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Occurrence of TTX in a Brackish Water Puffer "Midorifugu", Tetraodon nigroviridis, Collected from Thailand

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Screening tests were carried out on the toxicity of a brackish water puffer, Tetraodon nigroviridis, collected from Thailand through November, 1996 to May, 1998. Among the different tissues, the skin showed the highest toxicity scores, ranging from 49.2-286 MU/g, followed by muscle (5.4-11 MU/g), while liver and intestine mostly gave scores under 5 MU/g. The toxin was partially purified by three kinds of column chromatography consisting of activated charcoal, Bio-Gel P-2 and Bio-Rex 70. Analyses by HPLC, TLC, electrophoresis, 1H-NMR and LC/MS identified the purified toxin as tetrodotoxin.

Key words: brackish water puffer; Tetraodon nigroviridis; TTX; 1H-NMR; LC/MS; Thailand

Introduction

Puffer fish has been reported to possess a potent neurotoxin which consists mainly of tetrodotoxin (TTX), although the toxicity varies markedly depending on the geographical location1 and season21. TTX is accumulated by puffer fish and other TTX-bearing organisms through the food chain, in which bacteria are the primary origin3,4. Its pharmacological action is to block the sodium channel of biological membranes4.

Puffer fish causes the largest death toll from food poisoning in the world, especially in some Asian countries. In Thailand, there have been several reports6-11 on food poisoning, including some fatal cases. Most of them involve marine puffer, and a few involve freshwater species. TTX as a toxic principle in marine puffer and paralytic shellfish poison (PSP) or TTX in freshwater puffer of Thailand have been described7, 8, 10, 11, while brackish water puffer is still unexplored as regards lethal potency and toxin profile. Due to the increasing trend of puffer consumption in Thailand, it is desirable to elucldate the toxicity as well as the toxic principle of brackish water puffer. In this context, we collected some specimens of a brackish water puffer, Tetraodon nigroviridis, from Thailand, and examined the toxicity and toxic principle. The present paper deals with the findings.

Materials and Methods

Puffer fish specimens

Four hundred and forty-eight specimens of T. nigroviridis (Japanese name "midorifugu") were collected from the brackish water habitat of the Mekong River of Thailand in November, 1996 through May, 1998 and transported alive by air to our laboratory at Nagasaki University.

Screening test of toxicity

The specimens were immediately dissected into skin, muscle, liver and intestine. Each tissue was combined and minced and to a small portion of the mince (1-5 g) was added an equal volume of 0.1% acetic acid. The mixture was heated in a boiling water bath for 5 min and centrifuged at 11,000 g for 10 min. The supernatant thus obtained was examined for toxicity by
the standard assay method for TTX\textsuperscript{12}, since the toxin principle was suspected to be TTX. Lethal potency was expressed in mouse unit (MU), where one mouse unit is defined as the amount of toxin that kills an 18–20 g male mouse of the ddY strain in 30 min after i.p. injection.

**Partial purification of toxin**

The frozen remaining portions (355 g) of the minced tissues (skin, muscle, liver and intestine) of the “midorifugu” specimens collected in May, 1998 were pooled and extracted with 80% ethanol adjusted to pH 2 with HCl. The extract was then centrifuged at 5,700 g for 20 min. These steps were repeated twice for the residue. The supernatants were combined and defatted with an equal volume of dichloromethane, and the aqueous layer was concentrated to remove the residual dichloromethane. The resulting concentrate (20,700 MU) was loaded on a water-washed activated charcoal (Wako Pure Chemical Industries Ltd.) column (2.5 x 40 cm), which was washed with 1 L of water. The adsorbed toxin was eluted with 1 L of 1% acetic acid in 20% ethanol. The eluate (19,200 MU) was concentrated, freeze-dried and rechromatographed on the same column with the same eluent. The toxin (17,400 MU) obtained was further purified on a column (0.8 x 90 cm) of Bio-Rex 70 (Bio-Rad Laboratories; H\textsuperscript{+} form) by means of a two-step linear gradient of 0–0.05 and 0.5–1.5 mol/L acetic acid. Most of the toxin was eluted (16,100 MU) with the gradient solution of 0–0.05 mol/L acetic acid as a single fraction. The toxin was concentrated, freeze-dried and rechromatographed in the same manner to afford the partially purified toxin (13,300 MU, 3 mg).

**High-performance liquid chromatography**

Reversed-phase high-performance liquid chromatography (HPLC) was performed on a Waters LC Module 1 HPLC system using a LiChroCART Superspher RP-18 (e) column (4 x 250 mm, Merck) for analysis of both TTX and PSP. For TTX, 10 mmol/L ammonium phosphate buffer (pH 7.0) containing 2 mmol/L heptanesulfonic acid (HSA) as an ion-pairing reagent was used as a mobile phase at a flow rate of 0.8 mL/min. The eluate was continuously mixed with an equal volume of 4 mol/L NaOH and heated in a reaction coil at 110°C. The fluorescence intensity of fluorophors formed was measured at 505 nm with 384 nm excitation. For analysis of PSP, the following two mobile phases were used: (I) 10 mmol/L ammonium phosphate buffer (pH 7.3) with 2 mmol/L HSA for gonyautoxins (GTXs) and (II) 4% acetonitrile–30 mmol/L ammonium phosphate buffer (pH 7.3) with 2 mmol/L HSA for saxitoxins (STXs)\textsuperscript{14,15}. The eluate was mixed with both of 50 mmol/L periodic acid (flow rate 0.4 mL/min) and 0.2 mol/L KOH containing 1 mol/L ammonium formate in 50% formamide (flow rate 0.4 mL/min) and heated at 65°C for 1.5 min\textsuperscript{16}. The fluorophors formed were monitored at 392 nm with 336 nm excitation. The toxin was iden-

<table>
<thead>
<tr>
<th>Month of collection</th>
<th>Number of specimens</th>
<th>Average total length (cm)</th>
<th>Average body weight (g)</th>
<th>Toxicity (MU/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nov. '96</td>
<td>10</td>
<td>2.8</td>
<td>1.9</td>
<td>133</td>
</tr>
<tr>
<td>Feb. '97</td>
<td>23</td>
<td>3.7</td>
<td>2.1</td>
<td>49.2</td>
</tr>
<tr>
<td>Mar. '97</td>
<td>22</td>
<td>3.1</td>
<td>1.7</td>
<td>212</td>
</tr>
<tr>
<td>Jun. '97</td>
<td>32</td>
<td>3.2</td>
<td>1.1</td>
<td>286</td>
</tr>
<tr>
<td>Jul. '97</td>
<td>61</td>
<td>3.9</td>
<td>2.3</td>
<td>262</td>
</tr>
<tr>
<td>May '98</td>
<td>300</td>
<td>3.2</td>
<td>1.2</td>
<td>197</td>
</tr>
</tbody>
</table>

Mean±SD 3.3±0.4  1.7±0.5  189.9±87.2  7.8±2.0  3.3±1.0  3.9±3.5

Table 1. Toxicity of *Tetraodon nigroviridis* Collected from Thailand
Thin-layer chromatography (TLC)

TLC was performed on silica gel-60 F_{254} pre-coated plates (Merck), using two solvent systems of pyridine-ethyl acetate-acetic acid-water (15:5:3:4) and 3-butanol-acetic acid-water (2:1:1). After the run, the plate was sprayed with 10% KOH or 1% H_{2}O_{2}. The toxin was visualized under UV light (365 nm).

Electrophoresis

Electrophoresis was conducted on 5×18 cm cellulose acetate membranes (Chemetron) using 0.08 mol/L Tris-HCl buffer (pH 8.7) under a constant current of 0.8 mA/cm width for 30 min. The toxin was detected as in TLC.

1H-NMR spectrum

"Midorifugu" toxin was dissolved in 0.6 mL of 4% CD_{3}COOD (purity of 99.5%) in D_{2}O and subjected to 1H-NMR analysis with a VARIAN UNITY plus 500 spectrometer at 500 MHz.

Mass spectral analysis

The purified toxin was submitted to LC/MS analysis on a Hitachi M-1000 equipped with a HPLC system (L-6200). For HPLC, an ODS-3 (1.5×150 mm) column with the mobile phase of 50% acetonitrile (flow rate 70 μL/min) was used.

Results and Discussion

Toxicity scores

The results of toxicity examination of different organs in "midorifugu" are shown in Table 1.
The skin exhibited the highest toxicity score, with the maximum level (286 MU/g) in June, 1977, followed by muscle (5.4–11 MU/g). These findings coincide well with those for two species of Thai fresh water puffer fish. Toxicity scores of the liver and intestine of "midorifugu" were always under 5 MU/g, except for that of the intestine in November (11 MU/g). The skin of T. nigroviridis showed seasonal variation of toxicity. The highest level of toxicity score was monitored in June (286 MU/g), followed by July (262 MU/g), while the lowest was in February (49.2 MU/g). The increased level of toxicity through June to July might be due to a higher metabolic rate of "midorifugu" in the hot season, resulting in more ingestion of the dietary toxic organisms available in that period. The toxicity score of skin in March (212 MU/g) was similar to that in May (197 MU/g), while the specimens of November showed a relatively low level of toxicity (133 MU/g).

The minimal lethal dose of TTX to humans has been estimated to be 10,000 MU. On the basis of that, 10 MU (2 μg TTX) per 1 g of edible portion was regarded as a suitable criterion to judge the acceptability of puffer fish as food. In this context, people might be advised not to ingest the whole body of "midorifugu" throughout the year, since its skin was recognized to be a toxic part.

**Toxin analyses**

The HPLC pattern of the purified T. nigroviridis toxin is shown in Fig. 1. The toxin clearly featured a peak whose retention time (18.4 min) coincides with that of authentic TTX. No peak corresponding to the authentic STXs and GTXs was observed (Fig. not shown).

In TLC analysis, as shown in Fig. 2, the "midorifugu" toxin was visualized by KOH as one fluorescent yellow spot with the same Rf value as authentic TTX in two solvent systems. The Rf values were 0.71 and 0.50 with pyridine:ethyl acetate: acetic acid: water and 3-butanol: acetic acid: water and 3-butanol: acetic acid.

The 1H-NMR Data of Authentic TTX and T. nigroviridis Toxin

<table>
<thead>
<tr>
<th>H (Carbon No.*)</th>
<th>Authentic TTX**</th>
<th>T. nigroviridis toxin</th>
</tr>
</thead>
<tbody>
<tr>
<td>H (4)</td>
<td>5.49 (9.9)</td>
<td>5.41 (9.4)</td>
</tr>
<tr>
<td>H (4a)</td>
<td>2.34 (9.5)</td>
<td>2.33 (9.4)</td>
</tr>
<tr>
<td>H (5)</td>
<td>4.25</td>
<td>4.25</td>
</tr>
<tr>
<td>H (7)</td>
<td>4.08</td>
<td>4.08</td>
</tr>
<tr>
<td>H (8)</td>
<td>4.29</td>
<td>4.29</td>
</tr>
<tr>
<td>H (9)</td>
<td>3.96</td>
<td>3.96</td>
</tr>
<tr>
<td>2 H (11)</td>
<td>4.02</td>
<td>4.02</td>
</tr>
<tr>
<td>(11)</td>
<td>4.04</td>
<td>4.04</td>
</tr>
</tbody>
</table>

* See Fig. 4.
** Reference 13

Chemical shifts are expressed as ppm. Coupling constants in parenthesis are given as Hz.

The HPLC pattern of the purified T. nigroviridis toxin is shown in Fig. 1. The toxin clearly featured a peak whose retention time (18.4 min) coincides with that of authentic TTX. No peak corresponding to the authentic STXs and GTXs was observed (Fig. not shown).

In TLC analysis, as shown in Fig. 2, the "midorifugu" toxin was visualized by KOH as one fluorescent yellow spot with the same Rf value as authentic TTX in two solvent systems. The Rf values were 0.71 and 0.50 with pyridine: ethyl acetate: acetic acid: water and 3-butanol: acetic acid.
acid: water, respectively. As shown in Fig. 3, the
toxin also showed one fluorescent (yellow) spot
with Rm of 0.62 in electrophoresis, as did authen-
tic TTX.

The $^1$H-NMR spectral data of the toxin are
shown in Table 2. Its proton chemical shifts ($\delta$value) are at 2.33 (d, $J=9.4$ Hz), 3.96 (s), 4.02 (s),
4.04 (s), 4.08 (s), 4.25 (s), 4.29 (s) and 5.41 ppm (d,
$J=9.4$ Hz). Comparing our data with those of
authentic TTX$^{13}$ (2.34 (d, $J=9.5$ Hz), 3.96 (s),
4.02 (s), 4.04 (s), 4.08 (s), 4.25 (s), 4.29 (s) and 5.49
ppm (d, $J=9.9$ Hz)) the signals at 2.33, 3.96, 4.02,
4.04, 4.08, 4.25, 4.29 and 5.41 ppm can be assig-
and C4-H, respectively.

The spin-spin coupling constant of 9.4 Hz ob-
served at C4a-H in this toxin agrees with that (9.4
Hz) of C4-H (refer to Fig. 4), which indicates
coupling of these protons, as seen in the spec-
trum of authentic TTX (Table 2).

Chromatograms and mass spectra in LC/MS
analysis are also shown in Fig. 5. In the MS, a
protonated molecular ion peak (M+H)$^+$ appea-
red at m/z=320, which coincides well with the
corresponding data of authentic TTX$^{13}$.

From the above toxin analysis it was un-
ambiguously concluded that the $T$. nigroviridis
toxin is TTX. It is generally accepted that TTX
is accumulated in TTX-bearing animals through
the food chain, starting from bacteria. This may
also be the case in freshwater and brackish
water puffer.

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analysis of the “midorifugu” toxin.

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