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Two new ASP toxin production types in strains of *Nitzschia navis-varingica* from the Philippines

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Abstract — *Nitzschia navis-varingica*, a diatom widely distributed in brackish waters of Japan and Southeast Asia, was found to produce domoic acid (DA) and its derivatives isodomoic acids A (IA) and B (IB) as the major toxin components. The toxin composition of *N. navis-varingica* has previously been classified into three types namely DA type (produces DA and IB with an IB ratio of less than 10%), DA-IB type (the IB ratio is 20–80%) and IA-IB type (produces no DA). In the latter type, the ratio of IB tends to be higher in southern areas. In order to investigate more detailed distribution of ASP toxin types of *N. navis-varingica*, a screening was performed in three localities on Luzon Island, the Philippines. All isolates from one locality, Bulacan, were the DA-IB type. Isolates from the other two localities, Alaminos and Cavite, were found to make up two different and never described toxin composition types, the IB and the DA-IA-IB types, respectively. Sub-strains established from a representative parent of each new toxin composition type showed the same toxin composition as the parental strains. These results suggest that ASP toxin composition types of *N. navis-varingica* are more complex than previously reported.

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

Introduction

Amnesic shellfish poisoning (ASP) toxin was traced and identified as domoic acid (DA) at the poisoning that occurred in Canada (Wright et al. 1989). The causative organism was also traced and identified as the pennate diatom *Pseudo-nitzschia multiseries* (Bates et al. 1989). After this, DA-producing *Pseudo-nitzschia* strains from all over the world have been screened, resulting in the finding of many different *Pseudo-nitzschia* spp. as DA-producers. Only three *Pseudo-nitzschia* species (*P. multiseries*, *P. australis* and *P. seriata*) are known as major ASP toxin producers producing high level of DA (Bates 2000, Kotaki 2008, Trainer et al. 2008).

During a screening of DA-producing diatoms in tropical waters, *N. navis-varingica* isolated from Vietnam was found to produce high level of DA (Kotaki et al. 2000). The species has been shown to be widely distributed in brackish water areas of the Philippines, Thailand and Japan as in Vietnam (Kotaki et al. 2004, Romero et al. 2008) and has been found to produce not only DA but also isodomoic acids A (IA) and B (IB) as major toxin components (Kotaki et al. 2005) (Fig. 1). The toxin composition type identified are 1) DA with trace of IB where IB ratio is less than 10% (named DA type) and 2) DA with substantial amounts of IB, with the IB ratio of 20 to 80% (named DA-IB type). In southern areas IB ratio tends to increase except in isolates from Thailand which showed similar toxin composition to that of northern Japan (Romero et. al. 2008). Isolates from limited areas in Luzon Island (Bulacan and Iba Estuary) showed the special toxin composition of IA-IB (Kotaki et al. 2008, Kotaki 2008).

In order to obtain a more detailed insight in the distribution of *N. navis-varingica* that produces ASP toxins, screening of *Nitzschia*-like diatoms isolated from the estuarine areas of Luzon Island, the Philippines was performed followed by analysis through culture experiments.

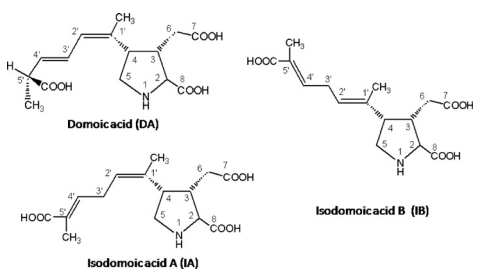

Fig. 1. Structure of domoic acid and isodomoic acids A and B.

Materials and Methods

Collection of diatoms

Nitzschia samples were collected by a scoop net (mesh size: 20 μm) and pre-cultured before isolation by capillary washing method. Several brackish water sites were selected in the collection areas of Cavite and Bulacan in Manila Bay and Alaminos in Pangasinan, the Philippines (Fig. 2).

Screening of DA-producing diatoms

Established isolates of *Nitzschia*-like diatoms were analyzed for major ASP toxins of DA, IA and IB by culture experiment using f/2 medium. Culture condition was set at 25°C under irradiance level of 70 μmol photons m^{-2} sec^{-1} with light : dark cycle of 16 : 8. Three ml of whole culture was harvested into test tubes at 3-week culture period measuring the cell concentration. Each sample was extracted by boiling for 8 min. The extract was centrifuged and its supernatant was analyzed for ASP concentration (DA, IA and IB) by HPLC-fluorescence analysis with pre-column derivatization using 9-fluorenylmethylchloroformate (FMOC-Cl) according to slightly modified method of Pocklington et al. (1990) in which a Develosil ODS 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).

Representative strain positive for ASP toxin production was cultured in one-litter scale and the cell fraction on the filter was extracted by sonication and analyzed by LC-MS/MS using multiple reaction monitoring (MRM, *m*/*z* 312-266, 312-248 and 312-161) (Takata et al. 2009).

Species identification of the diatoms

Representative strains positive for the ASP toxin production were randomly selected and observed by transmission electron microscopy (TEM), and morphological characteristics were examined for the identification according to Lundholm and Moestrup (2000) and Kotaki et al. (2004) under a light microscope and TEM.

ASP toxin composition in the sub-strains

To confirm the ASP toxin composition of *N. navis-varingica*, sub-strains were newly established from one representative parental strain of isolates that showed different ASP toxin composition as before. These were cultured, harvested, extracted and analyzed as described above.

Results and Discussion

Isolation of N. navis-varingica*-like diatoms*

Seven isolates of *N. navis-varingica*-like diatoms (PBULD strains) were obtained from Bulacan Estuary, one isolate from Cavite (PCAV strain), and ten isolates (1 in 2007 and 9 in 2008) from Alaminos (PALM 07/08 strain) (Fig. 2.).

ASP toxin production of the isolates

As shown in Fig. 3, the isolates from Bulacan Estuary (PBULD strains) showed the typical DA-IB type toxin composition (average whole toxin content was 1.9 pg $cell^{-1}$, DA : IB = 36.7:63.3). Interestingly, the isolates from Alaminos (PALMC strains) showed a new type of toxin composition of only IB with the average toxin content of 2.4 pg $cell^{-1}$ and that (PCAVA strain) from Cavite also showed a new type of toxin composition of DA-IA-IB with the average whole toxin content of 1.7 pg $cell^{-1}$ (DA : IA : IB = 32.7 : 12.1 : 53.2). The presence of these toxins in the new isolates was confirmed by LC-MS/MS.

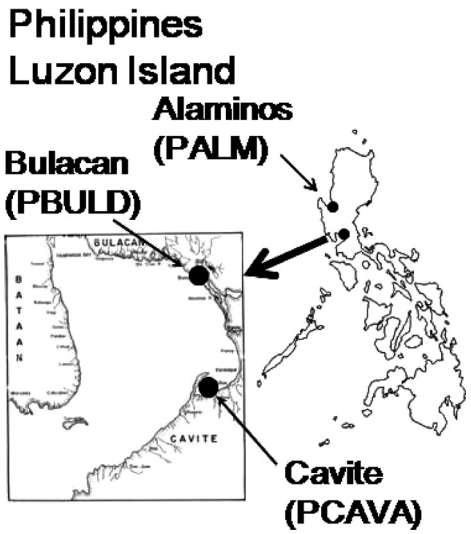

Fig. 2. Map of sampling areas in the Philippines.

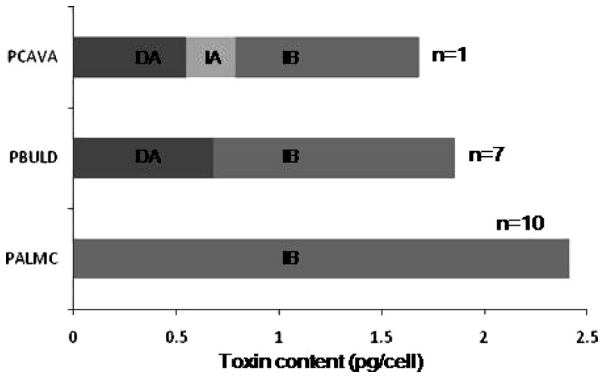

Fig. 3. ASP toxin composition of *Nitzschia navis-varingica* isolated from Luzon Island, the Philippines. Strains tested: PCAVA (Cavite, Manila Bay, 2007), PBULD (Bulacan, Manila Bay, 2007), PALMC (Alaminos, Pangasinan, one isolate in 2007 and 9 isolates in 2008). DA, Domoic acid; IA, Isodomoic acid A; IB, isodomoic acid B.

Species identification of the isolates

Observation under TEM of representative strains of the isolates (PALMC 07-1, PCAVA 07-2, PBULD 07-4) were morphologically identified as *N. navis-varingica*.

ASP toxin production of the sub-strains

Sub-strains were established from representative strains of the newly found toxin types (IB and DA-IA-IB type) and analyzed by HPLC with fluorescence detection. Thirty-three sub-strains were established from the parental strain (PALMC 07-1) that showed IB type toxin composition. These sub-strains also showed toxin composition of 100% IB with average toxin content of 4.6 pg cell^{-1} similar to the parent strain. Ten sub-strains were established from a parental strain from Cavite (PCAVA 07-2) that showed DA-IA-IB type toxin composition. Similarly the sub-strains showed the same toxin composition as that of the parent strain with average toxin content of 1.12 pg cell^{-1} and with toxin ratio of DA:IA:IB=29.4:16.8:53.8. These results show that toxin composition in each strain is stable and that toxin composition of *N. navis-varingica* is more complex than reported before (Kotaki et al. 2008a, Kotaki 2008b). Interestingly, only *N. navis-varingica* isolates from northern Luzon Island showed the curious toxin composition of IA-IB, IB and DA-IA-IB, although they are morphologically included in the same species. Five types of ASP toxin composition, namely DA, DA-IB, IA-IB, IB, DA-IA-IB type, have now been confirmed in total.

Based on the obtained results, it can be inferred that there are several factors that influence toxin composition including the ratio of the major toxin components DA, IA and IB. As part of a more detailed screening of *N. navis-varingica* toxin production and distribution, investigations of these factors are under way.

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