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Determination of pectenotoxin-2 after solid-phase extraction from seawater and from the dinoflagellate *Dinophysis fortii* by liquid chromatography with electrospray mass spectrometry and ultraviolet detection
Evidence of oxidation of pectenotoxin-2 to pectenotoxin-6 in scallops

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Abstract

Solid-phase extraction of pectenotoxin-2 (PTX2) and pectenotoxin-6 (PTX6) from seawater samples using a nonpolar cartridge column Sep-Pak C_{18} was investigated for determination of PTXs in toxic phytoplankton. PTX2 and PTX6 were almost completely recovered from the seawater samples. Determinations of PTX2 from the toxic phytoplankton and scallops were carried out by liquid chromatography (LC) on UV trace equipped with an atmospheric pressure electrospray ionization mass spectrometry (ESI-MS). PTX2 obtained from the toxic phytoplankton and scallops yielded a mass spectra exhibiting abundant $[M+H]^+$, $[M+NH_4]^+$ and $[M+Na]^+$ indicating that ESI-MS is useful for the identification of PTX2. Determinations of PTX6 from the toxic phytoplankton and scallops were carried out by LC fluorescence detection. A significantly higher content of PTX6 compared to PTX2 in scallops was observed, suggesting that a transformation of PTX2 to PTX6 in tissues of the scallops occurs rapidly.

Keywords: Diarrhetic shellfish poisoning; *Dinophysis fortii*; Pectenotoxins; Toxins

1. Introduction

Diarrhetic shellfish poisoning (DSP) was first described in Japan by Yasumoto et al. [1] as a new type of seafood poisoning resulting from the ingestion of shellfish contaminated by toxic dinoflagellates. There are three different groups of toxins which show toxicity in the mouse bioassay applied for diarrhetic shellfish toxin analysis; okadaic acid (OA) and its derivatives; macrolide toxins, called pectenotoxins (PTXs) (Fig. 1); and yessotoxins [2]. PTX1 is highly hepatotoxic [3], and the other PTXs are presumed also to be hepatotoxic [4,5]. Only PTX2 has been detected from the toxic dinoflagellate *Dinophysis fortii*, and it has been suggested that the other PTXs including PTX6 are produced in bivalves by the oxidation of 43-methyl group of PTX2 [4,6,7].

In a previous study [8], we developed a solid-phase extraction (SPE) method for OA and

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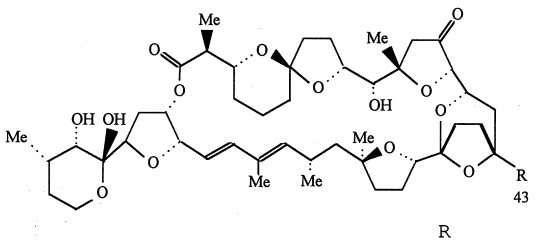

	R
Pectenotoxin-1 (PTX1)	CH_2OH
Pectenotoxin-2 (PTX2)	CH_3
Pectenotoxin-3 (PTX3)	CHO
Pectenotoxin-6 (PTX6)	COOH

Fig. 1. Chemical structures of PTXs.

dinophysistoxin-1 (DTX1) from seawater samples containing toxic phytoplankton by using a nonpolar cartridge column in order to recover the toxins which were eluted from the phytoplankton cells during the storage and experimental procedures. In the present study, we have investigated the SPE of PTX2 and PTX6 in seawater samples, and applied the results to the determination of PTXs in toxic phytoplankton. Determination of PTX2 and PTX6 in scallops collected at the same sampling site as the phytoplankton sample was also carried out to evaluate the kinetics of the transformation of PTX2 to PTX6 in the scallops.

Generally, the determination of PTX2 is carried out by UV trace analysis at 235 nm [5–7], however, UV detection is not specific for the analyte from natural products. The first investigation by mass spectrometry (MS) to confirm PTX2 in the toxic phytoplankton *D. fortii* was carried out using fast atom bombardment (FAB) negative ion by Lee et al. [7]. Recently, ion spray (nebulizer-assisted electrospray) ionization techniques have been demonstrated to be well suited to liquid chromatography–mass spectrometry (LC–MS) analysis of OA and derivatives [9–14], and has been applied to the confirmation of PTX2 in *D. fortii* collected in the northern Adriatic Sea [15]. In the present study, confirmation of PTX2 in toxic phytoplankton and scallops by LC–MS equipped with an atmospheric pressure electrospray ionization (ESI) was carried out.

2. Experimental

2.1. PTX2 and PTX6 standards

PTX2 and PTX6 standards obtained from the midgut glands of scallops (*Patinopecten yessoensis*) sampled at Mutsu Bay, Japan [16], were provided by Professor T. Yasumoto of Tohoku University (Sendai, Japan).

2.2. Sample materials

Toxic phytoplankton and scallops (*Patinopecten yessoensis*) were collected at the monitoring site in Mutsu Bay, Japan, on the 8 July 1996 during a DSP outbreak. Toxic phytoplankton sample was collected from 20-m depth with a sampling pump as described in [8]. A 2000-l volume of seawater was subjected to concentrate toxic phytoplankton by size fractioning using plankton nets, the 20–100 μm fraction which contains toxic phytoplankton was collected and resuspended in 1000 ml of filtered seawater (condensed toxic phytoplankton sample). The toxic phytoplankton in an aliquot of the condensed toxic phytoplankton sample were identified using a microscope. The cell density of *D. fortii* and *D. acuminata* in the condensed toxic phytoplankton sample was 1729 and 6 cells/ml, respectively. Other toxic phytoplankton were not observed. The condensed toxic phytoplankton sample was kept frozen at −30°C until used for the extraction of the toxins.

Scallops were cultured at the monitoring site in Mutsu Bay at a 20-m depth. The midgut glands from five individuals were combined and used for the toxin analysis. The selected samples were within a size range of 10–11 cm in shell length.

2.3. SPE of PTXs from seawater sample containing the toxic phytoplankton

Extraction of PTX2 and PTX6 from the seawater sample containing toxic phytoplankton was carried out by SPE on a Sep-Pak C_{18} cartridge as previously described [8]. An aliquot (20 ml) of the condensed toxic phytoplankton sample in seawater was filtered through a 1 μm filter paper (No. 5C). The residue was washed with 5 ml of methanol three times (toxic

phytoplankton residue extract). The filtrate of the condensed toxic phytoplankton sample was transferred to a Sep-Pak C_{18} cartridge column (Waters, Milford, MA, USA) which had been previously conditioned with 10 ml of methanol and distilled water. The Sep-Pak C_{18} cartridge column was subsequently washed with 10 ml of distilled water and finally with 10 ml of methanol. The methanol eluate and the toxic phytoplankton residue extract were combined and then evaporated. The residue was dissolved in 2.5 ml of 80% aqueous methanol and extracted twice with 2.5 ml of hexane to remove lipid components, then 1 ml of 0.2% acetic acid was added. The toxins were extracted with 4 ml of chloroform (twice). The combined chloroform extracts were made up to 10 ml with chloroform. In the analysis of PTX2, an aliquot (1 ml) of the chloroform extract was evaporated, then dissolved in 100 μl methanol, and 2 μl of the solution was directly analyzed by LC coupled with an ESI-MS detector. In the analysis of PTX6, an aliquot (0.5 ml) of the chloroform extract was subjected to a preparation of g-anthryldiazomethane (ADAM) derivative of PTX6 according to [6,17]. The purified ADAM derivative of PTX6 was dissolved in 100 μl of methanol, then 10 μl of the solution was injected into the LC–fluorescence detection (FLD) system.

To determine the recovery percentage of the extraction of PTX2 and PTX6 by the Sep-Pak C_{18} cartridge, the PTX2 and PTX6 standards (200, 400, 600 and 800 ng of each) were dissolved in ca. 300 μl methanol, then spiked into 5 ml filtered seawater obtained from the blank condensed phytoplankton sample. Extraction of the spiked PTX2 and PTX6 standards by the Sep-Pak C_{18} cartridge were carried out according to the present method. An aliquot of the extract was analyzed by LC.

2.4. Determination of PTXs in scallops

Extraction of PTXs from the midgut glands of scallops was carried out according to the method previously reported [6,17]. In the PTX2 analysis, an aliquot of chloroform extract corresponding to 0.050 g midgut gland was put into a colored vial, and dried under nitrogen gas. The residue was dissolved in 50 μl of methanol. A 5-μl aliquot of this solution was then directly analyzed by LC–MS. In the PTX6 analysis, 0.025 g of midgut gland extract was derivatized with ADAM under the conditions described in [6,17]. The purified ADAM derivative of PTX6 was then dissolved in 100 μl of methanol and 10 μl of the solution was analyzed by LC–FLD.

2.5. LC–MS of PTX2

LC–MS was performed using a Hewlett-Packard Model 1050 Series liquid chromatograph coupled to a Finnigan MAT SSQ-7000 mass spectrophotometer (San Jose, CA, USA) equipped with an atmospheric pressure ESI interface and an ICIS data system. The LC flow was introduced into the ESI interface without any splitting after detection by UV absorption at 235 nm. Separation of PTX2 was achieved on a Mightysil RP-18 column containing an octadecyl phase bonded to 3-μm silica gel particles (100 mm× 2 mm I.D.; Kanto, Tokyo, Japan) at 35°C. Acetonitrile–water (7:3, v/v) containing 0.1% acetic acid was used as the mobile phase at a flow-rate of 200 μl/min. The voltage on the ESI interface was maintained at approximately 4.5 kV. The temperature of the heated capillary was 175°C. High-purity nitrogen gas was used as the sheath gas at operating pressure of 70 p.s.i. and an auxiliary gas at 5 units, respectively (1 p.s.i.=6894.76 Pa). Positivly-charged ions with m/z values of 600–1200 were scanned with a scan time of 1.0 s.

2.6. LC–FLD of PTX6

The analysis of the ADAM-PTX6 derivative by LC–FLD was carried out according to [6,17], with some slight modifications. Separation was performed with a Hitachi (Tokyo, Japan) L-6000 pump equipped with a Develosil ODS-5 column (250 mm×4.6 mm I.D., Nomura, Seto, Japan) with acetonitrile–water (8:2, v/v) as a mobile phase and a flow-rate of 1.1 ml/min. The column temperature was maintained at 35°C. Peaks of the fluorescent derivatives were monitored with a Hitachi F-1050 fluoromonitor. The excitation and emission wavelengths were set at 365 and 412 nm, respectively. Fluorescence peaks with retention times that agreed with or were close to those of the standard toxins

were collected from the outlet of the fluoromonitor and rechromatographed on a Capcell Pak CN SG120 column (250 mm×4.6 mm I.D., Shiseido, Tokyo, Japan) with acetonitrile–water (53:47, v/v) as mobile phase at 35°C and a flow-rate of 1.1 ml/min, as previously reported [18]. The excitation and emission wavelengths of the fluoromonitor were set at 365 and 412 nm, respectively.

3. Results and discussion

3.1. SPE of spiked PTX2 and PTX6 standards by Sep-Pak C_{18} cartridge column

The percentage recovery of PTX2 and PTX6 standards calculated from the four extracts differing by the amount of spiked toxin (200, 400, 600 and 800 ng) was 99±2 and 100±3% (mean±S.D.), respectively. These results indicate that Sep-Pak C_{18} column is applicable for the extraction of PTX2 and PTX6 from the seawater samples. We suggest that SPE on Sep-Pak C_{18} may be also applicable for the extraction of other PTXs in seawater considering their similar chemical structures.

3.2. LC–MS analysis of PTX2 in toxic phytoplankton and scallops

Fig. 2 shows a chromatogram of the toxic phytoplankton extract on a Mightysil RP-18 column detected by UV trace at 235 nm as well as the total ion chromatogram (TIC) obtained by full-scan positive ion ESI detection. A peak with a retention time identical to the standard PTX2 was detected in both chromatograms. The Mightysil RP-18 stationary phase provided an excellent peak shape for PTX2. Capacity factor (k') and height equivalent to a theoretical plate (HETP) of the Mightysil RP-18 column for PTX2 were 1.96 and 0.028 mm, respectively.

Fig. 3 shows the full-scan positive ion ESI mass spectrum of PTX2 obtained after HPLC separation of the toxic phytoplankton extract. PTX2 yielded a mass spectra exhibiting abundant $[M+H]^+$, $[M+NH_4]^+$ and $[M+Na]^+$ at m/z 859, 876 and 881,

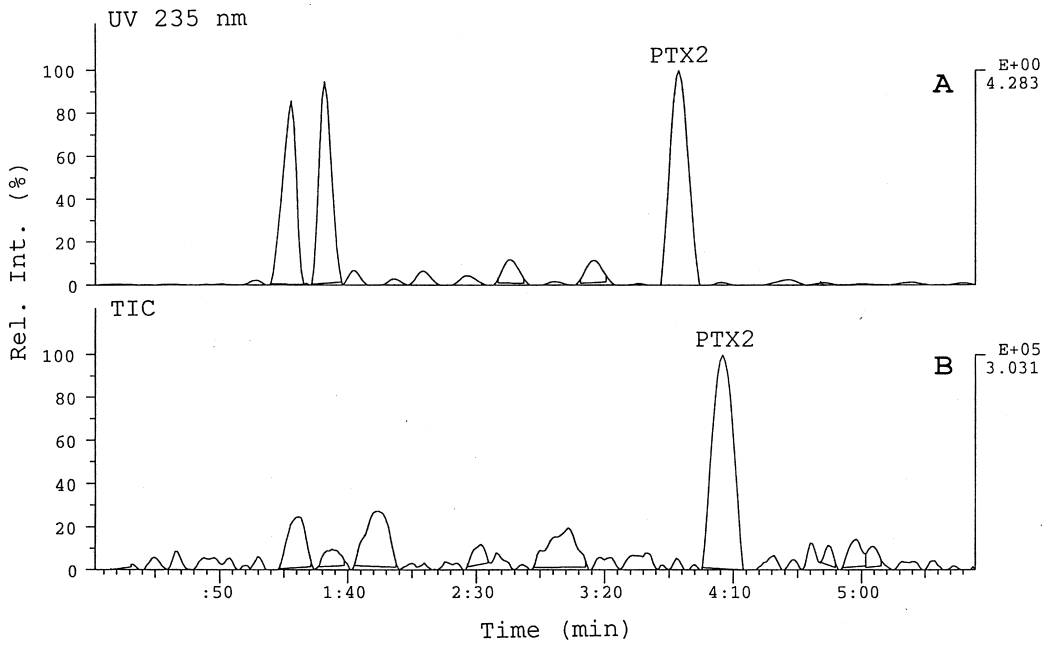

Fig. 2. Chromatograms of PTX2 obtained from extracts of the toxic phytoplankton. (A) UV trace at 235 nm (B) total ion chromatogram (TIC) obtained by positive mode full-scan monitoring. Scan range: m/z 600–1200. Column: Mightysil RP-18 (100 mm×2.0 mm I.D.). Mobile phase: MeCN–water (7:3, v/v) with 0.1% acetic acid; flow-rate: 200 μl/min; column temperature: 35°C.

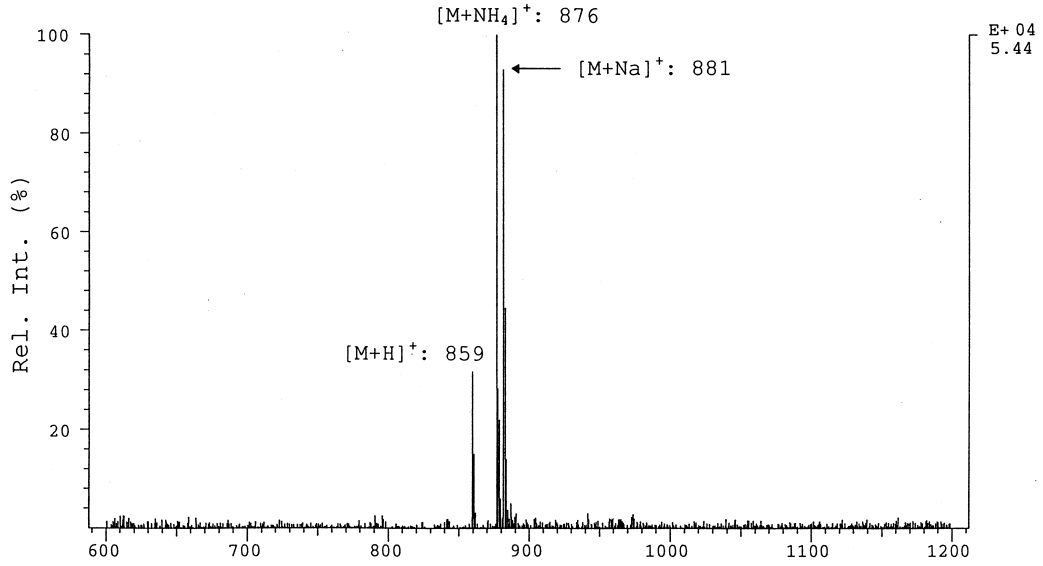

Fig. 3. Background-subtracted positive ion mass spectra of PTX2 obtained from LC–MS analysis of toxic phytoplankton extracts. Chromatographic conditions as described in Fig. 2.

respectively. These typical mass spectra on ESI confirmed the presence of PTX2 in the toxic phytoplankton sample. In the present study, different mobile phases were tested to improve sensitivity. Formic acid at 0.1% gave almost equal sensitivity to 0.1% acetic acid. Mobile phase without acid modifier or with 5 m*M* ammonium acetate also gave ionization for PTX2, however, the efficiency of the ionization was not sufficient. During the optimization of the MS parameters, it was observed that the ionization efficiency of PTX2 increased with increasing heated capillary temperature, however, the relative abundance of $[M+H]^+$ and $[M+NH_4]^+$ decreased and $[M+Na]^+$ increased at higher temperatures.

Previous study by LC–MS on PTX2 extracted from *D. fortii* showed a single molecular ion peak of $[M+H]^+$ by ion spray MS on PE-SIECX API III equipment [15]. In the present study, other ion peaks $[M+NH_4]^+$ and $[M+Na]^+$ in addition to $[M+H]^+$ were also observed using our ESI-MS equipment. This is probably due to differences in the droplet desolvation process during the ionization of the analyte [19].

The presence of PTX2 in scallops was confirmed by both chromatogram using UV trace and MS monitoring. The LC–MS analysis showed the mass spectra exhibiting $[M+H]^+$, $[M+NH_4]^+$ and $[M+Na]^+$, however, content of PTX2 in scallops was too low to allow quantitative analyses by UV trace.

3.3. PTXs in D. fortii and scallops

Table 1 shows the PTX2 and PTX6 contents in *D. fortii* and in the midgut glands of scallops determined by LC. The *Dinophysis* species composition in the condensed toxic phytoplankton sample used in the SPE of PTXs was approximately *D. fortii* (100%), therefore, PTX2 and PTX6 contents in *D. fortii* were estimated from both the cell density of *D. fortii* and the toxin content in the seawater sample. In a previous study, the PTX2 content in *D. fortii* was 42.5 pg/cell [7]. In the present study, the PTX2 content obtained was higher than that reported

Table 1
PTXs contents in *D. fortii* and the midgut glands of scallops collected from Mutsu Bay on 8 July 1996

D. fortii (pg/cell)		Scallops (μg/g)	
PTX2[a]	PTX6[b]	PTX2[a]	PTX6[b]
182±4[c]	ND[d]	<2.0	13±1[c]

[a] Determined by LC on UV trace at 235 nm.
[b] Determined by LC–FLD.
[c] Mean±S.D. obtained for triplicate analyses of a single extract.
[d] Not detected.

previously. Since a wide range variation in the DTX1 content in *D. fortii* has been reported [7,8], similar variation in the PTX2 contents were presumed.

Although PTX6 was detected in the midgut glands of scallops, it was not detected in *D. fortii*; this indicates that PTX6 is produced in scallops by the oxidation of the 43-methyl group of PTX2 as previously suggested [4,6,7]. It is noteworthy that the content of PTX6 in scallops is significantly higher than that of PTX2. This suggests that the oxidation of PTX2 to PTX6 in scallops occurs rapidly.

In conclusion, Sep-Pak C_{18} cartridge is well suited for the extraction of PTX2 and PTX6 from seawater. PTX2 yielded a typical mass spectra on ESI exhibiting abundant $[M+H]^+$, $[M+NH_4]^+$ and $[M+Na]^+$, indicating that ESI-MS is useful for the identification of PTX2. Although PTX6 was detected in the midgut glands of the scallops, it was not detected in *D. fortii*, confirming that PTX6 is produced in the scallops by the oxidation of PTX2. A significantly higher content of PTX6 in scallops compared to that of PTX2 suggests that the transformation of PTX2 in scallops occurs rapidly.

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