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Short communication

Paralytic toxins in three new gastropod (Olividae) species implicated in food poisoning in southern Taiwan

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Abstract

The toxins in the new gastropods *Oliva miniacea*, *O. mustelina* and *O. nirasei* implicated in a food paralytic poisoning incident in South Taiwan in Feburary 2002 were studied. It was found that the three species of gastropods contained moderate amounts of toxin in edible portion only, and the highest toxicity score was 18 MU/g for *O. miniacea*, 10 MU/g for *O. mustelina*, and 27 MU/g for *O. nirasei*. The toxin was partially purified from the toxic specimens of each species by ultrafiltration using a YM-1 membrane, followed by chromatography on Bio-Gel P-2 column. Analyses by HPLC, GC–MS and LC–MS showed that the toxin from *O. miniacea*, *O. nirasei* and *O. mustelina* contained TTX, and related compounds 4-*epi* TTX and anhydro-TTX. The paralytic shellfish poisons were not found.

Keywords: Food poisoning; Olive shell; Toxicity; Tetrodotoxin

Food poisoning caused by olive shells (*Oliva miniacea*, *O. mustelina* and *O. nirasei*) was first reported and involving one victim (male, 51 years old) occurred in South Taiwan in February, 2002. The symptoms exhibited by the victim were paresthesia of the lips, tongue and pharynx, papillary constriction, increased neuromuscular symptoms, coma, nausea, vomiting, diarrhea, respiratory failure, impaired mental faculties and loss of deep tendon. Therefore, the victim was treated with emetics, and gastric lavage, especially with 2% sodium bicarbonate, followed by activated charcoal and ventilatory support. After 1 week, the victim recovered and now is healthy. The olive shells were collected from the coastal areas of Pingtung. Tetrodotoxin and/or related compounds have been reported in gastropod mollusks including trumpet shell *Charonia sauliae* (Narita et al., 1981), frog shell Tutufa lissostoma (Noguchi et al., 1984), Japanese ivory shell *Babylonia japonica* (Noguchi et al., 1981; Yasumoto et al., 1981), rock shells *Rapana rapiformis* and *R. venosa venosa* (Hwang et al., 1991a,b), several species of Naticidae such as lined moon shell *Natica lineata*, banded moon shell *N. vitellus* and bladder moon shell *Poilnices didyma* (Hwang et al., 1990a; Hwang et al., 1991a,b), and several species of Nassariidae such as basket shells *N. clathrata*, *Zeuxius scalaris* and *Z. siquijorensis* (Hwang et al., 1992; Hwang et al., 1995; Jeon et al., 1984). Among them, all these gastropods contain only TTX and/or its related components, except for the gastropods of Naticidae and Nassariidae collected from South Taiwan containing TTX and paralytic shellfish poisons (PSP) (Hwang et al., 1994; Hwang et al., 1995). As known, poisoning due to ingestion of TTX-containing puffer has frequently occurred in Japan, and also in Taiwan, Hong Kong, Thailand, Singapore, Malaysia, Kiribati, Fiji, Australia, Papua New Guinea, Bangladesh, U.S.A. with much fewer victims (Noguchi and Ebesu, 2001). The incident consuming TTX-containing gastropods was first reported in Japan (Narita et al., 1981). Then Nassariidae, including *Z. scalaris* and *N. clathrata*, associated poisoning has been reported in Taiwan (Hwang et al., 1995), and also caused a similar poisoning.

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The responsible toxin in the gastropods has been established to be TTX and PSP. Similarly, we also identified that the gastropod poisoning, more than 40 incidents, in Mainland China since 1977 was caused by TTX (Sui et al., 2003). These facts prompted us to examine the responsible toxin and compare this toxin with PSP and TTX in the remaining gastropods, which had been frozen.

The specimens of each gastropod were more than 10 specimens for *O. miniacea*, *O. mustelina* and *O. nirasei* remaining from the food poisoning case. The edible parts of 10 specimens from each gastropod species were dissected into the digestive gland and edible portions. The dissected tissue was weighed, homogenized with 10 volumes of 1% acetic acid in methanol for 5 min, and centrifuged (2000*g*, 20 min). The operation was repeated twice. The supernatants were combined, concentrated under reduced pressure at 45 °C, and examined for toxicity by the mouse assay for TTX (Hwang and Jeng, 1991; Yasumoto, 1991). Authentic TTX and anhydrotetrodotoxin (anh-TTX) (Goto et al., 1965; Hwang et al., 1988) obtained from the liver of the puffer *Fugu oblongus* were used as reference standards. Authentic GTX-1-4, saxitoxin (STX) and neoSTX obtained from the purple clam *Soletellina diphos* and the crab *Zosimus aeneus* (Daigo et al., 1985; Hwang et al., 1987) and

Table 1
Anatomical distribution of toxicity in specimens of *Oliva miniacea*, *O. mustelina*and *O. nirasei* collected from Pingtung in February, 2002

Specimens	Gastropod weight (g)	Gastropod length (cm)	Digestive gland		Edible portion		Total toxicity (MU/specimen)
			Weight (g)	Toxicity (MU/g)	Weight (g)	Toxicity (MU/g)	
Oliva miniacea							
1	65.92	75.41	5.75	ND[a]	14.88	3	45
2	57.54	72.69	4.68	ND	12.28	18	216
3	80.34	81.67	7.79	ND	15.21	13	203
4	49.92	78.02	4.46	ND	10.26	13	135
5	74.32	84.26	4.73	ND	14.47	4	53
6	74.05	78.45	6.89	ND	10.81	12	129
7	34.34	66.13	4.37	ND	5.97	14	84
8	137.51	101.41	10.93	ND	25.58	3	79
9	111.82	95.86	10.50	ND	24.03	15	363
10	44.36	69.27	5.23	ND	9.45	17	159
Mean ± SD	93.01 ± 29.76	80.27 ± 10.58	6.57 ± 2.31		14.29 ± 5.90	11 ± 5	146 ± 91
O. mustelina							
1	29.40	56.62	2.61	ND	7.20	5	35
2	26.67	55.91	0.83	ND	6.35	6	39
3	18.27	51.93	1.09	ND	3.51	16	56
4	19.49	54.62	1.08	ND	3.88	5	21
5	21.70	51.07	1.03	ND	4.68	13	63
6	20.37	53.41	2.09	ND	2.83	10	29
7	24.71	58.94	2.56	ND	3.96	4	17
8	22.65	55.86	2.75	ND	3.76	10	37
9	21.16	52.21	3.09	ND	3.67	6	23
10	22.47	56.20	3.21	ND	4.34	6	25
Mean ± SD	22.69 ± 3.21	54.65 ± 2.35	2.03 ± 0.89		4.42 ± 1.28	8 ± 4	35 ± 14
O. nirasei							
1	21.58	59.18	1.74	ND	3.29	8	28
2	24.07	57.46	2.54	ND	4.21	27	112
3	15.04	51.53	1.18	ND	3.71	28	103
4	18.38	50.31	1.86	ND	3.87	14	55
5	20.52	54.43	2.20	ND	3.73	16	60
6	22.14	56.39	3.55	ND	3.83	22	84
7	16.65	50.45	1.12	ND	3.43	14	49
8	24.93	61.93	3.08	ND	5.61	8	47
9	21.75	53.64	2.22	ND	5.02	4	21
10	23.34	54.41	3.04	ND	5.20	3	16
Mean ± SD	20.84 ± 3.06	54.93 ± 3.61	2.25 ± 0.76		4.19 ± 0.76	14 ± 8	58 ± 31

[a] ND (not detected) means less than 3 MU/g.

used as reference standards. Toxicity was expressed in mouse units. The toxicities of the *O. miniacea*, *O. mustelina* and *O. nirasei* specimens are shown in Table 1. All of the specimens detected were toxic. The mean value of toxicity was 146 ± 91 MU/specimen (mean ± SD) in *O. miniacea*, 35 ± 14 MU/specimen in *O. mustelina*, and 58 ± 31 MU/specimen in *O. miniacea*. Toxicity of digestive gland was not detected (<3 MU/g).

After the bioassay, the remained extract (840 MU for *O. miniacea*, 270 MU for *O. mustelina* and 420 MU for *O. nirasei*) from the toxic specimens of each species were mixed, concentrated under reduced pressure at 45 °C, and defatted with dichloromethane. The aqueous layer was concentrated and filtrate through YM-1 membrane and purified by Bio-Gel P-2 column (Hwang et al., 1988; Hwang et al., 1990a). Toxic fractions were combined, freeze-dried, dissolved in a small amount of water, and submitted to the analyses described below. High-performance liquid chromatography (HPLC) was performed on a reversed-phase column (Merck Lichromsper 100 RP-18, 4 mm ID × 20 cm; E. Merck, Darmstadt, Germany). The mobile phase for TTX and GTX analysis was sodium 1-heptane sulfonate (2 mM) in methanol (1%)-potassium phosphate buffer (0.05 M, pH 7.0). For STX analysis, the mobile phase was 1-heptane sulfonate (2 mM) in methanol (20%)-potassium phosphate buffer (0.05 M, pH 7.0). The TTX was detected by mixing the elute with 3N NaOH at a ratio of 1:1, followed by heating at 99 °C for 0.4 min, and monitoring the fluorescence at 505 nm with 381 nm excitation. In the case of GTX and STX analysis, the elutes were mixed with an equal volume of periodate reagent. The periodate reagent was according to the methods of Nagashima et al. (1987). In gas chromatography-mass spectroscopy (GC–MS), a part (50 MU) of purified toxin from each gastropod species was mixed with 2 ml of 2N NaOH, and heated in a boiling water bath for 45 min. After cooling, the solution was examined for the UV absorption spectrum characteristics to the C_9-base, 2 amino-6-hydroxymethyl-8-hydroxyquinazoline that should have been derived from TTX and/or related substances, if present. The above hydrolyzate was adjusted to pH 4 with 1N HCl and extracted three times with 5 ml each of 1-butanol. The extracts were combined, freeze-dried, trimethylsilylated (Narita et al., 1981), and subjected to GC–MS on a Shimadzu QP-2000A. In liquid chromatography-mass spectroscopy (LC–MS), a part (50 MU) of purified toxin from each gastropod species was used. In this method, combined HPLC–MS is performed using Agilent 1100 series LC/MSD Trap system coupled to a mass spectrometer. HPLC system is equipped with an ZORAX, 300SB-C3, ID 4.6 × 150 mm column. The mobile phase for TTX analysis was 1% acetonitrile, 10 mM TMA, 10 mM ammonia formate (pH = 4.0, flow rate 0.4 ml/min).

Each toxin extracted from *O. miniacea, O. mustelina* and *O. nirasei* was purified by Bio-Gel P-2 column chromatography, the amount and specific toxicity of the toxin obtained were 11.4 mg and 67 MU/mg for *O. miniacea*, 4.4 mg and 56 MU/mg for *O.mustelina*, and 6.5 mg and 54 MU/mg for *O. nirasei*, respectively. With HPLC (Fig. 1), the toxin from each gastropod species afforded three signals, which had the same retention (13.1, 14.8 and 16.9 min) as those of TTX, 4-*epi* TTX and anh-TTX, respectively. After alkali degradation, the absorption maximum of each gastropod toxin appeared at around 274 nm, indicating the presence of C_9-base specific to TTX or related substance. The trimethylsilyl (TMS) derivative from the toxin gave rise to ion peaks at *m/z* 407, 392, and 376 at the same retention time as the TMS derivative from authentic TTX. Both TMS derivatives displayed a parent peak at *m/z* 407, a base peak at *m/x* 392, and a fragment peak at *m/z* 376 (Fig. 2). In the LC–MS, a protonated molecular ion peak $(M + H)^+$ appeared at *m/z* = 320 showing the molecular weight of authentic TTX of 319 (data not show). Judging from these data, the causative agent of this food poisoning incident due to ingesting three new gastropod species was identified as TTX and related substances. The toxin did not exhibit any PSP peak with HPLC, GC–MS and LC–MS analysis.

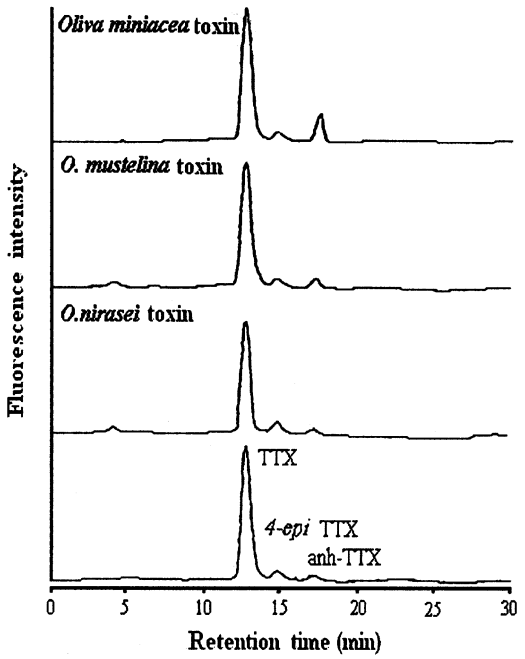

Fig. 1. HPLC of *Oliva miniacea*, *O. mustelina* and *O. nirasei* toxins, along with authentic TTX, 4-*epi* TTX and anh-TTX.

Tetrodotoxin was originally discovered and isolated from puffer fish (Yokoo, 1950). However, this toxin has also been isolated from or detected in the following marine gastropods: *Charonia sauliae* (Narita et al., 1981), *Babylonia japonica* (Noguchi et al., 1981; Yasumoto et al., 1981), *Tutufa lissostoma* (Noguchi et al., 1984), *Zeuxis siquizorensis* (Narita et al., 1984), *Niotha clathrata* (Jeon et al., 1984), and *Natica lineata* (Hwang et al., 1990a). Now, we have shown for the first time that the Olive shells (*O. miniacea*, *O. mustelina* and *O. nirasei*) also contain

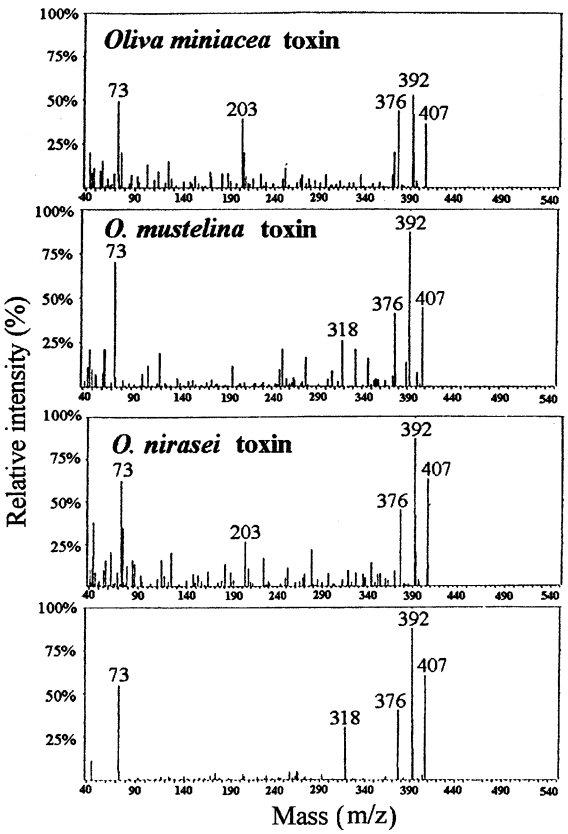

Fig. 2. Mass spectra of the trimethylsilylated (TMS) derivative of alkali-hydrolyzed toxin from *Oliva miniacea*, *O. mustelina* and *O. nirasei*, along with authentic TTX (the lowest).

TTX and related substances. As mentioned earlier, those specimens collected from southern Taiwan showed lethal toxicity in mice, and TTX occurred in the edible portion only. The olive shells live in sand deeply, therefore it is not easy to capture. Usually, people cannot take the gastropods as food, meanwhile the yield is low and does not sale in the market, so we do not find the species to be toxic, in our previous experiments.

The amount of TTX in these three gastropods ranged from 16–363 MU/specimen. The minimum lethal dose of TTX for human on oral administrarion is assumed to be 10,000 MU (Tani, 1945). If the consumer ingests more than 1000 MU of TTX, clinical symptoms will appear. Hence, the victim has ingested more than three specimens of these gastropods.

Yamamori and Nakamura (1988) reported that TTX stimulated the gustatory nerve of fish in fairly low concentration and fish did not eat a diet containing TTX. The localization of TTX in skin of brackishwater puffer (*Tetradon steindachnerion*) have been demonstrated (Tanu et al., 2002) and Tsuruda et al. (2002) noted that the newt possesses TTX secreting glands in the skin. The gastropod *Natica lineata* contained high amount of toxin in the muscle and could excrete toxin as a defense agent (Hwang et al., 1990b). According to the results of this study, *O. miniacea*, *O. mustelina* and *O. nirasei* contained TTX in the muscle, they may secrete TTX as a defense agent when they encounter predators, or as a paralyzing agent when they attack prey.

It was reported that TTX-containing animals may absorb and accumulate TTX and related substance produced by bacteria (Noguchi et al., 1987; Simidu et al., 1987; Hwang et al., 1989). Besides bacteria, poisonous starfish may also contribute to the TTX source of gastropod (Maruyama et al., 1984). To clarify the problem of toxin source, the origin of TTX in *O. miniacea*, *O. mustelina* and *O. nirasei* is currently being investigated.

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