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Tetrodotoxin in a Pufferfish, *Fugu xanthopterus* (Korean Name, Ggachibog)

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Abstract

A total of 24 specimens of the pufferfish, *Fugu xanthopterus*, purchased at a fish market in Pusan, Korea were examined for toxicity using the assay method of tetrodotoxin (TTX). Also, the toxins isolated from the puffer liver were partially purified and analyzed for their chemical composition by instrumental behaviors. On the whole, when the level of toxicity in each organ was analyzed compared to that of liver, they were 100% for the liver, 92% for the intestine, 75% for the skin, 17% for the muscle, 78% for the testis, 87% for the ovary, and 71% for bile. The highest and average scores of toxicity for the liver were 917 and 231±51MU/g liver, respectively. The toxins of the puffer gave four peaks in HPLC whose retention times (10, 20, 22 and 25min) were close to those of TDA, TTX, 4-epi-TTX, and anh-TTX, respectively.

Key words : *Fugu xanthopterus*, assay method, toxins

INTRODUCTION

Tetrodotoxin (TTX) or pufferfish toxin, along with paralytic shellfish poison (PSP), ciguatera toxin, scombroid-related toxin, and diarrhetic shellfish poison (DSP) have been considered typical marine toxins. Tetrodotoxin is an alkaloid, a derivative of aminoperhydroquinazoline with a molecular weight of 319. It is also sparingly soluble in water, in acid solution, and unstable above pH 7 or below pH 3[1]. The lethal potency of tetrodotoxin is comparable to that of saxitoxin, and is about one-thousand times as high as that of sodium cyanide[2]. Puffer toxin has been extensively studied in Japan. Present quarantine regulations were established on the basis of work done by Tani[3] on pufferfish toxicity and its public health aspects. The purification was further developed by Tsuda[4] so that the necessary amounts of tetrodotoxin for structural and chemical studies could be obtained from purification. Furthermore, the toxic substances in pufferfish have been chemically characterized and its structure determined by Woodward[5]. A puffer, *Fugu xanthopterus*, is one of the popular species in Korea. The edible portion, mostly muscle is generally nontoxic, whereas tissues such as liver and intestine are often toxic due to the TTX. Actually, food poisoning cases have sporadically been caused by those toxic tissues in Korea.

This present paper is undertaken to elucidate the toxicity of the puffer, *Fugu xanthoperus*, and to examine the isolation and identification of the tetrodotoxin, based on its thin-layer chromatographic and electrophoretic behaviors, gas chromatography mass spectrometry of C_9 base trimethysilyl derivative, and high performance liquid chromatography from the pufferfish.

MATERIALS AND METHODS

Materials

Twenty-four specimens of pufferfish, *Fugu xanthopterus* (called Ggachibog in Korean), were purchased at a fish market in Pusan, Korea from July 1991 to August 1991. The pufferfishes were immediately frozen, packed in ice boxes and transported to our laboratory. Each specimen was in a half thawed state and liver, intestine, ovary or testis, skin, and muscle were separated before assay. Each part of pufferfish was

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homogenized and examined for toxicity. The toxin was partially purified, by a purification method of tetrodotoxin (TTX) and compared with an authentic specimen of TTX.

Assay of lethality

The official method for TTX[6] was applied to assay for lethal potency of each specimen. Briefly, about 1g of each part was added with 0.1N acetic acid after homogenization and heated for 10min in the boiling water bath. The extract was injected *i.p.* into ddy strain male mice (18~20g body weight) with 1ml and dealth times were recorded. One mouse unit (MU) was the amount of TTX which killed a mouse in 30 min after injection.

Purification of toxin

The procedure for preparation of toxin was followed by the method of Hwang *et al.*[7]. Toxic livers of each specimen were excised and homogenized for 3 ~5min in 3 volumes of 1% acetic acid in methanol. The combined supernatant was concentrated and washed several times with an 1/3 volume of chloroform. The aqueous layer was concentrated under reduced pressure to remove chloroform, and filtered through a Diaflo YM-2 membrane (Amicon). The extract was applied to a Bio-Gel P-2 (Bio-Rad Lab., Richmond, CA, U.S.A.) column (6.5×70cm), and placed on the second Bio-Gel P-2 column (2.0×90cm). Toxic fractions were collected, concentrated and freeze-dried. Each preparation was submitted to various analyses as described below.

Thin layer chromatography

TLC was performed on silica gel precoated plates (E. Merck, Darmstadt, F.R.G.) using a solvent system of *tert*-butanol-acetic acid-water (2 : 1 : 1). Toxin was found as a blue fluorescent spot under ultra violet light (365nm) after spraying the plate with 10% KOH and drying it for 5min[8].

Electrophoresis

Electrophoresis was carried out on 5×18cm cellulose acetate membranes (Chemetron, Milano, Italy) in 0.08M Tri-HCl buffer (pH 8.7) under a constant current of 0.8mA/cm width for 15min[9]. Toxins were confirmed by TLC.

High performance liquid chromatography

Analytical high performance liquid chromatography (HPLC) was performed on a Hitachi 638~50 system with a 650-10M fluorescence spectrometer equipped with a 90μl microflow cell. A silica ODS column (6×30mm) of YMC AM-314 (Yamamura Kagaku, Tokyo, Japan) was developed. The resulting fluorescent mixture was monitored at 505nm with 380 nm excitation[10].

Gas chromatography-mass spectrometry

The C_9 base, 2-amino-6-hydroxymethyl-8-hydroxyquinazoline, was derived from TTX by alkaline degradation and trimethylisilylated according to the method of Narita *et al.*[11]. The samples were submitted to gas chromatography mass spectrometry (GC-MS) on a JEOL JNS DX-300 GC-mass spectrometer. A column (0.3×200cm) of chromosorb coated with 1.5% OV 101 was used and the temperature was raised 190 to 220°C at a rate of 5°C/min. Scanning was carried out in the mass range of m/z 50-600 at 2 sec intervals.

RESULTS AND DISCUSSION

Toxicity of the pufferfish, *Fugu xanthopterus*

As shown in Table 1 and 2, there were total toxicity, individual and an antomical variations of toxicity for seven tissues of twenty-four specimens. The mean of total length and body weight were 28cm and 550 g, respectively. The highest scores were 917, 312, 79, 27, 72, 459, and 101MU/g for liver, intestine, skin, muscle, testis, ovary, and bile, respectively, and average toxicity values were 231.0±51.0 (mean±S.E.), 78.8±16.8, 3.3±1.4, 21.9±7.8, 175.0±38.0 and 21.6±5.1MU/g, respectively. Also, livers of 16 specimens were moderately toxic and those of 8 specimens were weakly toxic among 24 samples. Meanwhile, the ovary and bile were moderately toxic. It showed that these results were similar to those of other researchers[12,13]. From these results, the muscle and skin of *Fugu xanthopterus* was judged to be non-

Table 1. Anatomical analytical results of tetrodotoxin of the pufferfish, *Fugu xanthopterus* "Ggachibog", collected in Pusan, Korea

Specimen NO.	Sex	Toxicity(MU/g)							Total toxicity (MU)
		Liver	Intestine	Skin	Muscle	Testis	Ovary	Bile	
1	F	203	51	12	6	–	193	10	21,868
2	F	10	12	ND	ND	–	5	ND	1,099
3	F	125	21	20	ND	–	12	9	12,101
4	F	75	10	11	ND	–	42	11	6,168
5	F	40	13	10	ND	–	32	9	3,065
6	M	21	11	10	ND	16	–	ND	1,981
7	F	251	118	45	ND	–	265	11	167,694
8	F	124	59	15	12	–	118	19	10,938
9	M	57	42	18	ND	21	–	12	4,125
10	F	25	7	ND	ND	–	ND	ND	2,480
11	M	215	87	42	15	14	–	28	27,463
12	F	107	64	21	MD	–	72	12	7,812
13	M	57	24	12	7	ND	–	ND	6,044
14	M	47	ND	ND	ND	ND	–	ND	3,619
15	F	227	85	21	ND	–	251	11	24,678
16	F	512	312	79	27	–	401	79	69,054
17	M	414	251	32	ND	15	–	31	40,599
18	M	112	45	ND	ND	12	–	12	9,324
19	M	817	201	18	ND	72	–	52	71,907
20	F	651	172	ND	ND	–	307	28	54,442
21	F	201	18	11	ND	–	259	41	26,245
22	F	917	126	72	ND	–	459	101	97,996
23	F	125	92	15	13	–	212	20	11,092
24	M	213	71	ND	ND	47	–	22	11,798

M : Male, F : Female, – : Not assayed, ND : Not detected

Table 2. Toxicity data of pufferfish, *Fugu xanthopterus* "Ggachibog" as classified by tissue

Tissue	Frequency of toxic specimens (%)	No. of Specimens[a]			Toxicity range (MU/g)	Average[c] toxicity±S. E. (MU/g)
		moderately toxic	weakly toxic	non toxic		
liver	100 (24/24)[b]	16	8	0	10–917	231.0±51.0
Intestine	92 (22/24)	6	16	2	0–312	78.8±16.8
Skin	75 (18/24)	0	18	6	0– 79	19.3± 4.3
Muscle	17 (4/24)	0	4	20	0– 27	3.3± 1.4
Testis	78 (7/ 9)	0	7	2	0– 72	21.9± 7.8
Ovary	87 (13/15)	9	4	2	0–459	175.0±38.0
Bile	71 (17/24)	1	16	7	0–101	21.6± 5.1

[a] Symbols of toxicity : Strongly toxic, lethal at less than 10g. Moderately toxic, not lethal at less than 10g. Weakly toxic, not lethal at less than 100g. Negative, not lethal than 1,000g

[b] Numbers in parenthesis represent toxic specimens/total specimens. "Toxic" defined here is>10MU/g

[c] Calculated on the assumption that the toxicity of all the non detected specimen was zero

toxic or weakly toxic.

Purification by Bio-Gel P-2 column chromatography

The elution diagrams of pufferfish toxins, the liver of *Fugu xanthopterus*, by Bio-Gel P-2 chromatography were illustrated in Fig. 1. The liver was homogenized, centrifuged, and defatted with dichloromethane. After being treated with Diaflo YM-2 membrane, the extract was applied to a Bio-Gel P-2 with 500ml of 0.03M acetic acid after the gel column was washed with 1,000ml of water. When monitored by lethality to mice, the peak of bound fraction was observed from 104 to 144 of fraction number.

Instrumental behaviors of the toxin

As shown in Fig. 2, trace amounts of toxins isolated from the puffer appeared in TLC and electrophor-

esis, respectively. The toxins isolated from the puffer gave three spots on TLC, whose Rf values coincided well with those of anh-TTX, TTX, and TDA with a solvent system of *tert*-butanol-acetic acid-water (2 : 1 : 1), respectively. The toxin of the puffer gave two spots at migration distances of 5.0 and 7.0cm along with a clear spot at 2.5cm when electrophoresed and detected with 10% KOH. Therefore, the two spots agreed well with the authentic anh-TTX and TTX at migration distance while the clear spot was close to TDA.

The toxin of *Fugu xanthopterous* gave four peaks in HPLC whose retention times (10, 20, 22 and 25 min) were in agreement with those of the TDA, authentic TTX, 4-epi-TTX, and anh-TTX, respectively as shown in Fig. 3.

Fig. 4 showed the ion-monitored chromatograms of the trimethylsilyl (TMS) derivatives prepared from TTXs in livers of *Fugu xanthopterous*. Mass fragment ions at m/z 376, 380, 392 and 407 which are characteristic of the TMS derivative of the C9-base, appear-

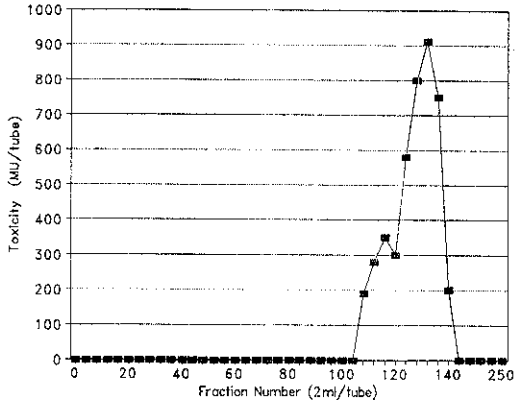

Fig. 1. Elution pattern from a Bio-Gel P-2 column of the toxin in the liver of *Fugu xanthopterus*.
The toxin (23,000 MU) was applied to a Bio-Gel P-2 column (2 × 90cm) equilibrated with water. After washing with 1,000ml of water, the column was eluted with 0.03M acetic acid, 500ml total volume. Fraction were collected at a flow rate of 2ml/min.

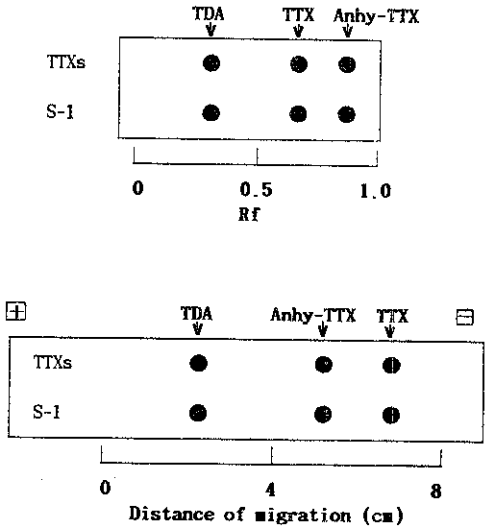

Fig. 2. Thin layer chromatography (upper) and electrophoresis (lower) of the puffer toxin along with authentic toxin.
Toxin of S-1 was purified from liver of *Fugu xanthopterus* by a method consisting of ultrafiltration and column chromatography using Bio-Gel P-2. TLC was carried out on a silica gel precoated plate (E. Merck) with a solvent system of *tert*-butanol-acetic acid-water (2 : 1 : 1). Electrophoresis was conducted on a cellulose acetate strip (Chemetron) in 0.08M Tris-HCl buffer (pH 8.7) at 0.8mA/cm width for 15min.

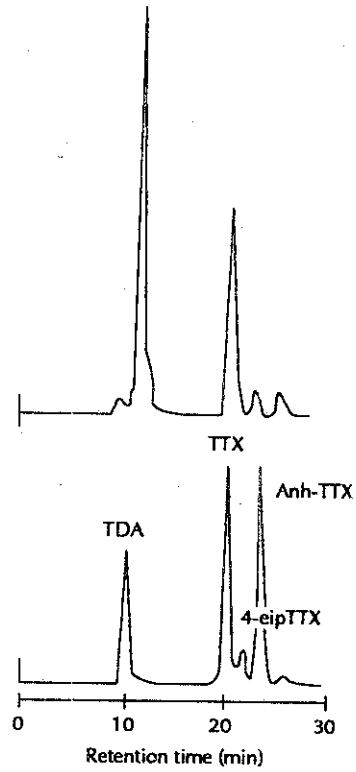

Fig. 3. HPLC of TTXs from the liver of *Fugu xanthopterus* (upper) along with that of authentic TTXs (lower).
HPLC was conducted on a reversed-phase ODS column of YMC-A 314 (Yamaura Riken), using a mixture of 0.05M potassium phosphate (pH 7.0) containing 5mM heptanesulfonic acid and methanol (99 : 1). The eluate was mixed with an equal volume of 3N NaOH, heated at 100° C, monitored at 505nm with 380nm excitation.

ed at almost the same Rt(12 : 33min) in the chromatogram. Therefore, both peaks from TDA and TTX revealed essentially the same mass spectra which were featured by fragment ions at m/z 407 (molecular ion peak), 392 (base), 380, and 376 which are characteristic of the C9-base TMS derivative.

Meanwhile, chromatographic or electrophoretic patterns of tetrodotoxin or paralytic shellfish poison differ from each other, depending on the species of causative plankton, the species of bivalves infested,

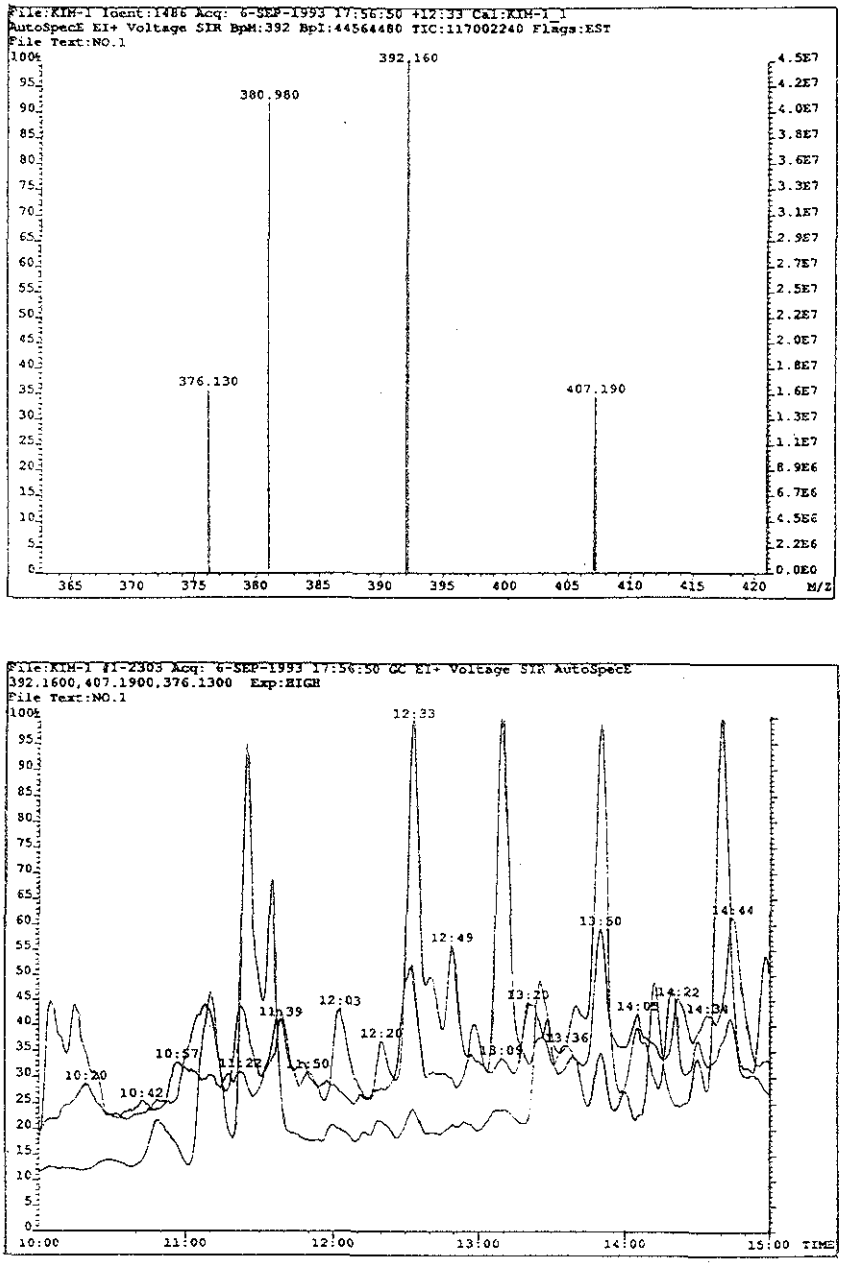

Fig. 4. GC-MS of the trimethylsilyl derivative of the alkaline degradation product from TTXs in *Fugu xanthopterous*.
The C9-base derived from TTXs by alkaline degradation was trimethylsilylated with a mixture of N,O-bis (trimethylsilyl) acetamide, trimethylchlorosilane and pyridine (2 : 1 : 1).

and their enviroment according to a report of Onoue *et al.*[14]. Therefore, TTX-related and other toxins[15] along with TTX have been detected as minor component, in the liver or other tissues of puffer : e.g., saxitoxin and an unknown toxin in *Fugu pradalis*, 4-epi TTX, anh-TTX, and TDA in *Fugu pradalis* and *Fugu poecrlonotus*. Recently, chemical structures of tetrodotoxin and related substances were clarified by great efforts of researchers in Japan and United States using the advanced physical techniques.

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까치복(*Fugu xanthopterus*)의 독성

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요 약

부산 공동어시장에서 구입된 까치복어, *Fugu xanthopterus*의 조직부위별 독력을 mouse bioassay법에 의하여 비교 검사하였다. 또한 bio gel P-2 column chromatography으로 복어독을 부분 정제하여, 박층 chromatography, 전기영동, GC-MS 및 HPLC에 의하여 독의 조성을 분석하였다. 즉, 까치복의 개체당 전체 독력은 1,099~167,694 MU로서 개체에 따라 뚜렷한 독력 차이가 있었다. 간, 난소 그리고 내장의 평균독력은 각각 231.0±51.0, 175.0±38.0 및 78.8±16.8MU/g(평균값±표준오차)으로 나타난 반면에 근육과 껍질조직의 평균독력은 각각 3.3±1.4와 19.3±4.3MU/g으로 개체에 따라 약독 내지 무독인 것으로 나타났다. 또한 각 부위별 최고독력의 순서는 간(917MU/g), 난소(459MU/g), 내장(312MU/g), 담즙(101MU/g), 껍질(79 MU/g), 정소(72MU/g), 그리고 근육조직(27MU/g)의 순이었다. 한편, TLC, 전기영동, HPLC로 분석한 결과 복어 개체에 따라 독의 조성이 다름을 알 수 있었고, TTX를 알칼리분해 시켜 생성된 C_9-base를 TMS화 시켜 GC-MS에 주입시킨 결과 C_9-base TMS 유도체의 특성인 407(molecular ion peak), 392(base), 380 및 376 m/z에서 fragment ions에 의해 TTX의 peak는 동일한 mass spectra로 나타났다. 따라서, 이들의 독성에 관한 구체적인 연구자료는 최근들어 수입 복어류의 증가, 양식어업의 발달, 식생활의 다양화 등에 따른 수산 식품의 위생적 안정성을 확보하는데 절실히 필요한 것이라 생각된다.