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Effective Toxification of Nontoxic Puffer by Feeding with Live Flatworms

By

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Abstract

To elucidate the mechanism involved in toxification of puffer, a feeding test was carried out in which nontoxic specimens of the puffer Fugu rubripes rubripes were fed with live flatworms; a tetrodotoxin-containing animal, and assayed for tetrodotoxin accumulated. The results showed that TTX was effectively accumulated in various tissues except muscles; e.g., on the 14 th day, the lethal potency was increased to 225 MU/g skin, 537 MU/g liver, and 205 MU/g intestine. Regardless of tissue, the toxin consisted mainly of tetrodotoxin, 4-epitetrodotoxin and anhydrotetrodotoxin.

Introduction

Recent studies disclosed that not only puffers but also various distantly-related species of animal contain tetrodotoxin (TTX) and/or related substances. In this connection, it was found that some intestinal bacteria of marine organisms, as well as marine bacteria, produced TTX and/or related substances (Noguchi ***et al.***, 1986, 1987; Narita ***et al.***, 1987; Yotsu ***et al.***, 1987). On the other hand, toxification of some TTX-containing animals through the food chain was demonstrated: e.g., trumpet shell **Charonia sauliae** was, at least partly, toxified by feeding a toxic starfish **Actropecten polyacanthus** (Noguchi ***et al.***, 1982; Narita ***et al.***, 1984). On the other hand, the puffer **Fugu rubipes rubripes** was, though slowly, toxified by feeding with toxic puffer ovaries (Matsui ***et al.***, 1981; Saito ***et al.***,). These and other findings made it possible to assume the mechanism involved in toxification of puffer and other TTX-bearing animals. In order to elucidate the mechanism-further, a feeding experiment of puffers was carried out with live flatworms; another TTX-bearing animal. The results obtained showed that the puffer was effectively toxified much more than with puffer ovaries.

Materials and Methods

Puffers

Eight live specimens (4-6 g body weight) of the puffer ***Fugu rubripes rubripes*** (torafugu) which were hatched and cultured in a floating net system at the Research Institute of Fisheries, Kiniki University, Japan; transported to the Laboratory of Marine Biochemistry, University of Tokyo in July 1988 and acclimatized in an aquarium for at least one month.

Flatworms

Flatworms (0.2 - 0.5 g body weight) were collected from the intertidal zone at Shimoda, Shizuoka Prefecture in August and September 1988 and kept alive in an aquarium.

Feeding experiment

Three puffer specimens were fed with a live flatworm every day, along with fish feed **ad libitum** for four days, and one specimen for two weeks. As the control, three puffer specimens were fed with fish feed alone for four days, and one specimen for two weeks.

Bioassay

After feedding, the puffers were dissected into the skin, liver, intestine inclusive the content, and muscle. A portion of each tissue was assayed for lethal potency by the mouse bioassay method for TTX (Kawabata, 1978). One mouse unit is defined here as the amount of toxin which kills a 20 g ddy-strain male mouse in 30 min after intraperitoneal adminstration.

Purification and identification of toxin

The remaining portions of each tissue were combined and homogenized with five volumes of 1% acetic acid and centrifuged at 3000 rpm for 20 min. The pellet was extracted again in the same manner. Both supernatants were combined, defatted with dichloromethane, fillered through a Diaflo membrane YM-2 (Amicon) which cut off over 1.000-dalton substances. The filtrate was freeze-dried, dissolved in a small amount of 0.03 M acetic acid, and applied to a Bio-Gel P-2 (2.5 X 95 cm) column. The column was developed with 0.03 M acetic acid, and toxic fractions were combined and freeze-dried. The toxins, thus, partially purified were submitted to TLC, electrophoresis and HPLC. Parts of the toxins were alkali-degraded, and analyzed for the Cg-base by gas chromatorgraphy-mass spectrometry (GC-MS) following the procedures described previously Noguchi ***et al.*** (1986, 1987). The toxin was also partially purified from the flatworms and analyzed similarly.TTX and anhydrotetrodotoxin (anh-TTX) were extracted from the ovaries of a puffer ***Fugu vermicularis prophyreus*** by the method of Goto et al. (1965). The tetrodonic acid (TDA) was derived from TTX by the method of Tsuda ***et al.*** (1964).

Results and Discussion

Accumulation of tetrodotoxin in puffers

Ten flatworms tested showed a lethal potency of 120+10 MU/g (mean+ S.E.). In an aquarium, live flatworms were immediatly ingested by the puffer when transferred from another aquarium. The puffer showed no abnormal signs. Saito ***et al.*** (1985) reported that several hundred specimens of puffer ***F. rubripes rubripes,*** which were cultured in

a floating net system, were nontoxic in all tissues including liver and ovary. Actually, none of the control puffers reared for 4 or 14 days showed any lethal potency, irrespective of tissue, Table (1).

The three puffers fed with flatworms for four days showed lethal potencies of 9-19 MU/g skin, 8-100 MU/g liver, and 25-35 MU/g intestine, Table (1). When fed with flatworms for 14 days, the puffer showed a higher lethal potency: the highest score was 537 MU/g liver, followed by 225 MU/g skin and 205 MU/g intestine, Table (1). In contrast, the muscles of all the four puffers were nontoxic.

Table 1: Fugu rubripes: Toxification of nontoxic specimens by feeding toxic flatworms.

	Feeding period (days)	Specimen No.	Body weight*1 (g)	Skin			
				Skin	Liver	Intestine	Muscles
	4	1	9.6	9	48	26	ND *2
		2	6.5	19	8	25	"
		3	4.7	12	100	35	"
	14	4	25.8	225	537	205	"
	4	1	7.2	ND	ND	ND	ND
		2	6.4	"	"	"	"
		3	5.2	"	"	"	"
	14	4	25.9	"	"	"	"

*1 Body weight after feeding experiment.
*2 <4 MU/g.

Matsui ***et al.*** (1981) reported that nontoxic specimens (20-30 MU/g body weight) of puffer ***F. rubripes rubripes*** showed a lethal potency of 134+ 29 MU/g liver and 9+ 5 MU/g skin when daily fed with diet containing toxic puffer livers (one weight of toxic liver containing 120 MU: two weights of fish food) for 20 days.

Saito ***et al.*** (1985) found that nontoxic puffer specimens of the same species (20 g body weight) showed lethal scores of 52 MU/g liver, 13 MU/g skin and 29 MU/g intestine when fed with diet containing toxic puffer ovary (80 MU for each specimen/day) for 45 days. Compared to their results, although the daily toxic dose contained in flatworm was relatively low (about 40 MU), our puffers accumulated the toxin several times more quickly. This may partly be accounted for by differences in puffer size, but mostly by those in TTX-containing feed.

Toxin composition in various tissues of puffer

The toxin from the liver of flatworm-fed puffer showed three spots both in TLC and electrophoresis as did the flatworm toxin, Fig. (1). The three spots agreed well with TTX, anh-TTX, and TDA in Rf, Fig. (1). Essentially, the same results were obtained for the toxins from other tissues of the toxified puffer (data not shown).

Regardless of tissue, the puffer toxin elicited a HPLC pattern which was composed of TTX, anh-TTX and 4-epiTTX, as was the flatworm toxin, Fig. (2). It was noticed that the proportions of anh-TTX and 4-epiTTX were reversed after ingesting by puffers. In the puffer toxins, on the other hand, a cluster of peaks whose retention times were similar to that of TDA. In the case of intestinal toxin, another major peak with a retention time of 25 min was detected, but remained unidentified.

The trimethylsilyl (TMS) derivative of the alkali-decomposition product of skin or liver toxin gave an ion-monitored chromatogram in which peaks at m/z 407, 392 and 376 agreed well with each other at retention time of 11.6 min. as did the Cg-base, Fig.(3).

As described above, the flatworms, and possibly other TTX-containing small invertebrates as well, could be involved in the web of toxification of puffer through the food chain. In this connection, U et al. (1978) mentioned that a toxic species of puffer ***Arothron stellatus*** preferentially feeds flatworms in nature. Further studies are necessary to substantiate the above hypothesis.

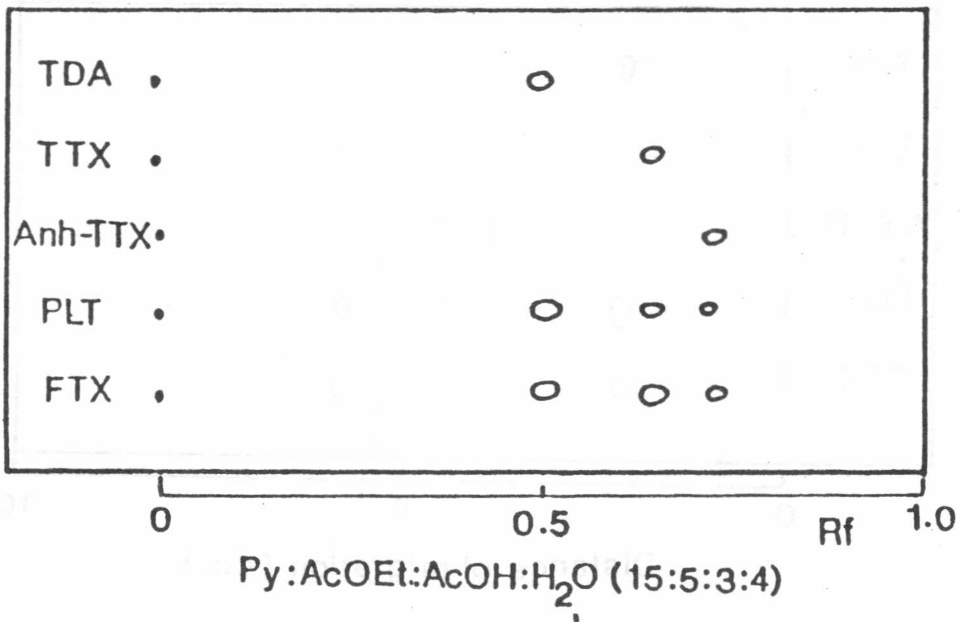

Fig (1)

TLC of the puffer liver toxin (PLT) and the flatworm toxin (FTX), along with authentic TTXS.

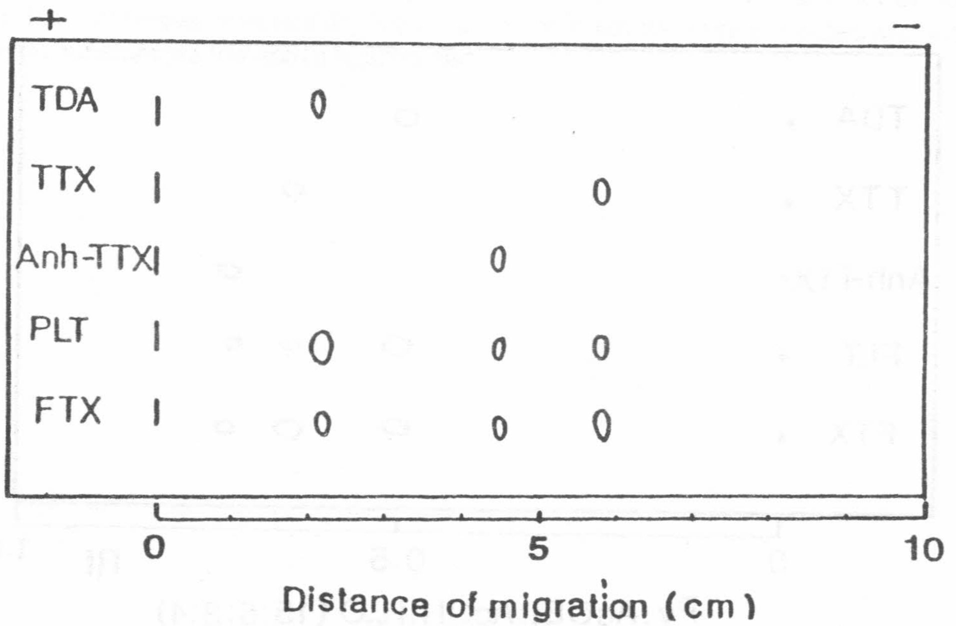

Fig (2)

Electrophoresis of the puffer liver toxin (PLT) and the flatworm toxin (FTX), along with authentic TTXs.

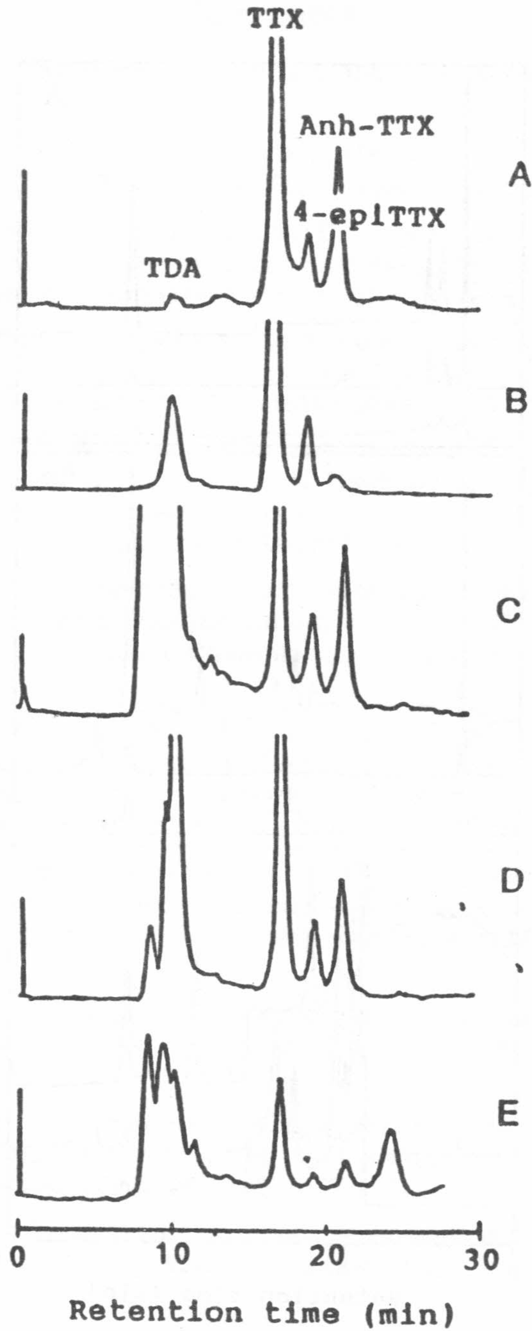

Fig (3)

HPLG of the toxins from puffer liver (C), skin (D), and intestine (E), along with authentic TTXs (A) and flatworm toxin (B).

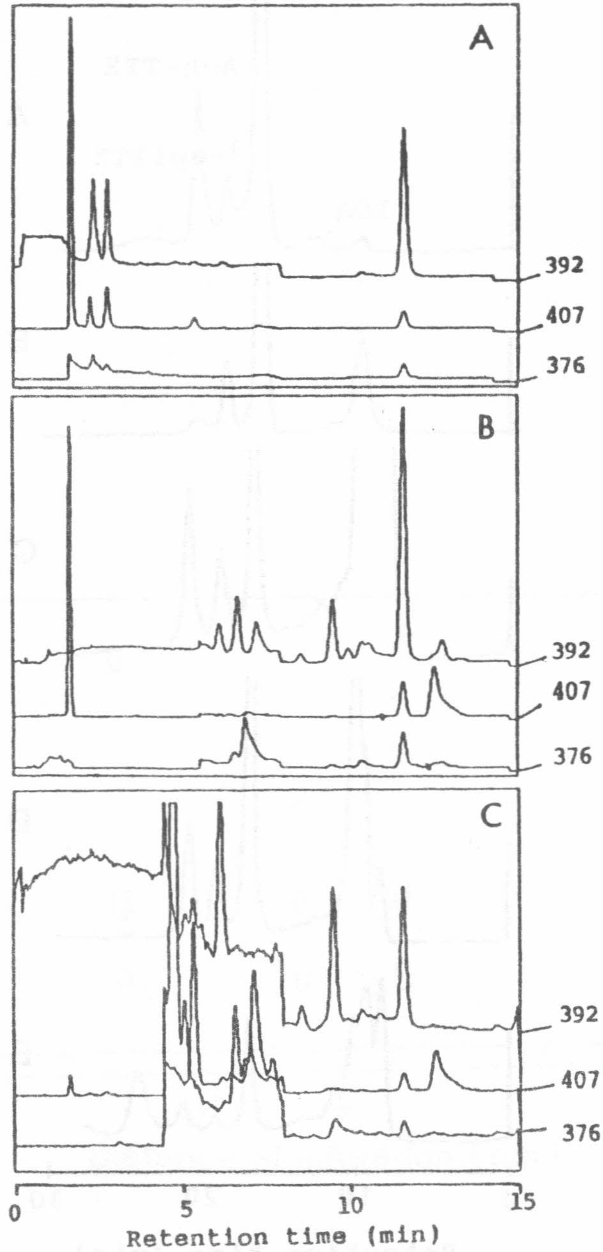

Fig (4)

Mass chromatogram of the TMS derivative of the alklai-decomposition prodduct from TTX (A), and of the corresponding derviative of toxins from puffer skin (B) and liver (C).

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