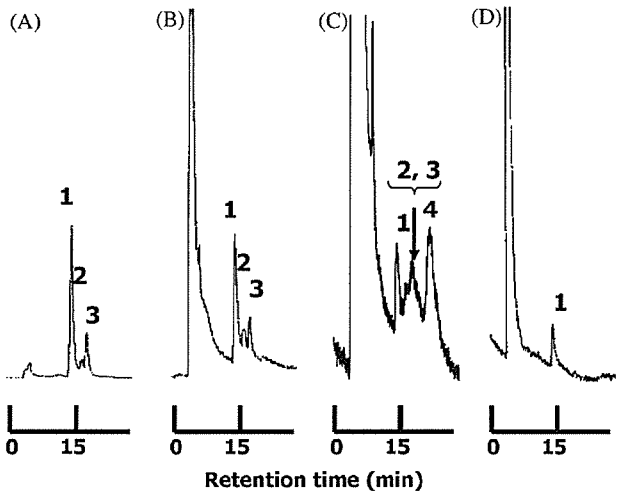

Fig. 2. HPLC analysis of TTX and its derivatives contained in the parasitic copepods and skin mucus of *Takifugu niphobles*. (A) TTXs standard: (1–3) tetrodotoxin, 4-epi-tetrodotoxin, anhydrotetrodotoxin. (B) Skin mucus of *Takifugu niphobles*; (C) *Pseudocaligus fugu*; (D) *Taeniacanthus* sp.

depicted in Fig. 3. Mass fragment ion peaks at m/z 376, 392, and 407, which are characteristic of the quinazoline skeleton (C9 base), appeared at the same retention times (*P. fugu* 10.29 min; *Taeniacanthus* sp. 10.30 min), respectively, along with TMS-C9 base derived from authentic TTX with a retention time of 10.26 min. All of these peaks from alkali-hydrolyzed extracts of these two parasitic copepods and authentic TTX revealed essentially the same mass spectra which were featured by fragment ions at m/z 407 (molecular peak), 392 (base peak), 376, 320, 318 and 230 (data not shown).

It can be concluded from the results of HPLC and GC–MS analysis that the extracts from the parasitic copepods contained TTX and its derivatives. As far as we know, this is the first report concerning the presence of TTX and its derivatives in the bodies of copepods. In this connection, it was indicated that the transmission of paralytic shellfish poison (PSP), which is the same Na^+ channel blocker as TTX, of the dinoflagellate toxins through herbivorous zooplankton as vectors to higher trophic levels and that they can reach sufficient levels in zooplankters to cause fish and its larvae kill(White, 1979, 1980, 1981; White et al., 1989). It is reported that planktonic copepods, *Acartia tonsa* and *Eurytemora herdmani* accumulate PSP by feeding on toxic dinoflagllates (Teegarden and Cembella, 1996). Hence not only parasitic but also planktonic copepods generally have a capability to be resistant to Na^+ channel blockers such as PSP and TTX.

On the other hand, some intestinal bacteria of TTX-bearing animals were demonstrated to produce TTX (Miyazawa and Noguchi, 2001). It suggests that TTX-bearers become toxic through the food chain in which TTX is transferred from lower to higher strata animals. This, along with the phylogenetically irregular occurrence of TTX, suggests that some microorganisms could be true producers of this toxin.

Relationships between the number of the two parasitic copepods on *T. niphobles* and the toxicity of its skin mucus of *T. niphobles* are depicted in Fig. 4. The numbers of *P. fugu* and *Taeniacanthus* sp. per host ranged from 0 to 94 individuals (average $\pm$ standard deviation $= 13.9 \pm 22.6$) and from 0 to 8 individuals (2.7 ± 2.8), respectively. The toxicity of the skin mucus of *T. niphobles* had a range of 108.5–1070.4 MU/g (average $\pm$ standard deviation $= 342.1 \pm 208.2$). Toxicity was detected from the skin mucus of all the hosts. Some evidence that may elucidate the physiological significance of this toxin in puffers has been recently reported. Saito et al. (1985) observed that puffers released large amounts of TTX from the skin when lightly wiped with gauze, and suggested that TTX in the mucus layer covering the integument of them may act as self-defense agent against predators. Kodama et al. (1985) found a similar phenomenon with a puffer stimulated by electric shock. In this connection, Kodama et al. (1986) also reported that unique exocrine glands or gland-like structures were found in the skin of several species of the puffer genus *Takifugu*. The glands of *T. pardalis* and *T. vermiculare porphyreum* consisted only of secretary cells with large vacuole.

Relationships between the more and less than the average number of the two parasites and the toxicity of its skin mucus of the host were examined by student's t-test (Table 1). In *P. fugu*, the average number per host was 13.9, and those are 520.7 ($n = 9$) and 269.0 MU/g ($n = 22$), respectively. A highly significant difference between them was detected at p-value 0.0011. In contrast, as for *Taeniacanthus* sp., the average number was 2.7, and those were 338.0 ($n = 14$) and 345.5 MU/g ($n = 17$), respectively. No significant difference was detected in *Taeniacanthus* sp.

The present study clearly coincides with the presence of TTX in the body of the parasitic copepod *P. fugu* on *T. pardalis*, which has been revealed by an immunoenzymatic technique (Ikeda et al., 2006). The fish ectoparasitic copepods are

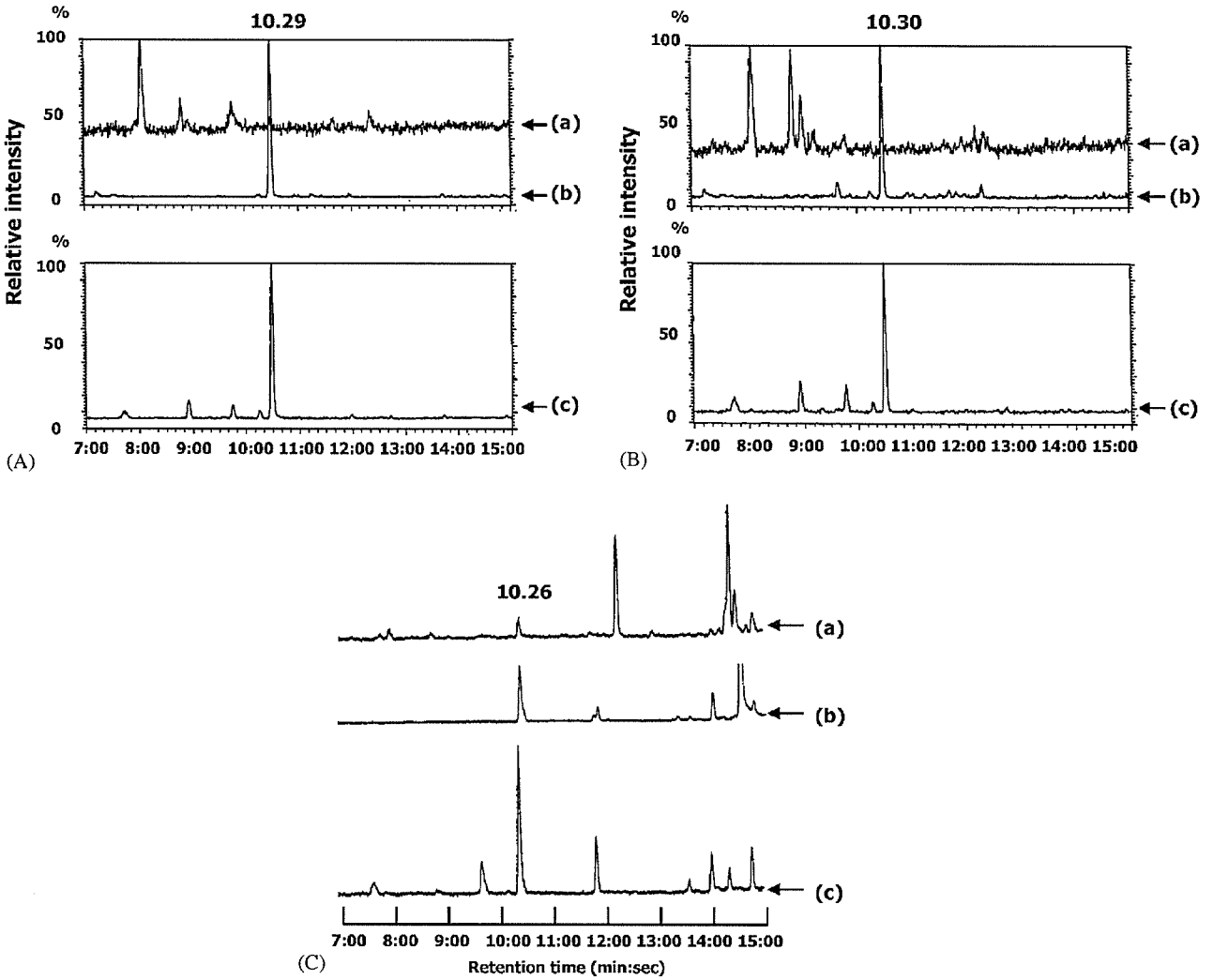

Fig. 3. Ion-monitored chromatograms of the trimethylsilyl derivative of the C9 base from the toxins contained in the parasitic copepods. (A) *Pseudocaligus fugu*; (B) *Taeniacanthus* sp.; (C) TTX standards (a–c); $m/z = 376, 407, 392$.

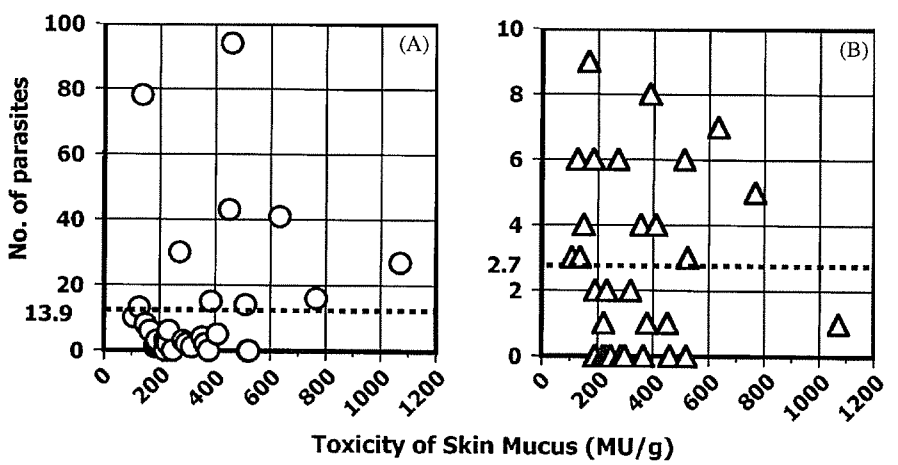

Fig. 4. Relationships between toxicity of mucus on the skin of the grass puffer *Takifugu niphobles* and the number of parasites on hosts. Dotted line in these figures shows average number of parasites. (A) *Pseudocaligus fugu*; (B) *Taeniacanthus* sp.

Table 1
Two groups of comparison divided by the average of parasites by student's *t*-test

Copepods	Over average		Under average		*p*-value
	No. of samples of *T. niphobles*	Mean toxicity (MU/g)	No. of samples of *T. niphobles*	Mean toxicity (MU/g)	
Pseudocaligus fugu	$n = 9$	520.7	$n = 22$	269.0	0.0011
Taeniacanthus sp.	$n = 14$	338.0	$n = 17$	345.5	0.9222

considered to feed on mucus, tissue and blood of host (Kabata, 1984). Since TTX and its derivatives were detected from both the host mucus and *P. fugu* in the present study, this skin parasite seems to have taken the mucus as food and accumulated TTXs in the body. The life cycle of the family Caligidae accommodating *P. fugu* is well investigated, which consists of two free-swimming naupliar stages, a single infective copepodid stage, four to six chalimus stages, one to two preadults (without molt), and one adult (Ho and Lin, 2005). The life cycle of *P. fugu* is incompletely addressed by us, but there are, at least, two naupliar and a single copepodid stages as free-swimming stages (Okabe, 2003). Since TTX is not accumulated in the ovary and eggs of the adult female of *P. fugu* (Ikeda et al., 2006), acquisition of this toxin seems to occur through feeding on the mucus and tissues of the host from the chalimus to the adult after attachment of the infective copepodid stage on it. The present HPLC result suggests that the gill parasite *Taeniacanthus* sp. has a different composition of toxin in the body. This may be explained by the following reasons: (1) toxic composition differs between the gill and skin mucus and/or (2) chemical conversion occurs in the body of the parasite.

The skin parasite *P. fugu* is found exclusively from the toxic puffer such as *T. niphobles*, *T. oblongus*, *T. pardalis*, and *T. poecilonotus* (Ho and Lin, 2005; Ikeda et al., 2006). The high host-specificity of *P. fugu* on the TTX-bearing puffer and the present bioassay strongly suggest a possibility that TTX may play a role in attracting the infective copepodid stage. In addition, preadults and adults of caligids detaching from the host can swim freely in water column (Ohtsuka, unpublished data). This may be only accidental and/or for active host switching. Also in that case, free-swimming preadults and adults of copepods of *P. fugu* may be re-attracted by TTX released from the host. Behavioral reaction of *P. fugu* to TTX will be observed in a laboratory in the future. However, the biological meanings of accumulation of TTX in the body and resistance mechanism against TTXs for the copepods are still unknown.

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徳島県浅川湾産スベスベマンジュウガニの毒の性状

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Toxin Profiles of a Xanthid Crab *Atergatis floridus* Collected from Asakawa Bay in Tokushima Prefecture, Japan

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Tamiko Hashimoto, and Naoyoshi Nishibori

ABSTRACT

A xanthid crab, *Atergatis floridus*, which lives along the coasts of Asakawa, in the southeastern parts of Shikoku Island, is known as a toxic marine animal. The crab contained both tetrodotoxin (TTX) and paralytic shellfish toxin (PST). The toxin was purified by Bio-Gel P-2 column chromatography and was shown to consist of TTX, 4-*epi*TTX, 6-*epi*TTX, 4, 9-anhydroTTX, and 11-saxitoxinehtanoic acid (SEA) by means of liquid chromatography mass spectrometry analysis.

Keywords : *Atergatis floridus*, TTX, 4-*epi*TTX, 6-*epi*TTX, 4, 9-anhydroTTX, 11-saxitoxinehtanoic acid (SEA)

緒　言

スベスベマンジュウガニ(*Atergatis floridus*)はオウギガニ科に属し，成長すると甲長が35 ㎜，甲幅が52 ㎜に達する小型の毒ガニである。甲殻の表面は名前のとおり平滑で体色は濃紫色，褐色又は淡緑色で明るい雲状紋があり，鋏の先は黒い。雑食性で，神奈川県三浦半島産のスベスベマンジュウガニ胃内容物から，紅藻類，海綿類，環形動物の組織，動物の卵及び砂が検出されている[1]。

紅海，東及び南アフリカ海岸，オーストラリア，タヒチ，ハワイなどに広く分布するインド太平洋種で，岩礁地帯やサンゴ礁の浅瀬などに普通にみられる。日本では，千葉県房総半島以南の太平洋沿岸各地，沖縄県南西諸島まで分布し，徳島県では県南部の浅川湾と県北部の小鳴門水道近くで確認されている[2]。

スベスベマンジュウガニは生息地域によって毒組成が著しく異なり，徳島県浅川湾産のものはフグ毒Tetrodotoxin(TTX)と麻ひ性貝毒Paralytic Shellfish Poison(PSP)の両方をあわせもち，冬期採取試料にTTX含有割合が高くなる傾向がある[2]。他の地域では，TTXを主成分とするもの[3]とPSPを主成分とするもの[4]の2つのタイプにほぼ限られ，浅川湾産のものの様に明らかに両者が共存しているものはほとんどみられない。

本研究は，徳島県浅川湾産スベスベマンジュウガニに含まれる毒素を精製し，高速液体クロマトグラフィー（HPLC），液体クロマトグラフィー／質量分析法（LC/MS）による毒成分の超精密分析を行い，これまでの結果と比較する事を目的とした。

材料及び方法

材料

1991年12月に徳島県浅川湾で採取し，−20℃で長期間凍結保存したスベスベマンジュウガニを試料とした。内臓は取り除き，殻と筋肉部を試料とした。

毒の抽出

スベスベマンジュウガニの殻及び筋肉部約2,000 gを磨砕後，3倍量の1％酢酸−80％メチルアルコールを加え，磨砕抽出した。3,000 ×gで20分間遠心分離して上清と残渣に分けた。残渣に同様の操作を2回繰り返し，得られた上清を合一して減圧濃縮した。濃縮物にジエチルエーテルで2回，ジクロ

ロメタンで1回，分液ロートにて脱脂操作を加え，水層を有毒画分とした。有機溶媒層に蒸留水を注いで分配し，水層を有毒画分に合一した。これらの操作で得た水層を減圧下で濃縮し，2,000 mlとした。限外ろ過膜を用いて，分子量1,000以下の画分を得て試料とした。

マウス毒性試験法

フグ毒及び麻ひ性貝毒のマウス毒性試験は公定法[5]に準じて行った。抽出及び精製の各段階でマウス毒性試験を行った。体重20 gのddY系雄マウスに，試験溶液1 mlを腹腔内投与して，投与が終了した時間からマウスの呼吸が完全に停止するまでの時間（致死時間）を測定した。TTXの場合は，呼吸が30分で停止する毒力を1マウスユニット(MU)とし，PSPの場合は，呼吸が15分で停止する毒力を1 MUとした。上記の方法で測定した致死時間から，TTX及びPSPの換算表を用いて毒性値を求めた。

活性炭処理

限外ろ過処理した毒抽出液を1 M水酸化ナトリウムでpH 5.5に調整した。よく水洗した活性炭1,000 mlを撹拌し，毒を活性炭に吸着させた。毒成分を吸着した活性炭に2,000 ml蒸留水を加え2分間撹拌してろ過した。集めた活性炭に1％酢酸－20％エチルアルコール4,000 mlを注加し1時間撹拌しろ過，さらに5％酢酸－40％エチルアルコール4,000 mlで1時間撹拌して，毒を活性炭から溶出した。得られた有毒脱着液をそれぞれFra. Ⅰ及びⅡとした。

Bio-Gel P-2カラムクロマトグラフィー

得られた有毒画分Ⅰ及びⅡをそれぞれ減圧濃縮後，Bio-Rad社製Bio-Gel P-2カラム（40 ㎜φ×800 ㎜）クロマトグラフィーに供した。毒溶液をカラムに吸着させ，蒸留水1,000 ml次いで0.1 M酢酸2,000 mlを流して毒成分を溶出させた。得られた毒溶液を減圧濃縮し，酢酸を除去した後，蒸留水でよく洗浄して0.15 M酢酸で緩衝化させたBio-Gel P-2カラム（10 ㎜φ×900 ㎜）により精製した。

HPLC用の試料調製

試料を，蒸留水とメタノールで活性化させたWaters社製Sep Pak C18カートリッジカラムに通し，妨害成分を吸着除去した。これで得た溶液を，Millipore社製ウルトラフリーを用いて15,000 ×gで20分間遠心ろ過し，適当な濃度に調製してHPLC用サンプルとした。

TTX分析用HPLC

カラムにGLサイエンス社製Inertsil ODS-3(4.6 ㎜φ×250 ㎜)を，移動相に10 mMヘプタンスルホン酸を含む2％アセトニトリル－60 mMリン酸アンモニウム緩衝液(pH 5.0)を用い，流速は0.6 ml/minとした。カラムからの溶離液に，4 M水酸化ナトリウムを同じ流速で混合させ，110℃に制御した10 mテフロンチューブで加熱して発色させ，励起波長384 nm，蛍光波長505 nmの蛍光強度を測定した[6]。

HPLCによるPSPの分析

全PSP成分一括分析のカラムに日立社製HG3013 N（4.6 ㎜φ×50 ㎜）と野村化学社製Develosil C-30UG-5（4.6 ㎜φ×250 ㎜）を，STX及びGTXそれぞれの分析に野村化学社製Develosil C-8を使用した。

移動相Aに5 mMヘプタフルオロ酪酸を含む10 mM酢酸アンモニウム緩衝液(pH 3.8)，移動相Bに10 mMヘプタフルオロ酪酸を含む10％アセトニトリル－30 mM酢酸アンモニウム緩衝液(pH 7.1)を用いた。分析開始時から25分まで移動相A，26分から45分を移動相B，46分から分析終了の70分まで移動相Aを流して分析した[7]。

STX分析の移動相には，カラムに野村化学社製Develosil C-8(4.6 ㎜φ×250 ㎜)を，移動相に2 mMヘプタンスルホン酸を含む4％アセトニトリル－30 mMリン酸アンモニウム緩衝液(pH 7.3)を用いた。

GTX分析の移動相には，カラムにDevelosil C-8(4.6 ㎜φ×250 ㎜)を，移動相に2 mMヘプタンスルホン酸を含む10 mMリン酸アンモニウム緩衝液

(pH 7.3)を用いた。いずれの分析も流速は0.6 ml/minとした。

カラムからの溶離液に，7 mM過ヨウ素酸を含む50 mMリン酸緩衝液（pH 10.0）を同じ流速で混合させ，65℃で加熱して蛍光化させ，その後0.5 M酢酸を同じ流速で混合して蛍光強度を増感させ，励起波長340 nm，蛍光波長410 nmの蛍光強度を測定した。

液体クロマトグラフィー／質量分析法（LC/MS）

カラムに日立社製HG3013N (4.6 ㎜φ×50 ㎜)と野村化学社製Develosil RP-AQUEOUS-AR(4.6 ㎜φ×250 ㎜)を使用した。移動相Aに5 mMヘプタフルオロ酪酸を含む10 mM酢酸アンモニウムを，移動相Bに5 mMヘプタフルオロ酪酸を含む10%メチルアルコール−30 mM酢酸アンモニウムを用いた。HPLCの全PSP成分一括分析と同様に，分析開始時から25分までを移動相A，26分から45分を移動相B，46分から分析終了の70分までを移動相Aとした。溶離液を音速噴霧イオン化法sonic spray ionization (SSI)[8]を装備した日立M-8000型LC/MSに導き，質量分析を検出器としたHPLCを実施した。MS測定条件をポジティブモード，第一細孔温度170℃，シールド温度300℃，検出器400V，フォーカス電圧30V，ドリフト電圧30Vとした。

結果及び考察

1．有毒画分の抽出と精製

スベスベマンジュウガニ試料約2,000 gからTTX換算で約45,000MUの粗毒を抽出した。これらを限外ろ過して，分子量1,000以下の画分に30,000 MUを回収した。全量を活性炭にて処理し，希酢酸エチルアルコール溶液で着脱した。1%酢酸−20%エチルアルコール溶液で18,000 MU(Fra. Ⅰ)を，そして5%酢酸−40%エチルアルコール溶液で3,000 MU（Fra. Ⅱ）を得た。こうした活性炭処理での毒の回収率は70%と低かったが，茶褐色に着色し濃縮すると粘性が高くなっていた抽出液が，凍結乾燥により淡黄色パウダー状になるほど不純物が取り除かれ，毒の純度は飛躍的に上昇したと思われた。

18,000 MUのFra. Ⅰを水洗したBio-Gel P-2カラム（40 ㎜φ×800 ㎜）に展開して精製した。カラムから水で溶出した画分をFra. A，0.1 M酢酸溶出画分をFra. Bとした。同様に3,000 MUのFra. ⅡもBio-Gel P-2カラムで精製し，0.1 M酢酸溶出画分をFra. Cとした。カラムから溶出した各画分の毒性は，それぞれFra. Aが5,000 MU，Fra. Bが34,000 MU，Fra. Cが2,000 MUとなった。Fra. AとBの合計した毒量は39,000 MUであり，Bio-Gel P-2カラム処理前と比較すると217%毒量が増大した。一方，Fra. Cは67%と減少した。Fra. AとBでの毒量増大は，精製の過程で低毒性成分が一部分解して高毒性成分へ変換したためと推測された。これは，deoxyTTXに酸素原子が付加してTTXになる場合などが考えられる。

続いて，カラムサイズ10 ㎜φ×900 ㎜でのBio-Gel P-2カラムクロマトグラフィーを行った。Fra. A，B，Cをそれぞれ別々に精製した。10 mlずつの画分に分けた結果，Fra. AはFra. 10〜16にPSP換算で約2,000 MU，Fra. BはFra. 28〜35にTTX換算で約30,000 MU，Fra. CはFra. 25〜33にPSP換算で約1,000 MUの毒性を示した（図1）。

2．HPLC分析

Fra. A〜Cそれぞれの第2回Bio-Gel P-2カラムクロマトグラフィー結果から，有毒成分溶出の各ピークよりFra. AのFra. 14（A-Fra. 14），Fra. BのFra. 31（B-Fra. 31），Fra. CのFra. 29（C-Fra. 29）を選び，HPLCの試料とした。A-Fra. 14，C-Fra. 29からは，PSP成分として保持時間31〜32分に強いピークが検出された。後に述べるLC/MSの結果から，11-saxitoxinethanoic acid（SEA）と同定された。一方，B-Fra. 31から保持時間15.4分，17.3分，19.9分にピークが検出され，前記と同様にLC/MSの結果から，それぞれを溶出順にtetrodotoxin（TTX），4-*epi*TTX，anhydroTTXと判断した。B-Fra. 31にはTTXが最も高濃度で溶出しており，これは約8,500 MUの毒性を示した。以上の結果を図2に示す。

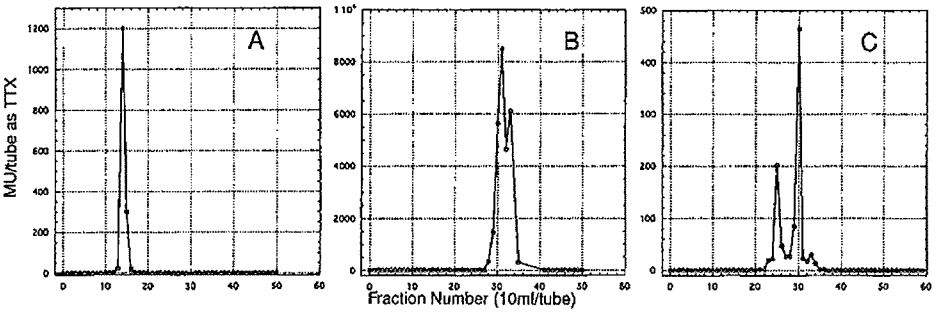

図1　浅川湾産スベスベマンジュウガニの1回目 Bio-GelP-2カラムクロマトグラフィーで溶出した有毒画分A,B及びCを2回目 Bio-Gel P-2カラムクロマトグラフィーに付した有毒成分の溶出結果
A：Fraction A (5,000 MU) を試料としたもの，B：Fraction B (18,000 MU) を試料としたもの，C：Fraction C (2,000 MU) を試料としたもの

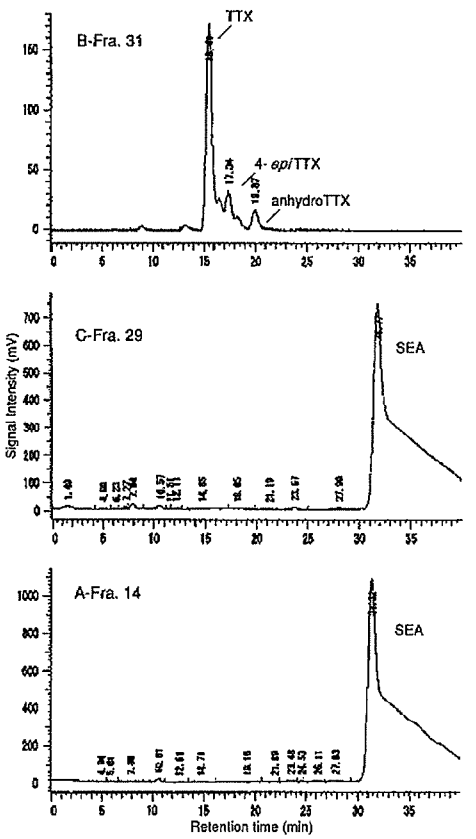

図2　2回目 Bio-Gel P-2カラムクロマトグラフィーで精製されたスベスベマンジュウガニ有毒フラクションのHPLC分析結果

3．LC/MS分析

HPLC分析においてTTXが検出されたB-Fra.31について，日立M-8000型LC/SSI-MSにて質量分析を行った。結果を図3及び図4に示す。図3のAはTTX，4-*epi*TTXの $[M+H]^+=320$ のマスクロマトグラムを示したものであり，12.8分と15.0分に2つのピークが出現した。それぞれのマスクスペクトルを図3のB，Cに表示した。両成分とも m/z 320の $[M+H]^+$ を与える事から，溶出の順に

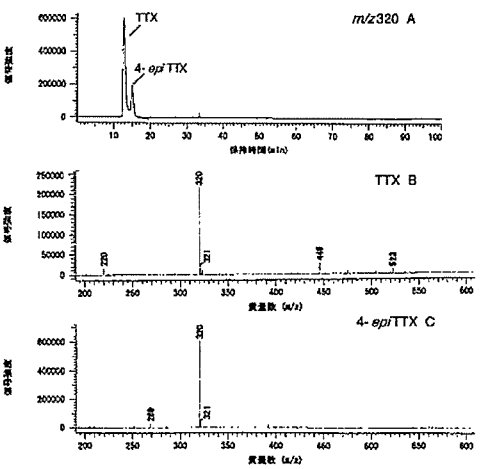

図3　B-Fra. 31のLC/MS分析結果
A：m/z 320によるマスクロマトグラム
B：保持時間12.73分に溶出したTTXのマススペクトラム
C：保持時間14.98分に溶出した4-*epi*TTXのマススペクトラム

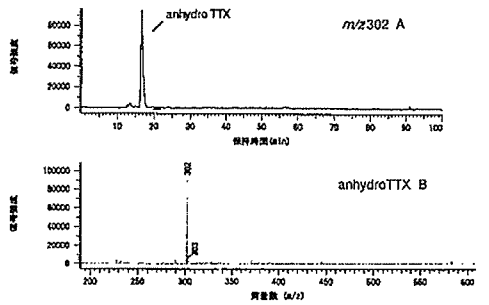

図4　B-Fra. 31のLC/MS分析結果
A：*m/z* 302によるマスクロマトグラム
B：保持時間16.58分に溶出したanhydroTTXのマススペクトラム

TTX，4-*epi*TTXと同定した。

また，TTXから脱水したanhydroTTXを*m/z* 302で検出した（図4－A）。保持時間17.8分に強いピークがみられ，そのマススペクトル図4－Bが*m/z* 302の単独シグナルを与える事から，本成分をanhydroTTXと同定した。

次に、HPLC分析においてSEAと思われるピークが検出されたA-Fra. 14についてLC/MS分析を行った結果を図5に示す。主要PSP成分として認められたSEAはLC/MSの分析条件で保持時間41～42分に溶出する事が*m/z* 358でのマスクロマトグラム図5－Aで明らかとなった。ピークトップが割れており，2成分存在するとの推測もできるが，イオントラップ型質量分析の特徴である成分過剰による飽和が原因と判断した。そのマススペクトログラム図5－Bは$[M+H]^+$=358，$[M+H-H_2O]^+$=340の2本が検出されており，PSP成分で11位にカルボキシル基が結合した11-saxitoxinethanoic acid（SEA）[9]と同定された。C-Fra. 29についても同様の結果が得られた。

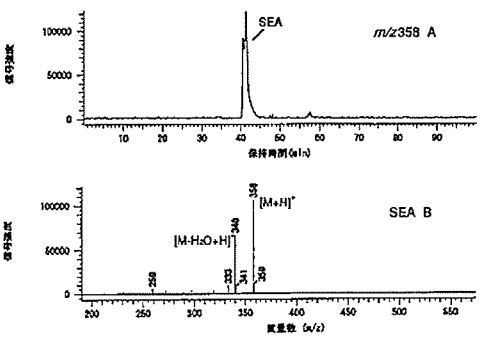

図5　A-Fra. 14のLC/MS分析結果
A：*m/z* 358によるマスクロマトグラム
B：保持時間41.35分に溶出したSEAのマススペクトラム

まとめ

1991年12月の浅川湾産スベスベマンジュウガニはTTX換算で約22.5 MU/gとかなり低い毒性値であったが，これは長期間保存していたため，毒性が著しく低下したのではないかと考えられる。Bio-Gel P-2カラムクロマトグラフィーを行った後毒性値が大きく跳ね上がったが，これは低毒性成分が高毒性成分に変換された事を意味しており，結果としてTTX，4-*epi*TTX，anhydroTTX，SEAが測定されたと結論した。同定された4種類の毒成分を母体とした各種前駆体の存在が推測される。

徳島県浅川湾産スベスベマンジュウガニは，TTXとPSPの両方をあわせもち，その比率は7：3であった。有毒成分の精製を行った結果，TTX群の大部分をTTXが占め，PSPはほぼSEAのみで構成されていた。SEAはPSP標品として入手しがたい貴重な物質である。PSP産生種として知られている渦鞭毛藻や毒化した二枚貝のPSP代謝は主としてスルホン化である。SEAはSTXにカルボキシル基が導入されたものであり，この様な特殊なPSP代謝酵素群がオウギガニ科スベスベマンジュウガニに分布する事が推測された。

本研究では，今までの浅川湾産スベスベマンジュウガニの分析結果と同様の傾向がみられ[2)10)]，数種のTTX関連物質を確認する事ができた。冬期採取試料のためか，TTXの含有割合が高かった。今後もこの地域のスベスベマンジュウガニの毒成分組成について詳しく調査すべきであると考える。

なお，本研究の一部は学術研究高度化推進費ならびに四国大学学術助成をうけ行われたものである。

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研究ノート

徳島県産ニホンイモリの毒性について

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Toxicity of the Newt *Cynops pyrrhogaster* Collected in Tokushima Prefecture, Japan

Takefumi SAGARA, Naoyoshi NISHIBORI, Tamiko HASHIMOTO, and Sachio NISHIO

緒　言

天然トラフグなどのフグ科魚類には，致死性神経毒であるフグ毒（tetrodotoxin：TTX）を蓄積するものがある。フグ科魚類以外にもスベスベマンジュウガニ，トゲモミジガイヒトデ，ハナムシロガイ，ツムギハゼ，ヒョウモンダコ，ヒモムシなど，多様の海産生物においてTTXの保有が確認されている[1]。一方，両性類にもTTXを保有するものがあり，中南米やバングラディッシュ産のカエル，国内では沖縄産シリケンイモリや本州産ニホンイモリなどが知られているが[2]，その国内における報告例は少ない。TTXを保有する生物は，その生息環境によって毒性値が大きく異なるとされており，ニホンイモリについても同様の報告がなされている[3]。徳島県にもニホンイモリが生息しているが，その毒性に関する報告が無かったため，本研究では，徳島県那賀川水系に分布するニホンイモリを採取し，地域別毒性を検討した。

試料及び方法

試料

2004年6月5日に徳島県羽ノ浦で採取したニホンイモリ15個体及び，同年6月26日に徳島県十八女で採取した16個体を試料とした。試料は採取後，実験に供するまで－30℃で冷凍保管した。

毒の抽出

毒の抽出は，旧厚生省生活衛生局監修食品衛生検査指針理化学編のフグ毒の項に準じて行った[4]。各個体をそれぞれ個別に磨砕し，等量の0.1%酢酸を加えて沸騰水中で10分間加熱抽出し，3,000 ×gで20分間の遠心分離によって得られた上清を粗毒抽出液とした。得られた粗毒抽出液は，前処理を施してマウス毒性試験及び機器分析に供した。

マウス毒性試験

粗毒抽出液を蒸留水で適当な濃度になるよう希釈して，ddY系雄マウスに1 ml腹腔内投与して致死時間を測定した。マウス単位（MU）は，得られた致死時間から既定の換算表により求めた。尚，TTX換算で1 MUとは，体重 20 gのマウス1匹を30分間で死亡させる毒量である[4]。

高速液体クロマトグラフィー（HPLC）分析

粗毒抽出液をMillipore製Ultra free C3LGCで処理し，高速液体クロマトグラフィー（HPLC）分析試料とした。分析の固定相には，Waters製Puresil C18（4.6 mm×250 mm）を，移動相には10 mMヘプタンスルホン酸を含む60 mMリン酸アンモニウム緩衝液を用い，流速を0.8 ml/minとした。毒を蛍光化させるために，カラム通過後の液に4 M水酸化ナトリウム溶液を流速0.8 ml/minで混入し，恒温槽内において110℃で1分間加熱し，励起波長384 nm，蛍光波長505 nmで検出した[2]。

液体クロマトグラフィー／質量分析計(LC/MS)分析

HPLC分析試料を用いた。固定相にはShiseido製C-18MG-Ⅱ（3.0 mm×250 mm）を，移動相には0.4%ヘプタフルオロ酪酸を含む5 mM酢酸アンモニウム緩衝液を用い，流速を0.4 ml/minとした[5]。

MSは音速噴霧イオン化法（sonic spray ionization：SSI）を装備したHitachi製M-8000を用い，極性ポジティブ，第一細孔温度170℃，シールド温度300℃，検出器400V，フォーカス電圧30V，ドリフト30Vで測定した。

結果及び考察

マウス毒性試験

マウス毒性試験の結果を表1に示す。羽ノ浦で採取した15個体中14個体，十八女で採取した16個体中9個体が有毒であった。有毒個体の毒性は羽ノ浦のもので24～247 MU/g，十八女のもので18～146 MU/gであった。地域別での平均毒性値は羽ノ浦で130±79 MU/g（mean±S.D.），十八女で33±42 MU/gであり，地域による毒性の違いがみられ，十八女産の個体では無毒個体の出現割合も高かった。最も毒性が高かったのは羽ノ浦産の個体で，247 MU/g，1047 MU/個体であった。

HPLC分析

羽ノ浦産イモリ試料のHPLC分析結果を図1に示す。ピークa及びcは標品のTTX及び4,9-anhydroTTXの保持時間と一致した。このHPLCの分析条件でTsurudaらは6-*epi*TTXをTTXの直後に検出している[2]ので，試料に検出されたピークdが6-*epi*TTXではないかと推定される。また，ピークeはそれに伴い検出される4,9-anhydro-6-*epi*TTXと考えられる[2]。

LC/MS分析

羽ノ浦産イモリ試料のLC/MS分析結果を図2,3に示す。TTX,4-*epi*TTX及び6-*epi*TTXの$[M+H]^+$が検出される*m/z* 320，及び4,9-anhydroTTXの[M+H]＋である*m/z* 302において試料から検出されたピークa,b及びcの保持時間が，標品のTTX，4-*epi*TTX及び4,9-anhydroTTXと一致した。また，サンプルのクロマトグラムにおいて，TTXのピークの直後に，6-*epi*TTXと思われるピークが検出された[5]。

サンプルの*m/z* 288，*m/z* 290，*m/z* 304及び*m/z* 336にピークが検出され，それぞれの保持時間のマススペクトラムを確認したところ，対象のスペクトラムを確認することができ，これらは5,11-dideoxyTTX（$[M+H]^+=288$，$[M-H_2O+H]^+=270$，）[6]，*nor*TTX-6-ol（$[M+H]^+=290$），deoxyTTX（$[M+H]^+=304$）及びoxoTTX（$[M+H]^+=336$）である可能性が示唆された。しかしながら，ピーク

表1　徳島県産ニホンイモリのマウス毒性試験結果

採取地	採取年月	雌雄	体長 (mm)	体重 (g)	毒性 (MU/g)	総毒量 (MU/個体)
羽ノ浦	2004年6月	♂	80	4.2	247	1047
		♂	85	4.1	228	937
		♂	90	3.9	223	863
		♂	92	2.8	215	591
		♂	85	3.5	201	708
		♂	82	2.9	156	457
		♀	150	5.9	135	794
		♀	110	6.1	120	730
		♂	80	2.7	117	318
		♂	87	3.0	88	265
		♀	100	4.7	86	408
		♂	85	2.7	56	153
		♂	93	4.6	56	255
		♀	102	8.6	24	206
		♂	88	3.9	<2	−
十八女	2004年6月	♂	77	2.6	146	380
		♂	85	3.2	95	302
		♀	105	5.1	65	332
		♂	80	2.3	61	139
		♂	90	2.7	56	150
		♀	100	3.8	35	133
		♂	85	2.8	29	82
		♀	95	3.3	27	89
		♀	103	4.5	18	81
		♀	110	4.6	<2	−
		♀	105	4.1	<2	−
		♀	100	4.2	<2	−
		♀	100	3.6	<2	−
		♂	89	3.9	<2	−
		♂	85	2.8	<2	−
		♂	80	3.1	<2	−

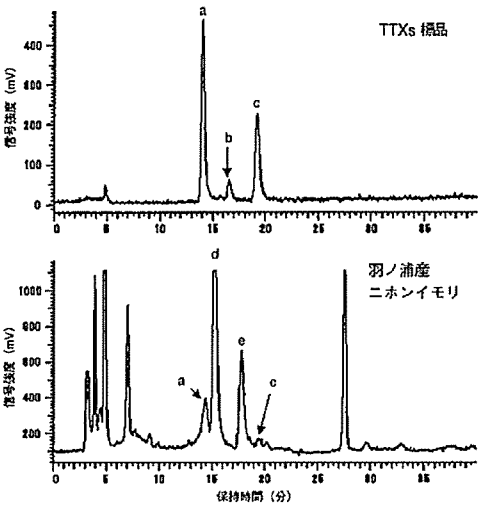

図1　羽ノ浦産ニホンイモリのHPLC分析結果
a:TTX, b：4-*epi*TTX, c：4,9-anhydroTTX, d:6-*epi*TTX, e：4,9-anhydro-6-*epi*TTX

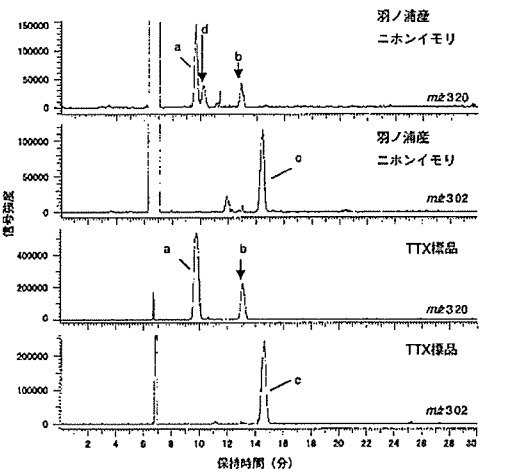

図2　羽ノ浦産ニホンイモリのLC/MS分析結果
a:TTX, b：4-*epi*TTX, c：4,9-anhydroTTX, d:6-*epi*TTX

が明瞭でないため，この分析には精製等の更なる前処理が必要と思われる。

まとめ

徳島県那賀川周辺に生息するイモリは，有毒個体の出現割合が高く，地域によって毒性値が異なることがわかった。HPLC及びLC/MS分析の結果から，その有毒成分は，TTX, 4-*epi*TTX及び4,9-anhydroTTXを含むことがわかり，他地域の個体と同様に6-*epi*TTXも保有していると判断された。その他の成分として5,11-dideoxyTTX, *nor*TTX-6-ol, deoxyTTX及びoxoTTXを保有する可能性が示唆されたが，このことを明確にするためには，精製等の更なる前処理が必要であると思われた。

日本にはイモリを食する習慣は無いが，イモリの黒焼きは惚れ薬になるという言い伝えがあり[7]，喫食されるケースが稀にある。本研究で調査したものの中で，最も高い毒性であった個体であれば5匹でヒトの最小致死毒量を上回ることから，極めて危険な生物であることを認識しなければならない。

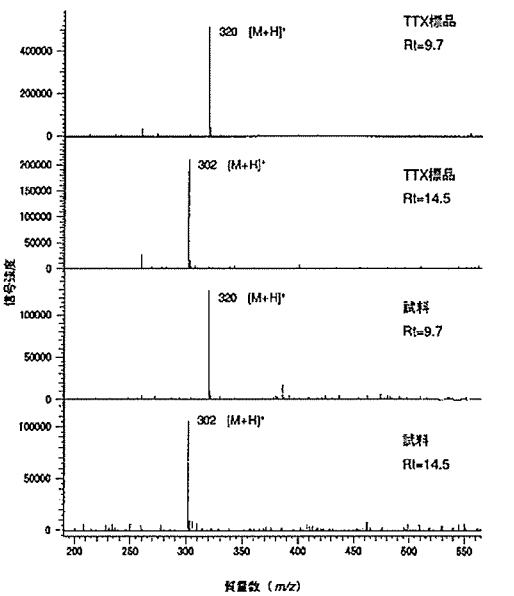

図3　羽ノ浦産ニホンイモリのマススペクトラム

謝　辞

実験に協力していただいた西岡祐一氏ならびに森藤美弥氏に感謝する。また，本研究の一部は四国大学の研究費助成を受け行われたものである。

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研究ノート

Cyclin Box Sequence in *Skeletonema costatum*

Naoyoshi NISHIBORI, Ryuji KONDO, Takefumi SAGARA and Sachio NISHIO

KEYWORDS : cyclin box, *Skeletonema* costatum, DNA sequenceing

Recently, blooms cause a significant threat to human health and fishery resources through mass mortality of marine animals and/or shellfish poisoning in humans around the world. As these blooms are the consequence of the vigorous and unusual growth of phytoplanktons, we consider that studies on the regulation of cell cycle in phytoplankton has great importance in the formation of blooms by these phytoplanktons. Cell cycle progression is well investigated and known to be regulated mainly by the activity of cyclin-dependent protein kinase (CDK) in yeast and mammalian cells[1]. Although the CDK is expressed at constant levels throughout the cell cycles, it is activated periodically by a cyclin subunit that is expressed in G1/S and G2/M phases during cell cycles. It is known that the cyclin gene has a conserved region called the cyclin box and that this region is important for binding to CDK[2]. Recently, cyclin genes have been cloned from a number of organisms such as sea urchin eggs[3] carrots and soybeans[4], and *Arabidopsis*[5]. In phytoplankton cells, the existence of a cdc 2 -kinase-like protein in *Crypthecodinium cohnii*[6] and *Gambierdiscus toxicus*[7] has been reported, but little is known about the cyclin gene and its expression in the cell cycle control of phytoplanktons thus far. In this study we describe the nucleotide sequence of the cyclin box of a bloom-forming diatom *Skeletonems costatum.*

An axenic clonal strain of *S.costatum* (NIES 324) was purchased from the National Institute for Environmental Studies of Japan. The algae was cultured in SWM- 3 medium at 20℃, under 6000lx and an L : D cycle 12 : 12. A 100ml stationary phase culture was centrifuged and the cell pellet obtained was used for the extraction of genomic DNA using Isoplant (NIPPON GENE) according to the instructions of the manufacturer. The primer set used to amplify the cyclin box gene were forward (5'-ATGCGNGGNATHYTNRTNGAYTGG-3') and reverse (5'-GGRTANATYTCYTCRTAYTT-3') primers encoding the amino acids MRGILI/VDW and KYEEYIP, respectively. A polymerase chain reaction (PCR) was performed using a DNA thermal cyclar (PCR 2400, Perkin Elmer) with a 100 μl reaction buffer mixture containing 30-50μg of template DNA, 100pmol of each primer, 10mM dNTPs and 2. 5U of *Taq* DNA polymerase (Nippon Gene). The thermocycling was as follows : 35cycles of 1min at 94℃, 2. 5min at 55℃, and 2min at 72℃ with a final elongation step of 7min at 72℃. The PCR products were analyzed using electrophoresis with 2%agarose gel. Unpurified PCR products were cloned into PCR II vector using a TA cloning kit (Invitrogen) and four clones were selected randomly. Each clone was sequenced using Thermo sequence core sequencing kit (Amersham) and a DNA sequencer SQ-5500 (HITACHI) with a pair of Texas Red M13 primes.

The PCR amplified fragment analyzed by agarose gel electrophoresis revealed a single product of approximately 200bp in length corresponding well to the length of cyclin box gene described previously[8].

The cyclin box sequence of *S costatum* (NIES324) is shown in Fig. 1 with some other cyclin box sequences obtained from the DNA database GenBank. The sequence obtained here was slightly longer than the sequence of *S.costatum* submitted

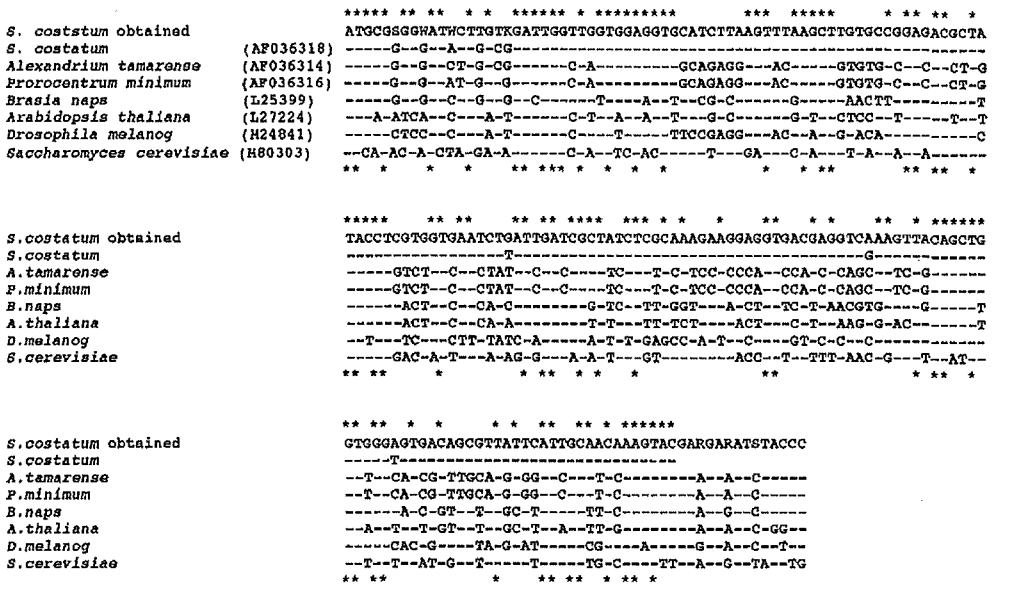

Fig. 1. Nucleotide sequence of S.costatum (NIES324) cyclin box aligned with cyclin box sequences obtained from DNA database GenBank. Upper and lower asterisks show identical nucleotides in phytoplanktons and in all sequences aligned here, respectively. Nucleotides identical to those of *S.costatum* (NIES324) obtained are indicated by (−). Accession numbers are also listed.

directly by Lin and Carpenter (GenBank Accession number. AF036318) and agreed well with other cyclin box sequences obtained from GenBank. The sequences of *S costatum* (NIES324) obtained here contained some mixed sequences shown by the letter S,W,K, and R and these mixed sequences might have been provoked by the use of degenerate primer. Except for these nucreotides, only five nucreotides were different from those of the *S. costatum* sequence from GenBank. The deduced amino acid sequences are listed in Fig. 2. with consensus amino acid sequences of A-type and B-type cyclin boxes. Only one amino acid of the deduced sequence obtained in this experiment was different from the sequence of *S.costatum* obtained from GenBank and agreed well with other deduced amino acid sequences. The amino acid sequence of *S costatum* (NIES324) in this experiment shared 68% and 69% homology with those of *B.napus* and *A. thaliana* and these scores were higher than that of *A. tamarense* (61%) and *P.mimimun* (63%). From the cyclin box sequence, cyclin in *S.costatum* could not

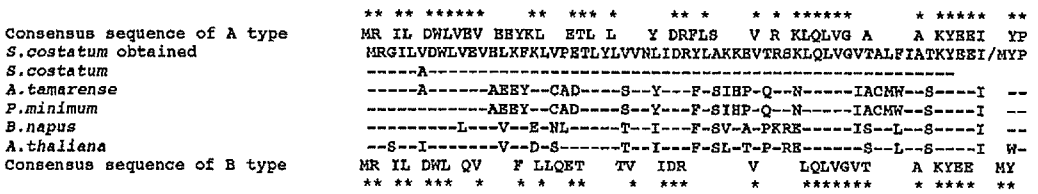

Fig. 2. Deduced amino acid sequence of *S.costatum* (NIES324) cyclin box aligned with cyclin box sequences of phytoplanktons and plants. Consensus sequences in cyclin A and cyclin B are also listed. Amino acids identical to those of *S.costatum* (NIES324) obtained are indicated by (−).

be assigned to the A-type or B-type group as shown in higher plants[5].

In yeast and mammalian cells, the cyclin is divided into some subgroups and different types of cyclin are expressed according to the cell cycle. Some subgroups of cyclins are also reported in higher plants[9] and the importance of transcriptional regulation of these genes for cell division and development were suggested[10-12].

This experiment has shown that the eucaryotic planktonic algae possessed cyclin that controls the cell cycle just like the other eucaryotic organisms. The expression of cyclin is still being investigated.

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(西堀尚良：四国大学短期大学部生活科学科)

Original

Occurrence of Paralytic Shellfish Poison (PSP)-Producing Dinoflagellate *Alexandrium tamarense* in Hiroshima Bay, Hiroshima Prefecture, Japan, during 1993–2004 and Its PSP Profiles

(Received February 1, 2005)

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To assess levels of shellfish intoxication by the paralytic shellfish poison (PSP)-producing dinoflagellate *Alexandrium tamarense*, potential health risks to human shellfish consumers and the possible need for regulatory intervention, yearly variations of maximum cell density of this species were examined from 1993 to 2004 in Kure Bay and Kaita Bay, which are located within Hiroshima Bay, Hiroshima Prefecture, Japan. The seawater temperature was determined concomitantly. In Kure Bay, maximum concentrations of 1,400 and 1,300 cells/mL at 0 and 5 m depths were observed on 21 and 24 April 1997. In Kaita Bay, remarkably high concentrations above 1,000 cells/mL of *A. tamarense* were observed in two out of three years investigated. These facts suggest that the environment in both bays is favorable for the propagation of *A. tamarense*. The temperature range at which the natural population of *A. tamarense* blooms was generally from 12 to 16℃. Four strains (ATKR-94, -95, -97 and -01) from Kure Bay and one strain (ATKT-97) from Kaita Bay were established. The strain ATKR-94, cultured in modified SW-2 medium at 15℃ for 15 days, showed a specific toxicity of 33.8×10^{-6} MU/cell. The toxins in all five strains exist almost exclusively as β-epimers (C2 (PX2 or GTX8), GTX3, dcGTX3 and GTX4), which accounted for 54.9 to 73.0 mol% of the total. The corresponding α-epimers (C1 (PX1 or epi-GTX8), GTX2, dcGTX2 and GTX1) accounted for 6.0 to 28.9 mol%. The toxin profiles of ATKR-97 and ATKT-97 were characterized by unusually high proportions of low-potency sulfocarbamoyl toxin, which comprised 62.4 and 68.2 mol%, respectively, of total toxins. In the toxic bivalves, the low-toxicity sulfocarbamoyl components, major components of *A. tamarense*, were present in amounts of only a few percent, suggesting that ***in vivo*** conversion of PSP occurs after ingestion.

A comparison of the toxin profiles of the causative dinoflagellate and contaminated bivalves showed that PSP components exist in the bivalves in the form of α-epimers, presumably owing to accumulation or storage of the toxins.

Key words: paralytic shellfish poison; *Alexandrium tamarense*; dinoflagellate; oyster; mussel; Hiroshima Bay; gonyautoxin; saxitoxin

Introduction

Contamination of bivalves with paralytic shellfish poison (PSP), produced by a number of toxic dinoflagellate species, such as *Alexandrium catenella*, *Gymnodinium catenatum* and *Pyrodinium bahamense* var. *compressum*, poses a serious problem to the shellfish culture industry, as well as to public health, in various parts of the world[1]. This toxin, once produced, can accumulate in filter-feeding shellfish that feed on the dinoflagellates, resulting in illness to humans at higher trophic levels in the food chain, mainly in the form of paralysis in parts of the body, followed by death in severe cases. *Alexandrium tamarense* is one of the toxigenic dinoflagellates responsible for several well-documented episodes of PSP in widely separated geographical countries in the world. In a previous paper, we reported on the first infestation (in 1992) of shellfish with PSP in Hiroshima Bay, Hiroshima Prefecture, which is one of the largest oyster culture areas in Japan[2,3]. Since then, *A. tamarense* has been periodically associated with episodes of PSP toxicity. Subsequent monitoring for toxins contained in commercial shellfish by mouse bioassay showed that short-necked clams, mussels and oysters were contaminated with PSP from the end of March to May in association with

the appearance of the toxic dinoflagellate *A. tamarense* in this bay. Fortunately, there have been no cases of food poisoning connected with the present PSP infestation of bivalves in Hiroshima Bay. In the 1993–2004 survey on the occurrence of PSP-producing dinoflagellates in Hiroshima Bay, five strains of *A. tamarense* were isolated and their PSP profiles were investigated. This investigation, developed as an extension of previous studies in which the authors participated, was undertaken to assess levels of shellfish intoxication by *A. tamarense*, potential health risks to human shellfish consumers and the possible need for regulatory intervention.

Materials and Methods

Dinoflagellate

Figure 1 shows Hiroshima Bay, and the relevant localities of Kure Bay and Kaita Bay. The relation between seawater temperature and maximum cell density of *A. tamarense* was examined throughout early spring to early summer from 1993 to 2004. Seawater samples were collected for cell counts from 0 and 5 m depths in Kure Bay and Kaita Bay, which are located within Hiroshima Bay, in this study period, simultaneously with the trial to isolate the toxic dinoflagellate identified as *Alexandrium tamarense* on the basis of the morphological characteristics[3]. Samples of seawater were taken with a Van Dorn water sampler and then concentrated appropriately. The number of *A. tamarense* cells in 1 mL of the concentrate was counted under a microscope. Five clonal cultures of *A. tamarense* were established and used for toxin analysis. Four strains isolated from Kure Bay in 1994, 1995, 1997 and 2001 were designated ATKR-94, -95, -97 and -01, respectively. One strain from Kaita Bay in 1997 was designated ATKT-97. The culture method, toxicity assays and toxin composition analysis of these strains were essentially the same as reported previously[3].

Shellfish

Prior to a year's survey, non-toxic oysters and mussels were collected, divided into cages created of mesh netting (2–3 dozen mussels per cage) and hung at 5 m depth at the experiment station in Kure Bay on 11 March 1997 so that aliquots could be collected routinely throughout the study period. Specimens of oysters and mussels collected from the cages at 5 m depth on 21 April 1997 were brought to our laboratory on ice for mouse assay of PSP toxicity by an official Japanese method[4], and immediately used for purification of toxins as described below.

Assay of toxicity

In the assay of toxicity of the dinoflagellates, cells were suspended in 0.5 mol/L acetic acid and ultrasonicated for 10 min. The lysate was centrifuged at 2,000×*g* for 20 min and the supernatant was obtained. A series of test solution was prepared by dilution with a small amount of distilled water and assayed for PSP toxicity by an official Japanese method[4]. The PSP toxicity of the shellfish samples was measured by the same method, using 0.1 mol/L hydrochloric acid as the extraction solvent[4]. The activity was expressed in mouse units (MU); 1.0 MU is defined as the dose of toxin required to kill a 20 g ddY strain male mouse in 15 min after intraperitoneal injection.

Purification of toxins from A. tamarense cells and bivalves

The acetic acid extract of *A. tamarense* cultured cells was concentrated and loaded onto a Sep-Pak Plus C18

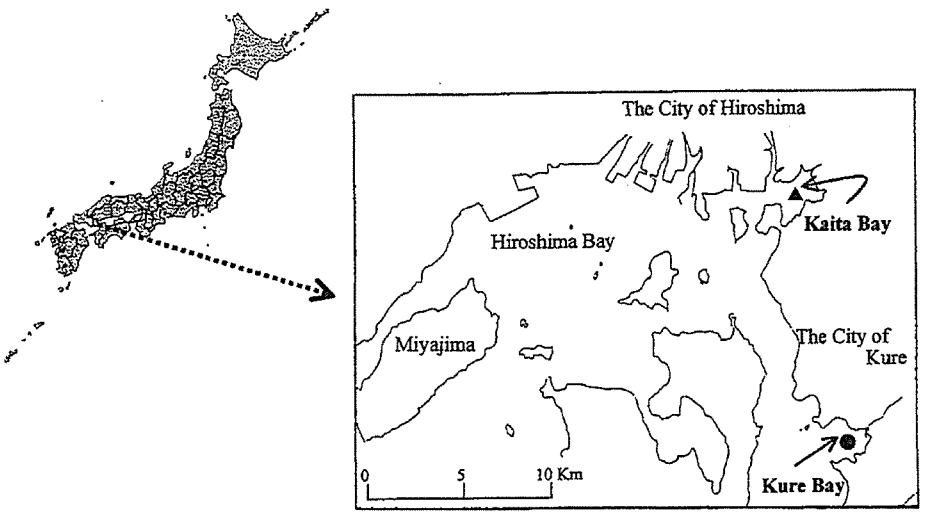

Fig. 1. Map showing Kure Bay and Kaita Bay along with Hiroshima Bay
●: Kure station; ▲: Kaita station

Environmental Cartridge (Waters). The unbound portion was collected and concentrated to dryness *in vacuo*. The residue was dissolved in a small volume of water and injected into the HPLC-fluorometric system[2),3)].

Specimens of oysters and mussels were collected from the cages at 5 m depth on 21 April 1997. Toxins from the toxic specimens of oysters and mussels were partially purified essentially according to the procedures previously described[2),3)]. The reference standards of PSP used in this study were prepared from the digestive glands of PSP-infested scallops *Patinopecten yessoensis* in Ofunato Bay, Iwate Prefecture[5)] and from a xanthid crab *Zosimus aeneus* from Kabira in Ishigaki Island, Okinawa Prefecture[6)]. Contents of *N*-sulfocarbamoyl derivatives (C1 (PX1 or epi-GTX8), C2 (PX2 or GTX8), C3 (PX3), C4 (PX4), GTX5 (B1) and GTX6 (B2)) were estimated from the increased amounts of corresponding carbamate toxins (GTX2, GTX3, GTX1, GTX4, STX and neoSTX) after acid treatment, due to lack of standards. Acid treatment was performed with 0.1 mol/L hydrochloric acid for 15 min in boiling water.

Results and Discussion

In the present study, the appearance of *A. tamarense*, in association with infestation of bivalves in Hiroshima Bay, was monitored in more than 70 sampling stations every two weeks. As a result, the abundance of this species in Kure Bay and Kaita Bay was found to be remarkably high in comparison with that in other stations. In this report, we analyze the data on the abundance and PSP production of *A. tamarense* and toxification of bivalves, focusing on these two areas.

Figure 2 shows the yearly variation of the maximum natural population density of *A. tamarense* and of the seawater temperature when the density reached maximum in Kure Bay and Kaita Bay. In Kure Bay, concentrations above 100 cells/mL of *A. tamarense* were observed almost every year during the investigation period except for 1999, 2003 and 2004. *A. tamarense* was recorded at maximum concentrations of 1,400 and 1,300 cells/mL at 0 and 5 m depths on 21 and 24 April 1997. In that year, the seawater temperature at 0 and 5 m depths was about 11℃ in the middle of March, and rose to almost 14 to 15℃ by the end of April. In Kaita Bay, remarkably high concentrations above 1,000 cells/mL of *A. tamarense* were observed in two out of three years investigated. On 20 May 1996, the maximum density reached 2,500 cells/mL at 0 m depth, when the seawater temperature was 18.1℃ (0 m depth) and 15.2℃ (5 m depth). Following this red tide, on 21 April 1997, a maximum density of 1,100 cells/mL in 0 m was observed, when the seawater temperature was 15.2℃ (0 m depth) and 13.4℃ (5 m depth). In many cases, judging from the results shown in Fig. 2, the seawater temperature when the maximum cell density of *A. tamarense* was recorded, was within the range of 12–16℃. This would explain why *A. tamarense* tends to occur from April to May in this area.

It is well known that *A. tamarense* is widely distributed in coastal waters throughout the world and is found in localities ranging from arctic to tropical[7)]. Its appearance is monitored all over the world[8)]. In comparison with the monitoring data in the past, the abundance of this species in Kure and Kaita Bays was high. This fact suggests that the environment in both bays is favorable for the propagation of *A. tamarense*. On the other hand, the abundance of the natural population of *A. tamarense* in 0 m depth was mostly higher than that in 5 m depth in the same water column. In this instance, the seawater temperature in 0 m depth tended to be high. This suggests that one of the key factors controlling the time when *A. tamarense* appears and forms a bloom is the seawater temperature. On the other hand, there were years when the maximum population density was low even though the seawater temperature was within the range mentioned above. In those years, the difference of seawater temperature between 0 and 5 m depths was small in comparison with that in years when a dense bloom of *A. tamarense* was observed. These phenomena suggest that stratification and the stability of the water column are also factors that support dense blooms of this species.

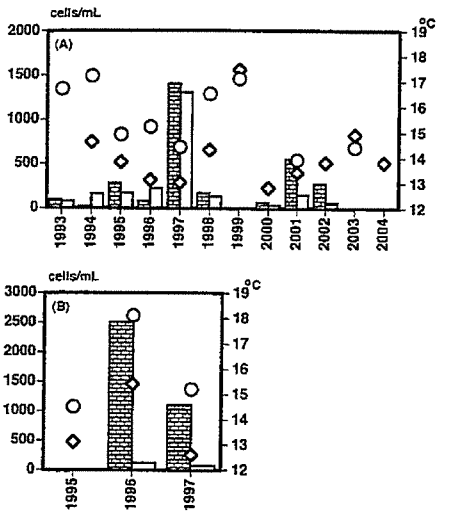

Fig. 2. Maximum cell density of *Alexandrium tamarense* in Kure Bay (A) and Kaita Bay (B), Hiroshima Prefecture
▤: Density (0 m depth); ○: Temperature (0 m depth); □: Density (5 m depth); ◇: Temperature (5 m depth)

Four strains (ATKR-94, -95, -97 and -01) were established from Kure Bay in 1994, 1995, 1997 and 2001 and one strain (ATKT-97) from Kaita Bay in 1997. All of these strains were identified as *A. tamarense* by the basis of the morphological characteristics, according to the criteria of Balech[9)]. The cultured ATKR-94 showed a specific toxicity of 33.8×10^{-6} MU/cell. This value was almost the same as that of the strain isolated in