研究成果の刊行に関する一覧表

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ミニシンポジウム記録　熱帯/亜熱帯産有毒魚類と底生性有毒微細藻に関する緊急の課題

中毒発生海域より分離した *Ostreopsis* sp. のパリトキシン様物質産生能

相良剛史

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Profiles of palytoxin-like compounds from the dinoflagellate *Ostreopsis* sp. isolated from the areas where poisonous fishes were collected

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1. はじめに

近年，わが国ではブダイ科魚類やハタ科魚類，ハコフグ科魚類の喫食によるパリトキシン（PTX）中毒に類似した食中毒が相次いで発生し，問題となっている。[1,2] PTXは軟質サンゴの一種である *Palythoa toxica* より見出された毒であるが，[3] 底生性渦鞭毛藻の一種である *Ostreopsis siamensis* や *O. ovata* などからPTXおよびその類縁体が検出され，[4,5] 魚類の毒化原因は *Ostreopsis* 属等の渦鞭毛藻であるとの見方が強まっている。本項では，魚類へのPTX様毒の蓄積機構解明に資するため，中毒発生海域より分離した *Ostreopsis* sp. のPTX様物質産生能について検討をおこなったので，その概要について述べる。

2. *Ostreopsis* sp. より抽出した有毒成分の生化学的性状

2004年5月に宮崎県沿岸，2005年6月に長崎県沿岸から採取し単離した *Ostreopsis* sp. をESM培地[6]を用い，培養温度を20℃，光強度を40 μmol photon$\cdot$m$^{-2}\cdot$s^{-1}，明暗周期を12時間明/12時間暗の条件下で培養した。得られた培養藻体に3倍量の50%メタノールを加えて超音波破砕機でホモジナイズし，2,000×gで10分間遠心分離して上清を抽出液とした。残渣については，同様の操作を2回繰り返して上清を合一した。抽出液を減圧濃縮後，同量のジエチルエーテルで2回脱脂した。得られた水画分を再び減圧濃縮し，蒸留水で定容して水溶性画分とし，毒性および毒の性状を調べた。[7,8] いずれも，1.0×10^4 cells相当量の粗抽出液をマウスに投与したところ，遅延性致死活性が認められた。さらに，両者はマウス赤血球に対して，インキュベーション1時間では濃度1.0×10^3 cells相当量/mlでもほとんど溶血しなかったが，インキュベーション4時間において，前者で86.5%，後者で68.2%と，いずれも同濃度で高い溶血率を示した。また，ヒト赤血球に対しても同濃度，同時間でそれぞれ溶血率47.8%および36.2%の活性が認められ，これらの活性はウワバインにより特異的に抑制された。従って，宮崎県産ならびに長崎県産 *Ostreopsis* sp. は，マウスならびにヒト赤血球に対して遅延性溶血活性を示す毒を産生し，本毒の性状はPTX標準品に酷似していたことから，両株のPTX様物質産生能が確認された。

3. *Ostreopsis* sp. より抽出した有毒成分の分析

長崎県産 *Ostreopsis* sp. の培養藻体から調製した試験液につき，Waters社製Quattro *micro*（MS/MS）およびHITACHI社製NanoLC/Linear-Trap-TOF Nano-Frontier LD（TOF-MS）で分析した。Quattro *micro* を用いてマルチプルリアクションモニタリング（MRM）法でMS/MS分析すると，2価PTXのカリウム付加の脱水イオンと推察される $[M+K+H-5H_2O]^{2+}=1314$ からフラグメンテーションにより生じた m/z 327.4（図

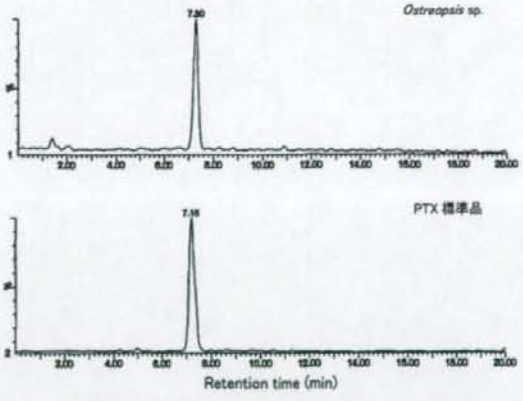

図1　PTX特有の付加イオン $[M+K+H-5H_2O]^{2+}=1314$ からフラグメンテーションにより生じた m/z 327.4をモニターした *Ostreopsis* sp. の部分精製毒（上）とPTX標準品（下）のMRMクロマトグラム

1)，PTXの3価のナトリウム，カリウム付加イオンと推察される $[M+Na+K+H]^{2+}=913.5$ から生じた m/z 327.4 およびPTX特有のフラグメントイオンである m/z 327 を前駆イオンとしてフラグメンテーションにより生じた m/z 75.9 のMRMクロマトグラムに，PTX標準品と一致する保持時間でピークが得られた。TOF-MS分析では，PTX標準品から検出された $[M+3H-3H_2O]^{3+}=875.834$ のピークは検出されなかったが，$[M+3H-3H_2O]^{3+}=977.596$ と $[M+2H-2H_2O]^{2+}=1474.915$ を強く検出し，それらを含めた複数の3価および2価の脱水イオン等，PTX関連物質と推測されるピークがPTXのHPLC保持時間に検出された。このため，本種の毒はPTXそのものではなく，PTX類似構造をもつ物質であると推定された。これにより，*Ostreopsis* sp. が魚介類の毒化原因となっていることが改めて示唆された。

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ノート

腐肉食性巻貝キンシバイ *Nassarius* (*Alectrion*) *glans* に認められたフグ毒の毒性と毒成分

(平成20年8月22日受理)

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Toxicity and Toxin Profile of Tetrodotoxin Detected in the Scavenging Gastropod *Nassarius* (*Alectrion*) *glans* "Kinshibai"

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From September 2007 to January 2008, a total of 66 specimens of 7 gastropod species, *Nassarius* (*Alectrion*) *glans* ($n=22$), *Bufonaria rana* ($n=11$), *Ficus subintermedia* ($n=10$), *Stellaria* (*Onustus*) *exutus* ($n=8$), *Tonna luteostoma* ($n=7$), *Hemifusus tuba* ($n=4$) and *Semicassis bisulcata persimilis* ($n=4$), were collected from Tachibana Bay, Nagasaki Prefecture, Japan, and their toxicity was determined by mouse bioassay. Among the gastropods tested, all *N. glans* specimens were toxic, whereas no other species showed toxicity of more than 5 MU/g. The toxicity scores of *N. glans* were very high; 48-2,730 MU/g (775±615 MU/g) in the muscle, and 16-10,200 MU/g (1,490±2,530 MU/g) in the viscera, including digestive gland. Interestingly, toxin was localized in the muscle in 13 of 22 specimens, where the total toxicity of the muscle (725-9,860 MU/individual) was 5.9-110 times higher than that of the viscera. LC/MS analysis demonstrated that the toxin of *N. glans* consisted mainly of TTX, which accounting for about 60-65% of the total toxicity. As for the remaining toxicity, participation of 11-oxoTTX was suggested. No paralytic shellfish poison was detected in HPLC-FLD analysis.

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Key words: 腐肉食性巻貝 scavenging gastropod; キンシバイ *Nassarius* (*Alectrion*) *glans*; 食中毒 food poisoning; フグ毒中毒 pufferfish toxin poisoning; フグ毒 pufferfish toxin; テトロドトキシン tetrodotoxin; 11-オキソテトロドトキシン 11-oxotetrodotoxin

緒 言

2007年7月下旬，長崎県長崎市において，同県橘湾産の小型巻貝を喫食した60歳の女性1名が舌のしびれ，四肢の麻痺，呼吸困難などを呈した後，一時呼吸停止に陥るという極めて重篤な食中毒が発生した[*1]．事件発生直後に本中毒の残品である調理済みキンシバイ *Nassarius* (*Alectrion*) *glans*，および未調理のアカニシ *Rapana venosa*，テングニシ *Hemifusus tuba*，ミガキボラ *Kelletia lischkei* を入手して毒性を調べたところ，キンシバイの筋肉と中腸腺から最高4,290 MU/gに達する強い麻痺毒性が検出された．さらにLC/MS分析により毒の本体がtetrodotoxin (TTX) であることが明らかとなり，本中毒はキンシバイを原因とするTTX中毒であると断定された．

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[*1] 厚生労働省医薬食品局食品安全部監視安全課長通知（平成19年8月16日，食安監発第0816003号）「ムシロガイ科キンシバイ（巻貝）での食中毒の発生事例について」，厚生労働省．

キンシバイは，ムシロガイ科の腐肉食性小型巻貝で，相模湾以南の潮間帯ないし水深 20 m の砂泥底に生息しているが[1]，日本では食習慣がなく，食中毒や毒性に関する知見もほとんどない．このような状況の下，本研究では中毒検体が採捕された橘湾における小型巻貝類の毒化状況を把握し，中毒の未然防止に資することを目的として，キンシバイを中心に同湾産小型巻貝類の毒性と毒成分について検討した．

実験方法

1. 試　　料

2007 年 9 月～11 月および 2008 年 1 月に長崎県橘湾 (Fig. 1) で採集されたキンシバイ (Fig. 2) 22 個体，および 2007 年 9 月と 10 月に同海域で採集されたミヤコボラ *Bufonaria rana* 11 個体，ビワガイ *Ficus subintermedia* 10 個体，キヌガサガイ *Stellaria* (*Onustus*) *exutus* 8 個体，ヤツシロガイ *Tonna luteostoma* 7 個体，テングニシ 4 個体，ウラシマガイ *Semicassis bisulcata persimilis* 4 個体を試料とした．試料は採集後，直ちに氷蔵にて長崎大学水産学部水産食品衛生学研究室に持ち帰り，－20℃で凍結保存した．供試の際，試料を流水中で急速解凍し，筋肉と中腸腺を含む内臓に分けて用いた．

2. 毒性試験

毒性試験は，食品衛生検査指針理化学編フグ毒検査法[2] (公定法) に準じて行った．

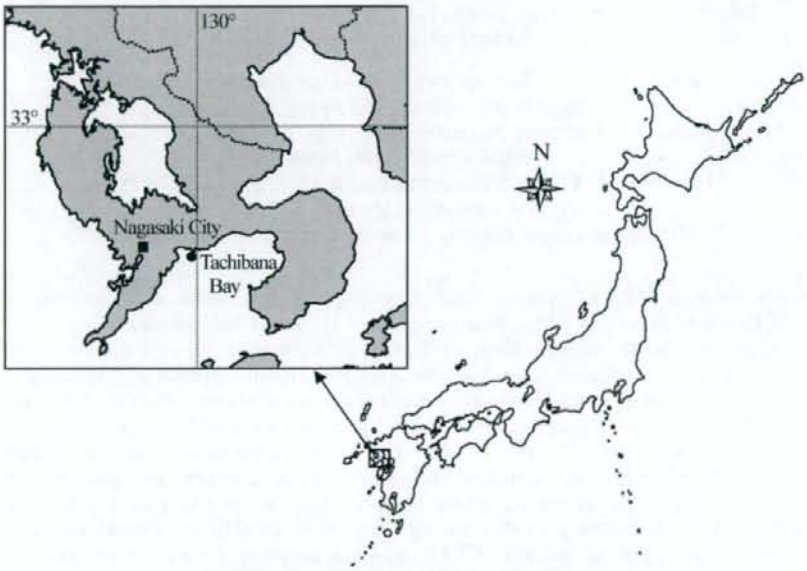

Fig. 1. Map showing Tachibana Bay (●) where gastropods specimens were collected.

Fig. 2. *Nassarius* (*Alectrion*) *glans* "kinshibai"

3. 毒成分分析

毒成分の分析はTTX成分を対象としてNakashimaら[3]のLC/MS法に準拠して行った．一方，麻痺性貝毒(paralytic shellfish poison; PSP)成分については，既報[4,5]のHPLC蛍光分析法にて分析した．

結果および考察

1. 小型巻貝類の毒性

供試した7種の巻貝のうち，キンシバイのみが有毒で，その他6種計44個体はいずれも無毒（5 MU/g未満）であった．

キンシバイの部位別毒力をTable 1に示す．供試22個体の筋肉と内臓はいずれも有毒であった．毒力は総じて強く，筋肉で48～2,370 MU/g（平均毒力±標準偏差：775±615 MU/g，以下同様），内臓で16～10,200 MU/g（1,490±2,530 MU/g）と測定された．特に2007年9月には供試10個体中8個体において，筋肉と内臓のどちらか一方，または両方が食品衛生上"猛毒"となる1,000 MU/gを上回り，最高毒力は筋肉で2,370 MU/g，内臓で10,200 MU/gに達した．また，同時期における筋肉の平均毒力は1,010 MU/gで採集期間を通じて最も高い値となり，その後は徐々に減少し，2008年1月に276 MU/gにまで低下した．一方，内臓の平均毒力は2007年9月に最高値2,450 MU/gを示した後，急激に減少し，2008年1月には65 MU/gとなった．これらの結果から，食中毒の発生した2007年7月から同年9月にかけて，橘湾ではキンシバイのみが毒化して高濃度の毒を筋肉および内臓に蓄積し，それらの毒力はその後しだいに減少したと考えられた．

日本では，1979年に静岡県静岡市で発生した肉食性巻貝ボウシュウボラ *Charonia saulia* による食中毒を契機として，同種や類似の巻貝オオナルトボラ *Tutufa lissostoma*，さらにはキンシバイと同じ腐肉食性巻貝であるハナムシロガイ *Zeuxis siquijorensis* やアラレガイ *Niotha clathrata* からTTXが検出された[6)~9)]．しかしながら，ハナムシロガイとアラレガイの毒力は低く（それぞれ可食部で3.4 MU/g，4～35 MU/g），日本ではこれらによる食中毒は発生していない．一方，台湾では小型巻貝による食中毒が1994年から2006年にかけて少なくとも9件発生し，46名が中毒，うち3名が死亡している[10~18)]．特に，2004年4月には，キンシバイにより患者6名中2名が喫食後30分で死亡するという深刻な事例が発生した[16)]．関連の調査では，中毒検体と同じ海域で採取したキンシバイから筋肉で1,170±557 MU/g（最高2,990 MU/g），中腸腺で538±608 MU/g（最高2,050 MU/g）に及ぶ極めて高い毒力が検出された[16)]．これらの値は，台湾産有毒巻貝類（ムシロガイ科9種，タマガイ科1種およびマクライガイ科3種）[10~18)]の中でも際立って高い．他方，中国

Table 1. Toxicity of *Nassarius* (*Alectrion*) *glans* specimens collected at Tachibana Bay, Nagasaki Prefecture

Month of collection	Specimen No.	Shell length (mm)	Shell width (mm)	Body weight (g)	Muscle			Viscera		
					Weight (g)	Toxicity*1 (MU/g)	Total toxicity*1,2 (MU/individual)	Weight (g)	Toxicity*1 (MU/g)	Total toxicity*1 (MU/individual)
Sept. 2007	1	44	20	9.8	3.9	360	1,420	1.6	5,580	9,150
	2	41	16	7.9	4.2	1,470	**6,150**	0.8	73	57
	3	40	21	6.7	3.1	494	**1,540**	1.4	36	50
	4	45	23	8.5	3.6	491	1,770	1.8	1,880	3,380
	5	43	21	8.8	4.8	591	2,860	1.4	1,980	2,830
	6	42	22	6.6	2.7	1,200	3,230	1.1	4,300	4,730
	7	42	17	8.1	4.0	1,970	**7,880**	1.4	285	410
	8	43	24	7.3	3.1	542	1,660	1.5	10,200	15,100
	9	40	21	7.7	4.2	2,370	**9,860**	1.1	119	133
	10	35	13	5.0	2.2	589	**1,300**	1.1	41	44
	Mean±SD	43±2.8	20±3.4	7.6±1.4	3.6±0.80	1,010±711	3,770±3,090	1.3±0.30	2,450±3,350	3,590±4,500
Oct. 2007	11	38	20	6.4	2.8	1,260	**3,520**	1.2	53	62
	12	38	21	6.5	2.8	48	132	1.4	154	216
	13	41	22	8.5	3.6	862	3,070	1.6	3,850	6,120
	14	35	20	5.3	3.0	245	**725**	0.8	72	57
	15	37	21	6.1	2.2	307	682	0.8	1,910	1,430
	16	36	22	6.0	2.7	416	**1,130**	1.1	61	68
	Mean±SD	38±1.9	21±0.89	6.5±1.1	2.8±0.43	523±451	1,540±1,400	1.1±0.33	1,020±1,570	1,330±2,410
Nov. 2007	17	38	23	7.0	3.2	1,250	**3,980**	1.4	28	38
	18	47	23	7.3	3.1	1,360	**4,180**	1.0	102	98
	19	41	23	8.4	3.7	288	1,070	1.6	1,890	3,080
	20	39	21	7.0	3.3	394	**1,310**	1.3	16	21
	Mean±SD	41±4.0	23±1.0	7.4±0.66	3.3±0.28	823±560	2,640±1,670	1.3±0.28	509±921	809±1,510
Jan. 2008	21	46	24	9.1	4.5	216	**976**	1.5	113	165
	22	41	22	8.1	3.9	336	**1,320**	1.3	17	22
	Mean	44	23	8.6	4.2	276	1,150	1.4	65	94

*1: Toxicity scores were determined by mouse bioassay.
*2: Bold numbers show the specimens in which total toxicity of the muscle was 5.9–110 times higher than that of viscera.

大陸では古くからムシロガイ科巻貝の食習慣があり，これに伴う食中毒も頻発している．2001年6月には，*Z. samiplicutus* の喫食により31名が中毒し，その中毒検体の可食部から307±192 MU/g（最高688 MU/g），中腸腺から370±118 MU/g（最高532 MU/g）の毒力が検出されている[19]．さらに最近では，オオハナムシロ *Z. siquijorensis* による同様の食中毒も相次いで発生しており，その可食部に数十 MU/g の毒性が認められたとの報告もある[20]．

一方，今回調査したキンシバイ1個体当たりの総毒力を見ると，22個体中13個体で筋肉が内臓よりも5.9～110倍高い値を示した (Table 1)．すなわち，これらの個体では毒の86～99%が筋肉に偏在していたことになる．日本産のTTX保有巻貝はいずれも中腸腺に毒が局在している[6)~9)]．しかしながら，Hwang ら[21]は，台湾産マサメダマ *N. lineata* の部位別毒性を詳細に調べ，同一個体では筋肉の毒力（最高毒力720 MU/g）が中腸腺（同12 MU/g）やその他の部位（同28 MU/g）より高く，筋肉に高濃度のTTXが含まれていたと報告している．さらに，台湾産キンシバイについても，85%の個体で筋肉の毒力が中腸腺より1.7～8.3倍高かったと述べている[16]．日本産キンシバイの毒蓄積パターンは，これら台湾産巻貝類と酷似している．

TTX保有生物のうち，クサフグ *Takifugu niphobles* やナシフグ *T. vermicularis* では，いったん凍結後に緩慢解凍すると，有毒部位から毒が筋肉に移行することが知られているが，急速解凍ではこのような毒の移行はほとんど起こらない[22~24]．今回調査したキンシバイについては，いずれも急速解凍のうえ毒性試験に供した．さらに，生きたキンシバイを用いた予備実験においても，今回と同様の毒分布（筋肉あるいは内臓への毒の偏在）が認められている．したがって，本調査において，凍結解凍による部位間の毒の移行は，あっても無視しうるレベルと考えられた．

2. キンシバイの毒成分

キンシバイの筋肉と内臓につき，LC/MSにて毒成分を分析したところ，*m/z* 320 のクロマトグラムにおいてすべての個体からTTX([M+H]$^+$=320) 標品と保持時間の一致するピークが検出された．Fig. 3a および 3c にその一例を示す．また，*m/z* 336 のクロマトグラムにおいては，TTXに対する相対的な溶出位置から[25), 26)]，11-

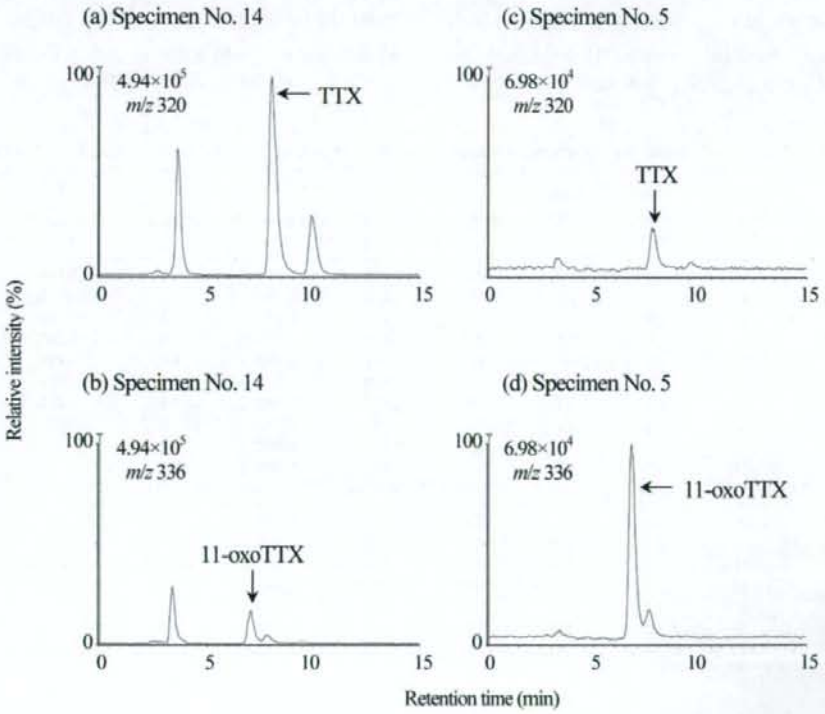

Fig. 3. LC/MS chromatograms at *m/z* 320 (a, c) and 336 (b, d) obtained from muscles of specimen No. 14 (a, b) and No. 5 (c, d) (see Table 1 for specimen numbers).

LC/MS[3)] was carried out on an Alliance LC/MS system (Waters) equipped with a Zspray™ MS 2000 detector, using a reversed-phase column with 30 mmol/L heptafluorobutyric acid in 1 mmol/L ammonium acetate buffer (pH 5.0) as the mobile phase, and the flow rate was set at 1.0 mL/min. As for MS conditions, about 20% of the eluate was introduced *via* a splitter into the ion source of MS, ionized by means of positive-mode electrospray ionization (ESI), and monitored through a MassLynx™ NT operating system.

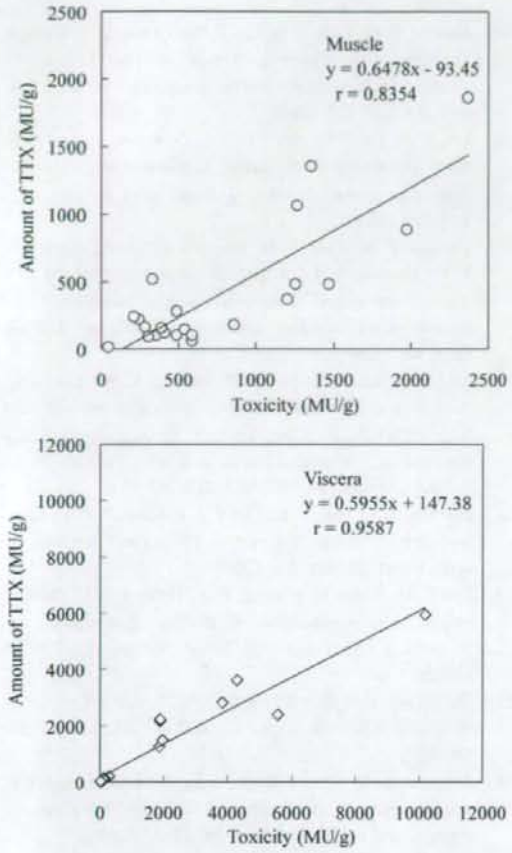

Fig. 4. Comparison between toxicity scores determined by mouse bioassay and amounts of TTX by LC/MS

oxoTTX($[M+H]^+=336$) と推定されるピークが認められた (Fig. 3b および 3d).

'LC/MS 分析から算出された TTX の毒力' と '公定法で測定された毒力' の相関について検討したところ，筋肉と内臓における相関係数がそれぞれ 0.8354 および 0.9587 となり，ともに良好な正の相関を示すことが分かった (Fig. 4). 両者の回帰直線は，それぞれ $y=0.6478x-93.45$ と $y=0.5955x+147.4$ で，平均的には筋肉で総毒力の約 65%，内臓では約 60%を TTX が占めると判断された.

一方，11-oxoTTX と推定される成分につき，マウスに対する比毒性が TTX の 2 倍で，かつ LC/MS 分析における単位量当たりのイオン強度が TTX と同等と仮定して毒力を算出し，'当該毒力と TTX の毒力の和' と '公定法で測定された毒力' の相関について検討したところ，筋肉，内臓ともに極めて良好な正の相関が認められ (相関係数はそれぞれ 0.9073 および 0.9763)，回帰直線はそれぞれ $y=1.060x+75.97$ および $y=0.9664x+176.8$ となった．したがって，前述の仮定が正しければ，TTX と 11-oxoTTX でほぼ 100%マウス毒性を説明できることになる．11-oxoTTX は，ヨーロッパアカガエル *Rana temporaria* の骨格筋の細胞膜における Na チャンネル阻害作用が TTX の 4～5 倍強い[27]．これに基づき，マウスに対する比毒性も TTX の 4～5 倍と仮定すると，TTX と 11-oxoTTX の毒力の和がマウス毒性を大きく上回るという矛盾が生じるが，生物種による差や活性測定法の違いを考慮すれば，問題の比毒性が TTX の 2 倍以下である可能性は否定できないであろう．あるいは，2 倍以上であったとしても，単位量当たりのイオン強度が TTX より十分に高ければ，当該矛盾は起こらない．いずれにしても，この点を明らかにするためには，11-oxoTTX を分離・同定・定量する必要がある.

11-oxoTTX は，これまでコクテンフグ *Arothron nigropunctatus*，スベスベマンジュウガニ *Atergatis floridus*，ブチイモリ *Notophthalmus viridescens*，コガネガエル科カエル *Brachycephalus ephippium* から分離されているが[25),26),28),29)]，巻貝からの検出例はない．したがって，キンシバイは，内臓のみならず筋肉に極めて高濃度の毒を保持することを含め，特異な TTX 蓄積機構を備えているものと推察される．この点については，毒の起源や 11-oxoTTX の分離・同定・定量，他の TTX 関連成分の存否などと併せて現在検討中である.

ムシロガイ科巻貝のうち，日本産のハナムシロガイやアラレガイの毒成分は TTX またはその関連物質であることが知られているが[8),9)]，台湾に生息する同種の巻貝は，TTX に加え，副成分として PSP 成分である gonyautoxin 1～4 および neosaxitoxin を保有するという[10)]．キンシバイについても，PSP を対象として HPLC 蛍光分析を行ったが，同成分は全く検出されなかった．台湾産キンシバイも毒の主体は TTX であり，PSP 成分は保有しない[16)].

ま と め

長崎県橘湾に生息する小型巻貝 7 種 66 個体につき，マウスに対する毒性を調べたところ，キンシバイ (22 個体) のみが有毒であった．本種の毒力は，これまでに報告のある腐肉食性巻貝[8)~21)]の中で最も強く，内臓で 10,000 MU/g を上回る個体も見られた．また，半数以上の個体で筋肉に毒が偏在しており，内臓を除去しても数個体の喫食でヒトの最小致死量 (10,000 MU)[30)] に達する可能性のあることが示された．一方，LC/MS 分析により，キンシバイでは総毒力の 6～7 割を TTX が占めることが明らかとなり，さらに残余毒力の相当部分を 11-oxoTTX が占めると推定された．以上の結果から，キンシバイは食品衛生上極めて危険な種であると結論した.

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Note

Distribution of Tetrodotoxin in Pufferfish Collected from Coastal Waters of Sihanouk Ville, Cambodia

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In Cambodia, fatal food poisonings associated with the consumption of pufferfish have occurred for decades, but the causative species or toxins have never been documented. Herein, we investigated the toxicity of three pufferfish species of the genus *Lagocephalus* collected from the coastal waters of Sihanouk Ville, one of the main regions where poisonings have occurred. *L. wheeleri* and *L. spadiceus* were non-toxic, whereas *L. lunaris* was toxic and all of its body tissues exhibited toxicity levels exceeding the safety limit for human consumption (10 mouse units/g). Tetrodotoxin (TTX) was identified as the main toxin in this species; no paralytic shellfish poison(s) were detected. Consequently, we can confirm pufferfish to be a hazardous reservoir of TTX in Sihanouk Ville. It is likely that *L. lunaris* is one of the causative species of past pufferfish poisonings that have occurred in Cambodia.

Key words: pufferfish; genus *Lagocephalus*; *Lagocephalus wheeleri*; *Lagocephalus spadiceus*; *Lagocephalus lunaris*; food poisoning; tetrodotoxin; Cambodia

Introduction

Tetrodotoxin (TTX; $C_{11}H_{17}N_3O_8$, molecular weight = 319) is a lethal marine toxin with no known antidote, and TTX poisoning, which is mainly caused by consumption of certain species of pufferfish, is a serious health issue in many coastal areas of the Asia-Pacific region[1), 2)]. In addition to pufferfish, TTX is also contained in other aquatic animals, such as octopus, gastropod, xanthid crab, and horseshoe crab, which are thought to bioaccumulate TTX through the food chain, starting from bacteria that naturally produce TTX in marine environments[2), 3)].

In Cambodia, poisoning incidents arising from the consumption of pufferfish are common and sometimes result in human fatalities (at least 5 fatal poisoning incidents occurred between 2003 and 2007, involving a total of 57 patients with 9 deaths), but the causative species or toxins have never been documented. In Sihanouk Ville (Fig. 1), one of the main regions where pufferfish poisonings have occurred so far, large quantities of morphologically similar pufferfish of the genus *Lagocephalus* (Fig. 2) are sporadically on sale in local fish-landing markets, even though some of these fish are known to be toxic, and to have caused many human fatalities in Asian-Pacific countries, especially Japan and Taiwan[2), 4)-6)]. Therefore, this study was undertaken in order to clarify the toxicity of Cambodian marine pufferfish species.

Materials and Methods

Pufferfish specimens

All the pufferfish specimens of the genus *Lagocephalus* ($n = 117$) were collected along the inshore fish-landing sites in Sihanouk Ville (Fig. 1) from April–May 2005 (rainy season), December 2005–January 2006 and December 2006–January 2007 (dry season). Different species were identified according to their morphological characteristics, *i.e.*, distribution patterns of small spines in their dorsal bodies (*L. wheeleri*: elliptical shape that is apart from the base of the dorsal fin ray; *L. spadiceus*: tadpole-like shape whose tail reaches the base of the dorsal fin ray; *L. lunaris*: elliptical shape that extends to the base of the dorsal fin ray) (Fig. 2). The collected specimens were immediately frozen, transferred by air to our Laboratory of Food Hygiene in Nagasaki University, Japan, and subsequently stored below −20℃ until required for toxin analyses.

Toxicity assessment

After thawing, the specimens were dissected into different anatomic tissues, and then the toxicity of each tissue was assessed by mouse bioassay, according to the official guidelines of the Japan Food Hygiene Association[7)]. Lethal potency was expressed in mouse units (MU), where 1 MU is defined as the amount of

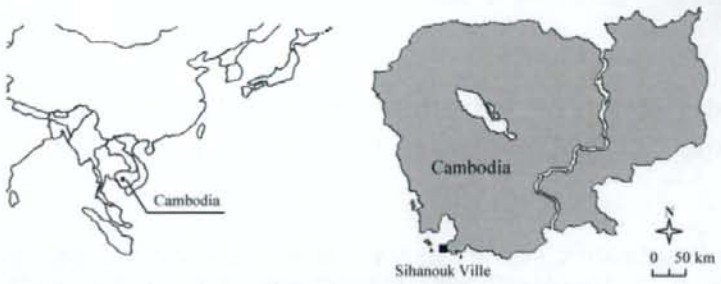

Fig. 1. Maps showing the location where marine pufferfish specimens were collected (■).

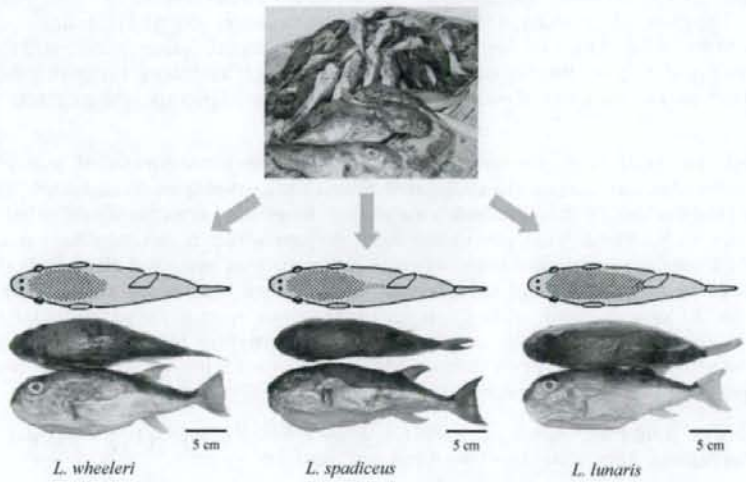

Fig. 2. Pictures of pufferfish of the genus *Lagocephalus* collected from the coastal water of Sihanouk Ville (Fig. 1). The species identification for *L. wheeleri*, *L. spadiceus* and *L. lunaris* are based on the morphological characteristics, *i.e.*, distribution patterns of small spines in their dorsal bodies.

toxin that kills a 20-g male mouse of the ddY strain within 30 min after intraperitoneal injection.

Toxin analyses

After mouse bioassay, a small amount (1–2 mL) of each tissue extract was filtered through a cellulose acetate membrane (0.45 μm; Toyo Roshi Co., Ltd., Japan), and submitted to toxin profile analyses as described below.

For detection of TTX, liquid chromatography/mass spectrometry (LC/MS) was performed on an Alliance separation module (Waters Corporation) equipped with a ZSpray™ MS 2000 detector (Micromass Limited) as reported by Ngy *et al.*[8] Reference of TTX (purity more than 90%; Wako Pure Chemical Industries Ltd., Japan), whose specific toxicity had been calibrated previously by mouse bioassay, was used as an external standard to identify and quantify the TTX contained in each sample.

For the detection of paralytic shellfish poison(s) (PSP), high-performance liquid chromatography with post-column fluorescence derivatization (HPLC-FLD) was conducted on a Hitachi L-7100 HPLC system using the methods described previously[9, 10], with some modifications. Reference samples of gonyautoxins 1–4 (GTX1–4), decarbamoylgonyautoxins 2, 3 (dcGTX2, 3), and neosaxitoxin (neoSTX), which were provided by the Fisheries Agency, Ministry of Agriculture, Forestry and Fisheries of Japan, as well as saxitoxin (STX) and decarbamoylsaxitoxin (dcSTX), prepared as reported previously[11], were used as standards to identify and quantify the individual analogues.

Results

The pufferfish specimens ($n=117$) collected in this study were identified as three different species, *Lagocephalus wheeleri* ($n=20$), *L. spadiceus* ($n=15$) and *L. lunaris* ($n=82$), which are common species in Cambodian and Asian-Pacific coastal waters.

Toxicity assessment by mouse bioassay revealed that *L. wheeleri* and *L. spadiceus* were non-toxic (less than 2 MU/g; data not shown), whereas *L. lunaris* was toxic (Table 1), irrespective of the collection season. No marked individual or seasonal variation in toxicity was observed, except for some specimens whose internal tissue toxicities were noticeably higher than those of the other specimens. Overall, the highest toxicities were generally found mainly in the liver (2–257 MU/g),

Table 1. Anatomic distribution of toxicity in Cambodian specimens of *L. lunaris*

Collection data	Specimen no.	Sex	Body size		Toxicity assessed by mouse bioassay (MU/g)					
			Weight (g)	Length (cm)	Skin	Muscle	Liver	Intestine	Testis	Ovary
Apr–May 2005	1	♂	350	21.8	3	3	9	5	—	—
(rainy season)	2	♂	340	19.0	14	34	48	63	18	—
	3	♂	300	20.3	3	9	9	<2	—	—
	4	♂	130	14.7	24	67	45	34	52	—
	5	♀	420	22.0	<2	<2	<2	<2	—	43
	6	♀	310	19.5	3	<2	5	3	—	62
	7	♀	305	19.5	21	62	46	14	—	222
	8	♀	255	19.3	2	<2	12	3	—	—
	9	♀	205	18.0	3	<2	<2	<2	—	19
	10	♀	187	16.8	7	41	16	28	—	—
	11[a]	♂/♀	56±5	10.8±1.2	3	2	2	2	—	—
	12[a]	♂/♀	54±7	9.7±1.1	3	3	<2	<2	—	—
	13[a]	♂/♀	50±6	11.1±1.0	3	15	8	6	—	—
	14[a]	♂/♀	47±5	10.3±0.9	<2	6	3	7	—	—
	15[a]	♂/♀	41±4	10.6±0.8	20	65	257	123	—	—
	16[a]	♂/♀	39±3	10.2±0.6	3	8	8	4	—	—
	17[a]	♂/♀	36±3	10.0±0.8	21	44	24	37	—	—
Dec 2005–Jan 2006	18	♀	180	16.3	3	2	4	<2	—	140
(dry season)	19	♀	35	10.1	6	33	17	11	—	17
	20[b]	♂/♀	61±6	15.0±1.4	<2	4	4	<2	—	—
	21[b]	♂/♀	51±5	13.3±1.3	<2	3	<2	2	<2	<2
	22[b]	♂/♀	36±5	11.0±0.9	3	7	3	<2	<2	<2
Dec 2006–Jan 2007	23	♂	223	22.0	<2	<2	<2	3	—	—
(dry season)	24	♂	125	17.9	3	5	3	3	<2	—
	25	♂	59	13.7	<2	3	<2	2	—	—
	26	♀	163	20.3	25	29	25	38	—	238
	27	♀	95	15.5	5	8	8	10	—	50

—: Not available nor applicable.

[a] Pooled specimens of 5 individuals due to their small size. Values presented are means ± standard deviation ($n=5$).

[b] Pooled specimens of 10 individuals due to their small size. Values presented are means ± standard deviation ($n=10$).

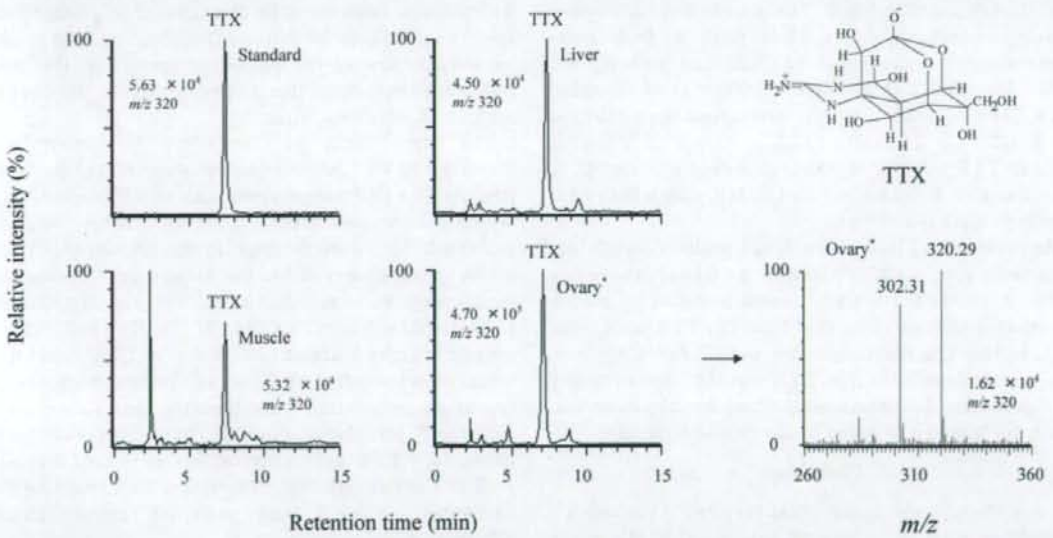

Fig. 3. LC/MS analysis for toxin profile of various toxic tissues extracted from *L. lunaris*. Selected ion (m/z 320) mass chromatograms of standard TTX [$(M+H)^+=320$], and of extracts of muscle, liver and ovary* (and selected mass spectrum). The ion intensity of the base peak (100%, relative intensity) is presented in each mass chromatogram.

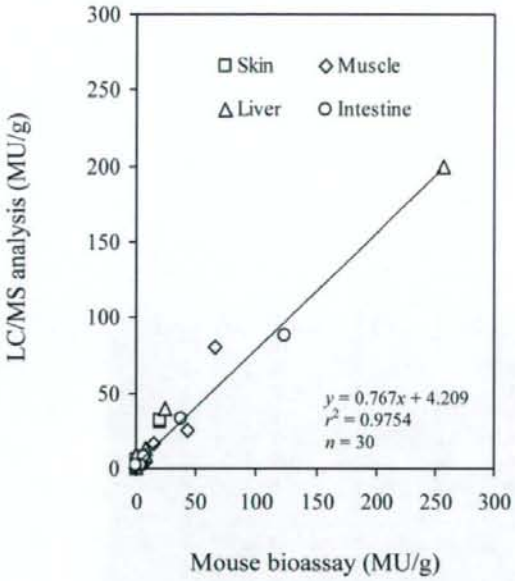

Fig. 4. Comparison of toxicity scores obtained from mouse bioassay and LC/MS analysis.

ovary (17–238 MU/g) and intestine (2–123 MU/g), followed by the muscle (2–67 MU/g), testis (18–52 MU/g) and skin (2–25 MU/g). These results indicate that the Cambodian *L. lunaris* is a hazardous species, as toxicity levels above 10 MU/g are considered as unsafe for human consumption[7].

The toxin profile of various toxic tissues from *L. lunaris* is depicted in Fig. 3. The muscle, liver and ovary tissue extracts showed a clear peak in their mass chromatograms scanned at *m/z* 320, and the retention time (7.34 min) was consistent with that of standard TTX ($[M+H]^+=320$). No PSP analogues were detected by HPLC–FLD in this study (data not shown). Likewise, neither TTX nor PSP was detected in any tissues of *L. wheeleri* and *L. spadiceus* in LC/MS and HPLC–FLD analyses (data not shown).

As depicted in Fig. 4, a significant positive correlation (Pearson's test; $n=30$, $r=0.9876$, $p<0.001$) was established between the toxicity scores assessed by mouse bioassay and those calculated from the TTX peak areas in LC/MS. The regression line, $y=0.767x+4.209$ ($r^2=0.9754$), demonstrates that TTX was the toxic principle in Cambodian *L. lunaris*, accounting for about 80% of the total toxicity detected in the mouse bioassay.

Discussion

According to the Japan Food Hygiene Association[7], pufferfish with a toxicity of less than 10 MU/g are considered as "non-toxic" and those with a toxicity above 10 MU/g are considered as "toxic", *i.e.*, unsafe for human consumption. In this context, the Cambodian *L. wheeleri* and *L. spadiceus*, whose toxicities were less than 2 MU/g, were determined to be non-toxic, irrespective of the collection season. Similarly, the toxicities detected in Japanese, Malaysian, Hong Kong, Bangladeshi and Thai specimens were less than 10 MU/g and these specimens were also considered to be non-toxic,[4),12–15]. On the contrary, the Cambodian *L. lunaris*, whose toxicity often exceeded 10 MU/g, was determined to be toxic. The morphology of *L. lunaris* is very similar to *L. wheeleri* and *L. spadiceus* (Fig. 2), so correct identification is important for consumers. Poisoning incidents due to the mistaken consumption of *L. lunaris* in the belief that it was non-toxic *L. wheeleri* or *L. spadiceus* has already been documented in Japan and Taiwan, involving more than 10 poisoning victims with one death[4)–6)].

As in many species of marine pufferfish reported previously[2),3)], the toxin was contained mainly in the liver and ovary (Table 1), and the toxicity scores, which ranged from less than 2 to 257 MU/g, were somewhat similar to those found in Taiwanese (less than 4 to 1,300 MU/g)[16] and Thai (less than 5 to 2,920 MU/g)[15] specimens, though not Bangladeshi species, whose toxicity scores were reported to be always less than 5 MU/g[14]. Interestingly, in the months (December to February) when low toxicity of less than 5 MU/g was reported for Thai specimens[15], the Cambodian ones were highly toxic. These findings clearly confirm that the levels and tissue distribution of the toxin in pufferfish vary with season and location, even for the same species in the same region[2),3),17),18)]. Because the minimal lethal dose of pufferfish (TTX) toxicity in humans is estimated to be 10,000 MU[4], consumption of only about 50 to 200 g of the edible tissues (*i.e.*, liver, ovary, or muscle) of *L. lunaris* (with toxicity above 50 MU/g) can be fatal. Therefore, *L. lunaris* can be desingnated as a hazardous species unsuitable for human consumption, and might have been one of the causative species in the past pufferfish poisonings that have occurred in the coastal regions of Sihanouk Ville.

The main toxin in Cambodian *L. lunaris* was identified as TTX, accounting for about 80% of the total toxicity. No PSP components, such as STXs and GTXs, which were detected in some other marine pufferfish[19)–23)], were detected in the present study. A minor peak observed in the electrospray ionization mass spectrum at *m/z* 302 of LC/MS (Fig. 3) appeared to be due to anhydroTTX ($[M+H-H_2O]^+=302$), whose specific toxicity is about 1/50 of that of TTX[24)], and this might be responsible for a part of the remaining toxicity. Many researchers have reported that Taiwanese[2)], Japanese[3)], Bangladeshi[14)] and Thai[15)] specimens contained only TTX and its derivatives, as in the currently studied Cambodian ones. Therefore, TTX could be the causative toxin in past pufferfish poisonings in Sihanouk Ville.

Consequently, we can confirm pufferfish in the coastal water of Sihanouk Ville to be a hazardous reservoir of TTX. Health authorities and the general public must be made aware of the correct identification of toxic and non-toxic pufferfishes, as well as other poten-

tially TTX-contaminated aquatic animals. Although *L. wheeleri* and *L. spadiceus* were tentatively identified as two different species based on their morphological characteristics, further studies on their identification using protein and genome-based techniques[4] are in progress.

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Transfer profile of intramuscularly administered tetrodotoxin to non-toxic cultured specimens of the pufferfish *Takifugu rubripes*

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ABSTRACT

Tetrodotoxin (TTX) was intramuscularly administered to non-toxic cultured specimens of the pufferfish *Takifugu rubripes* to investigate TTX transfer/accumulation profiles in the pufferfish body. In two groups of test fish administered either 50 MU/individual of TTX standard (purified TTX; PTTX) or crude extract of toxic pufferfish ovary (crude TTX; CTTX), TTX rapidly transferred from the muscle via the blood to other organs. The toxin transfer profiles differed between groups, however, from 4 to 72 h. In the PTTX group, little TTX was retained in the liver, and most (>96%) of the toxin remaining in the body transferred/accumulated in the skin after 12 h, whereas in the CTTX group, a considerable amount of toxin (15%–23% of the administered toxin or 28%–58% of the remaining toxin) was transferred/retained in the liver for up to 24 h, despite the fact that 89% of the remaining toxin transferred/accumulated in the skin at the end of rearing period (168 h). The total amount of toxin remaining in the entire body at 1–4 h was approximately 60% of the administered toxin in both groups, which decreased at 8–12 h, and then increased again to approximately 60%–80% at 24–168 h. Immunohistochemical observation revealed that the toxin accumulated in the skin was localized at the basal cells of the epidermal layer.

1. Introduction

The pufferfish *Takifugu rubripes*, as well as many marine pufferfish of the family Tetraodontidae, possess a potent neurotoxin, tetrodotoxin (TTX). In wild adult *T. rubripes*, the liver and ovary usually have strong toxicity, whereas the muscle, skin, and testes are non-toxic and are safe for human consumption (Noguchi and Arakawa, 2008). TTX is originally produced by marine bacteria, and distributed over a wide variety of animals other than pufferfish, including gobies, blue-ringed octopuses, carnivorous gastropods, starfish, toxic crabs, horseshoe crabs, flat worms, and ribbon worms (Miyazawa and Noguchi, 2001). The facts that pufferfish become non-toxic when fed non-toxic diets in an environment in which the invasion of TTX-bearing organisms has been eliminated (Matsui et al., 1982; Saito et al., 1984; Noguchi et al., 2006), and that such non-toxic pufferfish become toxic when orally administered TTX (Matsui et al., 1981; Yamamori et al., 2004; Honda et al., 2005; Kono et al., 2008a), indicate that TTX is exogenous in pufferfish and is derived from the food chain that starts from bacteria (Noguchi and Arakawa, 2008). The transfer, accumulation, and elimination mechanisms of TTX taken up into the pufferfish body via food organisms remain unclear. In our studies to clarify this point, we investigated the short-term transfer and accumulation profiles of TTX intramuscularly administered to non-toxic cultured specimens of *T. rubripes*. In oral administration

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experiments, long-term toxin accumulation is observed, but these experiments are not suitable for tracing short-term inter-tissue toxin transfer, because it is difficult to accurately administer a single large dose of toxin (Honda et al., 2005). To overcome this problem in the present study, we administered the TTX intramuscularly. Matsui et al. (1981) reported that when non-toxic cultured specimens of *T. rubripes* are fed diets containing crystalline TTX or crude toxic pufferfish ovary extract, only the test fish fed the crude extract of toxic pufferfish ovary accumulated TTX in their liver. Based on this information, we administered two types of toxins, 'purified TTX' and 'crude TTX', to evaluate whether the transfer profiles differed after entering the pufferfish body.

2. Materials and methods

2.1. Pufferfish specimens

Non-toxic cultured specimens of *T. rubripes* (approximately 4 months old; body weight, 13.2 ± 3.4 g; body length, 7.1 ± 0.6 cm; $n = 80$) (Noguchi et al., 2006) were purchased from a culture farm in Toishi, Nagasaki Prefecture, Japan. The specimens were acclimatized in aerated tanks for several days before administration of the toxin.

2.2. Preparation of toxin solutions

Toxic ovaries of the pufferfish *Takifugu vermicularis* were extracted with 1% acetic acid in 80% methanol, and the extract was defatted with dichloromethane and evaporated to make a condensed toxin solution (designated crude TTX). The toxicity of the crude TTX was evaluated using a mouse bioassay according to the official guidelines of the Japan Food Hygiene Association (2005). Lethal potency was expressed in mouse units (MU), where 1 MU was defined as the amount of toxin required to kill a 20-g male ddY strain mouse within 30 min after intraperitoneal administration. Liquid chromatography/mass spectrometry (LC/MS) analysis (Nakashima et al., 2004) revealed that the crude TTX was composed mainly of TTX and its analogs, such as 4-*epi*TTX and 4,9-anhydroTTX; TTX alone accounted for more than 90% of the total toxicity (data not shown).

Both TTX standards, purchased from Wako (purity > 90%; designated purified TTX) and crude TTX, were dissolved or diluted individually with a physiologic saline solution containing 1.35% NaCl, 0.06% KCl, 0.025% $CaCl_2$, 0.035% $MgCl_2$, and 0.02% $NaHCO_3$ at a concentration of 500 MU/ml and used in the following toxin administration experiments.

2.3. Toxin administration experiments

The acclimatized pufferfish specimens were divided into two groups of 40 individuals; one group was administered purified TTX (PTTX group) and the other was administered crude TTX (CTTX group). The groups were then maintained separately in two aerated 90-l tanks. Each fish was intramuscularly administered 0.1 ml (50 MU) of either purified or crude TTX solution and immediately returned to the tank (total handling time <30 s/individual to minimize stress to the fish). Then, 5 fish from each group were randomly collected at 1, 4, 8, 12, 24, 72, 120, and 168 h after toxin administration and toxin quantification was performed as described below.

2.4. Toxin quantification

Using a syringe precoated with sodium heparin, blood was withdrawn from the portal vein of each fish and centrifuged at 4200 g for 10 min. As TTX is partially binding to the TTX/PSP-binding protein in pufferfish blood plasma (Matsui et al., 2000; Yotsu-Yamashita et al., 2001), the supernatant (blood plasma) obtained was added with acetic acid at a final concentration of 0.1% to cut the binding, ultrafiltered through an Ultrafree-MC 5000 NMWL (Millipore Corp., Bedford, MA), and then submitted to enzyme-linked immunosorbent assay (ELISA) for TTX. After blood collection, all specimens were dissected into different anatomic tissues (liver, skin, and muscle), which were extracted with 0.1% acetic acid (Japan Food Hygiene Association, 2005). Each tissue extract was filtered through a USY-1 membrane (0.45 μm; Toyo Roshi Co., Ltd., Japan) and submitted to ELISA.

ELISA was performed according to the previously reported method (Ngy et al., 2008) using a monoclonal anti-TTX antibody developed by Kawatsu et al. (1997). The amount of TTX (in ng) determined by ELISA was converted to MU based on the specific toxicity of TTX (220 ng/MU). In a preliminary experiment using crude liver extracts ($n = 10$) of the pufferfish *Takifugu poecilonotus*, a significant and positive correlation (Pearson's test; $r = 0.9641$, $p < 0.01$) was observed between the TTX amounts determined by ELISA and those calculated from the TTX peak areas in LC/MS (Nakashima et al., 2004) (Fig. 1). The regression line, $y = 0.9874x + 7.301$ ($r^2 = 0.9295$) indicated that TTX was selectively quantified by ELISA in the presence of some TTX analogs including 4-*epi*TTX, 4,9-anhydroTTX, and deoxyTTXs (Yotsu-Yamashita, 2001) that were detectable in the extracts by LC/MS (data not shown).

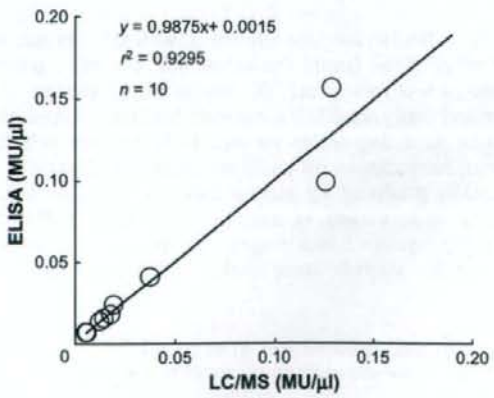

Fig. 1. Comparison of TTX amounts determined by LC/MS and ELISA.

2.5. Immunohistochemical observation

A part of the skin of each fish collected at 120 h after toxin administration was submitted to the immunohistochemical observation under light microscope according to the previously reported method (Tanu et al., 2002; Mahmud et al., 2003a,b) using the anti-TTX antibody.

3. Results

Changes in the toxin content (MU/g or MU/ml) of each pufferfish tissue during the rearing period are shown in Fig. 2. Changes in the toxin content of the liver differed between the PTTX and CTTX groups. In both groups, the toxin content was 12 MU/g at 1 h after administration. In the PTTX group, the toxin content gradually decreased until only a small amount (0.5–1.5 MU/g) remained after 12 h. In the CTTX group, however, the toxin content increased, reaching a maximum (around 21 MU/g) at 8–12 h; thereafter, the toxin content decreased, but 6.4 MU/g remained at the end of the rearing period (168 h). Changes in the skin toxin content also differed between groups. In both groups, the toxin content increased remarkably between 1 and 72 h and then remained at about 15 MU/g. The onset of the increase in the PTTX group occurred earlier than that in the CTTX group (after 12 h). The toxin content of the muscle, which was the site of administration, rapidly decreased and after 8 h was below the detection limit (0.01 MU/g) in both groups. The toxin content of the blood plasma was highest at 1 h (5.9 MU/ml in the PTTX group, 9.8 MU/ml in the CTTX group), and rapidly decreased thereafter.

Changes in the anatomic distribution of TTX, demonstrated by the relative amount of toxin retained in each tissue [% of the administered toxin (50 MU/individual)], are shown in Fig. 3. The total amount of toxin remaining in the whole body at 1–4 h was around 60% of the administered toxin (50 MU/individual) in both groups. The amount first began to decrease at 8–12 h, and then increased to approximately 60%–80% at 24–168 h. Changes in the amount of toxin in the liver tissues differed between the PTTX and CTTX groups. In the PTTX group, the amount of

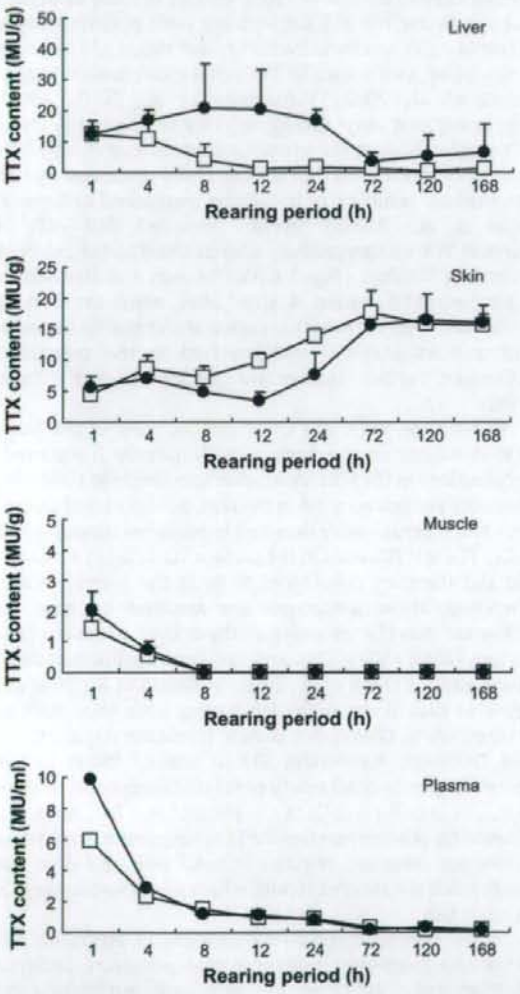

Fig. 2. Changes in the content of TTX (MU/g or MU/ml) retained in each tissue of the *T. rubripes* specimens during the rearing period after toxin administration. —□—: PTTX group; —●—: CTTX group. The toxin content in blood plasma was determined using a combined sample of 5 individuals for each point.

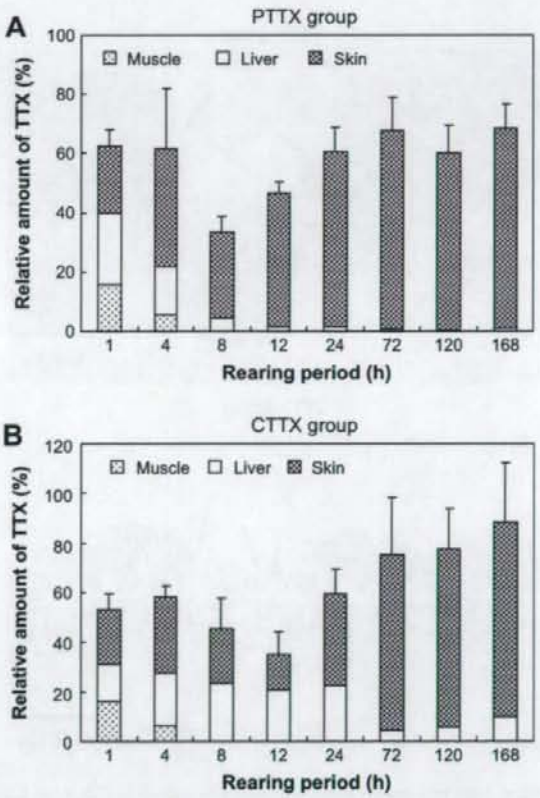

Fig. 3. Changes in the relative amount of TTX [% of the administered amount (50 MU/individual)] retained in each tissue of the *T. rubripes* specimens during the rearing period after toxin administration. A: PTTX group; B: CTTX group.

toxin in the liver rapidly decreased from 1 to 8 h, becoming less than 1.6% of the administered toxin (<3.2% of the remaining toxin) after 12 h. In the CTTX group, the amount of toxin in the liver did not decrease for up to 24 h and accounted for 15%–23% of the administered toxin (28%–58% of the remaining toxin). The amount of toxin in the skin gradually increased during the rearing period in both groups, and accounted for most (PTTX group, 98%; CTTX group, 89%) of the remaining toxin at 168 h.

Light micrographs of representative skin sections at 120 h after toxin administration are shown in Fig. 4. The epidermal layer of the skin was comprised of two distinct cell types, basal cells and succiform cells (Tanu et al., 2002; Mahmud et al., 2003a,b), and no gland or gland-like structure was observed. In both the PTTX and CTTX groups, positive reactions for TTX (brown color) were localized at basal cells along the basement membrane. No positive reaction was observed in the succiform cells, or in the skin sections of negative control, i.e., the fish without toxin administration (data not shown).

4. Discussion

TTX intramuscularly administered to non-toxic cultured *T. rubripes* rapidly transferred to other body tissues, and the toxin content of the liver and skin exceeded that of muscle within as little as 1 h after administration. At 1 h after intramuscular administration, a high concentration of TTX was present in the blood plasma, indicating that TTX transferred mainly via the bloodstream. The fact that muscles in toxic wild specimens of *T. rubripes* are not toxic indicates that the muscles of this species either do not retain and accumulate TTX or have a mechanism for eliminating TTX.

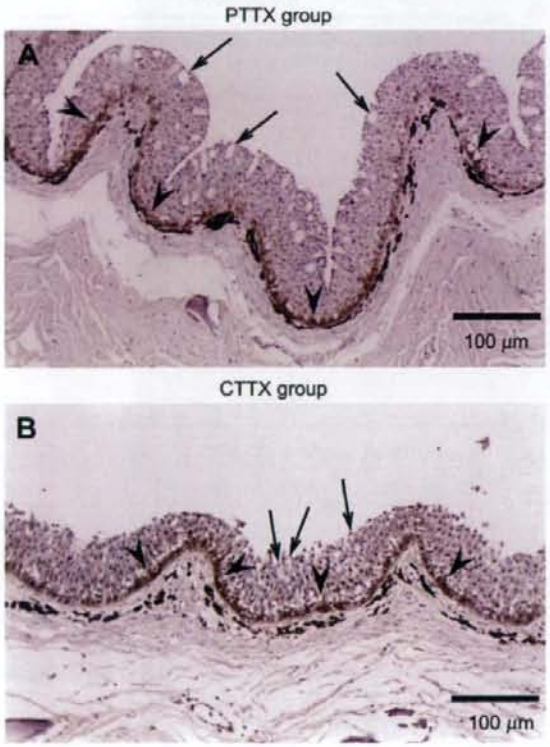

Fig. 4. Light micrographs of representative skin sections (×100) of the test fish collected at 120 h after toxin administration, showing TTX-positive basal cells (arrow heads) and TTX-negative succiform cells (arrows). A: PTTX group; B: CTTX group.

The toxin transfer profiles from 4 to 72 h differed between the PTTX and CTTX groups. In the PTTX group, little TTX was retained in the liver, and most of the toxin transferred to, and accumulated in, the skin after 12 h, whereas in the CTTX group, a considerable amount of toxin was transferred to, and retained in, the liver for up to 24 h. Although most of the toxin transferred to, and accumulated in, the skin thereafter, some toxin remained in the liver even at 168 h. Matsui et al. (1981) reported that when non-toxic cultured specimens of *T. rubripes* are fed diets containing crystalline TTX or crude extract of toxic pufferfish ovary, only the test fish fed with the toxic pufferfish ovary accumulate TTX in their liver. The liver tissue of *T. rubripes* is equipped with a specific TTX uptake mechanism (Nagashima et al., 2003; Matsumoto et al., 2005, 2007), suggesting that some substance(s) coexisting in the crude TTX might enhance the uptake mechanism or change TTX molecules into a form that is more easily processed by this mechanism, resulting in the above mentioned difference. Kono et al. (2008b) recently reported that 40% of purified TTX intramuscularly administered to the cultured pufferfish *Takifugu* (*Fugu*) *niphobles* was transformed to 4,9-anhydroTTX within 4 days after administration. It is unclear, however, whether such transformation between TTX and its analog(s) was involved in the concerned difference. Further studies are needed to clarify these points.

In both the PTTX and CTTX groups, most of the toxin that remained in the body was eventually transferred/accumulated in the skin. Wild adult specimens of *T. rubripes* generally possess no toxin in the skin, but toxicity of several tens MU is occasionally detected in juveniles (unpublished data). The test fish used in the present study were 4 months old and therefore considered to be in the juvenile stage. Therefore, these specimens are assumed to have an immature skin TTX-excreting ability or liver and ovary TTX-accumulating ability. Our previous immunohistochemical investigations (Tanu et al., 2002; Mahmud et al., 2003a,b) revealed that in the pufferfish having toxic skin, such as *T. vermicularis*, *Chelonodon patoca*, *Tetraodon steindachneri*, and *Tetraodon nigroviridis*, TTX is mainly found in the secretory glands or secretory cells (succiform cells) of their skin. The succiform cells of the present test fish, however, showed no positive reaction for TTX, suggesting that TTX, in *T. rubripes* juveniles, remains at basal cells and does not easily reach the succiform cells, which provably excrete TTX in adult fish.

When non-toxic cultured specimens of *Tetraodon turgidus*, the freshwater pufferfish that possesses paralytic shellfish toxin (PST) in the skin, are intramuscularly administered PST, a toxin similar to that used in the present study, the PST rapidly transfers from muscle to other tissues and accumulates mostly in the skin at the end of rearing

period (Ngy et al., 2008). Interestingly, when *T. turgidus* specimens were administered the same dosage of TTX, all died within 3–4 h, and more than half of the TTX administered remained in the muscle in the dead specimens. It is therefore inferred that marine pufferfish that ingest TTX are thus endowed with a mechanism by which they transport TTX specifically and actively, and freshwater pufferfish that ingest PST are endowed with a mechanism that processes PST. TTX/PST-binding proteins have been isolated from the blood plasma of marine pufferfish (Matsui et al., 2000; Yotsu-Yamashita et al., 2001), and may be involved in the transportation mechanism.

In both the PTTX and CTTX groups, a temporary decrease in the total amount of toxin remaining in the whole body was observed at 8–12 h. A similar decrease was observed when *T. turgidus* was administered PST (Ngy et al., 2008). Watabe et al. (1987) reported that when *T. rubripes* was intraperitoneally administered [^{3}H]-TTX, the amount of TTX in the gallbladder was greatly increased 6 days after administration. The temporary decrease may be due to the temporary storage of a large amount of TTX in a particular organ or tissue other than muscle, liver, and skin. This point, along with the properties of the TTX/PST-binding proteins and specific toxin transportation/accumulation mechanisms, remains to be elucidated. Further studies are in progress.

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Conflict of interest

The authors declare that there are no conflicts of interest.

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