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Tetrodotoxin in the Horseshoe Crab *Carcinoscorpius rotundicauda* Inhabiting Thailand

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Further attempts were made to identify the toxins of the horseshoe crab *Carcinoscorpius rotundicauda* which causes sporadically food poisonings in Thailand.

Toxic *C. rotundicauda* eggs were crushed and extracted with water repeatedly. After being defatted with dichloromethane, the combined extracts were ultrafiltered to cut off more than 1,000 dalton-substances. Toxic fractions were obtained from the filtrate by column chromatography on Bio-Gel P-2 and Bio-Rex 70.

Cellulose acetate strip electrophoresis, TLC, HPLC, and other instrumental analyses demonstrated that the horseshoe crab toxin was essentially composed of tetrodotoxin and related substances, along with paralytic shellfish poisons as minor components.

Most species of horseshoe crab have disappeared through evolution except only four species remaining as live fossils in the world. Among them, two species *Carcinoscorpius rotundicauda* and *Tachypleus gigas* inhabit the Gulf of Thai,[1] and are often eaten by the people there. Since 1925, there have been several reports of food poisonings due to ingesting the horseshoe crab, especially the eggs.[2] The causative species was *C. rotundicauda* in all the cases reported. The symptoms are similar to those of paralytic shellfish poisoning.[2–5] The responsible toxin was supposed to be an alkaloid[2] or a polar substance.[4] On the other hand, one of the authors found that horseshoe crab toxin was, though partly, accounted for by paralytic shellfish poison.[6,7]

Since this toxin had not fully been characterized as yet, the present study was undertaken. The results so far obtained showed that the toxin was composed mainly of tetrodotoxin (TTX) and related substances.

Materials and Methods

Materials

Female specimens of the two horseshoe crabs, *C. rotundicauda* and *T. gigas*, were collected from Maha-chai (180 and 97 specimens, respectively), Bang-pakong (80 and 60 specimens), and Ang-sila (15 and 5 specimens) areas along the Gulf of Thai, in August 1984 through August 1985. They were frozen immediately after collection, transported to the Laboratory of Marine Biochemistry, and kept frozen at −20°C until used, not longer than one month.

Assay of Toxicity

Each frozen specimen was partially thawed. The eggs were excised individually and examined for toxicity by the mouse assay method for TTX[8] with slight modifications, as follows.

Two grams of the eggs were placed in a test tube. After adding 8 m*l* of 0.1% acetic acid (AcOH), the tube was heated in a water bath at 100°C for 10 min. After cooling, the mixture was centrifuged at 1,000×g for 10 min and one m*l* each of the supernatant or its dilutions was injected intraperitoneally into male mice of ddY strain (18–20 g in body weight). Toxicity was calculated from the dose-death time relationship for TTX.

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Isolation of Toxins

Pooled toxic eggs (300 g, *ca.* 1,200 MU) of *C. rotundicauda* were crushed in a mortar. After adding two volumes of water, the mixture was subjected to a Bransonic 52 ultrasonicator (Yamato) for 30 min. The homogenate was centrifuged at 3,000×g for 15 min. The pellet was extracted two more times in the same manner. The supernatants were combined, filtered through a Toyo filter paper No. 2, and defatted with dichloromethane. The aqueous layer was concentrated with a rotary evaporator, and filtered through a Diaflo ultrafiltration membrane YM-2 whose cut-off limit was 1,000 daltons. The filtrate was applied to a Bio-Gel P-2 column (6.6×55 cm). After washing with 1 *l* of water, the column was developed with 3 *l* of 0.1 M AcOH. Toxic fractions were combined and rechromatographed on a Bio-Gel P-2 column (2.5×90 cm), using 0.03 M AcOH. Toxic fractions were combined and chromatographed on a Bio-Rex 70 (H^+ form) column (0.9×94 cm). Toxins were eluted by a two-step linear gradient, the first step from 0–0.05 M AcOH and the second from 0.05–1.5 M AcOH. Two toxic fractions A and B (Fig. 1) were obtained and subjected to the following analyses. TTX, anhydro TTX (anh-TTX), saxitoxin (STX), neoSTX and gonyautoxins 1-4 (GTX_{1-4}) were prepared as reported previously,[9,10] and used as reference standards.

Electrophoresis

Electrophoresis was performed on 5×10 cm cellulose acetate strips (Chemetron) using 0.08 M Tris-HCl buffer (pH 8.7) under a constant current of 0.8 mA/cm for 30 min. Toxins were visualized under UV light at 365 nm after spraying successively with 1% H_2O_2 and 10% KOH, followed by heating at 110°C for 10 min.[11,12]

Thin Layer Chromatography

TLC was performed on 10×10 cm Whatman LHP-K plates with a solvent system of pyridine/ethyl acetate/acetic acid/water (15: 5: 3: 4). Toxins were visualized as in electrophoresis.

High Performance Liquid Chromatography (HPLC)

Analytical HPLC for TTX and related substances was performed by a Hitachi HPLC 638-50 system with a 650-10 M fluorescence spectrophotometer equipped with a 90 μl microflowcell by the method of Nagashima *et al.*[13] Briefly, a reversed phase ODS column of YMC AM-314 (Yamamura Riken; 6×300 mm) was developed with 1% methanol/0.05 M potassium phosphate (pH 7.0) containing 5 mM heptanesulfonic acid as an ion-pairing reagent. The eluate was mixed with an equal volume of 3 N NaOH at a flow rate of 1.0 ml/min and heated in a reaction coil at 100°C. The resulting fluorescent mixture was measured at 505 nm with 380 nm excitation.

HPLC of paralytic shellfish poisons (STX, neoSTX and GTXs) was carried out according to the method of Nagashima *et al.*[13]

Gas Chromatography-Mass Spectrometry (GC-MS)

The C_9-base, 2-amino-6-hydroxymethyl-8-hydroxyquinazoline, was derived from TTX by alkaline degradation and trimethylsilylated as described previously.[12] The corresponding trimethylsilyl derivative was prepared from the

Table 1. Toxicity of the eggs of horseshoe crab *C. rotundicauda* from Thailand

Place of catch	Date of catch	No. of toxic specimens/ No. of specimens assayed	Toxicity (MU/g)
Maha-chai	Aug. '84–Feb. '85	0/50	—
	Mar. '85	1/34	6
	Apr. 〃	2/7	5, 5
	May 〃	1/20	5
	June '85–Aug. '85	0/69	—
		(Sum 4/180)	
Bang-pakong	Aug. '85–Feb. '85	0/20	—
	Mar. '85	2/32	8, 16
	May '85–Aug. '85	0/28	—
		(Sum 2/80)	
Ang-sila	Aug. '84–June '85	0/14	—
	July '85	1/1	15
		(Sum 1/15)	

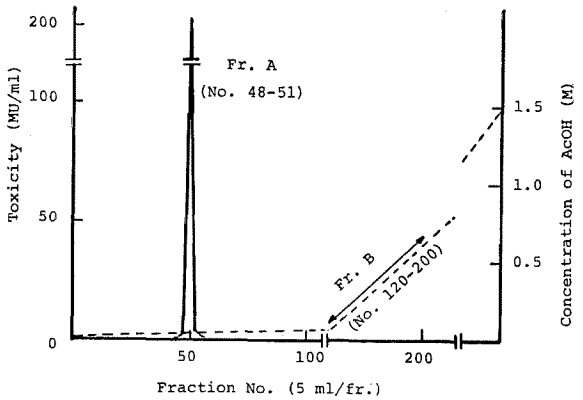

Fig. 1. Elution profile of horseshoe crab toxin from a Bio-Rex 70 column.

horseshoe crab toxin as follows. About 100 MU of the toxin was degraded in 2 N NaOH at 100°C for 45 min. After being cooled to room temperature, the mixture was adjusted to pH 3–5 with 10% HCl and repeatedly extracted with 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 convert the extracted "C_9-base" into its trimethylsilyl derivative. Both samples thus obtained were submitted to GC-MS on a Hitachi M-80A GC-mass spectrometer. A column (0.3 × 100 cm) of Chromosorb coated with 1.5% OV-101 was used and temperature was raised from 180°C to 250°C at a rate of 5°C/min. The ionizing voltage was 20 eV and the ion source temperature was kept at 280°C.

Results and Discussion

Toxicity

As Table 1 shows, the eggs of some *C. rotundicauda* specimens were found to be toxic, their toxicity scores ranging from 5 to 16 MU/g. The toxic specimens were from all the three areas. The frequency of toxic specimens was generally low: 2.2% at Maha-chai, 2.5% at Bang-pakong and 6% at Ang-sila. Since toxic specimens were recognized only in March to July, a seasonal variation of toxicity seems to be present. In contrast, all of the *T. gigas* eggs assayed were nontoxic.

Isolation and Characterization of Toxins

In Fig. 1 is shown the elution profile of the toxin from a Bio-Rex 70 column. A sharp peak appeared at around Fr. 50. Fractions 48–51 were combined and designated A (total toxicity, 1,100 MU). On the other hand, Frs. 120–200, corresponding to STXs, were combined and designated B (total toxicity, less than 10 MU). Fr. A was submitted to all analyses while Fr. B to HPLC alone.

As shown in Fig. 2, Fr. A gave a clear spot (yellow) at Rm 0.70 and a faint spot (yellow) at Rm 0.39, both of which agreed well with TTX and anh-TTX, respectively, in both Rm value and the color of spot.

As shown in Fig. 3, Fr. A gave two spots (yellow) whose *Rf* values (0.75, 0.80) coincided well with those of TTX and anh-TTX, respectively.

Fig. 4 shows HPLC of Fr. A, along with an authentic TTX and anh-TTX mixture containing a small amount of "4-epimer" of TTX. Fr. A gave

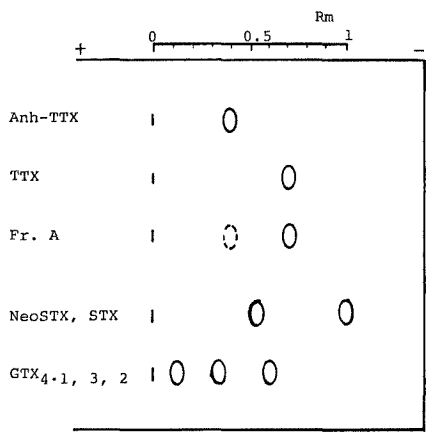

Fig. 2. Cellulose acetate membrane electrophoresis of Fr. A, along with authentic toxins.

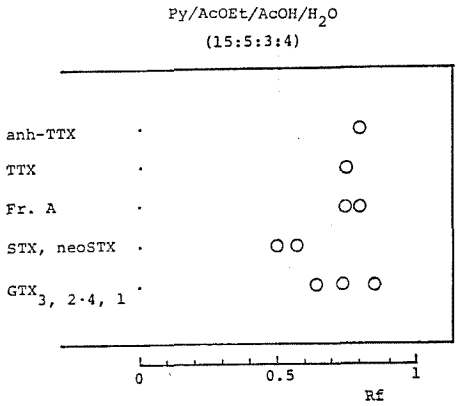

Fig. 3. TLC of Fr. A, along with authentic toxins.

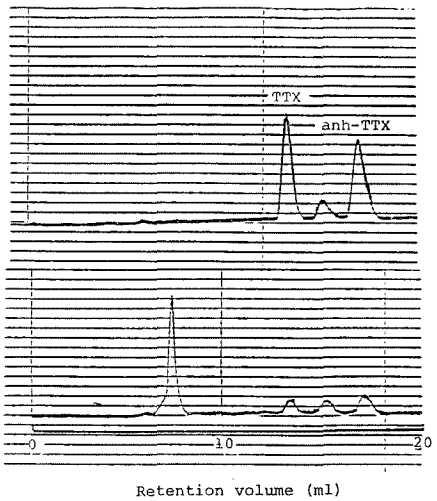

Fig. 4. HPLC of authentic TTX and anh-TTX (upper) and Fr. A (lower).

rise to four peaks, two of which exhibited the same retention volumes (Rv) as those of authentic TTX and anh-TTX (Rv 13.2 and 17.3 m*l*, respectively). Fr. B exhibited no peak corresponding to TTX or related compounds (data not shown).

As Fig. 5 shows, Fr. B gave rise to a big peak whose Rv (8.5 m*l*) agreed well with that of neoSTX, along with a small peak with the same Rv (10.2 m*l*) as that of STX. Fr. A did not show any clear GTX peak even when as much as 4 MU was injected (data not shown).

In Fig. 6 are shown the selected ion-monitored chromatograms of the trimethylsilyl derivatives

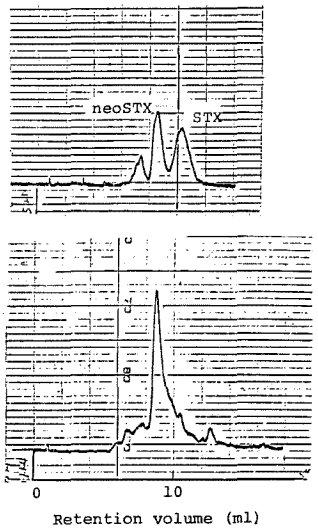

Fig. 5. HPLC of authentic STXs (upper) and Fr. B (lower).

prepared from TTX and the horseshoe crab toxin. Mass fragment ions at *m/z* 376, 392 and 407, which are characteristic of the tri-trimethylsilyl derivative of the C_9-base, appeared at almost the same retention time (14.0 min for TTX, 13.8 min for Fr. A). As shown in Fig. 7, both peaks revealed essentially the same mass spectra, which were featured by fragment ions at *m/z* 407 (parent peak) and 392 (base peak), indicating presence of the quinazoline skeleton in the horseshoe crab toxin.

On the basis of these results, it was concluded that the toxicity of the horseshoe crab *C. rotundicauda* was mostly accounted for by TTX, and only partly by neoSTX and STX.

Hashimoto and coworkers[6,7] previously detected PSPs such as GTX_1 and neoSTX, as major toxins in the extract of Thai *C. rotundicauda* specimens. That and the present results indicate that the toxin compositions of horseshoe crab widely differ depending upon the specimens.

In this connection, we[14] recently have noted in the xanthid crab *Atergatis floridus* that TTX and related compounds were major in low-toxic specimens whereas PSPs were major in highly toxic ones. In other words, TTX and related compounds were contained at a low level in all the xanthid specimens irrespective of toxicity, which may indicate that these toxins are essential for them to survive.

The toxicity of the horseshoe crab eggs used

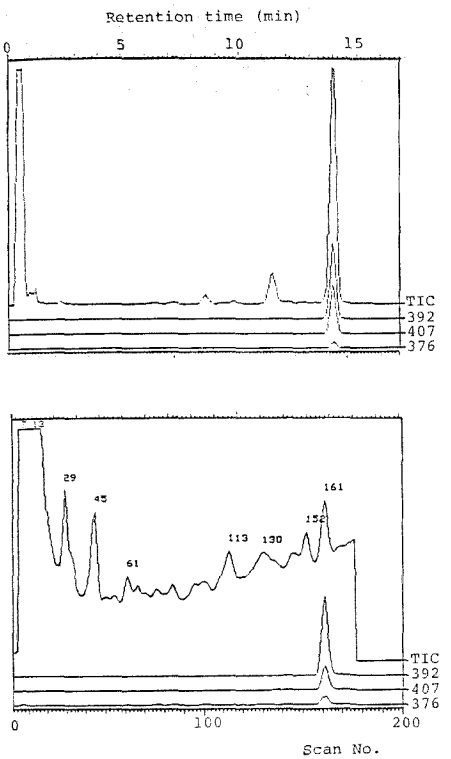

Fig. 6. Selected ion-monitored chromatograms of the C_9–base-$(TMS)_3$ derived from authentic TTX (upper) and from Fr. A (lower).

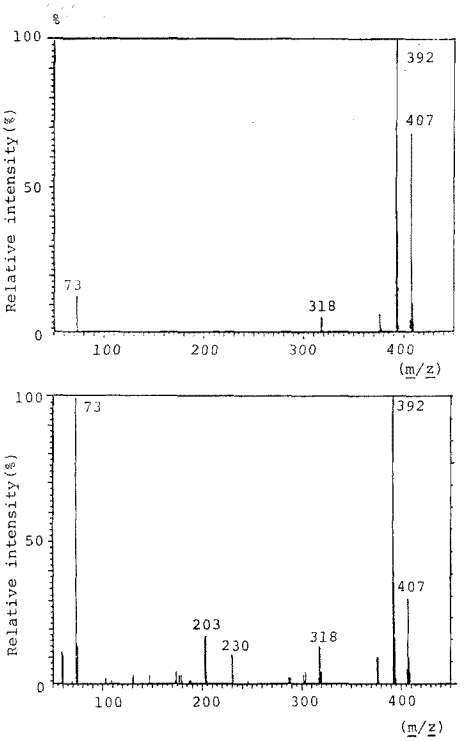

Fig. 7. Mass spectra of the C_9–base-$(TMS)_3$ derived from authentic TTX (upper) and Fr. A (lower).

for toxin extraction this time was low (below 16 MU/g), in contrast to that of the specimens previously used (about 65 MU/g eggs). This, along with their wide differences in toxin composition as described above, is supposed to indicate that TTX and related substances are also essential for the horseshoe crab.

This is the first finding of TTX and related compounds in the horseshoe crab. Elucidation of the mechanism of toxification is now in progress.

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