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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

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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	−

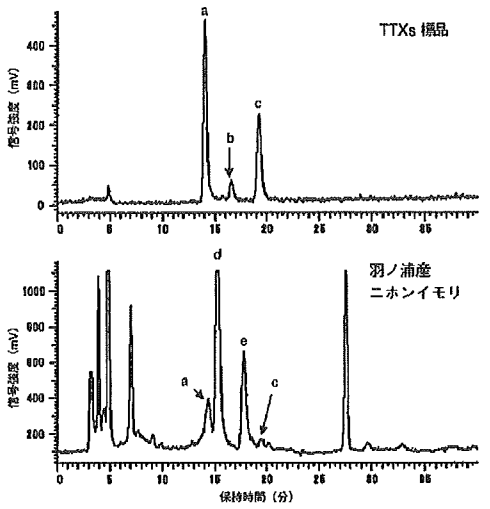

図1　羽ノ浦産ニホンイモリのHPLC分析結果
a:TTX, b：4-*epi*TTX, c：4,9-anhydroTTX, d:6-*epi*TTX, e：4,9-anhydro-6-*epi*TTX

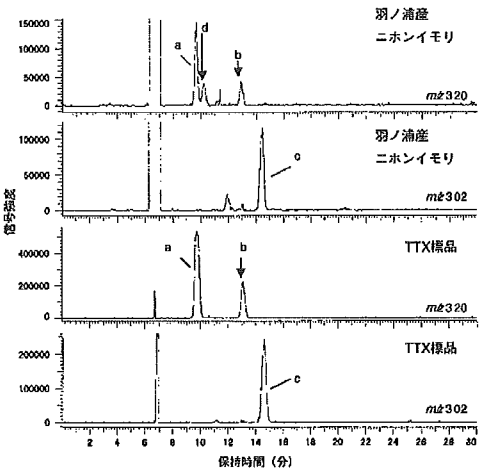

図2　羽ノ浦産ニホンイモリのLC/MS分析結果
a:TTX, b：4-*epi*TTX, c：4,9-anhydroTTX, d:6-*epi*TTX

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。

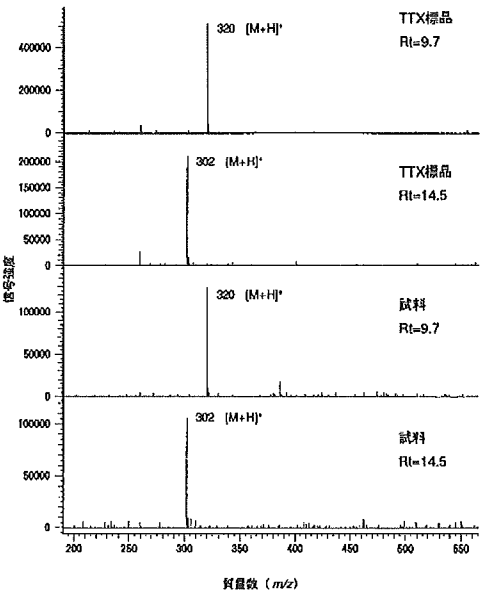

図3　羽ノ浦産ニホンイモリのマススペクトラム

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研究ノート

Cyclin Box Sequence in *Skeletonema costatum*

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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

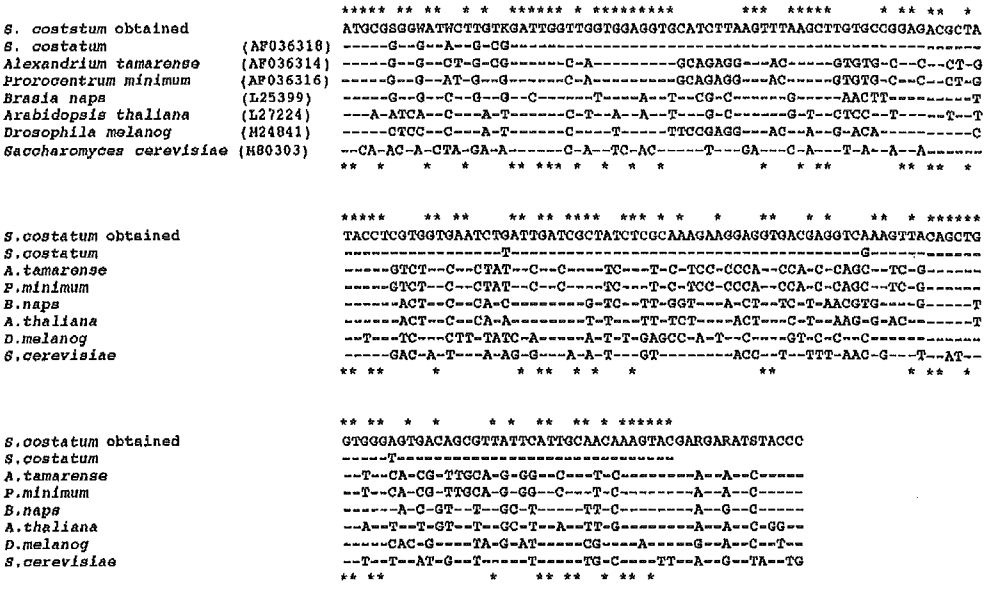

Fig. 1. Nucleotide sequence of S.costatum (NIES324) cyclin box aligned with cyclin box sequences obtained from DNA database GenBank. Upper and lower asterisks show identical nucleotides in phytoplanktons and in all sequences aligned here, respectively. Nucleotides identical to those of *S.costatum* (NIES324)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

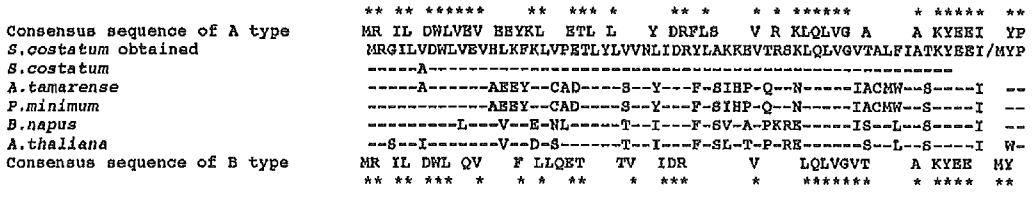

Fig. 2. Deduced amino acid sequence of *S.costatum* (NIES324) cyclin box aligned with cyclin box sequences of phytoplanktons and plants. Consensus sequences in cyclin A and cyclin B are also listed. Amino acids identical to those of *S.costatum* (NIES324) obtained are indicated by (−).

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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Original

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

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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

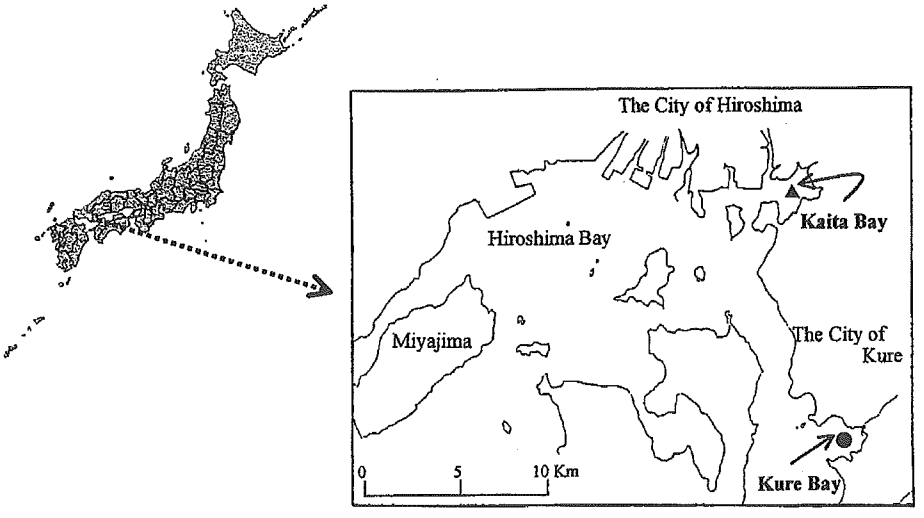

Fig. 1. Map showing Kure Bay and Kaita Bay along with Hiroshima Bay
●: Kure station; ▲: Kaita station

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.

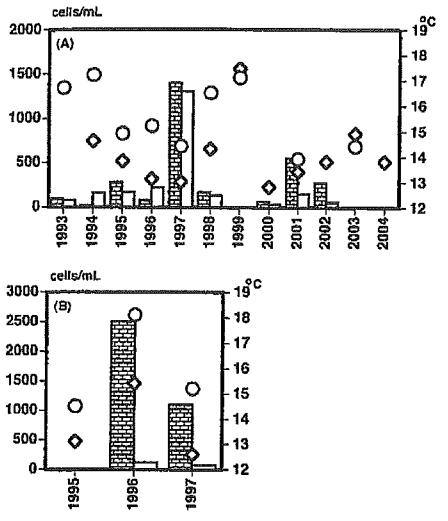

Fig. 2. Maximum cell density of *Alexandrium tamarense* in Kure Bay (A) and Kaita Bay (B), Hiroshima Prefecture
▤: Density (0 m depth); ○: Temperature (0 m depth); □: Density (5 m depth); ◇: Temperature (5 m depth)

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

Table 1. Toxin Profiles of *Alexandrium tamarense* Isolated in Hiroshima Bay and Toxic Bivalves

PSP component	Kure Bay						Kaita Bay
	Dinoflagellate	Bivalves (5 m depth)		Dinoflagellate			
	ATKR-97	Oyster	Mussel	ATKR-94	ATKR-95	ATKR-01	ATKT-97
GTX1	8.6	15.5	52.5	7.1	12.3	1.0	2.5
GTX2	7.6	12.0	13.1	0	0.9	1.4	0.6
GTX3	0.7	11.7	9.3	1.8	27.1	6.0	4.4
GTX4	4.2	4.8	11.5	40.8	11.6	34.4	0.6
dcGTX2	0.3	4.0	0.9	0	0	0	0.3
dcGTX3	0	3.8	1.2	0	0	0	0.3
C1 (PX1)	12.4	28.4	2.6	1.4	0.9	3.6	7.8
C2 (PX2)	50.0	7.6	1.8	30.4	32.2	20.4	60.4
C3 (PX3)	0	0	2.7	0	0.4	0	0
C4 (PX4)	0	0	0.7	0	0	0	0
neoSTX	16.2	7.0	2.7	18.5	13.3	32.5	16.6
STX	0	5.2	1.0	0	1.3	0.7	6.5

All results are shown in mol%

Kure Bay in 1993[3] and was also similar to the value of 39.9×10^{-6} MU/cell (Asakawa, one of the authors, personal communication) obtained from the natural population (160 cells/mL at 5 m depth) in Kure Bay on 6 May 1994. The strain isolated in the same bay in April 1992 possessed a specific toxicity of 53.4×10^{-6} MU/cell[3].

As for the toxicity of mussels and oysters in cages hung at 5 m depth in Kure Bay, their toxicity increased almost in parallel with the change in the natural population density of *A. tamarense*. On 21 April 1997 when the highest peak of cell density of *A. tamarense* was 1400 cells/mL at 0 m depth, toxicity levels in mussels and oysters collected from the mesh-netting cages reached the highest toxicity of 212 and 17 MU/g meat, respectively, and exceeded the quarantine limit.

The PSP profiles of the cultured cells of *A. tamarense* and contaminated shellfish are summarized in Table 1. The toxin profiles of *A. tamarense* (ATKR and ATKT) feature the presence of large amounts of C2 (20.4–60.4 mol%). Strains ATKR-97 and ATKT-97 contained higher proportions (50.0 mol%, 60.4 mol%) of C2 than the other strains. In addition, the toxins in all the strains exist almost exclusively as β-epimers (GTX3, GTX4, dcGTX3, C2 and C4), which account for 54.9–73.0 mol% of the total. On the other hand, the corresponding α-epimers (GTX2, GTX1, dcGTX2, C1 and C3) comprised only 6.0–28.9 mol%. DcGTX3 was observed in strain ATKT-97, though in only a trace amount (0.3 mol%). A similar toxin profile of cultured cells was recognized previously with two strains of *A. tamarense* (ATHS-92 and ATHS-93) isolated in Hiroshima Bay in 1992 and 1993[3]. Predominance of sulfocarbamoyl toxins such as C1 and C2 has also been reported for many strains of the dinoflagellates *A. tamarense* and *A. catenella*[10)-14)]. As for the configuration of 11-hydroxysulfate, Oshima *et al.* reported that contents of the α-epimers (GTX1, GTX2 and C1) were nearly three times higher than those of the corresponding β-epimers in the cysts of *A. tamarense*[15]. In contrast, toxins in vegetative cells were mostly composed of β-epimers. Based on this observation, Oshima *et al.* suggested stereospecific introduction of the moiety during biosynthesis[15]. The high proportion of β-epimers in vegetative cells of the present strains might be consistent with this idea.

Some fairly large differences were observed in the relative abundance of toxins between the responsible dinoflagellates and the contaminated shellfish (Table 1). The most notable difference was the change in the relative amounts of the low toxicity sulfocarbamoyl derivatives. In the toxic bivalves, C2, which is the major component of *A. tamarense* and is considered to be the precursor of GTX3[16),17)], was present in an amount of only a few percent. A substantial increase in the relative abundance of the carbamate toxin (GTX3) was recognized in bivalves, suggesting *in vivo* conversion of the *N*-sulfocarbamoyl toxins to corresponding carbamate toxins. It was also noted that PSP components exist in the bivalves in the form of the chemically more stable α-epimers. The α-epimers represent 59.9 and 71.8 mol% of the total for oysters and mussels, respectively. GTX1 was the major component in the bivalves, in spite of the lack of C3 in *A. tamarense* strains.

Noguchi *et al.* reported a possible bioconversion process from low-toxicity components such as C1 and C2 to high-toxicity ones, based on the results of feeding experiments[18]. Noguchi also reported that viscera homogenate of the "hiogi" scallop *Chlamys nobilis* has the ability to convert *N*-sulfocarbamoyl derivatives (C1, C2, GTX5 and GTX6) into decarbamoyl STX[19]. In contrast, it was reported that the toxin profiles of the Tasmanian dinoflagellate *Gymnodinium catenatum* and infested shellfish were characterized by unusually high proportions of low-potency sulfocarbamoyl toxin, which comprised 98–99% and 77–93%, respectively, of total toxins[20]. Shimizu *et al.* suggested the presence of enzymatic processes involving the transformation of GTXs to STX through the reductive elimination of the

C-11 hydroxysulfate and N-1 hydroxyl moieties in scallop homogenates[21]. Sullivan *et al.* showed that tissue extracts of the littleneck clam specifically eliminated the carbamoyl group of PSP[22]. Thus, these transformations of PSP components in bivalves can reasonably account for the large differences of toxin composition between the dinoflagellate and the filter feeders[23]. It has been reported that the toxin composition varies substantially among shellfish, suggesting complex metabolism of PSP in different species[10),24),25]. In this study, a large difference in the proportion of toxins such as GTX1 and C1 was recognized between oysters and mussels. The discrepancy may be due to environmental conditions which affect the metabolic pathways of bivalves.

The results obtained in this study suggest that the ultimate toxicity of bivalves may depend not only on the abundance and toxic potency of the dinoflagellates being filtered, but also on the *in vivo* transformations of the various toxins by the bivalves themselves. To clarify further the dynamic state of PSP in bivalve infestation, feeding experiments of *A. tamarense* to short-necked clam, mussel and oyster are in progress.

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Original

Paralytic Shellfish Poison (PSP) Profiles and Toxification of Short-necked Clams Fed with the Toxic Dinoflagellate *Alexandrium tamarense*

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As a part of our studies on paralytic shellfish poison (PSP) accumulation kinetics in bivalves, short-necked clam *Tapes japonia* was experimentally contaminated with PSP by being fed with the toxic dinoflagellate *Alexandrium tamarense* for 2, 4, 6, 8 and 10 days, and the processes of PSP accumulation and bioconversion were investigated: the toxicity level was determined by mouse bioassay and toxin components were identified by high-performance liquid chromatography (HPLC). The strain of *A. tamarense* used in this study possessed a specific toxicity of 186.7±81 (mean±S.D., $n=5$)×10^{-6} MU/cell. Total toxin concentration of this strain was 140.4±61 (mean± S.D., $n=5$) fmol/cell. The toxicity level of short-necked clams increased almost in parallel with the abundance of *A. tamarense*, reaching 1.8, 3.2, 3.8, 3.5 and 4.6 MU/g meat for 2, 4, 6, 8 and 10 days of feeding, respectively. The accumulation rates of PSP toxins, which are the ratio of the total amount of toxins accumulated in the bivalves to the estimated intake in each feeding experiment, were 7.5, 8.1, 5.7, 4.2 and 4.4% for 2, 4, 6, 8 and 10 days, respectively. At the end of each exposure period, many undigested algal cells were found in pseudofeces under microscopic observation. There was a remarkable difference in the relative proportions of the predominant toxin components between *A. tamarense* and short-necked clams. The most notable difference was the change in the relative amounts of C2 (carbamoyl-*N*-sulfo-11β-hydroxysaxitoxin sulfate), GTX1 and GTX 4 during the first two days. In the toxic bivalves, the amount of C2, which is dominant in *A. tamarense*, decreased to below half a percent after being ingested. Subsequently, the amount of GTX1 in the shellfish meat reached 50.1 mol%, while that of GTX4 decreased to about half of that in *A. tamarense*. As for the configuration of 11-hydroxysulfate, PSP components in *A. tamarense* exist almost exclusively as β-epimers (GTX3, GTX4, C2 and C4), accounting for 72.8 mol% of the total. This contrasts with the case of the short-necked clams, where the β-epimers represented 25.8, 33.8, 30.8, 36.8 and 28.5 mol% of the total after 2, 4, 6, 8 and 10 days, respectively. PSP components seemed to be converted rapidly at an early stage of the feeding of *A. tamarense*.

Key words: paralytic shellfish poison; *Alexandrium tamarense*; dinoflagellate; short-necked clam; pseudofeces; gonyautoxin; saxitoxin

Introduction

Paralytic shellfish poison (PSP), consisting of derivatives of saxitoxin (STX), are produced by a number of toxic dinoflagellates species, such as *Alexandrium tamarense*, *Alexandrium catenella*, *Gymnodinium catenatum* and *Pyrodinium bahamense* var. *compressum*, and to date more than 20 STX analogues have been reported[1]. These toxins can be accumulated in filter-feeding shellfish that feed on the dinoflagellates, resulting in illness to humans at higher trophic levels in the food chain, involving mainly paralysis in parts of the body, followed by death in severe cases. Hence, PSP potentially threatens human health, shellfish culture and related industries worldwide. *A. tamarense* is one of the toxigenic dinoflagellates responsible for several well-documented episodes of PSP in geographically widely separated countries around the world. Although accumulation of PSP in bivalves correlates with toxic dinoflagellate blooms, bivalves frequently contain a higher proportion of carbamate toxins (or a lower proportion of N-sulfocarbamoyl toxins) in comparison with the causative dinoflagellates[2)-7)]. The different toxin profiles of bivalves and toxic dinoflagellates have been partly explained by enzymatic and/or chemical transformation of toxins after accumulation in bivalve tissues[8)-10)]. Species-specific differences in PSP composition profiles have been recognized among bivalves[11)].

Blanco *et al.* suggested that it is very unlikely that any enzymatic reaction is involved in the reduction of the hydroxycarbamate to carbamate gonyautoxins[12]. Sekiguchi *et al.* showed that the sum of the amount of PSP toxins in the scallop and that released into the water cannot be explained in terms of the amount supplied by *A. tamarense*, suggesting that in addition to toxin accumulation from the food chain, another unknown mechanism is involved in toxin accumulation in the scallop[13].

However, research data on the mechanism of PSP toxins uptake and bioconversion in bivalves, especially in the important edible bivalves such as short-necked clams, oysters, *etc.*, are still insufficient. From a food-hygienic point of view, it is necessary to elucidate the mechanisms of PSP infestation of bivalves. In a previous paper, we reported on the first PSP infestation of shellfish (in 1992) in Hiroshima Bay, Hiroshima Prefecture, which is one of the largest oyster culture areas in Japan[2),3)]. We also reported that in the 1993–2004 surveys on the occurrence of PSP-producing dinoflagellates in Hiroshima Bay, five strains of *A. tamarense* were isolated, and their PSP profiles were investigated[4)]. A difference of toxin profiles between the bivalves and the toxigenic dinoflagellates was observed. Typically, *N*-sulfocarbamoyl toxins, such as C2 (PX2), are found in a lower proportions in bivalves than in toxic dinoflagellates[3)-6)].

In the present study, as a part of our studies on PSP accumulation kinetics in shellfish, short-necked clam was shown experimentally to accumulate PSP when fed with toxic dinoflagellate *A. tamarense* for 2, 4, 6, 8 and 10 days. The toxin contents and composition in the short-necked clams were determined by high-performance liquid chromatography (HPLC) and compared with those of the supplied *A. tamarense*.

Materials and Methods

Dinoflagellates

Strain ATHS-92 of the toxigenic dinoflagellate *A. tamarense* used in this study was isolated from Hiroshima Bay in April 1992. The strain was cultured batchwise in modified SW-II medium[3)] at 15℃ under a light intensity of 400 μE/m^{2}s (12 : 12 hr LD cycle). Algal cells of this toxic strain were used in the exponential growth phase.

Contamination of short-necked clams with PSP by feeding with A. tamarense

Non-toxic short-necked clams *Tapes japonica* were collected in the City of Onomichi, eastern part of Hiroshima Prefecture, in July 2000. They were acclimated to the experimental conditions for 3 days in a flow-through system provided with filtered ambient seawater at 18℃ with continuous aeration under a 12 : 12 L/D cycle until the beginning of the experiment. They were divided into 5 transparent plastic pails (60 clams per pail) filled with 20 L of aerated and filtered seawater at 18℃. Five pails (No. 1–5) were placed in one aquarium under the same experimental conditions. Sixty specimens of short-necked clam in No. 1–5 pails were fed with cultured cells for 2, 4, 6, 8 and 10 days (total 64, 119, 170, 220 and 295×10^5 cells of ATHS-92) at 10 am every day. The average amount of cells added to the pail was 29.5±6.1 (mean±S.D., n=10)×10^5 cells/day. On the first day of the feeding experiment, 500 mL of culture was added to the pail. After the second day, 250 mL of the culture was added every day. The cell number filtered by the short-necked clams in the pail was counted after removal of the bivalves.

Assay of toxicity

In the assay for toxicity level of the dinoflagellates, cells harvested by centrifugation at 3,000 rpm for 10 min were suspended in 0.5 mol/L acetic acid and ultrasonicated for 10 min. The lysate was centrifuged and the supernatant was diluted appropriately with water and assayed for PSP toxicity by an official Japanese method[14)]. The toxicity was expressed in mouse unit (MU); 1.0 MU is defined as the dose of toxin required to kill a 19–21 g ddY strain male mouse in 15 min after intraperitoneal injection. After completion of the feeding experiments, all clams in a pail were removed, shucked and immediately used for assay of PSP toxicity and purification of toxins as described below.

Purification of toxins from A. tamarense cells and bivalves

Extraction and purification of toxins from cultured *A. tamarense* cells and whole shucked short-necked clam tissues were carried out essentially as described before[2),3)]. An aliquot of the culture of *A. tamarense* was transferred to a glass vial and centrifuged at 3,000 rpm for 10 min. The supernatant was removed, and toxins in the cells were extracted with 0.5 mol/L acetic acid by ultrasonication. This acetic acid extract was concentrated and loaded onto a Sep-Pak Plus C18 Environmental Cartridge (Waters). The unbound portion was collected and concentrated to dryness *in vacuo*. The residue was dissolved in a small amount of distilled water and injected into the HPLC-fluorometric system[3)]. The PSP content of the samples was determined by comparing the peak area of each toxin with that of the standard. Weighed whole shucked meat of sixty short-necked clams in each group was combined and frozen immediately at −80℃ until toxins were extracted. Toxins from the toxic specimens of short-necked clams were partially purified essentially according to the procedures previously described[2)]. The reference standards of PSP used in this study were prepared from the digestive glands of PSP-infested scallops *Patinopecten yessoensis* from Ofunato Bay, Iwate Prefecture[15)] and from a xanthid crab *Zosimus aeneus* from Kabira, Ishigaki Island, Okinawa Prefecture[16)]. Contents of *N*-sulfocarbamoyl derivatives (C1 (PX1 or epi-GTX8)), C2 (PX2 or GTX8), C3 (PX3), C4 (PX4), GTX5 (B1) and GTX 6 (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 this feeding experiment, *A. tamarense* ATHS-92 possessed a specific toxicity of 186.7±81 (mean±S.D., $n=5$)$\times 10^{-6}$ MU/cell. Total toxin concentration of this strain was 140.4±61 (mean±S.D., $n=5$) fmol/cell. The relative amounts of individual toxins contained in the cells of *A. tamarense* were constant throughout the feeding period. Figure 1 shows the changes of toxicity and PSP accumulation rate in short-necked clams fed with *A. tamarense*. In this figure, accumulation rate is the ratio of total toxicity accumulated in the bivalves fed with this toxic dinoflagellate to the total toxicity of supplied cells in each feeding experiment. In the short-necked clams, toxicity levels reached 1.8 (0.9), 3.2 (1.6), 3.8 (2.0), 3.5 (1.9) and 4.6 (2.3) MU/g (nmol/g) shucked meat after 2, 4, 6, 8 and 10 days of feeding, respectively. The amounts of toxins of short-necked clams increased almost in parallel with the amounts of *A. tamarense* cells taken by them. However, the toxicity levels of these bivalves were extremely low in comparison with available toxicity during the exposure periods. It is generally accepted that filter-feeding activity becomes lower when bivalves are exposed to high densities of toxic dinoflagellates[17]. In the present study, the calculated clearance rates were 78.3, 98.4, 99.2, 98.9 and 98.9%, respectively. In all experimental groups, these rates were high. Therefore, the reason why the toxicity level of short-necked clams was low does not seem to be a decline of feeding activity. In this connection, the accumulation rates of PSP toxins were estimated to be 7.5, 8.1, 5.7, 4.2 and 4.4% after 2, 4, 6, 8 and 10 days of feeding, respectively. The rate decreased as the toxicity of the clams increased.

These data indicate that most of the toxins contained in *A. tamarense* cells were not accumulated in the short-necked clams. In other words, the low toxicity level of short-necked clams appeared to reflect a low accumulation rate. Both release of toxins from contaminated bivalves to the outside water and detoxification within bivalve tissue have been reported. These processes may partly explain the low accumulation rates in this feeding experiment. Interestingly, at the end of the each exposure period, many undigested algal cells were found in pseudofeces under microscopic observation (Fig. 2). This may be one of the major reasons why the accumulation rate of toxin was much lower than expected.

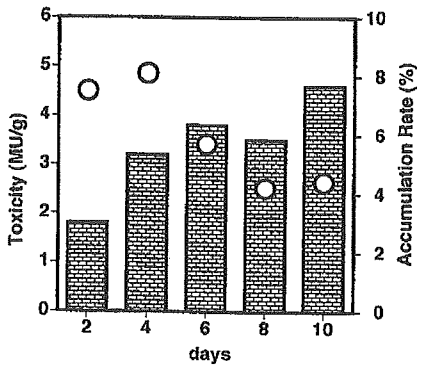

Fig. 1. Toxicity and PSP accumulation rate of short-necked clams fed with *Alexandrium tamarense*
▩: Toxicity; O: Accumulation rate

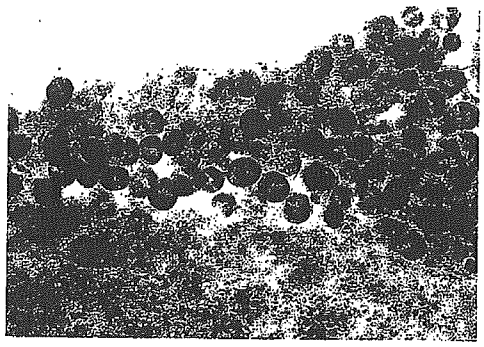

Fig. 2. Undigested cells of *Alexandrium tamarense* in the pseudofeces of short-necked clams

Table 1. Toxin Profiles of *Alexandrium tamarense* and Short-necked Clams Fed with *A. tamarense*

PSP components	*A. tamarense*	Short-necked clams fed with *A. tamarense*				
		2days	4days	6days	8days	10days
GTX1	10.4	50.1	38.9	38.7	37.5	48.1
GTX2	0	0.6	0.5	0.8	1.1	1.9
GTX3	1.6	2.6	3.6	3.5	5.3	7.3
GTX4	34.5	17.0	18.3	14.0	15.1	13.6
C1 (PX1)	0.8	2.3	1.5	2.5	2.2	1.5
C2 (PX2)	36.7	6.2	11.9	13.3	16.4	7.6
C3 (PX3)	1.3	0	0	0	0	0
neoSTX	14.7	21.2	23.7	26.5	20.7	18.7
STX	0	0	1.6	0.7	1.7	1.3

All results are shown in mol%.

The PSP profiles of the partially purified toxins from the cultured cells of *A. tamarense* and the short-necked clams fed with the plankton are summarized in Table 1. C2 and GTX4 were the dominant toxins in cultured *A. tamarense* cells. GTX1, GTX3, C1, C3, and neoSTX were also detected. Remarkable differences were observed in the relative abundance of the toxins between the responsible dinoflagellates and the contaminated shellfish. The most notable difference was the change in the relative amounts of C2 (carbamoyl-*N*-sulfo-11β-hydroxysaxitoxin sulfate), GTX1 and GTX4 during exposure. In the toxic bivalves, the amount of C2, which was dominant in *A. tamarense*, decreased less than half a percent, suggesting rapid conversion of PSP after feeding. Subsequently, the amount of GTX1 in the shellfish meat reached 50.1 mol%, while that of GTX4

decreased to about half of that in *A. tamarense*. As for the configuration of 11-hydroxysulfate, PSP components in ATHS-92 exist almost exclusively as β-epimers (GTX3, GTX4, C2 and C4), accounting for 72.8 mol% of the total. The same tendency was observed in the strains from Hiroshima Bay in 1993, 1994 and 1995[3),4)]. This contrasts with the case of the short-necked clams, where β-epimers represent 25.8, 33.8, 30.8, 36.8 and 28.5 mol% of the total after 2, 4, 6, 8 and 10 days of feeding, respectively. The contents of β-epimers in *A. tamarense* were approximately twice those in the bivalves fed with this dinoflagellate.

A comparison of the toxin profiles between the causative dinoflagellate and the contaminated bivalves showed that PSP components exist in the bivalves in the form of the chemically more stable α-epimers at an early stage (within two days) after the feeding of *A. tamarense*, suggesting rapid conversion of PSP components after feeding. Chen *et al.* reported rapid transformation of GTX4 to GTX1 in purple clams *Hiatula rostrata* fed with the toxic dinoflagellate *A. minutum*[18)]. Oshima *et al.* reported rapid degradation of N-1 hydroxy toxins and conversions of 11β-hydroxysulfate to 11α-epimer during the accumulation process in scallops, mussels and oysters[5)]. On the other hand, Ichimi *et al.* reported that there was a similar trend in the relative proportions of predominant toxins within *A. tamarense* and mussels experimentally contaminated with PSP by being fed with *A. tamarense*[19)]. Murakami *et al.* reported that the ratio of α-epimer (GTX1) to β-epimer (GTX4) at C11 tended to increase up to the equilibrium point of 3 : 1 in PSP-infested bivalves from Ibaraki Prefecture[11)]. Several reports have indicated that bivalves have higher proportions of carbamate toxins than the causative dinoflagellates. Noguchi *et al.* reported a possible bioconversion process from low-toxicity components such as C1 and C2 to high-toxicity ones, based on the results of feeding experiments[20)]. C1 and C2 in *A. catenella* were hardly detected in mussels, which attained a higher toxicity level than would have been expected from the uptake of *A. catenella* cells. In contrast, it was also reported that the toxin profiles of the Tasmanian dinoflagellate *Gymnodinium catenatum* and infested shellfish were characterized by unusually high proportions of low-potency sulfocarbamoyl toxin, which comprised 98–99% and 77–93%, respectively, of total toxins[21)]. The difference of toxin profiles between the bivalves and dinoflagellates has been partly explained by the enzymatic and/or chemical transformation of toxins after accumulation in bivalve tissues[8)–10)]. In this connection, a small amount of STX was detected in the short-necked clams after 4 days of feeding. There appear to be enzymatic processes leading to the transformation of GTXs to STX through the reductive elimination of the C-11 hydroxysulfate and N-1 hydroxyl moieties.

In this study, the toxin profiles of the short-necked clams and *A. tamarense* were not significantly different, except for epimerization for toxins from β- to α-type, and the remarkable change in the relative amounts of C 2 (Table 1). Interestingly, as shown in Table 1, the total ratio of β- and α-epimers in short-necked clams sampled after 2 days of feeding experiments was almost constant. This indicates that equilibrium is reached rapidly after the uptake of toxins by short-necked clams.

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