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$\times 10^5$ cells of ATHS-92) at 10 am every day. The average amount of cells added to the pail was 29.5$\pm$6.1 (mean$\pm$S.D., $n=10$)$\times 10^5$ 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., $n=5$)$\times10^{-6}$ 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.

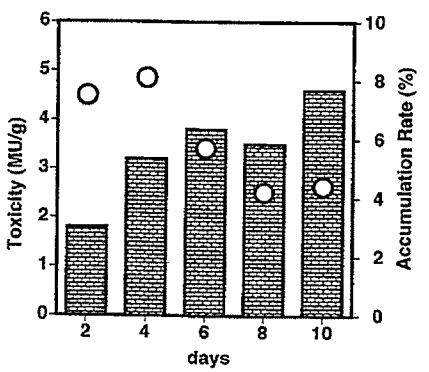

Fig. 1. Toxicity and PSP accumulation rate of short-necked clams fed with *Alexandrium tamarense*
■: Toxicity; ○: Accumulation rate

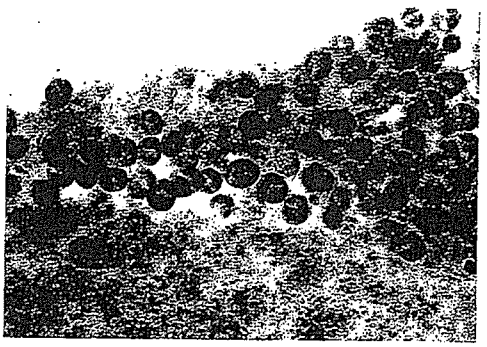

Fig. 2. Undigested cells of *Alexandrium tamarense* in the pseudofeces of short-necked clams

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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