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New stage of the study on domoic acid-producing diatoms—A finding of *Nitzschia navis-varingica* that produces domoic acid derivatives as major toxin components—

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Abstract—We reported *Nitzschia navis-varingica* as a new domoic acid (DA)-producing diatom, and furthermore reported its wide distribution in Asian waters. However the surveys were performed only in limited area of each country. In order to obtain more detailed information, screening of *N. navis-varingica* was primarily performed in the Philippines. During the survey, we confirmed that all strains of *N. navis-varingica* isolated from Bulacan, Manila Bay did not produce DA, but did produce isodomoic acids A (IA) and B (IB). The confirmation of IA and IB was done by the analyses of UV-spectra, LC-MS/MS, proton NMR and ^{13}C NMR. Re-observation of the toxin composition including IA and IB simultaneously with DA was performed on the former chromatograms. All the strains positive for DA and/or its derivatives isolated from estuary areas of Bacoor, Tanauan and San Roque, were confirmed to produced DA and IB, indicating that there is a local difference in toxin composition in the Philippines. All of the toxic strains were identified as *N. navis-varingica*. This is the first report of pennate diatom that produces IA and IB instead of DA as major toxin component. This finding might be a useful step in pursuing the DA production mechanism. Additionally fourty one *Pseudo-nitzschia* strains isolated from offshore areas of above mentioned estuaries were tested for DA, but all of them did not show any sign of DA production.

Key words: domoic acid, isodomoic acid A, isodomoic acid B, *Nitzschia navis-varingica*, pennate diatom, Philippines, amnesic shellfish poisoning

Introduction

Domoic acid (DA) is the toxin responsible for the amnesic shellfish poisoning (ASP) occurred in Canada in 1987 (Wright et al. 1989). The causative organism was traced and identified as *Pseudo-nitzschia multiseries* (formerly *P. pungens* f. *multiseries*) (Bates et al. 1989). After the finding of DA producing *Pseudo-nitzschia*, efforts to search for other *Pseudo-nitzschia* species that produces DA were performed by many researchers, followed by findings of several species mainly in temperate area (Cho et al. 2001, Garrison et al. 1992, Kotaki et al. 1999, Lundholm et al. 1994, Martin et al. 1990, Rhodes et al. 1996, 1998, Sarno and Dahlman 2000). DA in shellfish has also been monitored for the safe consumption mainly in Canada, USA and Europe, in order to prevent the ASP accidents afterwards.

DA was originally isolated from macroalga *Chondria armata* as an insecticidal agent (Takemoto and Daigo 1958) and its derivatives were also isolated from the same alga (e.g. isodomoic acids A, B, C, G and H) (Maeda et al. 1987, Zaman et al. 1997). Other derivatives were isolated from toxic shellfish and causative diatom *P. multiseries* (e.g. isodomoic acids D, E, F and 5′-epi-DA) (Wright et al. 1990). As only a limited information for the DA producing diatoms was available in tropical area (Hasle 2002), a survey for DA producing diatom was primarily performed in Vietnam and consequently a new species *Nitzschia navis-varingica* was first isolated as a major DA producer (Kotaki et al. 2000; Lundholm and Moestrup 2000). Then, distribution of *N. navis-varingica* was surveyed outside Vietnam, followed by reporting the isolation of the diatom at brackish water area in the Philippines and Japan, indicating the wide distribution of the diatom in Asian waters (Kotaki et al. 2004). However, as

the samplings were performed at only limited areas of each country, more detailed survey was needed to clarify the distribution of *N. navis-varingica* that produces DA.

We report here the isolation and establishment of some strains of *N. navis-varingica* that produces isodomoic acids A and B instead of DA, indicating the advantage for the study on the DA production mechanism by using the strains for culture experiments with comparing to diatoms that produce only DA. Screening of DA producing *Pseudo-nitzschia* is also reported in which all isolates were negative for the DA production.

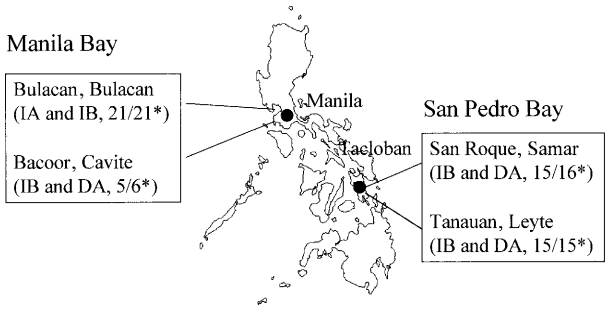

Fig. 1. Isolation of *N. navis-varingica* that produces domoic acid and its derivatives from four estuary area in the Philippines. DA; domoic acid, IA; isodomoic acid A, IB; isodomoic acid B. Number of toxic strains against the number of whole isolated strains.

Materials and Methods

Collection of diatoms

Nitzschia samples were collected by a scoop net (20 μm) at an estuary area of Tanauan and San Roque in San Pedro Bay near Tacloban, Leyte Island and at an estuary of Bacoor and Bulacan in Manila Bay (Fig. 1). Uni-algal cultures were established by capillary washing method from a crude culture of the net samples in f/2 medium (Guillard 1983). *Pseudo-nitzschia* samples were collected by a plankton net (20 μm) at offshore area of above mentioned estuaries.

Screening of DA producing diatoms

Established uni-algal cultures were analyzed for their DA production. Culture condition was set at 25°C under irradiance level of 70 μmol photons/m^2/sec with light : dark cycle of 16 : 8. Cell growth was monitored by *in vivo* chlorophyll *a* fluorescence using a hand-made fluorometer (Koike et al. 1994). A 10 ml of each culture at ten days after reaching stationary phase was taken out for measurement of DA concentration. Each sample was extracted by ultra-sonication using an ultrasonic disruptor (UD-201 Tomy Seiko, Tokyo, Japan output 7, 2 min). The extract was ultra-filtrated by an ultrafree-MC (Millipore Corporation, Bedford, MA. USA mw 10,000 cut-off). The filtrate was then analyzed for DA concentration by HPLC-fluorescence analysis with pre-column derivatization using 9-fluorenylmethylchloroformate (FMOC-Cl) according to Pocklington et al. (1990), in which a Develosil ODS-5 column (4.6×250 mm, Nomura, Seto, Aichi, Japan) and a mobile phase of 40% acetonitrile in 20 mM phosphate buffer (pH 2.5) were used (Kotaki et al. 2004).

Confirmation of DA derivatives in diatoms

A strain showing some peaks that react with FMOC-Cl reagent, appear around the DA peak at HPLC chromatogram and seem to be DA derivatives were mass cultured using f/2 medium under the same conditions described above. Cell fraction was obtained on the membrane filter, combined and extracted with 50% methanol by ultra-sonication as described above. DA derivative-like compounds were purified successively by Sep Pak C18 cartridge (Millipore), Wakosil ODS 25 C18 (Wako, Tokyo, Japan) column chromatography and Develosil ODS 5 (Nomura, Aichi, Japan) preparative HPLC. During the procedure, these compounds were monitored by HPLC analysis with UV detection (242 nm).

The purified compounds were analyzed by UV spectra (JASCO, V-550 spectrophotometer, Tokyo, Japan), LC-MS/MS (Applied Biosystem, API-2000, Column; Wako navi C18-5 2.0×150 mm, Wako), proton NMR and ^{13}C NMR (Varian INOVA 600 spectrometer at 20°C in D_2O) for the confirmation.

Species identification of the diatoms

Some *Nitzschia* strains among those positive for DA production from different sampling sites were randomly selected and observed by transmission electron microscopy (TEM), and morphological characteristics were examined for identification according to Lundoholm and Moestrup (2000). The remaining strains positive for DA and/or its derivative-like substances were identified according to characteristics by Lundholm and Moestrup (2000) and Kotaki et al. (2004) under a light microscope.

Some *Nitzschia* strains positive for DA and/or production of its derivatives were chosen randomly and analyzed for a region (D1–D3) of the nuclear large subunit rRNA gene (LSU rDNA) according to Lundholm et al. (2002) with the exception that primer sets N-D1R (5′-GAATTTAAGCATATAATTAA-3′) and N-D3F (5′-CCCAAATTTGACGATCGATTT-3′) were used for the polymerase chain reaction (PCR).

Results

Isolation of diatoms

Fifteen isolates of *Nitzschia*-like diatom were obtained from Tanauan, Leyte. Sixteen from San Roque, Samar, 6

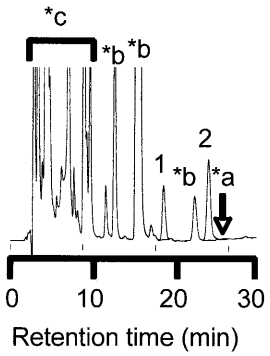

Fig. 2. HPLC chromatogram of whole culture (Cell+medium) of *N. navis-varingica* strain BLEC 03-17. 1 and 2; Unknown compounds. *a; Lack of DA, *b; Side products of the pre-column reaction derived from FMOC-Cl reagent, *c; Reaction products derived from contaminants in the medium and other compounds in the culture (eg. neutral amino acids) that react with FMOC-Cl reagent.

from Bacoor, Cavite and 21 from Bulacan, Bulacan were also obtained (Fig. 1). Forty-one isolates of *Pseudo-nitzschia* were obtained in total from offshore areas of those estuaries.

DA analysis in the isolates

DA was detected almost all isolates from three area except Bulacan. Their toxin ratio and average toxin value (pg/cell) were 15/15, 2.5 for Tanauan, 15/16, 3.6 for San Roque, 5/6, 3.8 for Bacoor, respectively. All of the 21 isolates from Bulacan did not show any sign of DA in HPLC analysis. However, two peaks (peaks 1 and 2) were detected near DA in HPLC chromatogram, indicating their possibility to be DA derivatives, because of the reactivity with the FMOC-Cl reagent and the similar affinity to ODS adsorbent (Fig. 2).

All the isolates of *Pseudo-nitzschia* did not show any sign of DA production by HPLC analysis.

Confirmation of DA derivatives in the diatom

For further study, extraction and purification of the compounds of the two peaks were performed after mass culture of a strain isolated from Bulacan estuary. Purified compounds were analyzed by UV-spectra, LC-MS/MS, proton NMR and ^{13}C NMR (data not shown) for the confirmation of the structure. Peak 1 and 2 compound showed an UV-spectra with maximum peak of 220 nm, indicating the lack of dien structure but multi carboxyl moieties, and showed a positive ion peak of 312.1, identical to the $[M+H]^+$ of DA or DA derivatives when analyzed by LC-MS /MS. The proton NMR spectra of the peak 1 and 2 compounds coincided well with those of isodomoic acids B (IB) and A (IA) respectively (Maeda et al. 1986) (Table 1, Fig. 3). Nuclear overhauser effects (NOE) were observed between 1′-Me and H3′ for peak 1 compound and between 1′-Me and H2′ for peak 2 compound. These indicate that the geometry of C1′=C2′ of the

Table 1. ^1H NMR (600 MHz) data of peaks 1 and 2 compounds.

Position	Peak 1[a] δH (J)[b]	Peak 2[a] δH (J)[b]
2	4.06 d (3.0)	3.96 d (7.8)
3	3.09 dddd (broad q)	2.99 dddd (broad q)
4	3.04 ddd (broad q)	3.63 ddd (7.2, 7.8, 7.2)
5	3.48 dd (11.4, 11.4)	3.47 dd (7.8, 11.4)
	3.62 dd (7.8, 12.0)	3.69 dd (7.8, 12.0)
6	2.26 dd (7.8, 16.2)	2.36 dd (9.6, 15.6)
	2.32 dd (7.2, 16.2)	2.52 dd (6.0, 15.6)
2′	5.25 dd (6.6, 7.2)	5.51 dd (7.2, 6.0)
3′	2.99 dd (7.2, 6.6)	2.76 m
		2.99 m
4′	6.75 dd (7.2, 7.2)	6.31 dd (7.8, 6.6)
1′-Me	1.75 s	1.76 s
5′-Me	1.85 s	1.81 s

Spectra were measured in D_2O.
[a] Chemical shifts are expressed in ppm.
[b] Coupling constants (Hz) are given in parentheses.
s=singlet, d=doublet, dd=double doublet, q=quintet, m=multiplet.

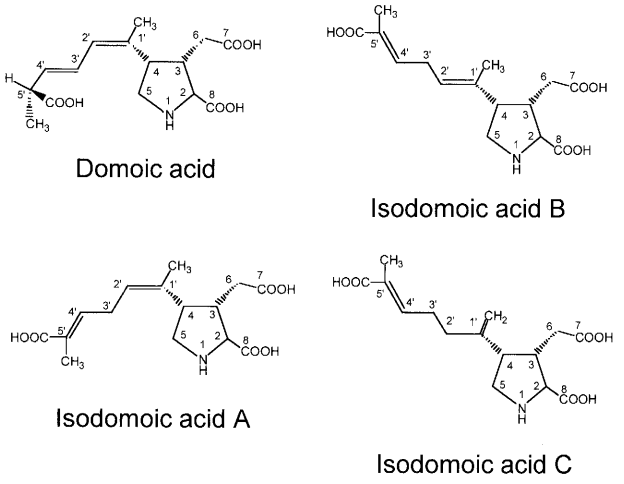

Fig. 3. Structure of domoic acid and isodomoic acids A, B and C.

two compounds were assignable to be *E* anad *Z*, respectively, supporting the above structure confirmation. These indicate that the compounds of peaks 1 and 2 are IB and IA respectively.

Species identification of diatoms

Microscopic observation on morphology of the *Nitzschia* isolates positive for DA and/or its derivative-like substances is shown as follows; cells were yellow-brown and possessed two chloroplasts at each end of the cell; the cells were lanceolate in valve view, 38–110 μm long and 9–11 μm wide; in girdle view, they were rectangular and slightly indented at the middle; the pervalvar axis was found to be wider than the transapical axis; most cells made ribbon-

shaped colonies whilst growing.

TEM observation of the representative strain of *Nitzschia*-like diatoms from each estuary area is shown as follows; length of the cells was in several of the isolates larger than that described by Lundholm and Moestrup (2000); the density of the interstriae as well as the density of the poroids in the striae and on the girdle bands corresponded to the description; the density of the fibulae, however, varied among strains. For the Tanuan, San Roque and Bacoor strains, the density corresponded with the description of *N. navis-varingica* (10–12, seldom 13, fibulae in 10 μm), whereas the strains from Bulacan had a slightly higher density (12–15, seldom 11, fibulae in 10 μm).

DNA sequencing was performed on the representative strains from Bacoor, Bulacan, and San Roque. The D1–D3 regions of the LSU rDNA sequence from the three strains determined in this study were all 804 base pairs long. The sequences of BLEC 03-17-2 and SREA 03-3 strains were completely identical, and two base substitutions separated them from BCEA 03-4-2.

These morphological observation and LSU rDNA sequencing studies showed that the characteristics of all the isolates that produce DA and its derivatives IA or IB were not indistinguishable from *N. navis-varingica* (Lundholm and Moestrup, 2000, Lindoholm et al. 2002, Kotaki et al. 2004).

Species identification was not done for the *Pseudo-nitzschia* isolates negative for DA production in the present study.

Toxin composition of the diatoms

Re-observation was done on the HPLC chromatogram of the strains isolated from other 3 areas, showing that all strains positive for DA production had also IB together. As a result, it was confirmed that there were two types of toxin composition among strains isolated from above 4 estuary areas. Strains isolated from Bulacan, northern part of Manila Bay, showed toxins composed of IA and IB. Other strains isolated from Bacoor (southern part of Manila Bay), from Tanauan and San Roque (more southern part from Manila Bay) showed toxins composed of DA and IB. *Nitzschia* isolates from Iba estuary, Zambales (north from Manila Bay) have toxins of IA and IB (Bajarias et al. this bull.). Other toxin composition types such as IA plus DA, IA plus IB plus DA, and DA only were not confirmed in the present survey. There existed two toxin composition types in the production of DA and its derivatives in *N. navis-varingica*.

Discussion

In the present study, we found that some strain of *N. navis-varingica* produced IA and IB instead of DA, and some others produced IB together with DA. As the *N. navis-varingica* strains that was found to produce DA before (Kotaki et al. 2000, 2004) had been screened for only DA production, it was uncertain whether they did produce only DA or IB together with DA. The next survey on this problem is now under way. Recently, some strains of *P. australis* was reported to produce isodomoic acid C (IC) simultaneously with DA and shellfish that fed on the diatom was reported to accumulate both toxins in New Zealand (Holland et al. 2003, Holland et al. 2005, Rhodes et al. 2003) (Fig. 3). Hence the present study first reported the presence of pennate diatoms that produce only DA derivatives (IA and IB) without DA.

The neuro-toxicity of IA and IB are uncertain as far. However, Holland et al. (2005) reported that the affinity of IC to glutamate receptors was 240-fold lower than DA because of the lack of 1′-2′ double bond with Z configuration (Hampson et al. 1992). This may indicate that the neuro-toxicity of IB is weaker than IA which has the 1′-2′ double bond with Z configuration. Measuring the affinity of IA and IB to glutamate receptor is needed to evaluate the potential of IA/IB against ASP occurrence. Insecticidal toxicities of IA and IB against American cockroach are reported to be 12-fold less toxic than DA (Maeda et al. 1986), and that of IC is 20-fold less toxic than DA (Holland et al. 2005). It is unknown whether there is any potential of *N. navis-varingica* on ASP accident or not. Because it has not been known yet how high the toxins from the diatoms are accumulated in the brackish water animals. Hence, it is uncertain whether we should monitor shellfish toxicity for IA/IB together with DA. However if the toxins including IA/IB are detected in *Pseudo-nitzschia* species, monitoring of IA/IB with DA in shellfish is highly recommended.

In the present study, *Nitzschia* strains that produce only DA derivatives have been isolated, indicating the advantage for pursuing the DA production mechanism in diatoms. Examination on stability of the toxin composition in each strain and some environmental factors that affect the toxin composition in *N. navis-varingica* is now under way.

Fortunately, all the *Pseudo-nitzschia* isolates were negative for DA production in the present study, suggesting the less potential of ASP occurrence in these areas. However, in Southeast Asian area, screening of DA producing *Pseudo-nitzschia* might be needed to obtain fundamental data for management to secure safety of shellfish for consumption.

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