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Occurrence of Tetrodotoxin in the Causative Gastropod *Polinices didyma* and another Gastropod *Natica lineata* Collected from Western Taiwan

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ABSTRACT

Attempts were made to elucidate the responsible toxins in the gastropod *Polinices didyma* which recently caused food poisoning incidents in western Taiwan. The remained (cooked) and captured (live) specimens of both gastropods were assayed for toxicity (as tetrodotoxin = TTX). Average toxicity of cooked and live specimens was 118 ± 105 and 47 ± 28 MU/specimen, respectively. In addition, another species *Natica lineata* collected from the same area was also found to be toxic. The toxin of each gastropod was partially purified from the methanolic extract of the gastropod by ultrafiltration and Bio-Gel P-2 column chromatography. HPLC and GC-MS analyses demonstrated that the toxin consisted of TTX. It was concluded that the causative agent of the above food poisoning was TTX.

Keywords: Tetrodotoxins, gastropod, food poisoning, *Polinices didyma*, *Natica lineata*

INTRODUCTION

Marine toxin poses serious problems to public health and the fishery industry throughout the world. Tetrodotoxin (TTX) has been detected widely in gastropod mollusks, including trumpet shell *Charonia sauliae*[1,2], frog shell *Tutufa lissostoma*[3], Japanese ivory shell *Babylonia japonica*[4,5], *Rapana rapiformis* and *R. venosa venosa*[6], several species of Naticidae such as *Natica lineata*[7,8], and several species of Nassariidae such as Niotha clathrata, *Zeuxis scalaris* and *Z. siquijorensis*[3, 9, 10, 11]. Moreover, some gastropods, such as *N. lineata* and *N. clathrata*, also contain paralytic shellfish poison (PSP)[9,12].

Though gastropod *Polinices didyma* has been investigated to possess weak tetrodotoxin (TTX) in southern Taiwan[8], it does not induce food poisoning. Thus, special regulation on its consumption to prevent food poisoning does not exist at the present. However, the first case of food poisoning with ingestion of cooked *P. didamy* occurred in Chiayi County, western Taiwan, in July 2000. These causative gastropods were examined for toxicity and toxin component.

On the other hand, consuming another gastropod *Natica lineata* is getting popular in western Taiwan. In our previous report, *N. lineata* collected in southern Taiwan contains both TTX and paralytic shellfish poisons (PSP), and the toxicity in muscle is higher than that in the digestive gland[13]. To protect consumers from TTX and/or other poisoning, the toxicity of gastropods in western Taiwan should be examined. In this study, we aim to provide the information regarding the toxin and seasonal variation of toxicity of the gastropod *P. didyma* and *N. lineata* in western Taiwan.

MATERIALS AND METHODS

I. *Materials*

The remaining causative cooked gastropod *Polinices didyma* of the food poisoning were collected in July, 2000 and frozen at –20°C until assay. The shells of the gastropods were removed and separated into the digestive gland and edible portions including muscle, salivary gland, brain and mouth organs. On the other hand, ten specimens of the gastropod *P. didyma* and *N. lineata* were collected monthly from Chiayi County, western Taiwan from October 1999 to September 2000. These specimens were kept alive until analyzing. The ICR (Institute of Cancer Research) strain mouse was purchased from National Laboratory Animal Breeding and Research Center, Taipei, Taiwan, R.O.C. Healthy mice weighing between 18 and 20 g were used. Authentic TTX, 4-*epi*-TTX and anhydrotetrodotoxin (anh-TTX), which were obtained from the liver of puffer *Takifugu oblongus*, were used as the reference standards[14].

II. *Assay of Toxicity*

The dissected tissue was weighed, extracted with ten folds of 0.1% acetic acid, and examined for toxicity by the

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mouse assay for TTX[15]. Toxicity was expressed in mouse units. One mouse unit (MU) was defined as the amount of toxin required to kill a 20 g ICR strain male mouse in 30 min after a single intraperitoneal injection.

III. *Purification of Toxin*

After bioassay, the edible portions and digestive gland from the cooked *P. didyma* were combined, and so do the live specimens of *P. didyma* and *N. lineata*. Each mixture was homogenized with three folds of 1% acetic acid in methanol for 5 min and centrifuged (2,000 g, 20 min). The extraction process was repeated twice. The extract (250 MU for cooked *P. didyma*; 300 MU for alive *P. didyma* and 400 MU for *N. lineata*) was concentrated under reduced pressure at 45°C and defatted with dichloromethane. The aqueous layer was concentrated and filtered through a Diaflo YM-1 membrane (Amicon, Beverly, MA, USA) to remove substance of more than 1,000 daltons. The filtrate was applied to a Bio-Gel P-2 column (2 × 94 cm; Bio-Rad, Hercules, CA, USA) that was developed with 0.03 M acetic acid. Toxic fractions were combined, freeze-dried, dissolved in a small amount of water, and applied to the analyses described below.

IV. *High-performance Liquid Chromatography*

High-performance liquid chromatography (HPLC) was performed on a reversed-phase column (Merck Lichromsper 100 RP-18, 4 mm I.D. × 20 cm; E. Merck, Darmstadt, Germany). The mobile phase for TTX analysis was 2 mM sodium 1-heptane sulfonate in the buffer system, methanol-potassium phosphate buffer (0.05 M, pH 7.0) (1:99, v/v). TTX was detected by mixing the eluate with equal amount of 3 N NaOH, heating at 99°C for 0.4 min, and monitoring the fluorescence at a 505 nm emission wave and a 381 nm excitation wave[16]. The identified toxin component was compared with the retention times of sample and authentic standards in HPLC. Furthermore, authentic standards were added into the sample, and the toxin components of the sample should have the same peaks of authentic standards.

V. *Gas-chromatography-mass Spectrometry (GC-MS)*

A small amount of toxins was dissolved in 2 mL of 3 N NaOH and heated in a boiling water bath for 45 min to obtain the C_9-base derivate. The above hydrolysate was adjusted to pH 4 with 1 N HCl and extracted three times with 5 mL of 1-butanol. The extracts were combined and evaporated to dryness. After trimethylsilylation, the products were subjected to GC-MS on a Shimadzu QP-2000A. A column (BPI, 0.22 mm × 12.5 m) was used, and the temperature was raised from 165 to 230°C at a rate of 3°C/min. The ionizing voltage was kept at 70 eV at an ion source temperature of 200°C.

RESULTS

I. *Toxicity of the Gastropods*

The toxicity of causative specimens of *P. didyma* collected from western Taiwan is shown in Table 1. Four out of seven specimens of the remaining cooked *P. didyma* were toxic. The average toxicity was 118 ± 105 (mean ± S.D.) MU/specimen. The highest scores of toxicity in the digestive gland and edible portion were 19 and 58 MU/g tissue, respectively. Additionally, 12.5% and 29.1% of the live specimens of *P. didyma* and *N. lineata* collected from

Table 1. Anatomical distribution of toxicity in toxic shellfish

Species	Toxic ratio (%)	Toxicity (MU/g)		Total toxicity*1 (MU/specimen)
		Digestive gland	Edible portion	
Polinices didyma	12.5 (15/120)*3	ND*2-10 (3 ± 3)*4	ND-14 (7 ± 3)	8-123 (47 ± 28)
Natica lineata	29.1 (35/120)	ND-11 (4 ± 4)	ND-26 (8 ± 6)	5-95 (22 ± 14)
Cooked *P. didyma*	57.1 (4/7)	ND-19 (10 ± 6)	ND-58 (28 ± 21)	30-261 (118 ± 105)

*1: Toxicity of all toxic specimens.
*2: ND means less than 3 MU/g.
*3: Toxic ratio = toxic specimen/total specimens.
*4: All data represent mean ± S.D.

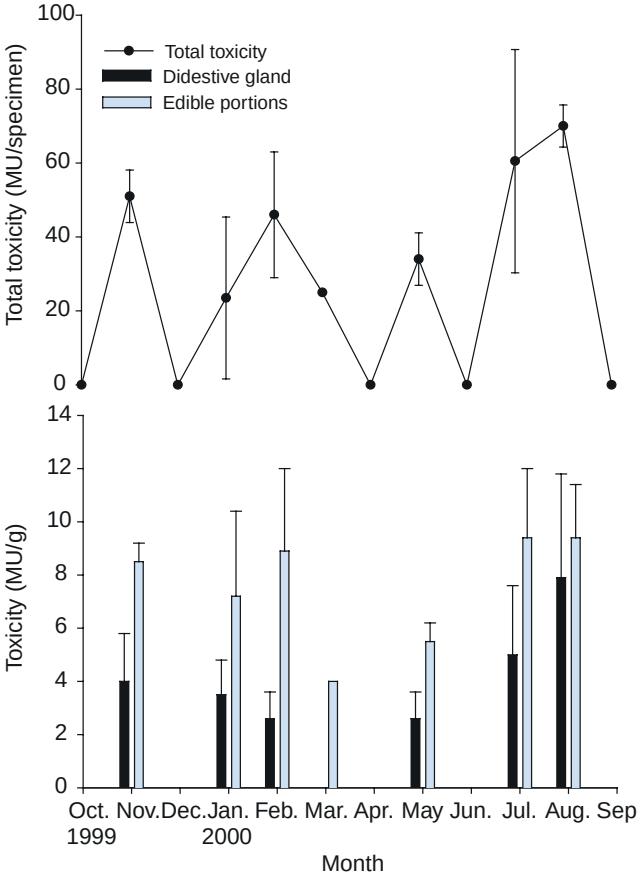

Figure 1. The seasonal variation of toxicity of *Polinices didyma*.

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Chiayi County during the period from October 1999 to September 2000 were found to be toxic. The average toxicities of *P. didyma* and *N. lineata* were 47 ± 28 and 22 ± 14 MU/g specimen. The highest scores of toxicity in the digestive gland and edible portion were 10 and 14 MU/g tissue for *P. didyma* and 11 and 26 MU/g tissue for *N. lineata*. Gastropod specimens showed variation in toxicity like other TTX-containing animals.

II. *Seasonal Variation of Toxicity in Gastropod*

Seasonal variations of toxicities in *P. didyma* and *N. lineata* are shown in Figures 1 and 2. It seems that the toxicity accumulated in *P. didyma* and *N. lineata* varies in a regular pattern every 3-4 months. It was found that the most toxic period of *P. didyma* was in July to August 2000 while that of *N. lineata* was November 1999. During the whole year, edible portions of both *P. didyma* and *N. lineata* are more toxic than the digestive gland. However, the percentage of toxic specimens of *P. didyma* and *N. lineata* throughout the year were lower than 20% and 50%, respectively. There were no seasonal variations in toxic ratio in *P. didyma* and *N. lineata*.

III. *Toxin Composition*

The HPLC profiles of the toxin in the gastropods *P. didyma*, *N. lineata*, and cooked *P. didyma* are shown in Figure 3. Each toxin displayed three peaks, whose retention times (Rt = 18.8, 21.9 and 23.8 min) corresponded well with those of TTX, 4-*epi*-TTX and anh-TTX. The other peak was identified as tetrodoic acid-like substance according to a previous paper[14, 17]. In GC-MS, the trimethylsilyl (TMS) derivatives from the gastropod toxins gave rise to ion peaks at 407, 392 and 376 *m*/*z* at the same retention time (8.2 min) as the TMS derivative from TTX. Both TMS derivatives displayed a parent peak at 407 *m*/*z*, a base peak at 392 *m*/*z*, and a fragment peak at 376 *m*/*z* (Figure 4). The toxins did not exhibit any PSP peak in HPLC analysis.

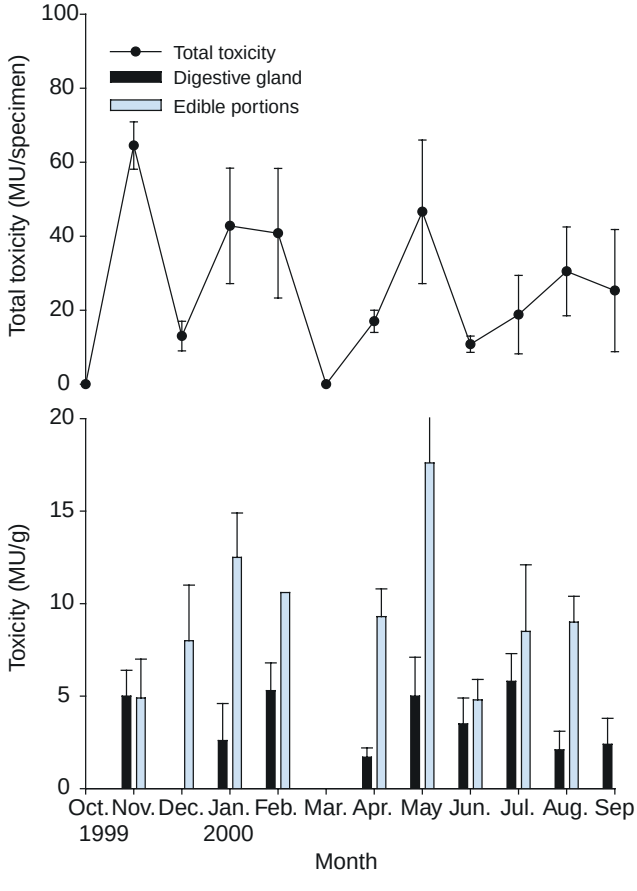

Figure 2. The seasonal variation of toxicity of *Natica lineata*.

DISCUSSION

The results allow us to conclude that TTX caused the first food poisoning incident of the gastropod *P. didyma* that occurred in Chiayi County, western Taiwan, in July 2000. The highest toxicity score of the remaining cooked *P. didyma* (261 MU/specimen) was higher than that of *P. didyma* collected from Chiayi County (123 MU/specimen).

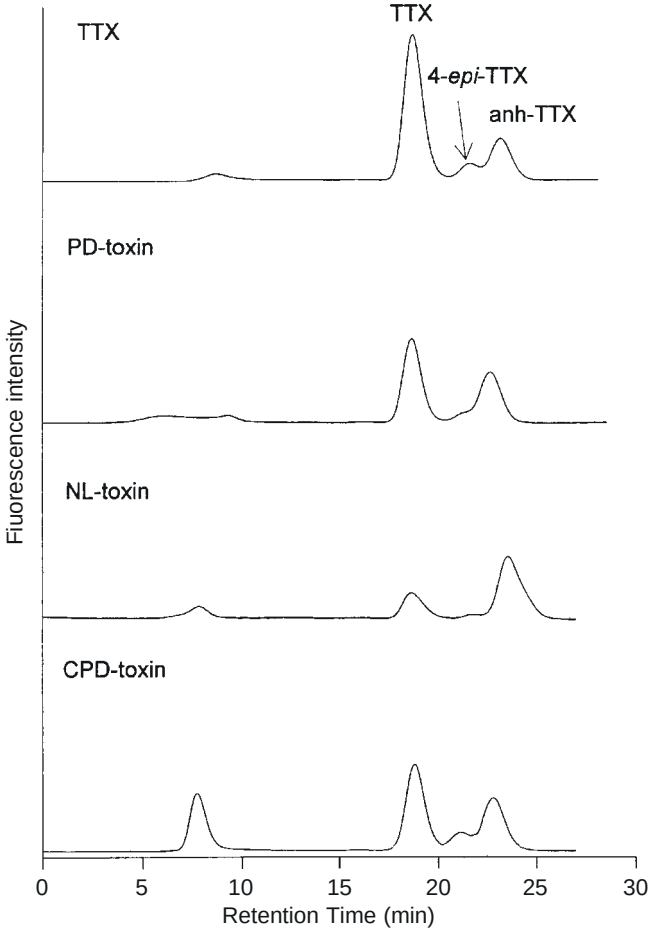

Figure 3. HPLC of *Polinices didyma* toxin (PD-toxin), *Natica lineata* toxin (NL-toxin) and cooked *Polinices didyma* toxin (CPD-toxin), along with authentic TTX, 4-*epi*-TTX and anh-TTX.

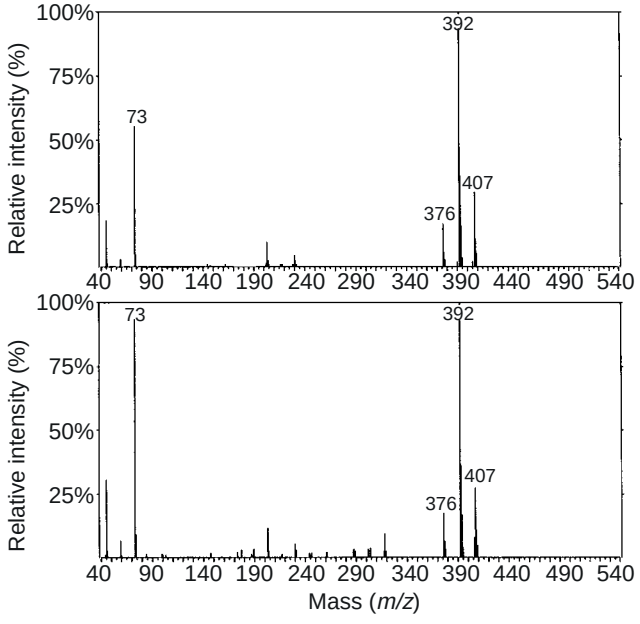

Figure 4. Mass spectra of the TMS derivatives from authentic TTX (upper) and the cooked *Polinices didyma* toxin (CPD-toxin)(lower). *Polinices didyma* toxin and *Natica lineata* toxin were similar as CPD-toxin.

In addition, the average toxicity of *N. lineata* was 21.8 ± 13.8 MU/g specimen. In our previous report[8], the toxicity score of *P. didyma* and *N. lineata* from Pingtung County, southern Taiwan was 1,563 and 2,590 MU/specimen. Therefore, toxicity of *P. didyma* and *N. lineata* specimens collected from western Taiwan was much lower than that of southern Taiwan.

In this study, the edible portion of *P. didyma*, *N. lineata* and cooked *P. didyma* were more toxic than the digestive gland. This result is the same as our previous work[8]. It is suggested that the muscle is the major tissue where *P. didyma* and *N. lineata* accumulated toxin. The composition of above three toxins was identified as only TTX. However, both TTX and PSP are found in *P. didyma* and *N. lineata* in southern Taiwan[13]. Nassariidae gastropods are carnivorous animals who like to inhabit at sandy coastal waters. The sources of TTX and PSP in these areas seem to be abundant[18, 19]. It indicates that these gastropods might accumulate TTX and/or PSP from the food chain. Although the TTX-producing bacteria have been found in the gastropod *N. clathrata*[20], the role of TTX-producing bacteria in the mechanism of TTX accumulation in TTX-containing animals should be considered.

On the other hand, *P. didyma* collected from Chiayi had the highest toxicity in July and August, corresponding with the time period of this incident. The toxicity of gastropod *N. clathrata* seem to have regional and seasonal variations in southern Taiwan[10]. In this study, the seasonal variation of gastropod toxicity was not found in western Taiwan. Accordingly, these data indicate that the gastropods *P. didyma* and *N. lineata* in western Taiwan are not completely safe to eat.

CONCLUSION

TTX was the toxin responsible for the first case of gastropod *P. didamy* associated food poisoning. The two common specimens of gastropods, *P. didamy* and *N. lineata*, in Western Taiwan were found to contain TTX. It is suggested that consumption of gastropods in Western Taiwan should be regulated.

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