 cells of each strain were directly disrupted in 0.5 ml of 40 mM Tris–HCl, and centrifuged at 12,000 × *g* for 15 min at 4 °C. The supernatant representing the ICF-CS and the pellet representing the ICF were collected.

2.3.2. Immunoblotting analysis

For each subcellular fraction, two separate 2-DE gels were performed, one for comparative analysis using silver staining (Yan et al., 2000), and the other for immunoblotting. After 2-DE, the gel was electrotransferred to a PVDF membrane (Millipore, Bedford, Massachusetts, USA) at a constant current of 100 mA overnight at 4 °C. The PVDF membrane was then blocked with PBS containing 5% (w/v) non-fat milk powder at room temperature for 4 h, washed with PBS-T (0.05% (v/v) Tween 20 in PBS) for five cycles (each for 10 min), incubated with primary antibody (the antiserum prepared using the different protocols, 1:5000 dilution) for 2 h, and then with the secondary antibody horseradish peroxidase-goat anti-rat (Santa Cruz Biotechnology, Santa Cruz, California, USA, 1:2000 dilutions). Immunospots were visualized with an enhanced chemiluminescence detection system (GE Healthcare Life Sciences, Piscataway, New Jersey, USA). The immunoblots and silver stained gels were imaged with an ImageScanner equipped with ImageMaster Platinum software (GE Healthcare Life Sciences, Piscataway, New Jersey, USA). For each treatment three replicates were performed, unless stated otherwise, and each gel shown in the results is representative of the three replicates. Protein spots were selected for quantitative analysis if they were consistently visible in all samples from one condition.

2.3.3. N-terminal amino acid sequencing using Edman degradation

400 μg of total proteins obtained from the two strains were applied for 2-DE preparative gels and then electro-transferred onto