Screening of Tetrodotoxin and Its Derivatives in Puffer-related Species

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Attempts were made to detect tetrodotoxin (TTX) and its derivatives in the following fish species which are phylogenically related to the family Tetraodontidae: spiny puffer “*harisenbon*” *Diodon holacanthus*, sunfish “*manbou*” *Mola mola*, “*uchiwafugu*” puffer *Triodon bursarius*, “*kawahagi*” filefish *Stephanolepis cirrhifer*, and “*umazurahagi*” filefish *Navodon modestus*. The liver of each species did not show toxicity (<5 MU/g) when assayed by the official method using mice. The liver was extracted with acidic methanol, and the TTX fraction was separated from the extract by the method consisting mainly of ultrafiltration and gel chromatography.

In UV, HPLC, and GC-MS analyses, TTX fraction from spiny puffer showed anhydroTTX, in addition to a small amount of TTX, which coincided with the phylogenically closest relationship of this species to the family Tetraodontidae.

Recent progress in analytical techniques for tetrodotoxin (TTX) and its derivatives (collectively abbreviated TTXs below) makes it possible to detect them at a much lower concentration than that by the mouse assay method. By means of these techniques, bacteria such as *Vibrio* spp. and *Staphylococcus*, isolated from the intestines of TTX-bearing organisms, were found to produce TTXs including anhydrotetrodotoxin (anh-TTX).¹⁾ This suggests that TTXs production by intestinal bacteria may be implicated, at least partly, in the toxification of TTX-bearers. Further, *Vibrio* spp. are widely distributed in seawater.²⁾ The possibility of toxification is therefore present in fishes other than toxic puffer.

As described previously,³⁾ anh-TTX was clearly detected in nontoxic species of puffer belonging to the family Tetraodontidae. It might also have originated from some intestinal bacteria. But no information is available on the distribution of TTXs in other species of fish.

In this situation, the present work was performed to detect TTXs, more widely, in fish species which are close to the family Tetraodontidae.

Materials and Methods

Materials

The following five species of fish, which are phylogenetically related to the family Tetraodontidae, were freshly caught or procured at a fish market. Spiny puffer “*harisenbon*” *Diodon holacanthus* (4 specimens), sunfish “*manbou*” *Mola mola* (3), “*uchiwafugu*” puffer *Triodon bursarius* (2), “*kawahagi*” filefish *Stephanolepis cirrhifer* (3), and “*umazurahagi*” filefish *Navodon modestus* (2). After being frozen immediately, they were transported to the Laboratory of Marine Biochemistry, and used. Details of those fish specimens are given in Table 1. In addition, TTX was purified from ovaries of “*mafugu*” puffer *Fugu vermicularis porphyreus* by the method of Goto *et al.*,⁴⁾ and used as reference standard. It contained small amounts of anh-TTX and 4-epitetrodotoxin (4-epiTTX).

Assay for Lethal Potency

Frozen specimens were partially thawed and the livers dissected. The livers were individually assayed for lethal potency by the official method for TTX using mice.⁵⁾

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Table 1. Details of fish species used

Fish	Place and date of procurement	No. of specimens	Body weight
Spiny puffer "Harisenbon" <i>Diodon holacanthus</i>	Okinawa Pref. in May '87	4	79~119
Sun fish "Manbou" <i>Mola mola</i>	Manazuru, Kanagawa Pref. in Sept. '87	3	Not weighed
"Uchiwafugu" puffer <i>Triodon bursarius</i>	Tokyo Wholesale Fish Market, Tokyo in Feb. '88	2	750~800
"Kawahagi" filefish <i>Stephanolepis cirrifer</i>	Manazuru, Kanagawa Pref. in Nov. '87	3	49~ 75
"Umazurahagi" filefish <i>Navodon modestus</i>	Manazuru, Kanagawa Pref. in Feb. '87	2	160~208

Separation of TTX Fraction

As shown in Fig. 1, the liver (usually 5 g) was homogenized with 3 volumes of 1% acetic acid in methanol and centrifuged at 3,000 rpm for

Fig. 1. Procedure of separation of TTX fraction.

20 min. The residue was extracted two more times in the same way. The supernatants were combined, concentrated *in vacuo*, and defatted with dichloromethane. The water layer was evaporated to dryness under reduced pressure and dissolved in a small amount of water. The solution was filtered through a Diaflo YM-2 membrane (Amicon, Danvers, MA., Ireland) whose cut-off limit was 1,000 Da. The filtrate was evaporated to dryness and dissolved in a small amount of 0.03 M acetic acid. The solution was chromatographed on a Bio-Gel P-2 column (2×95.5 cm) using 0.03 M acetic acid. The fractions corresponding to authentic TTXs were combined and lyophilized. "TTX fraction", or the fraction corresponding to authentic TTXs, was collected and lyophilized. TTX fraction thus obtained was analyzed for TTXs, as described below.⁸⁾

High Performance Liquid Chromatography (HPLC)

A portion of TTX fraction was examined by reverse phase HPLC on a YMC AM-314 (Yamamura Riken, Kyoto) column (0.6×30 cm) with heptanesulfonic acid as ion-pairing reagent, as reported previously.⁶⁾ The eluate was mixed with an equal volume of 3 N NaOH at a flow rate of 1 ml/min and heated in a reaction coil at 100°C for 0.4 min. The resulting fluorophors were monitored by a Hitachi 650-10 M fluorospectrometer at 505 nm with excitation at 380 nm.

UV Spectrophotometry

Another portion of TTX fraction was dissolved in 1 ml of 1.5 N NaOH and heated at 100°C for 45 min.⁷⁾ After being cooled to room tem-

perature, the degradation product was subjected to UV spectrophotometry to confirm the formation of the C₆-base, 2-amino-6-hydroxymethyl-8-hydroxyquinazoline.

Gas Chromatography-Mass Spectrometry (GC-MS)

The above alkali-degradation product was adjusted to pH 3–5 with 10% HCl and extracted

three times with 5 ml each of 1-butanol. The extracts were combined and evaporated to dryness *in vacuo*, and to the residue was added a mixture of *N*, *O*-bis (trimethylsilyl) acetamide, trimethylchlorosilane, and pyridine (2:1:1), in order to trimethylsilylate the C₆-base.⁷⁾ The derivative obtained was submitted to GC-MS on a Hitachi M-80 GC-mass spectrometer. A column (0.3×200 cm) of 1.5% silicon OV-101 Chromosorb W (60~80 mesh) was used. The temperature was programmed from 160°C to 250°C at a rate of 5°C/min and the flow rate of inlet helium gas was 40 ml/min. Ionizing voltage was 70eV and the ion was accelerated at 3kV. Ion source temperature was kept at 200°C.

Results

Spiny Puffer

None of the specimens of this species showed any lethal potency, when the liver extract was assayed by the official method for TTX.⁵⁾ In HPLC, the TTX fraction from each specimen

Fig. 2. HPLC of TTX fraction from spiny puffer (lower) and of authentic TTXs (upper).

Fig. 3. UV absorption spectra of the alkali-degradation product from spiny puffer TTX fraction (left) and from authentic TTXs (right).

Fig. 4. Ion-monitored chromatograms of the TMS derivative from alkali-degraded TTX fraction of spiny puffer (lower) and from alkali-degraded TTXs (upper).

Fig. 5. Mass spectra of the TMS derivative from alkali-degraded TTX fraction of spiny puffer (left) and from alkali-degraded TTXs (right).

gave rise to several peaks, two of which showed the same retention times as those of TTX and anh-TTX, respectively, though the peak of TTX was very small. An example of the HPLC pattern is depicted in Fig. 2.

Figure 3 shows a UV absorption spectrum of the alkali-degradation product from TTX fraction, along with that of the corresponding product from TTX. The degradation product from spiny puffer exhibited an absorption maximum at around 270 nm, indicating the formation of the C_9 -base.

Figure 4 shows the ion-monitored chromatograms of the trimethylsilyl derivatives prepared from authentic TTX and spiny puffer TTX fraction. Mass fragment ions at m/z 376, 392, and 407 which are characteristic of the C_9 -base, appeared at the same retention time (12.7 min) in both chromatograms. Both of those peaks from authentic TTX and spiny puffer TTX fraction elicited essentially the same mass spectra which were featured by fragment ions at m/z 407 (molecular peak), 392 (base peak), and 376 (Fig. 5).

Other Species of Fish

No specimen of other fish species showed any lethal potency in the mouse bioassay. Neither TTX nor its derivatives were detected at all, in HPLC, UV spectrophotometry, and GC-MS.

Discussion

In a previous paper,⁹⁾ we reported the occurrence of TTXs in the liver of two nontoxic puffer species, "*shirosabafugu*" *Lagocephalus wheeleri* and "*yoritofugu*" *Sphoeroides pachygaster*, both of which belong to the family Tetraodontidae. In the present study, we detected small amounts

of these substances only in spiny puffer of the five puffer-related species. However, any specimen of spiny puffer was nontoxic. It is interesting that the distribution of TTXs was extended to this diodontid species of fish.

As previously reported⁹⁾ we assayed some seven hundred specimens of cultured "*torafugu*" puffer *Fugu rubripes rubripes* for lethal potency and found no toxic specimen, regardless of collection site, age, and tissue of specimen. These specimens were, however, easily toxified when fed on a TTX-containing diet, indicating that toxification of puffer is closely associated with feeding or the food chain. Other fish species except for spiny puffer could feed on a diet which diodontid and tetraodontid fish do not feed. Much more physiological and ecological information on puffer and related species is needed to elucidate the physiological significance of TTXs in them. On the other hand, the process of toxification during evolution of puffer may give a clue in this respect. This is one of the reasons why puffer-related species were used in the present study.

Tyler⁹⁾ described that tetraodontid fish is phylogenetically close to diodontid fish. Fujita¹⁰⁾ suggested that spiny puffer and a puffer "*sabafugu*" *Lagocephalus lunaris spadiceus* phylogenetically predated another puffer "*torafugu*", on the basis of morphological and ecological comparisons. In other words, toxic pufferes as represented by "*torafugu*" evolved from the types such as spiny puffer or "*sabafugu*". As puffer changed habitat from the surface to bottom of the sea due to some unknown cause(s), they could have acquired biological structures for having TTX. The occurrence of TTXs in spiny puffer seems to support the Fujita's hypothesis.

As we reported previously,¹¹⁾ "*sabafugu*"

showed a TTX resistibility which was intermediate between toxic puffer species and other teleosts, supporting also the hypothesis. In this connection, it seems interesting to assay TTX resistibility of spiny puffer.

Further studies are now in progress.

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