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Tetrodotoxin in several species of xanthid crabs in southern Taiwan

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Abstract

Tetrodotoxin was detected by tetrodotoxin (TTX) bioassay in 28 specimens of six species of xanthid crabs collected from Tunkang, southern Taiwan, in May 2004. The average toxicity of crab specimens was 347 ± 276 (means ± SD) mouse units (MU) for *Demania cultripes*, 59 ± 17 MU for *Demania toxica*, 324 ± 114 MU for *Demania reynaudi*, 214 ± 65 MU for *Lophozozymus incisus*, 184 ± 85 MU for *Lophozozymus pictor* and 611 ± 471 MU for *Atergatopsis germaini*. Each toxin of the six species of crabs was extracted with acidic methanol, cleansed using a C18 solid-phase extraction (SPE) column, filtered through a microcentrifuge filter and determined by LC-MS and GC-MS. The recovery of TTX was above 82.9% and the detection limit was 0.005 μg with LC-MS analysis. A linear equation was obtained between 0.03 and 3.0 μg of TTX. Analyses by LC-MS and GC-MS showed that these crab species all contained TTX. The paralytic shellfish poisons were not found. Among samples, *D. cultripes*, *D. toxica* and *L. incisus* are the first to be recorded as toxic in Taiwan.

Keywords: Xanthid crab; Tetrodotoxin; Toxicity; LC-MS; GC-MS

1. Introduction

The xanthid crabs *Zosimus aeneus*, *Atergatis floridus* and *Platypodia granulosa* from the Indo-West Pacific region are known to contain potent neurotoxins, including paralytic shellfish poison (PSP) and/or tetrodotoxins (TTX) (Konosu, Inoue, Noughi, & Hashimoto, 1968; Koyama, Noguchi, Ueda, & Hashimoto, 1981; Noguchi et al., 1983). In addition, palytoxin has been reported to be present in *Lophozozymus pictor* and *Demania* spp. (Alcala, Alcala, Garth, Yasumura, & Yasumoto, 1988; Yasumoto et al., 1986a). In Taiwan, a weak toxicity has been reported to occur in Taiwanese crabs: *Z. aeneus*, *L. pictor*, *Atergatopsis germaini*, *A. floridus*, *Demania reynaudi* and *Xanthias lividus*. Among them, all toxic crabs contain TTX as the major toxin and PSP as the minor one, except for *A. germaini* which contains mainly PSP as the major toxin and TTX as the minor toxin (Hwang, Tsai, Chai, & Jeng, 1996; Hwang & Tsai, 1999; Tsai, Hwang, Chai, & Jeng, 1995, 1996, 1997, 2002).

Recently, a food poisoning incident involving one victim in Tungkang, southern Taiwan in February 2002, was reported. The causative gastropods were *Oliva miniacea*, *Oliva mustelina* and *Oliva nirasei*, which contained moderate amounts of TTX (Hwang, Tsai, Lu, & Hwang, 2003). Another food poisoning incident involving 5 victims (2 deaths) also occurred in the same place in April 2004. The causative gastropod was *Nassarius glans* which also contained a high amount of TTX

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(Hwang et al., 2004). Following these incidents, an extensive screening on the animals in benthos collected from the same coastal waters, using TTX bioassay, indicated that six species of xanthid crab were toxic. This study was to determine the toxicity of each toxic xanthid crab and to identify their toxic component by using high performance liquid chromatography (HPLC), liquid chromatography-mass spectrometry (LC-MS) and gas chromatography-mass spectrometry (GC-MS).

2. Materials and methods

2.1. Materials

The crab species, *Demania cultripes* (8 specimens), *Demania toxica* (2 specimens), *D. reynaudi* (5 specimens), *Lophozozymus incisus* (7 specimens), *L. pictor* (3 specimens) and *A. germaini* (3 specimens), were caught at 40–50 m in depth from 80 miles off the coast at Tungkang, southern Taiwan with crab cages, by fishing boat, in May 2004. The crabs were delivered live to our laboratory and dissected into appendage, cephalothorax and viscera to determine the anatomical distribution of toxicity.

2.2. Assay method for toxicity

The respective parts of each specimen were homogenized and examined for toxicity by the mouse assay method for TTX (Hwang & Jeng, 1991). Toxicity was expressed in mouse units (MU), where 1 MU is defined as the amount of toxin required to kill 20 g Institute of Cancer Research (ICR) strain male mice in 30 min after intraperitoneal injection.

2.3. Purification of toxins

After toxicity examination, the residues were combined, homogenized for 5 min with three volumes of 1% acetic acid in methanol, and centrifuged (3000*g*, 15 min). The operation was repeated twice. The supernatants were combined and concentrated under reduced pressure at 45 °C. The methanol extract was passed through a C18 solid-phase extraction (SPE) column (1000 mg, Zorax, Palo Alto, CA, USA), previously regenerated with 10 ml methanol and equilibrated with 10 ml water. Toxin was collected immediately and eluted by 10 ml 0.5% acetic acid. The eluates was freeze-dried, dissolved in 2 ml 0.5% acetic acid and filtered through a 3000 MW cut-off Ultrafree microcentrifuge filter (Micron YM-3, Millipore, Waters, MA). The filtrate was freeze-dried and dissolved in a small amount (1 ml) of water and submitted to subsequent analyses. Authentic TTX, 4-epi-tetrodotoxin (4-epi TTX) and anhydrotetrodotoxin (anh-TTX) were obtained from Wako Pure Chemical Industries (Tokyo, Japan). Authentic GTX-1-4, saxitoxin (STX) and neoSTX obtained from the purple clam *Soletellina diphos* and the crab *Z. aeneus* (Daigo et al., 1985; Hwang, Noguchi, Nagashima, Liao, & Hashimoto, 1987) are used as reference standards.

2.4. Liquid chromatography-mass spectrometry analysis for TTX

This method was modified from that of Tsuruda et al. (2002) and combined LC-MS was performed using an Agilent (Palo Alto, CA, USA) Model 1100 series LC/MSD Trap system coupled to a mass spectrometer with a positive ion electrospray ionization (ESI) interface. The electrospray interface was typically operated using the following settings: fragmentor, 120 V; nebulizer, N_2 (35 psi); drying gas, N_2 (12 l/min, 350 °C); V-cap, 3000 V. The LC system was equipped with a Insertsil ODS-3 column (5 μm, I.D. 2.1 × 150 mm, GL Science) at room temperature. The injection volume was 5 μl. The mobile phase for TTX analysis was 30 mM heptafluorobutyric acid in 1 mM ammonia acetate buffer (pH 5.0, flow rate 0.5 ml/min).

2.5. Standard curve, detection limit and recovery of LC-MS for TTX

The standard curve of TTX was prepared from 0.03, 0.15, 0.30, 1.5 and 3.0 μg, and peak area (y) vs. amount of TTX (x) was plotted. The determination was done in triplicate ($n = 3$). Data for the standard curve were subjected to linear regression analysis. The detection limit was evaluated according to the ratio of signal to noise (S/N); 3 was as the minimum. The treated samples were prepared by dissolving approximately 10, 50 and 100 μg of TTX in the sample (g) of toxic crab *D. cultripes*. Each spiked concentration was analyzed in triplicate, including a blank test to evaluate the average recoveries. The spiked samples and blank samples without the TTX standard, were extracted with methanol, centrifuged, passed through the SPE column, and filtered through microcentrifuge filters as described above. The samples were then analyzed by LC-MS.

2.6. Gas chromatography-mass spectrometry analysis for TTX

A small amount of each crab toxin was dissolved in 2 ml of 3 M NaOH and heated in a boiling water bath for 45 min to obtain the C_9 base derivative (2-amino-6-hydroxymethyl-8-hydroxyquinazoline) of TTX and/or related substances. The acidity of the above hydrolysate was adjusted to pH 4 with 1 M HCl and extracted three times with 5 ml of 1-butanol. The extracts were combined and evaporated to dryness. Then the trim-

ethylsilylation was applied and the products were subjected to GC-MSD on the Agilent (Palo Alto, CA, USA) Model 6890 series. The injector temperature was set at 180 °C. A column (BP1, 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 carrier gas used was He gas at a flow rate of 4 ml/min. The ionizing voltage was kept at 70 eV at an ion source temperature of 200 °C. Scanning was carried out in a mass range of *m*/*z* 46–560 at 3 s intervals. The injection volume was 1 μl in this study.

2.7. High-performance liquid chromatography analysis for PSP

High-performance liquid chromatography was performed on a reversed-phase column (Merck LiChrospher 100 RP-18, 4 mm I.D. × 25 cm; E. Merck,

Table 1
Anatomical distribution of toxicity in specimens of six species of xanthid crabs collected from Tungkang, southern Taiwan in May 2004

Specimens	Body weight (g)	Carapace length (mm)	Appendages		Cephalothorax		Viscera		Total toxicity (MU/specimen)
			Weight (g)	Toxicity (MU/g)	Weight (g)	Toxicity (MU/g)	Weight (g)	Toxicity (MU/g)	
Demania cultripes									
1	294.3	97	148.6	3.5	134.7	ND[a]	11.0	25	795
2	208.7	85	103.8	ND	92.5	5.3	12.4	25	800
3	211.3	86	112.7	ND	87.7	3.0	10.9	5.0	318
4	165.7	83	89.7	3.0	66.9	ND	9.1	5.9	323
5	72.3	64	36.2	ND	30.5	3.0	5.6	5.8	124
6	97.2	73	50.9	3.0	42.1	3.5	4.2	7.5	332
7	51.4	63	25.8	ND	22.2	ND	3.3	3.0	10
8	53.0	65	28.5	3.0	21.5	3.0	3.0	3.8	161
Means ± SD	144.2 ± 83.7	77 ± 12	74.7 ± 42.5	1.6 ± 1.6	62.4 ± 37.8	2.2 ± 1.9	7.2 ± 3.9	10.1 ± 8.7	347 ± 276
D. toxica									
1	38.6	58	20.7	3.1	15.8	ND	2.1	3.0	71
2	30.8	56	16.1	ND	13.2	3.0	1.5	5.0	47
Means ± SD	34.7 ± 5.5	57 ± 2	18.4 ± 3.3	1.6 ± 2.2	14.5 ± 1.8	1.5 ± 2.1	1.8 ± 0.4	4.0 ± 1.4	59 ± 17
D. reynaudi									
1	94.5	65	55.4	3.0	30.9	3.0	3.2	3.5	270
2	102.6	67	62.5	3.0	35.4	3.0	4.6	9.8	339
3	47.7	60	25.5	4.3	19.1	4.5	3.1	3.6	207
4	141.6	78	96.5	3.1	41.9	4.5	3.2	7.2	511
5	92.6	64	65.0	3.3	26.1	3.0	1.5	3.0	297
Means ± SD	95.8 ± 33.4	67 ± 7	61.0 ± 25.3	3.3 ± 0.6	30.7 ± 8.7	3.6 ± 0.8	3.1 ± 1.1	5.4 ± 3.0	324 ± 114
Lophozozymus incisus									
1	56.8	64	29.0	3.0	21.6	ND	6.2	8.4	139
2	57.8	67	28.4	6.5	24.1	3.9	5.3	4.0	300
3	77.9	70	42.4	3.5	30.9	3.0	4.6	7.0	273
4	61.1	63	33.5	4.0	22.9	3.0	4.7	5.2	227
5	90.1	89	42.1	ND	40.5	3.0	7.5	6.4	170
6	82.4	85	36.0	ND	42.1	3.0	4.3	3.0	140
7	138.2	98	68.9	3.2	60.1	ND	9.2	3.0	248
Means ± SD	80.6 ± 28.5	77 ± 14	40.0 ± 13.9	2.9 ± 2.3	34.6 ± 14.0	2.3 ± 1.6	6.0 ± 1.8	5.3 ± 2.1	214 ± 65
L. pictor									
1	80.5	71	36.9	3.1	41.1	3.0	2.5	5.2	247
2	72.0	75	28.7	3.0	40.2	3.0	3.1	3.5	218
3	63.7	70	26.4	3.0	35.2	ND	2.1	3.5	87
Means ± SD	72.1 ± 8.4	72 ± 3	30.7 ± 5.5	3.0 ± 0.1	38.8 ± 3.2	2.0 ± 1.7	2.6 ± 0.5	4.1 ± 1.0	184 ± 85
Atergatopsis germaini									
1	82.2	78	36.0	25	40.6	3.1	5.6	23	1148
2	77.9	79	35.9	6.8	37.3	3.0	4.7	5.0	380
3	69.2	58	35.3	5.0	29.5	3.0	4.4	8.0	300
Means ± SD	76.4 ± 6.6	71.7 ± 11.8	35.7 ± 0.4	12.3 ± 11.1	35.8 ± 5.7	3.0 ± 0.1	4.6 ± 0.6	11.9 ± 9.4	611 ± 471

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

Darmstadt, Germany). The mobile phase for gonyautoxin (GTX) analysis was sodium 1-heptanesulfonate (2 mM) in methanol (1%)–potassium phosphate buffer (0.05 M, pH 7.0). For saxitoxin (STX) analysis, the mobile phase was 1-heptanesulfonate (2 mM) in methanol (20%)–potassium phosphate buffer (0.05 M, pH 7.0) (Nagashima, Maruyama, Noguchi, & Hashimoto, 1987). The eluates were mixed with an equal volume of periodate reagent. The fluorogenic reaction was performed at 65 °C for 0.7 min. The fluorescence intensity was measured at 388 nm with 344 nm excitation (Nagashima et al., 1987).

3. Results

The toxicities of the *D. cultripes*, *D. toxica*, *D. reynaudi*, *L. incisus*, *L. pictor* and *A. germaini* specimens are shown in Table 1. All of the specimens examined were toxic. The respective values of the mean and the highest toxicities were 347 ± 276 MU/specimen (means ± SD) and 800 MU/specimen in *D. cultripes*, 59 ± 17 MU/specimen and 71 MU/specimen in *D. toxica*, 324 ± 114 MU/specimen and 511 MU/specimen in *D. reynaudi*, 214 ± 65 MU/specimen and 300 MU/specimen in *L. incisus*, 184 ± 85 MU/specimen and 247 MU/specimen in *L. pictor*, and 611 ± 471 MU/specimen and 1148 MU/specimen in *A. germaini*.

In LC-MS analysis for TTX, the total ion chromatogram (TIC) of standard TTX and related compounds showed three peaks with retention times of 2.56, 3.35 and 3.70 min (Fig. 1). The first two peaks, with retention times of 2.56 and 3.35 min, exhibited the protonated molecular ion peak $(M + H)^+$ appearing at $m/z = 320$ of authentic TTX and 4-*epi* TTX, respectively, whereas the last peak with a retention time of 3.70 min, exhibited the protonated ion peak $(M + H\text{-}H_2O)^+$ appearing at $m/z = 302$ of authentic anh-TTX. In LC-MS for toxic crabs analysis, each toxin of six crabs revealed one peak which had the same retention time (2.56 min) as that of authentic TTX (Fig. 2). In addition, the toxin of *D. cultripes* also contained another peak which had the same retention time (3.70 min) as that of anh-TTX (Fig. 2).

A standard curve of TTX was prepared in the range of 0.03–3.0 μg. The correlation coefficient (r^2) and linear regression equation for TTX were as follows: $y = 11302x + 6075$ ($r^2 = 0.9875$). The detecting limit was 0.005 μg of TTX. The recovery of TTX in this study is shown in Table 2. The recovery of TTX spiked with three amounts (10, 50, 100 μg/g) into the specimens of *D. cultripes* was in the range of 82.9–88.6%. The average recovery of TTX was 86.3% (Table 2). Hence, the procedure of C18 SPE column, microcentrifuge filters and LC-MS is a good, simple and rapid method for detecting the amount of TTX in the specimen of toxic crab.

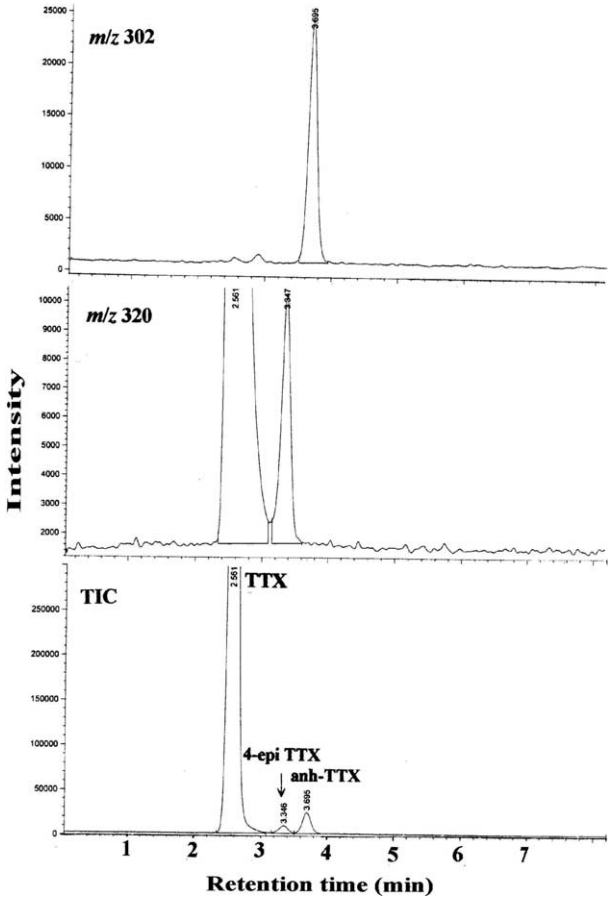

Fig. 1. LC-MS mass chromatograms in selected ion monitored (SIM) mode at *m/z* 302, 320 and TIC for authentic TTX, 4-*epi* TTX and anh-TTX.

Furthermore, all samples exhibited the C_9-base derivative of TTX by using GC-MS method. The GC-MS chromatograms and mass spectrum of the trimethylsilyl (TMS) derivative from the authentic TTX gave rise to three ion peaks at *m/z* 407, 392, and 376 at the retention time (21.0 min) (Fig. 3). The GC-MS mass spectrum of TMS derivative from the toxin of six crabs displayed a parent peak at *m/z* 407, a base peak at *m/z* 392, and a fragment peak at *m/z* 376 at the same retention time (21.0 min) as authentic TTX (Fig. 4).

The toxin of all toxic crabs did not exhibit any PSP peak in HPLC analysis. It was concluded from the results of LC-MS and GC-MS analyses that the toxins of six species of xanthid crabs were only composed of TTX. In addition, the toxin of *D. cultripes* also contained a small amount of anh-TTX.

4. Discussion

The weak toxicity of six species of xanthid crabs *D. cultripes*, *D. toxica*, *D. reynaudi*, *L. incisus*, *L. pictor*

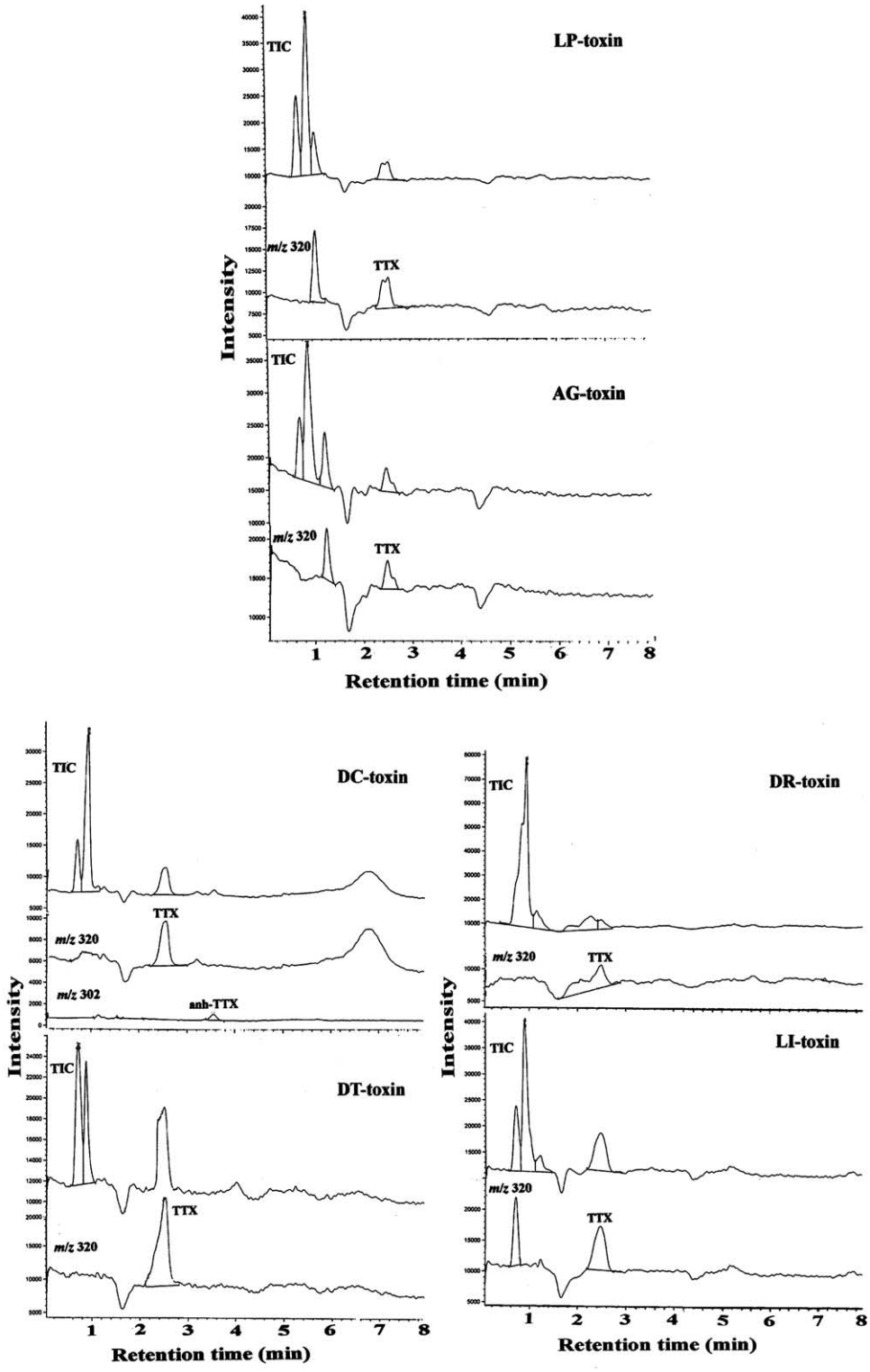

Fig. 2. LC-MS mass chromatograms in SIM mode at TIC and *m/z* 320 for crab toxins from *Demania cultripes* (DC-Toxin) (added SIM mode at *m/z* 302), *D. toxica* (DT-toxin), *D. reynaudi* (DR-toxin), *Lophozozymus incisus* (LI-toxin), *L. pictor* (LP-toxin) and *Atergatopsis germaini* (AG-toxin).

and *A. germaini* collected from Southern Taiwan was similar to that of *D. reynaudi* and *L. pictor*, while it was much lower than that of *A. germaini* collected from Keelung, northern Taiwan (Tsai et al., 1995, Tsai, Hwang, Chai, & Jeng, 1996, 1997). However, the toxicities of Philippine crabs, including *L. pictor*, *D. cultripes*, *D. reynaudi* and *D. toxica*, and a Singapore crab, *L. pictor*, are considerly higher when compared to those

Table 2
Recovery of TTX when spiked to a toxic crab sample, *Demania cultripes* and then treated with C18 SPE column, microcentrifuge filtration and LC-MS

Spiked concentration (μg/g)	Recovery (%)
100	88.6 ± 2.6
50	87.3 ± 2.8
10	82.9 ± 3.3
Means ± SD	86.3 ± 2.9

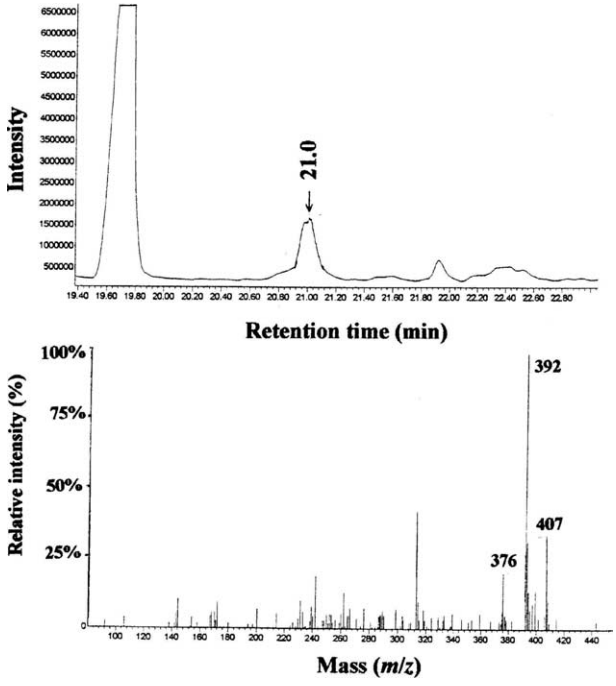

Fig. 3. GC-MS chromatograms (upper) and mass spectra (lower) of the TMS derivatives from authentic TTX. The retention time for TTX was 21.0 min.

of the same species examined in this work (Alcala & Halstead, 1970; Alcala et al., 1988; Lau et al., 1995; Yasumoto et al., 1986a). In this study, it was found that the six crabs, *D. cultripes*, *D. toxica*, *D. reynaudi*, *L. incisus*, *L. pictor* and *A. germaini*, collected from southern Taiwan contained mainly TTX, but no PSP. As reported previously, *A. germaini*, collected from Keelung, northern Taiwan was reported to contain PSP as the major toxin and TTX as the minor, whereas the profile of *L. pictor* and *D. reynaudi* toxin collected from the same place was mainly composed of TTX and GTX as the minor (Tsai et al., 1995, 1996, Tsai, Hwang, Chai, & Jeng, 1997). However, the crabs *D. reynaudi*, *D. cultripes* and *L. pictor*, in the Philippines, and *L. pictor*, in Singapore, were found to contain palytoxin, but toxin of *D. toxica* in the Philippines was unknown (Alcala & Halstead, 1970; Alcala et al., 1988; Lau et al., 1995; Yasumoto et al., 1986a). There were marked individual and regional variations in the toxicity and toxin compositions of these above xanthid crabs. This indicates that the crab toxin might be of exogenous rather than endogenous origin.

In our previous study, the five toxic crabs, *Z. aeneus*, *L. pictor*, *A. floridus*, *D. reynaudi* and *X. lividus*, collected from other areas of Taiwan were reported to contain TTX as the major toxin and PSP as the minor one (Hwang et al., 1996; Tsai et al., 1995, 1997, Tsai, Ho, Jeng, & Hwang, 2002). However, it was found that the six crabs collected from the southern coastal area of Taiwan in this study contained mainly TTX, but no PSP. The *A. floridus* specimens, living on reefs off Ishigaki Island, Okinawa Prefecture, contained mainly PSPs (Arakawa, Noguchi, & Onoue, 1995; Koyama et al., 1981; Yasumoto, Oshima, Hosaka, & Miyakoshi, 1981), whereas specimens inhabiting a small islet of Ishigaki Island and the Pacific coast of Kanto region contained predominantly TTX (Arakawa, Noguchi, Shida, & Onoue, 1994; Noguchi et al., 1983; Noguchi, Arakawa, Daigo, & Hashimoto, 1986a). Consequently, the toxic components of xanthid crabs were related to region. Further study is needed to elucidate this discrepancy. Except for *D. reynaudi*, *L. pictor* and *A. germaini*, three species, *D. cultripes*, *D. toxica* and *L. incisus*, were the first to be recorded as toxic in Taiwan.

In Taiwan, fishermen and coastal inhabitants may consume the larger crabs, *D. reynaudi*, *L. pictor*, *A. germaini*, *D. cultripes*, and *L. incisus*. The other crab *D. toxica* is not commonly used as food judged from the sizes of these specimens (less than 40 g). In all specimens tested, a specimen of *A. germaini* had the highest toxicity of about 1148 MU (Table 1), which is lower than the suggested lethal oral dose (10,000 MU) of TTX for humans. Nevertheless, people in coastal areas should be warned of the potential hazard of xanthid crabs to avoid either intentional or accidental consumption.

TTX was originally discovered and isolated from puffer fish (Yokoo, 1950). However, this toxin has also been isolated or detected in the following organisms: California newt (Mosher, Fuhrman, Buchwald, & Fisher, 1964), goby fish (Lin, Hwang, Shao, & Jeng, 2000; Noguchi & Hashimoto, 1973), atelopid toads (Kim, Brown, & Fuhrman, 1975), gastropods (Hwang, Chueh, & Jeng, 1990; Narita et al., 1981; Noguchi, Maruyama, Ueda, Hashimoto, & Harada, 1981) and xanthid crabs (Hwang & Tsai, 1999; Konosu et al., 1968; Koyama et al., 1981; Noguchi et al., 1983). The mechanism of the induction of toxicity in TTX-containing animals and the dynamic state of TTX have not been completely described. It is known that some intestinal bacteria of TTX-bearing crabs are endowed with a TTX-producing ability (Hwang & Tsai, 1999; Noguchi et al., 1986b; Yasumoto et al., 1986b). Do et al. (1991) also reported the production of TTX in actinomycetes isolated from

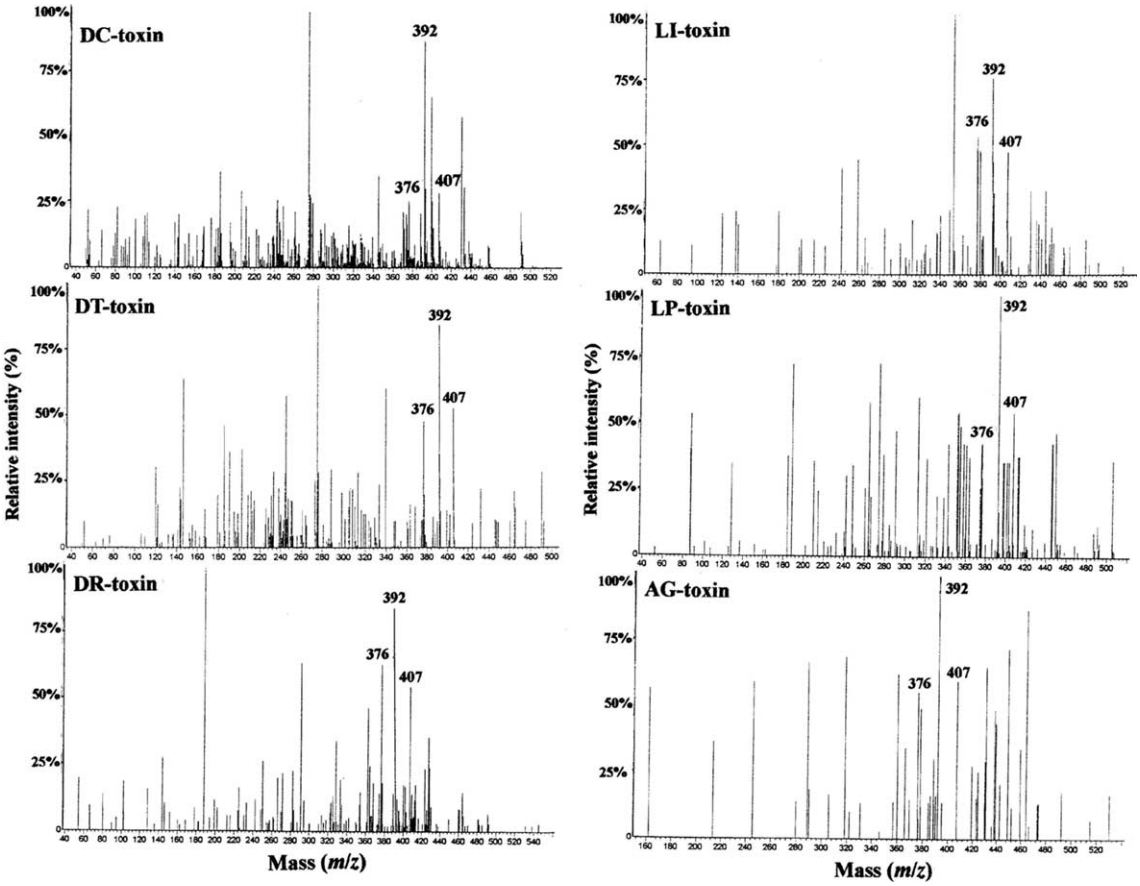

Fig. 4. Mass spectra of the trimethylsilylated (TMS) derivative of alkali-hydrolyzed crab toxins from *Demania cultripes* (DC-Toxin), *D. toxica* (DT-toxin), *D. reynaudi* (DR-toxin), *Lophozozymus incisus* (LI-toxin), *L. pictor* (LP-toxin) and *Atergatopsis germaini* (AG-toxin) in the retention time of 21.0 min for chromatogram.

marine sediments. Furthermore, the trumpet shell becomes toxic, mainly by feeding on TTX-containing starfish (Noguchi, Narita, Maruyama, & Hashimoto, 1982). We also indicated that the starfish *Astropecten scoparius* might mainly accumulate high amounts of TTX from the small gastropod *Umborium suturale* (Lin & Hwang, 2001). Therefore, the origin of TTX in the toxic xanthid crabs may be from toxic lower strata invertebrates such as gastropods (Lin & Hwang, 2001), starfish (Lin, Tsai, Lin, & Hwang, 1998; Tsai, Chao, Lin, Noguchi, & Hwang, 2004), flatworms (Miyazawa et al., 1986), ribbon worms (Ali et al., 1990; Asakawa, Toyoshima, Shida, Noguchi, & Miyazawa, 2000; Miyazawa et al., 1988), and/or TTX-producing bacteria inhabiting the intestines or the marine sediments.

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