Occurrence of Tetrodotoxin-related Toxins in the Gastropod Mollusk *Niotha clathrata* from Taiwan

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(Received March 28, 1991)

Paralytic toxicity was detected in the gastropod mollusk *Niotha clathrata* from Taiwan. The toxicity scores of specimens collected from Chiating, Kaohsiung Prefecture and Tungkang, Pingtung Prefecture in August 1989 were 13.1±5.9 and 7.9±4.0 (mean±S.E.) MU, respectively. The highest score was 107 MU at Chiating and 40 MU at Tungkang. The toxicity of specimens was not only detected in the digestive gland, but also detected in the other edible parts. The toxin was partially purified from toxic specimens of *N. clathrata* by ultrafiltration using a Diflo YM-2 membrane, followed by chromatography on a Bio-Gel P-2 column. Analyses by TLC, electrophoresis, and HPLC showed that the fraction I toxin of *N. clathrata* contained tetrodotoxin (TTX), and that the fraction II toxin was different from TTX and remained as an unidentified toxin.

Tetrodotoxin (TTX) and/or TTX-related compounds have been reported to occur in gastropod mollusks including the trumpet shell *Charonia sauliae*,1,2) the frog shell *Tutufa lissostoma*,3) the Japanese ivory shell *Babylonia japonica*,4,5) *Zeuxis siquijorensis*,6) and *Niotha clathrata*.7) Recently, we reported that the lined moon shell *Natica lineata*8) collected from Taiwan also contained TTX. During further extensive screening, we found that the gastropod *N. clathrata* collected from Taiwan also showed paralytic toxicity. Although the toxin component of *N. clathrata* have been identified as TTX in Japan, Taiwanese specimens have not been examined. This gastropod *N. clathrata* is consumed, and it is important to safeguard consumption of *N. clathrata* in Taiwan. We collected specimens of gastropod *N. clathrata*, and examined their toxicity. The gastropod was found to contain TTX and unidentified toxins.

**Materials and Methods**

**Materials**

About two hundred specimens of the gastropod *N. clathrata* were purchased from markets in Chiating, Kaohsiung Prefecture and in Tungkang, Pingtung Prefecture, South Taiwan in August 1989. These specimens were transported alive to our laboratory, and immediately dissected into the digestive gland and other edible parts.

**Assay Method for Toxicity**

Each part of each specimen was homogenized and examined for toxicity by modified mouse assay method for TTX.9) The dose-death time curve of authentic TTX was established by using ICR (Institute of Cancer Research) strain. Lethal potency 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 i. p. injection.

**Isolation of Toxin**

Specimens were combined, homogenized for 5 min with about three volumes of 1% acetic acid in methanol, and centrifuged (3,000 × g, 20 min). This operation was repeated two more times. The supernatants were combined, concentrated under reduced pressure at 40°C, and defatted with dichloromethane. The aqueous layer was concentrated and filtered through a Diaflo YM-2 membrane (Amicon), whose cut-off limit was 1,000 Da. The filtrate was applied to a Bio-Gel P-2 column (2 × 94 cm) which was developed with 0.03 M acetic acid. Toxic fractions were combined, freeze-dried, dissolved in a small amount of water, and submitted to the following analyses. Authentic TTX and anhydrotetrodotoxin (anh-TTX),10,11) which was obtained from the liver of the puffer *Fugu oblongus*, were used as reference standards.

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Thin-layer Chromatography

Thin-layer chromatography (TLC) was performed on 5 × 20 cm (thickness 2 mm) silica gel 60 F254 precoated plates (Merck) with two solvent systems, pyridine-ethyl acetate-acetic acid-water (15:5:3:6) and 1-butanol-acetic acid-water (2:1:1). Toxins were visualized as yellow fluorescent spots under a UV lamp (365 nm) after spraying the plate with 10% KOH and heating at 110°C for 10 min.

Electrophoresis

Electrophoresis was performed in 5 × 18 cm cellulose acetate strips (Chemetron) in 0.08 M Tris-HCl buffer (pH 8.7) under a constant current of 0.8 mA/cm for 1 h. Toxins were visualized as described in TLC.

High Performance Liquid Chromatography

High performance liquid chromatography (HPLC) was performed on a reversed phase column (YMC-Pack AM-314 ODS, 6 mm I. D. × 300 mm) with a mixture of 2 mM sodium 1-heptane sulfonate and 1% methanol in 0.05 M potassium phosphate buffer (pH 7.0) at a flow rate of 1 ml/min. The eluate was mixed with an equal volume of 3 N NaOH and heated in a reaction coil (Teflon tube, 3 mm I. D. × 10 m) at 99°C. The intensity of fluorescence was measured at 505 nm with 380 nm excitation.12)

Results and Discussion

Toxicity of N. clathrata

Toxicity of the gastropod N. clathrata specimens collected in Taiwan is shown in Tables 1 and 2, which indicated that twelve out of twenty specimens collected from Chiating, Kaohsiung Prefecture were toxic. The toxicity score was 13.1 ± 5.9 (mean ± S. E.) MU/specimen. The highest score was 107 MU/specimen. On the other hand, six out of ten specimens collected from Tungkang, Pingtung Prefecture were toxic. The toxicity score was 7.9 ± 4.0 (mean ± S. E.) MU/specimens and the highest score was 40 MU/specimen. Furthermore, it is noted that toxicity of specimens was not only detected in the digestive gland, but also detected in other edible parts.

It is reported that the toxicity score of N. clathrata specimens collected from Enshunada...
Sea was $5.1 \pm 2.2$ (mean \pm S. E.) MU/g edible part, but it was nontoxic in the specimens collected from Shimizu, Shizuoka Prefecture. Therefore, toxicity of *N. clathrata* specimens collected from Taiwan was higher than that of Japanese specimens.

### Table 2. Anatomical distribution of toxicity in *Niotha clathrata* specimens collected at Tungkang, Pingtung Prefecture in South Taiwan in August 1989

<table>
<thead>
<tr>
<th>Specimens No.</th>
<th>Total length (cm)</th>
<th>Total weight (g)</th>
<th>Digestive gland</th>
<th>Other edible parts</th>
<th>Total toxicity (MU)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>2.48</td>
<td>4.37</td>
<td>0.32 13</td>
<td>1.03 ND</td>
<td>4</td>
</tr>
<tr>
<td>2</td>
<td>2.21</td>
<td>2.58</td>
<td>0.21 ND</td>
<td>0.64 ND</td>
<td>0</td>
</tr>
<tr>
<td>3</td>
<td>2.45</td>
<td>4.23</td>
<td>0.52 ND</td>
<td>0.95 ND</td>
<td>0</td>
</tr>
<tr>
<td>4</td>
<td>2.30</td>
<td>2.77</td>
<td>0.25 ND</td>
<td>0.68 7</td>
<td>5</td>
</tr>
<tr>
<td>5</td>
<td>2.09</td>
<td>2.92</td>
<td>0.29 ND</td>
<td>0.56 ND</td>
<td>0</td>
</tr>
<tr>
<td>6</td>
<td>2.78</td>
<td>4.09</td>
<td>0.46 ND</td>
<td>0.98 ND</td>
<td>0</td>
</tr>
<tr>
<td>7</td>
<td>2.61</td>
<td>3.99</td>
<td>0.33 ND</td>
<td>1.09 17</td>
<td>19</td>
</tr>
<tr>
<td>8</td>
<td>2.46</td>
<td>3.46</td>
<td>0.18 13</td>
<td>0.78 ND</td>
<td>2</td>
</tr>
<tr>
<td>9</td>
<td>2.46</td>
<td>3.46</td>
<td>0.26 ND</td>
<td>0.78 11</td>
<td>9</td>
</tr>
<tr>
<td>10</td>
<td>2.17</td>
<td>2.63</td>
<td>0.28 ND</td>
<td>0.66 61</td>
<td>40</td>
</tr>
</tbody>
</table>

Mean $\pm$ S.E. 2.40 $\pm$ 0.07 3.45 $\pm$ 0.22 0.31 $\pm$ 0.03 2.6 $\pm$ 1.7 0.82 $\pm$ 0.06 9.6 $\pm$ 6.0 7.9 $\pm$ 4.0

*1,2* See footnotes of Table 1.

**Fraction I**

**Fraction II**

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Identification of *Niotha Clathrata* Toxin

The toxin was extracted and purified from about 180 specimens of *N. clathrata* by the procedures described above. The elution profile of the toxin in a Bio-Gel P-2 column chromatography is shown in Fig. 1. Two toxic fractions (fractions I and II) were obtained and their total toxicities were 42,000 and 14,000 MU, respectively. The specific toxicity calculated as TTX was 480 MU/mg for fraction I and 270 MU/mg for fraction II.

As shown in Fig. 2, the fraction I toxin exhibited three spots in TLC. One of the main spots...
coincided well with TTX in Rf values in two solvent systems used for detection. The fraction II toxin in N. clathrata exhibited one spot in TLC. The Rf value was 0.71 and 0.47 with pyridine-ethyl acetate-acetic acid-water (15:5:3:6) and 1-butanol-acetic acid-water (2:1:1), respectively.

Fig. 2. TLC of N. clathrata toxin (NC), along with authentic TTX and anh-TTX. Solvent systems: (A) pyridine-ethyl acetate-acetic acid-water (15:5:3:6); (B) 1-butanol-acetic acid-water (2:1:1). The plate was sprayed with 10% KOH.

Fig. 3. Cellulose acetate membrane electrophoresis of N. clathrata toxin (NC), along with authentic TTX and anh-TTX.

coincided well with TTX in Rf values in two solvent systems used for detection. The fraction II toxin in N. clathrata exhibited one spot in TLC. The Rf value was 0.71 and 0.47 with pyridine-ethyl acetate-acetic acid-water (15:5:3:6) and 1-butanol-acetic acid-water (2:1:1), respectively.

Fig. 4. HPLC of N. clathrata toxin (NC), along with authentic TTX and anh-TTX.
As shown in Fig. 3, the fraction I and fraction II of *N. clathrata* toxin presented four and two spots in electrophoresis, respectively. One of the four spots in fraction I was indistinguishable from TTX both in migration distance (6.4 cm) and in fluorescent color (yellow). The migration distance of the main spot of fraction II was 4.9 cm.

As shown in Fig. 4, the fraction I toxin contained one main peak which showed the same retention time (14.8 min) as that of TTX in HPLC, and this peak contained all lethal potency. Therefore, lethality of fraction I toxin was mainly composed of TTX. However, retention time of the highest peak in fraction II toxin was 12.2 min, which is different from those of TTX and anh-TTX.

In recent years, TTX-related substances along with TTX have been detected as minor components in puffer fish and other TTX-containing animals: e.g., 4-epi-TTX, anh-TTX, and tetrodine acid-like toxin in four species of Taiwan puffer fishes and a ribbon worm *Cephalothrix linearis*; and chiriquitoxin in the Costa Rican frog *Atelopus chiriquiensis*. We could not identify the fraction II toxin in this study. Further studies to identify the fraction II toxin are underway.

Acknowledgements

The authors express their sincere gratitude to Director C.-Y. Lai, Taiwan Museum, for identification of the gastropod species. This work was supported by a fund granted from National Science Council (Grant No. NSC 79-0409-B018-05), Republic of China.

References