Accumulation and depuration profiles of PSP toxins in the short-necked clam *Tapes japonica* fed with the toxic dinoflagellate *Alexandrium catenella*

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Abstract

A toxic dinoflagellate responsible for paralytic shellfish poisoning (PSP), *Alexandrium catenella* (*Ac*) was fed to the short-necked clam *Tapes japonica*, and the accumulation and depuration profiles of PSP toxins were investigated by means of high-performance liquid chromatography with postcolumn fluorescence derivatization (HPLC-FLD). The short-necked clams ingested more than 99% of the *Ac* cells (4×10^7 cells) supplied once at the beginning of experiment, and accumulated a maximal amount of toxin (185 nmol/10 clams) after 12 h. The rate of toxin accumulation at that time was 23%, which rapidly decreased thereafter. Composition of the PSP toxin accumulated in the clams obviously different from that of *Ac* even 0.5 h after the cell supply, the proportion of C1 + 2 being much higher than in *Ac*, although the reason remains to be elucidated. In contrast, a higher ratio of gonyautoxin (GTX)1 + 4 than in *Ac* was detected in the toxin profiles of clam excrements. The variation in toxin composition derived presumably from the transformation of toxin analogues in clams was observed from 0.5 h, such as reversal of the ratio of C1 to C2, and appearance of carbamate (saxitoxin (STX), neoSTX and GTX2, 3) and decarbamoyl (dc) derivatives (dcSTX and dcGTX2, 3), which were undetectable in *Ac* cells. The total amount of toxin distributed over *Ac* cells, clams and their excrements gradually declined, and only 1% of supplied toxin was detected at the end of experiment.
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1. Introduction

Intoxication of filter-feeding bivalves during blooms of toxic dinoflagellates responsible for paralytic shellfish poisoning (PSP) has become a global concern in recent decades (Hallegraeff, 1993). PSP toxins produced by dinoflagellates can be concentrated and retained in bivalve tissues, and pose a significant human health risk after consumption of the contaminated bivalves (Bricelj and Shumway, 1998). Every year, along the coastal

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water of Kyushu in Japan, many edible bivalves, such as the short-necked clam *Tapes japonica*, the mussel *Mytilus galloprovincialis* (formerly classified as *M. edulis*), the oyster *Crassostrea gigas*, and the scallop *Clamys nobilis* are intoxicated in association with the occurrence of the toxic dinoflagellates *Alexandrium catenella* (designated *Ac* below) and *Gymnodinium catenatum*, causing a serious problems in the local fishery industries (Onoue et al., 1981; Noguchi et al., 1988; Takatani et al., 1998a,b).

Accumulation and depuration profiles of PSP toxins in bivalves differ significantly according to different bivalve species (Hurst and Gilfillan, 1977; Oshima et al., 1982; Takatani et al., 1998a). During toxic algal blooms, mussels usually accumulate high levels of toxicity rapidly, which also declines in a short period of time (usually within several weeks) after the disappearance of the causative dinoflagellates. The scallops, *Patinopecten yessoensis* and *Chlamys nipponensis*, are known to become much more toxic than the mussel under the same environmental conditions, but they require several months until the toxicity drops down to the safety level. On the other hand, levels of toxicity in the short-necked clam and oyster are generally low, and their toxicity declines as quickly as in the mussel.

PSP toxin compositions of bivalves are not necessarily the same as those of the causative dinoflagellates, and interspecific differences are also observed among bivalves collected from the same area at the same time. For example, mussels and oysters better reflect the toxin compositions of the causative planktons (Onoue et al., 1981; Oshima et al., 1987; Bricelj et al., 1990), whereas the clams such as *Meretrix lamarckii*, *Pseudocardium sachalinensis* and *Protothaca staminea* often show largely different toxin compositions (Sullivan et al., 1983; Murakami et al., 1999). Although the differences in toxin profiles between bivalves and the causative planktons are considered to come from the enzymatic and/or chemical transformation of toxins in bivalve tissues (Shimizu and Yoshioka, 1981; Sullivan et al., 1983; Oshima, 1995a; Bricelj and Shumway, 1998; Fast et al., 2006), possible participation of selective uptake or excretion of specific toxin component(s) in bivalves cannot be excluded (Anderson et al., 1989; Oshima et al., 1989; Bricelj and Shumway, 1998; Suzuki et al., 1998). In this regard, negative results were obtained in the feeding experiments when mussels were fed with *Alexandrium tamarense* (Ichimi et al., 2001; Suzuki et al., 2003) or *Alexandrium minutum* (Blanco et al., 2003),

but similar investigations are needed for clams, which show larger differences in toxin profiles.

In the present study, *Ac* was fed to the short-necked clam *T. japonica*, whose accumulation and depuration profiles of PSP toxins were investigated in detail. Although toxic dinoflagellates were fed consecutively in similar feeding experiments previously reported (Lassus et al., 1989; Chen and Chou, 2001; Ichimi et al., 2001; Sekiguchi et al., 2001; Blanco et al., 2003; Suzuki et al., 2003), it is expected that if such a manner of feeding is applied to the clam, the kinetics of toxin uptake, transformation and elimination would become too complicated to analyze, as the clam could metabolize/transform PSP toxins very rapidly. To avoid this problem, *Ac* was fed only once but in a large amount at the beginning of the experiment in this study, in order to clarify the short-term accumulation/transformation profiles of PSP toxins, especially of C1, 2 and gonyautoxin (GTX)1, 4, the major toxin analogues produced by *Ac*.

2. Materials and methods

2.1. Experimental specimens

2.1.1. Toxin-producing dinoflagellate

The strain of *Ac* used in this study was originally isolated from the bloom which occurred in Miyanokawachi Bay, Kumamoto Prefecture, Japan in 2000. It was maintained in our laboratory in a modified SWM-3 culture medium (pH 7.7–7.8, salinity 33‰) at 21 °C under a 12:12 LD cycle with fluorescent illumination (light intensity 35 μmol/m^2/s). Before feeding to the clams, the strain was mass cultured in a 31 culture flask under the same conditions.

2.1.2. Short-necked clam

A total of 120 non-toxic specimens of the short-necked clam *T. japonica* (body weight 14.3–16.0 g and shell length 2.4–3.2 cm) were collected from mudflat near the mouth of Taira River, Nagasaki Prefecture. The specimens were immediately transported to our laboratory, and acclimatized for a week in a large aerated polyethylene tank filled with filtered seawater.

2.2. Feeding experiment

The clam specimens were divided into 12 groups of 10 individuals each. Each group was then placed

into a 1.5 l tank filled with 0.9 l filtered seawater, and supplied with 100 ml (4×10^7 cells) of *Ac* culture in early stationary phase once at the beginning of the experiment. Each group was reared at 21 °C inside an incubator, and harvested at 0.5, 1, 3, 6, 12, 24, 48, 72, 96, 120, 144 or 168 h after the supply of *Ac*. The whole edible tissues of 10 clams in each group were pooled, and their toxin profiles were analyzed as described below. In addition, the number of *Ac* cells that remained in reared seawater of each tank was counted. Finally, the toxin profiles of residue (*Ac* cells and clam excrements) were also examined.

2.3. Toxin extraction

2.3.1. Dinoflagellate cells

The toxin profiles of cultured cells of *Ac* used for the feeding experiment were determined similarly as reported by Ravn et al. (1995). Briefly, the dinoflagellate cells (10^6 cells) harvested at early stationary phase were first concentrated through a plankton net (mesh size 15–20 μm), and then centrifuged at 1500*g* for 10 min. The cell pellet obtained was mixed with 1.5 ml of 0.5 M acetic acid, and disrupted by ultrasonication for 30 s, which was centrifuged again at 3000*g* for 20 min. The above procedure was repeated two more times, and the supernatant thus obtained was pooled together. The final volume of supernatant was made up to 5 ml with water and then ultrafiltered (Ultrafree-PFL, Millipore, cut-off limit of 10,000 Da). The final filtrate thus obtained was subjected to high-performance liquid chromatography with postcolumn fluorescence derivatization (HPLC-FLD) analysis.

2.3.2. Clam tissues

The clam tissues were extracted according to the standard method recommended by the Association of Official Analytical Chemist (AOAC, 2003) with slight modifications as follows. The clam tissues (whole edible parts of 10 individuals in each group) were first homogenized with 5 ml of 0.1 M HCl, and then heated in a boiling water bath for 5 min. The homogenate was centrifuged at 1500*g* for 15 min, and the supernatant obtained was treated with a Sep-Pak C18 cartridge (Waters). The toxic eluate from the cartridge was ultrafiltered, and the filtrate was analyzed by HPLC-FLD.

2.3.3. Residue in rearing tanks

After each group of clams was harvested, the seawater remaining in each tank (total 1 l) was filtered through a 0.45 μm filter membrane under a negative pressure. The residue (*Ac* cells and excrements) retained on the membrane was extracted in the same manner as for the dinoflagellate cell pellet, and subjected to HPLC-FLD analysis.

2.4. HPLC-FLD analysis

HPLC-FLD analyses were carried out on a Hitachi L-7100 HPLC system, using a reversed-phase column (LiChroCART Superspher RP-18(e), 0.4×25 cm, Merck). Three types of mobile phases were used to detect different groups of toxin analogues; they were (i) 1 mM tetrabutylammonium phosphate (pH 5.8) for C toxins (Oshima, 1995b), (ii) 2 mM heptanesulfonic acid (HSA) in 10 mM ammonium phosphate (pH 7.3) for GTXs, (iii) 2 mM HSA in 30 mM ammonium phosphate (pH 7.3) with 4% acetonitrile for saxitoxins (STXs) (Arakawa et al., 1995). Each elute was subjected to a post column reaction with 50 mM periodic acid in 0.2 mM KOH containing 1 mM ammonium formate and 50% formamide at 65 °C. Ten microliters of each sample solution was injected in each analysis and the various PSP toxin analogues (Fig. 1) were detected with a fluorescence detector at 336 nm (excitation) and 392 nm (emission). The reference materials of C1, C2, GTX1–4, decarbamoylgonyautoxins 2, 3 (dcGTX2, 3) and neosaxitoxin (neoSTX), which were provided by the Fisheries Agency, Ministry of Agriculture, Forestry and Fisheries of Japan, as well as STX and decarbamoylsaxitoxin (dcSTX) prepared as reported previously (Arakawa et al., 1994), were used as external standards to identify/quantify each individual analogue. Since the reference materials of C3, 4, GTX5 and GTX6 were unavailable, they were quantified after being hydrolyzed into the corresponding carbamate derivatives (GTX1, 4, STX and neoSTX) (Takatani et al., 1998a). All toxins were quantified individually, but some of them are represented together in Figs. 2–4.

3. Results

3.1. Toxin profile of Ac culture

The original toxin profile of *Ac* culture at its early stationary phase is shown in Table 1. The toxin was
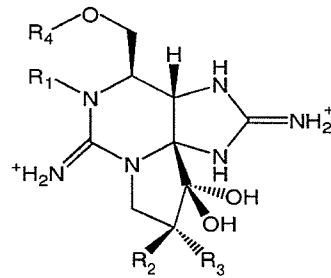
			Carbamate toxins	N-sulfocarbamoyl toxins	Decarbamoyl toxins
R1	R2	R3		R4	
			-CONH$_2$	-CONHSO$_3^-$	H
H	H	H	STX (2,483)	GTX5 (160)	dcSTX (1,274)
OH	H	H	neoSTX (2,295)	GTX6 (180)	dcneoSTX (33)
OH	OSO$_3^-$	H	GTX1 (2,468)	C3 (33)	dcGTX1 (1,500)
H	OSO$_3^-$	H	GTX2 (892)	C1 (15)	dcGTX2 (1,617)
H	H	OSO$_3^-$	GTX3 (1,584)	C2 (239)	dcGTX3 (1,872)
OH	H	OSO$_3^-$	GTX4 (1,803)	C4 (143)	dcGTX4 (1,080)

C: C toxin; GTX: gonyautoxin; STX: saxitoxin; dc: decarbamoyl

Value in parenthesis: specific toxicity in MU/μmol

Fig. 1. Structures of the known PSP toxin analogues and their specific toxicity values (Oshima, 1995b; Oshima, 1998).
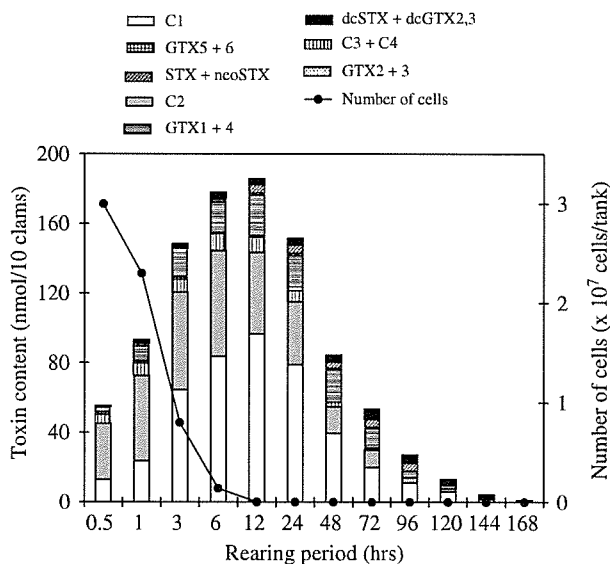

Fig. 2. Changes in the toxin profile of clams and the number of Ac cells remained in seawater during the feeding experiment.

found to consist of C1–4, GTX5, GTX6, GTX1 and GTX4, among which C2 and GTX4 were predominant. The total toxin content of Ac culture used to feed the short-necked clam was calculated to be 824.6 nmol/100 ml.

3.2. Toxin accumulation and depuration profiles in clams

Changes in the toxin profile of clams and the number of Ac cells remained in seawater during the whole rearing period are illustrated in Fig. 2. The toxin content rapidly increased as the cell number dropped, and reached the maximum level (185 nmol/10 clams) 12 h after the supply of Ac. During this period, the clams were found to ingest more than 99% of the supplied cells. The toxins once accumulated in the clams rapidly decreased from 12 to 168 h, and only 0.6% of the maximum

□ C1 ▨ GTX1 + 4
▥ GTX5 + 6 ■ dcSTX + dcGTX2,3
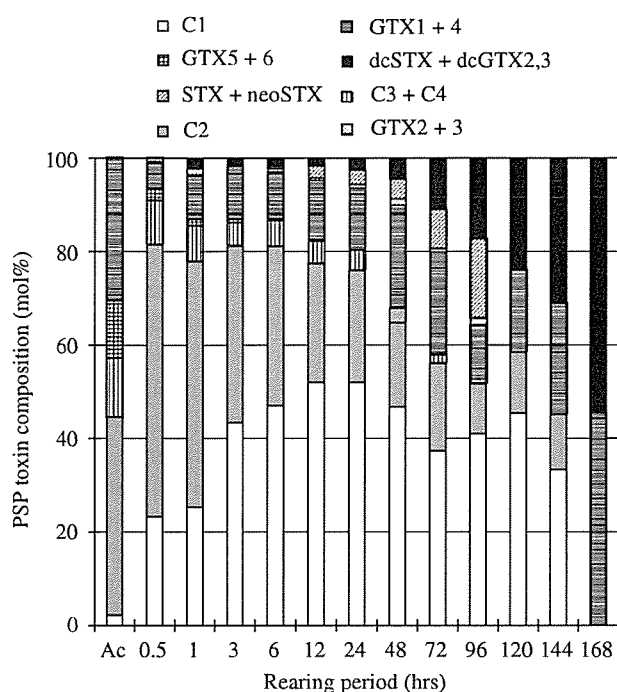▧ STX + neoSTX ▥ C3 + C4
▨ C2 □ GTX2 + 3

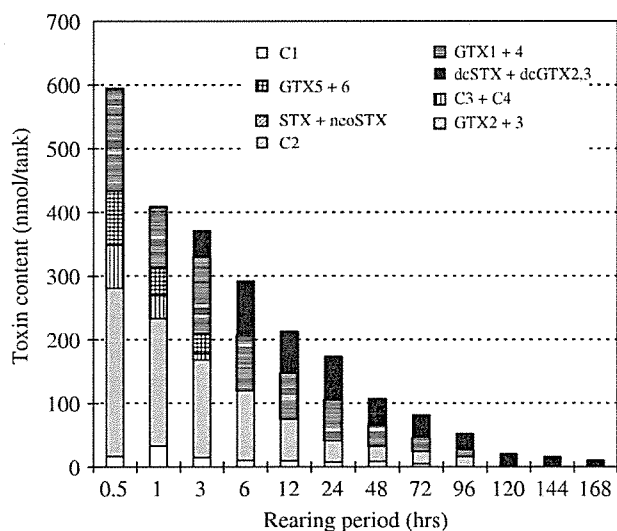Fig. 3. Changes in the PSP toxin composition of clams during the feeding experiment.

Fig. 4. Changes in the toxin profile of tank residue (remaining *Ac* cells and excrements) during the feeding experiment.

Table 1
Toxin profile of *Ac* culture used to feed the short-necked clams

PSP analogues	Toxin content (nmol/100 ml)	Mol%
C1	18.2	2.2
C2	349.7	42.4
C3	14.1	1.7
C4	90.2	10.9
GTX5	16.4	2.0
GTX6	84.3	10.2
GTX1	50.6	6.1
GTX4	201.1	24.4
Total	824.6	100.0

clams was much higher and lower, respectively, than that in *Ac*. However, the former gradually decreased, while the latter gradually increased thereafter. In addition, the ratio of C1 to C2 in the clams rapidly increased during the rearing period. Decarbamoyl (dc) toxins were first detected from 1 h, and their proportion gradually increased and exceeded 50% at the end of experiments. GTX2, 3 and STX, neoSTX appeared during 0.5–96 and 6–96 h, whereas GTX5, 6 and C3, 4 almost disappeared after 6 and 96 h, respectively.

3.3. Toxin profiles of residue in rearing tanks

Changes in the toxin profile of residue (remaining *Ac* cells and excrements) during the feeding experiment are illustrated in Fig. 4. The total toxin content of residue gradually decreased from 0.5 to 168 h. The toxin composition of residue was almost the same as that of the supplied *Ac* cells at 0.5 h, but dc toxins started to appear in a tiny amount at 1 h, and they increased remarkably at 3 h. In addition, C3, 4 and GTX5, 6 disappeared at 6 h, when the clams had ingested most of the *Ac* cells supplied. GTX1 + 4 and dc toxins became the dominant analogues thereafter until 96 and 168 h, respectively.

3.4. Distribution of toxins in clams and residue

Fig. 5 shows changes in the relative toxin contents among *Ac* cells, clams and excrements during the feeding experiment. As described in the figure legend, the toxin content of *Ac* cells (Tc-*Ac*) after each rearing period was estimated from the ratio of remaining cell number to initial cell number. The toxin content of excrements (Tc-E) was calculated
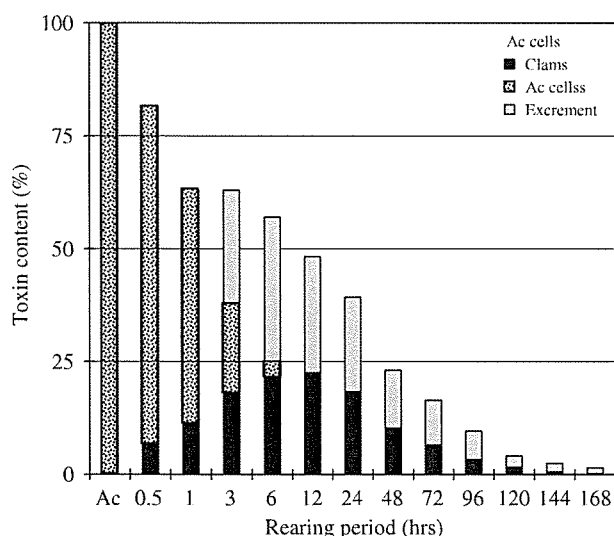
level remained in the clam tissues at the end of experiment (after 168 h).

Changes in the toxin composition of clams are shown in Fig. 3. The composition in clams varied greatly from that of *Ac*. Even 0.5 h after the supply of *Ac*, the proportion of C1 + 2 and GTX1 + 4 in

Fig. 5. Changes in the relative toxin contents among Ac cells, clams and excrements during the feeding experiment. The toxin content of Ac cells (Tc-Ac) after each rearing period was estimated from the ratio of remaining cell number to initial cell number. The toxin content of excrements (Tc-E) was calculated from Tc-Ac and the toxin content of residue (Tc-R) according to the following equation: Tc-E = Tc-R–Tc-Ac.

from Tc-Ac and the toxin content of residue (Tc-R) according to the following equation: Tc-E = Tc-R–Tc-Ac.

The total toxin content (Ac cells, clams and excrements combined) was gradually diminished during the rearing period, and only 1% of the toxin supplied originally remained after 168 h. The relative toxin content of Ac cells fell sharply and disappeared at 12 h, while the toxin content in excrements became noticeable at 3 h and reached the maximum (32%) at 6 h. The clams accumulated the maximal amount of toxin (23%) at 12 h, which, together with their excrements, decreased gradually thereafter.

4. Discussion

In the present study, the short-necked clam ingested more than 99% of the supplied Ac cells, and accumulated a maximal amount of toxin after 12 h (Fig. 2). The rate of toxin accumulation at that time was 23%, which was lower than 35% of the mussel fed with A. tamarense (Ichimi et al., 2001) or 50% of the purple clam fed with A. minutum (Chen and Chou, 2001), and most of the toxin accumulated disappeared after 168 h (7 days). The rate of intoxication in short-necked clams was reported to be generally low, and the acquired toxicity rapidly

declined after disappearance of the causative dinoflagellate (Oshima et al., 1982; Takatani et al., 1998a). Hence, the present findings in this study seemed to be consistent with the intoxication profiles of clams under the natural conditions. In this connection, Lassus et al. (1989) also reported that the accumulation rate and maximum burden of toxin in clams (Ruditapes philippinarum) when fed with A. tamarense (formerly classified as Protogonyaulax tamarensis) were lower than those in the mussels and scallops.

The PSP toxin composition accumulated in the clams obviously varied from that of Ac even after 0.5–1 h, the proportion of C1 + 2 being much higher than in Ac (Fig. 3). During this period, ingestion, digestion and/or absorption would be the main processes occurring in the clams, and there was no appreciable loss of toxin with excrements (Fig. 5). Therefore, the possible mechanisms that could produce the changes in toxin profile of the clams are expected as follows:

(1) Some toxins were lost, in soluble form, from the digestive system.
(2) Some toxins were transformed into C1, 2 during digestion.

However, (1) does not seem to be responsible for the enrichment in C1, 2 as they are the most soluble analogues of the PSP group. In addition, (2) also seems unlikely to happen in the digestive system in which the opposite transformation could be expected. It does not seem, therefore, that either of the mechanisms could be responsible for the change in the toxin profile detected. Further studies, including methodological verification (especially for collecting algal cells and residues), are needed to elucidate this point.

On the other hand, the variation in toxin composition derived presumably from the transformation of components during digestive and/or after absorption processes in clams was observed from 0.5 h, such as the reversal of the ratio of C1 to C2, and the appearance of carbamate (STX, neoSTX and GTX2, 3) and dc derivatives (dcSTX and dcGTX2, 3), which were undetectable in Ac cells (Figs. 2–4). The former could be attributable to the epimerization of C2 (11β-epimer) to C1 (11α-epimer) (Oshima et al., 1990, Oshima, 1995a), and the latter to the enzymatic hydrolysis of N-sulfocarbamoyl toxins (C1–4 and GTX5, 6) (Oshima, 1995a) or reductive conversion of GTX1,

4 (Shimizu and Yoshioka, 1981). The similar changes of composition were also observed in the naturally intoxicated short-necked clams (Oshima et al., 1990; Takatani et al., 1998a).

From 12 to 168 h, when toxin elimination would become the main process in clams, the proportion of C1 + 2 remaining in clams gradually declined, instead those of GTX1 + 4 and dcGTX2 + 3 increased steadily (Fig. 3). Since the toxin eliminated from clams with cell debris as excrements was also proportionally rich in GTX1 + 4 and dcGTX2 + 3 (Fig. 4), it inferred that these components could be retained longer in the bivalves and some indigestible cell tissues, whereas highly water-soluble C1, 2 would be released more rapidly into seawater possibly through branchial respiration or by elution.

The total amount of toxin distributed over *Ac* cells, clams and excrements gradually declined, and only 1% of supplied toxin was detected at the end of experiment (Fig. 5). A preliminary experiment suggested that a fairly large part of the lost toxin still existed, without being decomposed, in the seawater of the tanks, as a toxin mostly consisting of C1, 2. However, further studies are needed to elaborate this point, since the methodology used in this experiment for recovering toxin from seawater was not sufficiently reliable for quantitative analyses. Studies along this line are now in progress.

Acknowledgments

References

Anderson, D.M., Sullivan, J.J., Reguera, B., 1989. Paralytic shellfish poisoning in northwest Spain: the toxicity of the dinoflagellate *Gymnodinium catenatum*. Toxicon 27, 665–674.

AOAC, 2003. Paralytic shellfish poison. In: Horwitz, W. (Ed.), Official Methods of Analysis of AOAC International, vol. II, 17th ed. AOAC International, Gaithersburg, pp. 59–61 (Chapter 49).

Arakawa, O., Notuchi, T., Shida, Y., Onoue, Y., 1994. Occurrence of carbamoyl-*N*-hydroxy derivatives of saxitoxin and neosaxitoxin in a xanthid crab *Zosimus aeneus*. Toxicon 32, 175–183.

Arakawa, O., Noguchi, T., Onoue, Y., 1995. Paralytic shellfish toxin profiles of xanthid crabs *Zosimus aeneus* and *Atergatis*

floridus collected on reefs of Ishigaki Island. Fish. Sci. 61, 659–662.

Blanco, J., Reyero, M.I., Franco, J., 2003. Kinetics of accumulation and transformation of paralytic shellfish toxins in the blue mussel *Mytilus galloprovincialis*. Toxicon 42, 777–784.

Bricelj, V.M., Shumway, S.E., 1998. An overview of the occurrence and transfer kinetics of paralytic shellfish toxins in bivalve mollusks. In: Reguera, B., Blanco, J., Fernández, M.L., Wyatt, T. (Eds.), Harmful Algae. UNESCO, Santiago de Compostela, pp. 431–436.

Bricelj, V.M., Lee, J.H., Cembella, A.D., Anderson, D.M., 1990. Uptake kinetics of paralytic shellfish toxins from the dinoflagellate *Alexandrium fundyense* in the mussel *Mytilus edulis*. Mar. Ecol. Prog. Ser. 63, 177–188.

Chen, C.Y., Chou, H.N., 2001. Accumulation and depuration of paralytic shellfish poisoning toxins by purple clam *Hiatula rostrata* Lighttoot. Toxicon 39, 1029–1034.

Fast, M.D., Cembella, A.D., Ross, N.W., 2006. In vitro transformation of paralytic shellfish toxins in the clams *Mya arenaria* and *Protothaca staminea*. Harmful Algae 2, 201–206.

Hallegraeff, G.M., 1993. A review of harmful algal blooms and their apparent global increase. Phycologia 32, 79–99.

Hurst, J.W., Gilfillan, E.S., 1977. Paralytic shellfish poisoning in Maine. In: Wilt, E.S. (Ed.), Tenth National Shellfish Sanitation Workshop. US Department of Health, Education and Welfare, Food and Drug Administration, Washington, DC, pp. 152–161.

Ichimi, K., Suzuki, T., Yamasaki, M., 2001. Non-selective retention of PSP toxins by the mussel *Mytilus galloprovincialis* fed with the toxic dinoflagellate *Alexandrium tamarense*. Toxicon 39, 1917–1921.

Lassus, P., Fremy, J.M., Ledoux, M., Bardouil, M., Bohec, M., 1989. Patterns of experimental contamination by *Protogonyaulax tamarensis* in some French commercial shellfish. Toxicon 27, 1313–1321.

Murakami, R., Yamamoto, K., Noguchi, T., 1999. Toxicity and paralytic shellfish poison composition of three species of bivalves collected in Ibaraki, Japan. J. Food Hyg. Soc. Japan 40, 46–54.

Noguchi, T., Chen, S., Arakawa, O., Hashimoto, K., 1988. A unique composition of PSP in 'hiogi' scallop *Chlamys nobilis*. In: Natori, S., Hashimoto, K., Ueno, Y. (Eds.), Mycotoxins and Phycotoxins '88. Elsevier, Amsterdam, pp. 351–358.

Onoue, Y., Noguchi, T., Maruyama, J., Ueda, Y., Hashimoto, K., Ikeda, T., 1981. Comparison of PSP compositions between toxic oysters and *Protogonyaulax catenella* from Senzaki Bay, Yamaguchi Prefecture. Bull. Jp. Soc. Sci. Fish. 47, 1347–1350.

Oshima, Y., 1995a. Chemical and enzymatic transformation of paralytic shellfish toxins in marine organisms. In: Lassus, P., Arzul, G., Erard, E., Gentien, P., Marcaillou, C. (Eds.), Harmful Marine Algal Blooms. Lavoisier, Paris, pp. 475–480.

Oshima, Y., 1995b. Post-column derivatization HPLC method for paralytic shellfish poisons. In: Hallegraeff, G.M., Anderson, D.M., Cembella, A.D. (Eds.), Manual on Harmful Marine Microalgae. UNESCO, Paris, pp. 81–94.

Oshima, Y., 1998. Paralytic shellfish poison. Jpn. J. Toxicol. 11, 347–353.

Oshima, Y., Yasumoto, T., Kodama, M., Ogata, T., Fukuyo, Y., Matsuura, F., 1982. Features of paralytic shellfish poison occurring in Tohoku district. Nippon Suisan Gakkaishi 48, 525–530.

Oshima, Y., Hasegawa, M., Yasumoto, T., 1987. Dinoflagellate *Gymnodinium catenatum* as the source of paralytic shellfish toxins in Tasmanian shellfish. Toxicon 25, 1105–1111.

Oshima, Y., Hirota, M., Yasumoto, T., Hallegraeff, G.M., Blackburn, S.I., Steffensen, D.A., 1989. Production of paralytic shellfish toxins by the dinoflagellate *Alexandrium minutum* Halim from Australia. Nippon Suisan Gakkaishi 55, 925.

Oshima, Y., Sugino, K., Itakura, H., Hirota, M., Yasumoto, T., 1990. Comparative studies on paralytic shellfish toxin profile of dinoflagellates and bivalves. In: Graneli, E., Sundstrom, B., Edler, L., Anderson, D.M. (Eds.), Toxic Marine Phytoplankton. Elsevier, New York, pp. 391–396.

Ravn, H., Anthoni, U., Christophersen, C., Nielsen, P.H., Oshima, Y., 1995. Standardized extraction method for paralytic shellfish toxins in phytoplankton. J. Appl. Phycol. 7, 589–594.

Sekiguchi, K., Sato, S., Kaga, S., Ogata, T., Kodama, M., 2001. Accumulation of paralytic shellfish poisoning toxins in bivalves and an ascidian fed on *Alexandrium tamarense* cells. Fish. Sci. 67, 301–305.

Shimizu, Y., Yoshioka, M., 1981. Transformation of paralytic shellfish toxins as demonstrated in scallop homogenates. Science 212, 547–549.

Sullivan, J.J., Iwaoka, W.T., Liston, J., 1983. Enzymatic transformation of PSP toxins in the littleneck clam *Protothaca staminea*. Biochem. Biophys. Res. Commun. 114, 365–472.

Suzuki, T., Yamasaki, M., Ota, H., 1998. Comparison of paralytic shellfish toxin profiles between the scallop *Patinopecten yessoensis* and the mussel *Mytilus galloprovincialis*. Fish. Sci. 64, 850–851.

Suzuki, T., Ichimi, K., Oshima, Y., Kamiyama, T., 2003. Paralytic shellfish poisoning (PSP) toxin profiles and short-term detoxification kinetics in mussels *Mytilus galloprovincialis* fed with toxic dinoflagellate *Alexandrium tamarense*. Harmful Algae 2, 201–206.

Takatani, T., Morita, T., Anami, A., Akaeda, H., Kamijyo, Y., Tsutsumi, K., Noguchi, T., 1998a. Appearance of *Gymnodinium catenatum* in association with the toxification of bivalves in Kamae, Oita Prefecture, Japan. J. Food Hyg. Soc. Japan 39, 275–280.

Takatani, T., Akaeda, H., Kaku, T., Miyamoto, M., Mukai, H., Noguchi, T., 1998b. Paralytic shellfish poison infestation to oyster *Crassostrea gigas* due to dinoflagellate *Gymnodinium catenatum* in Amakusa Islands, Kumamoto Prefecture. J. Food Hyg. Soc. Japan 39, 292–295.

研究ノート

軟体動物ウミフクロウの毒性について

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Toxicity of the MOLLUSCA, *Pleurobranchaea maculata*

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緒　言

フグ毒（TTX）や麻ひ性貝毒（PSP）を保有する生物の毒化機構は，それらを産生する生物の摂取による食物連鎖と考えられているが[1]，徳島産スベスベマンジュウガニやニホンイモリ等，その毒の起源が明らかでない生物も存在する[2],[3]。それらの毒化機構を明らかにするためにはその餌生物の毒性を調査する必要がある。

ウミフクロウ（*Pleurobranchaea maculata*）は，体長6～11 cmで青森湾以南の本邦各地，中国（青島），ニュージーランド，南太平洋に分布する軟体動物門腹足網に属する後鰓亜網ウミフクロウ科の生物である[4]。スナヒトデ（*Luidia quinaria*）はスナヒトデ科に属し，腕の長さは14 cmに達し，体色は背面黄褐色ないし暗灰色で，中心より腕の中央を走る濃色の帯が認められ，水深5-50 mのあたりに生息し，北海道南部から九州までに分布している。モミジガイ（*Astropecten scoparius*）はモミジガイヒトデ科に属し，5本の腕が正しく星型に射出しており，体色は青灰色，淡褐色及び赤褐色で，我が国では北海道西南部以南の本州各地に分布しており，浅海から数10 mまでの海底から主として底引きで得られる。トゲモミジガイ（*Astropecten polyacanthus*）は大きなもので体長9 cm以上，通常浅海の砂汀に生息しており，我が国では富山湾及び房州以南に分布するモミジガイヒトデ科の生物である[5]。

いずれも，TTXとPSPを併せ持つ徳島県産スベスベマンジュウガニ等の餌生物になっていると考えられるため，本研究ではこれら生物の毒性スクリーニングを行うとともに，有毒なものについては，機器分析により毒成分を調べた。

試料及び方法

試料

2006年2月18日に徳島県松茂空港沖合（図1）で，底引き網により採取したウミフクロウ2個体，スナヒトデ2個体，モミジガイ2個体及びトゲモミジガイ2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。
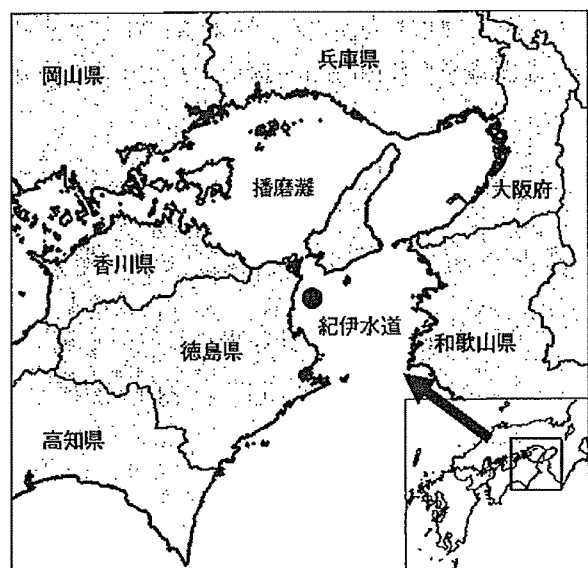

図1　試料の採取場所
●：試料採取地点

毒の抽出

毒の抽出は，厚生労働省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[6]。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分間加熱抽出した後，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

マウス毒性試験は厚生労働省監修食品衛生検査指針理化学編のフグ毒の項および麻ひ性貝毒の項に準じて行った[6,7]。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。TTX 換算では体重20 g のマウス1匹を30分間で死亡させる毒量を，また，PSP 換算では体重20 g のマウス1匹を15分間で死亡させる毒量を1 MU と定義した[6,7]。

高速液体クロマトグラフィー（HPLC）分析

カラムに日立社製 HG3013N（4.6 mmφ×50 mm）と野村化学社製 Develosil C-30UG-5（4.6 mmφ×250 mm）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[8]。

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。ウミフクロウから TTX 換算で4.2 MU/g および2.5 MU/g，PSP 換算では2.5 MU/g および1.8 MU/g のマウス毒性が検出された。HPLC による TTX の検出は検出限界が低く，5 MU 程度では検出できないため，PSP を対象とした機器分析を行った。一方，スナヒトデ，モミジガイ及びトゲモミジガイは，いずれの個体も無毒であった。

HPLC 分析

ウミフクロウ試料の HPLC 分析結果を図2に示す。ウミフクロウ1及び2は，ともにゴニオトキシン（gonyautoxin, GTX）2が最も強く検出され，次いで GTX3，デカルバモイルゴニオトキシン（decarbamoyl gonyautoxin, dcGTX）2，dcGTX3 の順の強度で検出された。ウミフクロウ2では，プロトゴニオトキシン（protogonyautoxin, PX）1及

表1 マウスによる毒性試験結果

| 試　料 | No. | 体重 (g) | 毒性（MU／g） | | 総毒量（MU／個体） | |
			TTX 換算	PSP 換算	TTX 換算	PSP 換算
ウミフクロウ *Pleurobranchaea maculata*	1	8.1	4.2	2.5	33.7	20.3
	2	5.8	2.5	1.8	14.3	10.4
スナヒトデ *Luidia quinaria*	3	13.9	<2		—	
	4	6.1	<2		—	
モミジガイ *Astropecten scoparius*	5	24	<2		—	
	6	20.5	<2		—	
トゲモミジガイ *Astropecten polyacanthus*	7	11.2	<2		—	
	8	10.8	<2		—	

図2　ウミフクロウの HPLC 分析結果
a：PX1（C1），b：PX2（C2），c：dcGTX3，
d：dcGTX2，e：GTX3，f：GTX4，g：GTX2，
h：GTX1，i：neoSTX

び2も標準品と一致する保持時間でピークを検出し
たが，両者ともに GTX1は検出されなかった。

　それぞれのピーク面積から毒成分を定量し，それ
らをマウス毒性値に変換したところ，2.2 MU/g お
よび1.4 MU/g であった。これらをマウス毒性試験
から求めた PSP 換算の毒性値と比較すると，88%
及び78%となり，動物試験の誤差を考慮するとウミ
フクロウに含有する有毒成分は PSP が主成分であ
ると判断された。ウミフクロウに PSP 成分が含有
されるという報告はこれまでに無いため，本研究結
果は，ウミフクロウが徳島産スベスベマンジュウガ
ニ等，毒化機構が明らかでない有毒生物の毒化に寄
与している可能性を示唆した。

まとめ

　2006年2月18日に徳島県松茂空港沖合で，底引き
網により採取したスナヒトデ，トゲモミジガイ，モ
ミジガイ及びウミフクロウの毒性を調べたところ，
ウミフクロウから PSP 換算で2.5 MU/g および
1.8 MU/g のマウス毒性が得られた。その毒成分組
成を HPLC で調べたところ，GTX 群を主体とする
PSP が主成分であることが解った。徳島県沿岸に
は PSP を保有しているものの，その毒化機構が明
らかになっていないスベスベマンジュウガニが生息
するが，ウミフクロウがその餌生物となっている可
能性が考えられることから，それがスベスベマン
ジュウガニの毒化に寄与している可能性が示唆され
る。しかしながら，それらの生息環境が多少異なる
ことと，その毒性値や食性にいくつかの疑問点が見
出されることから，引き続き調査を続ける必要があ
ると考える。

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引用文献

1）野口玉雄(1996)：フグはなぜ毒をもつのか-海洋生
　　物の不思議，日本放送出版協会，東京，pp.56-86.
2）西尾幸郎・相良剛史・黒田智久・橋本多美子・西堀
　　尚良(2006)：徳島県浅川湾産スベスベマンジュウガ
　　ニの毒の性状．四国大学紀要 B 自然科学編23，59-
　　64.
3）相良剛史・西堀尚良・橋本多美子・西尾幸郎
　　(2006)：徳島県産ニホンイモリの毒性について．四
　　国大学紀要 B 自然科学編23，65-68.
4）内海富士夫(1956)：原色日本海岸動物図鑑，保育社，
　　大阪，p.96.
5）内海富士夫(1956)：原色日本海岸動物図鑑，保育社，
　　大阪，pp.107-108.
6）厚生労働省監修(2005)：食品衛生検査指針（理化学

編), 日本食品衛生協会, 東京, pp.661-666.

7) 厚生労働省監修(2005)：食品衛生検査指針（理化学編), 日本食品衛生協会, 東京, pp.673-680.

8) S. Nishio, 2002. Occurrence of Toxic Oysters Crassostrea gigas Infested with *Alexandrium tamiyavanichii* in the Seto Inland Sea, Japan. *Proceedings of International Scientific Symposium on Marine Toxins and Marine Food Safety*, 78-87.

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研究ノート

高速液体クロマトグラフィーによるパリトキシンの微量分析

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Analysis Method of Palytoxin Using High Performance Liquid Chromatography

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緒　言

　わが国では，時として海洋生物毒（マリントキシン）のひとつで自然毒のなかでは最強の部類に属するパリトキシン（PTX）様物質による中毒が発生してきた。本中毒は，これまで有毒なアオブダイのみによって引き起こされる特異な中毒であると考えられていた[1,2]。しかしながら，近年，ハタ科魚類やハコフグ科魚類といったアオブダイとは異なる魚類を原因食品とする同様の食中毒事例が西日本各地で相次いで発生し，食品衛生上，大きな問題となっている。そのため，国民の食用魚介類の安全性に対する不安は広がりつつあり，それらのPTX様物質の検査体制の徹底や毒性の再評価が求められている。一方，有毒なアオブダイの毒の起源は長らく不明であったが，最近，付着性の有毒渦鞭毛藻 *Ostreopsis* 属であることが明らかとなった[2]。*Ostreopsis* 属渦鞭毛藻は，本来，熱帯または亜熱帯海域に生息することが知られているが[3]，温帯海域である西日本沿岸，特に徳島県を中心とする四国太平洋沿岸には，有毒種がほぼ周年的に分布していることが筆者らの研究により明らかにされつつある。

　他方，マリントキシンのなかでもフグ毒（tetrodotoxin：TTX），麻ひ性貝毒（paralytic shellfish poison：PSP），下痢性貝毒（diarrhetic shellfish poisoning：DSP）などは，わが国の公定法であるマウス毒性試験法[4]に加え，高速液体クロマトグラフィー（high performance liquid chromatography：HPLC）や質量分析法（MS）によって化学的な検出法が確立さ

れている[5]-[7]。しかしながら，PTX またはPTX 様物質の検出法はマウス毒性試験や溶血活性試験などの生化学的手法が主流であり，機器分析法に関しては，現在，多方面で種々の改良が行われているにもかかわらず煩雑な操作や熟練を要し，なお且つ，いずれの毒も検出感度は悪く，未だ発展途上の段階である。

　このような状況の下，本研究では，HPLC による PTX の簡便，迅速かつ高感度な分析法の確立を目的とし，カラムクロマトグラフィー，固相抽出および各種膜ろ過による前処理法についても検討を加えた。

方　法

PTX 標準品

　PTX 標準品は和光純薬工業株式会社製を使用し，100 μg を蒸留水 1 ml に溶解して以下の試験に供した。

マウス毒性試験

　マウス毒性試験は Taniyama らの方法に準じて行った[8]。試験液を ddY 系雄マウスに 1 ml 腹腔内投与して48時間観察し，生死を確認した。本研究において，1 マウス単位（mouse unit：MU）は供試マウス 1 尾を約48時間で死亡させる毒量と定義した[8]。

HPLC 分析

　カラムに Purospher　STAR　RP-8e（φ2 mm×250 mm, Merck）を使用した。移動相 A に0.1%

ギ酸－20％アセトニトリル，移動相Bに0.1％ギ酸－80％アセトニトリルを用い，移動相Aから移動相Bに60分間かけて切替えるリニアグラジエント法を用い，流速を0.2 ml/min とした。毒の検出にはPTX標品特有の紫外部極大吸収の263 nm[9],[10]を使用した。

カラムクロマトグラフィー

カラムクロマトグラフィーは前述のHPLC分析法を用いた。100 ppm のPTX標準品10 μl を注入し，PTXが溶出する保持時間のピークを分取した。分取した画分は減圧下で濃縮乾固してギ酸およびアセトニトリルを取り除き，蒸留水にて溶解して毒性を確認した。カラム以外の要因でのPTXの失活を考慮し，対照として分析系のカラムのみを取り除いたものでも同様の作業を行った。

固相抽出法の検討

メタノールと蒸留水で平衡化したOASIS MAX 3 cc (Waters) ミニカラムに100 ppm のPTX標準品1 ml を吸着させて2 ml の2％アンモニア水と100％メタノールを通過させ，1％酢酸－80％メタノールで毒を溶出させた。それらを減圧濃縮したものを少量の蒸留水に溶解し，毒性を確認した[8]。

ろ過膜の検討

ろ過膜材質に再生セルロース（RC）を用いたUltrafree-MC (MILLIPORE) 10,000 Dalton (RC 10 kDa)，ポリエーテルスルホン（PES）を用いたビバスピン500 (sartorius) 5,000 Da (PES 5 kDa) および10,000NMWL(PES 10 kDa)の3種類の限外ろ過ユニットと，ポリビニリデンフルオライド(PVDF)を用いたUltrafree-MC 0.45 μm(PVDF 0.45 μm) および親水性ポリテトラフルオロエチレン（PTFE）を用いたUltrafree-MC 0.5 μm(PTFE 0.5 μm)の2種類の精密ろ過ユニットを用いた。

RC 10 kDa は保湿剤と

して施されているグリセリンによるPTXへの影響を把握するため，蒸留水を用いてグリセリン除去処理したものを準備し，未処理のものと共にPTX標準品500 MU/100 μl をろ過して，得られたろ液の毒性を検討した。

次に，ろ過膜のドデシル硫酸ナトリウム（SDS）処理による吸着防止作用を確認するため，PES 5 kDa, PES 10 kDa, PVDF 0.45 μm および PTFE 0.5 μm のデバイスに5％SDS 0.5 ml を入れ一晩放置し，蒸留水にて2回洗浄する処理を施し，未処理のものと共にPTX標準品500 MU/100 μl をろ過して，得られたろ液の毒性を検討した。

結果および考察

カラムクロマトグラフィー

HPLC分析の結果，保持時間27分にPTXのピークが認められたので分取した。注入したPTXが50 MU であったのに対し，この分取した溶液の毒量は20 MU であった。一方，対照として得られたカラムを通過していないものの毒量も20 MU であった。このことより，本実験でのPTXの失活はカラム通過によるものではなく，酸性溶液下での濃縮操作に起因しているのではないかと推察された。

固相抽出法の検討

OASIS MAX 3 cc によるPTX標準品固相抽出法の検討を行った際の毒の回収率を表1に示す。供試5,000 MU 中，80％の回収率である4,000 MU が1％酢酸－80％メタノール画分で溶出された。このことより，OASIS MAX 3 cc を用いた固相抽出法はPTXの簡易精製に有用であることが示された。

表1 PTX標準品(5,000 MU)のOASIS MAX 3 cc 処理による溶出位置

画分	非吸着	2％NH₄OH	100％MeOH	1％AcOH-80％MeOH
毒量（MU）	＜100	＜100	＜100	4,000
回収率（％）	＜2	＜2	＜2	80

ろ過膜の検討

　RC 10 kDa のグリセリン除去処理の有無による PTX への影響を調べたところ，両者とも供試 500 MU 中，20%の回収率である100 MU の回収が確認できた。これらを HPLC で分析した結果，グリセリン未除去，グリセリン除去ともに PTX 標品と同じ保持時間にピークは見られたが，ともに小さくなっていた(図1)。ピーク面積より算出した PTX の毒量は20 MU であり，マウス毒性試験から算出したものと一致した。本処理により PTX の一部が RC 10 kDa を通過できなかったり，通過する際に膜に吸着して，20%程度しか回収できなかったものと推察された。

　次に，SDS 処理を施したろ過膜でろ過した PTX ろ液の HPLC 分析結果を図2に示す。

　PES 10 kDa では，SDS 処理，非処理ともに，PTX の保持時間に小さなピークが認められ，それよりも早い16分あたりに大きなピークが認められた。SDS 非処理のものには PTX 標準品には含まれない複数の明瞭なピークが認められた。このことより PTX は PES 10 kDa を通過すると他成分に変換するが，SDS 処理により変換成分が変わることがわかった。PES 5 kDa では PES 10 kDa のときに検出された

ピークと異なるものも検出されたが，全体的にピーク面積が小さくなっていた。

　これらの結果から，PTX は限外ろ過することにより失活，他成分への変換などを起こし，回収率が低くなるため，機器分析の前処理法としては不適切であると思われた。

　一方，PVDF 0.45 μm および PTFE 0.5 μm の精密ろ過では，SDS 処理の有無にかかわらず PTX 標準品と同様のピークが得られ，そのピーク面積も減少は無かった（図2-b）。

　以上の結果から，PTX の機器分析用試料を調整する際には，PVDF 0.45 μm または PTFE 0.5 μm ろ過が有効であることが示唆された。

まとめ

　PTX 標準品を用いた機器分析試料調製のための前処理法の検討結果より，逆相分配系でのカラムクロマトグラフィーは PTX の精製に有効であるが，酸性溶液下での乾燥で PTX が失活する恐れがあるため，有毒画分を分取した後，中和するなどして濃縮するような工夫が必要ではないかと思われた。

　固相抽出法の検討では，OASIS　MAX 3 cc を用いることにより，アンモニア水洗浄とメタノール洗浄で夾雑物質の除去が期待でき，80%以上の回収率が得られるため，PTX の簡易精製に有用であることが示された。

　ろ過膜の検討では，再生セルロースおよびポリエーテルスルホンを膜材料とした限外ろ過では，保湿用に膜に塗布されているグリセリンを取り除いても，ドデシル硫酸ナトリウム処理を施して膜への物質吸着を抑えてもろ液に PTX を効率よく回収することはできなかった。さらに，限外ろ過を行うことにより PTX が他成分に変換する事実が認められたため，PTX を精製する際に限外ろ過は用いてはならないものと思われた。この変換した成分については，今後，質量分析装置により確認する予定である。

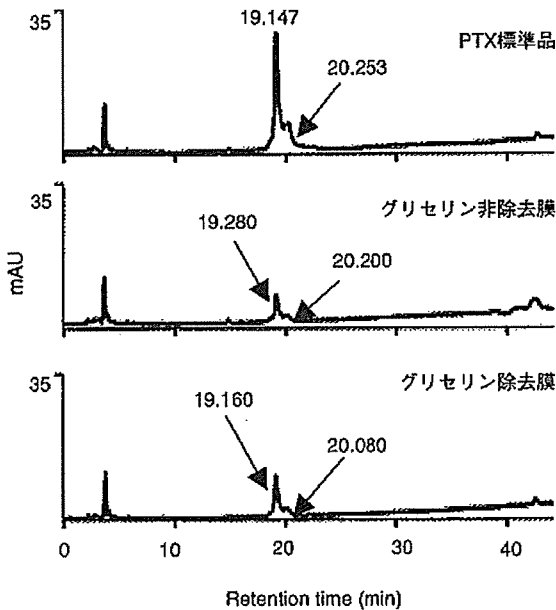　一方，ポリビニリデンフルオライドおよびポリテトラフルオロエチレンを膜材料とした精密ろ過では，ドデシル硫酸ナトリウム処理の有無に関わらず，
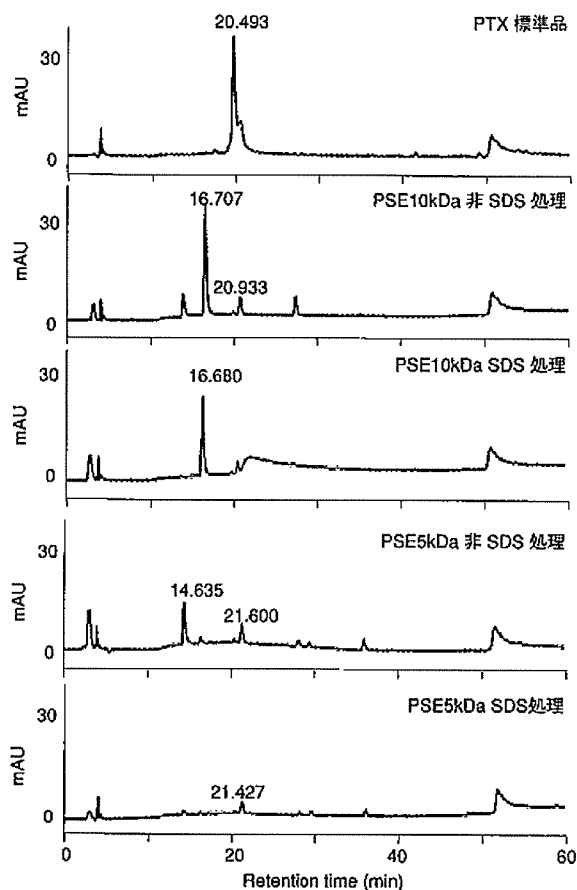

図1　再生セルロース膜で限外ろ過した PTX の HPLC 分析結果
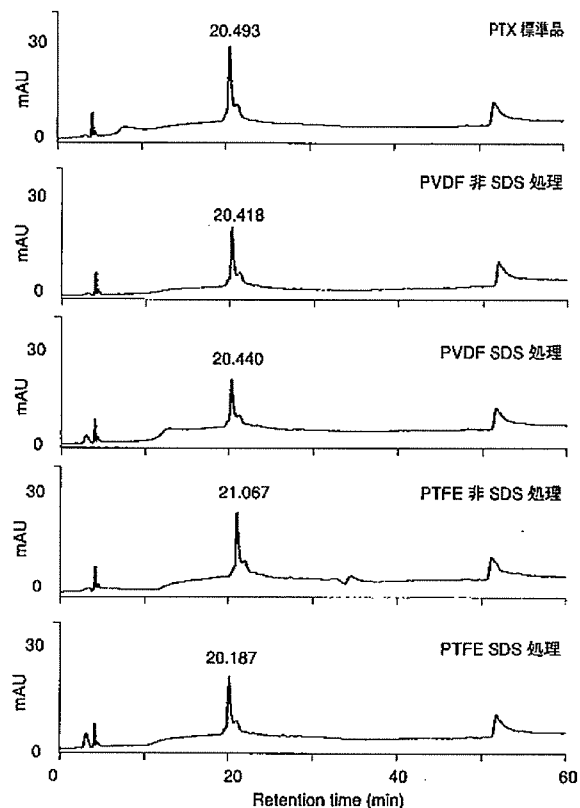
図 2 － a　各種フィルターろ過による PTX の
HPLC 分析結果

図 2 － b　各種フィルターろ過による PTX の
HPLC 分析結果

PTX をほぼ100％回収でき，成分の変換も認められ
なかったため，機器分析試料の調製のための前処理
に有効であると思われた。

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参考文献

1 ）T. Noguchi, D. F. Hwang, O. Arakawa, K. Daigo, S.
Sato, H. Ozaki, N. Kawai, M. Ito, and K. Hashimoto,
1987. Palytoxin as the causative agent in the
parrotfish poisoning. In : *Progress in Venom and Toxin*

Research (ed. by Gopalakrishnakone, P. and Tan, C.
K.), National University of Singapore, Kent Ridge,
Singapore : 325-335.
2 ）S. Taniyama, O. Arakawa, M. Terada, S. Nishio, T.
Takatani, Y. Mahmud, and T. Noguchi, 2003. *Ostre-
opsis* sp., a possible origin of palytoxin (PTX) in
parrotfish *Scarus ovifrons. Toxicon.* 42 : 29-33.
3 ）Y. Fukuyo, 1981. Taxonomical study on benthic
dinoflagellates collected in coral reefs. *Bull. Japan.
Soc. Sci. Fish.* 47 : 967-978.
4 ）厚生労働省監修，2005. 食品衛生検査指針（理化学
編），日本食品衛生協会，東京：661-685.
5 ）M. Yotsu, M. Iorizzi, and T. Yasumoto, 1990. Distri-
bution of tetrodotoxin, 6-*epitetrodotoxin*, and 11-
deoxytetrodotoxin in newts. *Toxicon,* 28 （ 2 ）：238-
241.
6 ）Y. Oshima, K Sugino, and T Yasumoto, 1989. Latest
advances in HPLC analysis of paralytic shellfish
toxins. In : Natori S, Hashimoto K, Ueno Y (eds).

Mycotoxins and Phycotoxins '88, Elsevier, New York. 319-329

7) T. Suzuki, T. Jin, Y. Shirota, T. Mitsuya, Y. Okumura and T. Kamiyama, 2005. Quantification of lipophilic toxins associated with diarrhetic shellfish poisoning in Japanese bivalves by liquid chromatography-mass spectrometry and comparison with mouse bioassay. *Fisheries Science* 71 : 1370-1378.

8) S. Taniyama, Y. Mahmud, M. Terada, T. Takatani, O. Arakawa and T. Noguchi, 2002. Occurrence of a food poisoning incident by palytoxin from a serranid *Epinephelus* sp. in Japan. *Journal of Natural Toxin,* : 11 （ 4 ） 277-282.

9) P. Riobo, B. Paz, and J. M. Franco, 2006. Analysis of palytoxin-like in *Ostreopsis* cultures by liquid chromatography with precolumn derivatization and fluorescence detection. *Analytica Chimica Acta.* 566 : 217-223.

10) D. Uemura, Y. Hirata, T. Iwashita, and H. Naoki, 1985. Studies on palytoxin. *Tetrahedron.* 41 （ 6 ） : 1007-1017.

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Detection of tetrodotoxin (TTX) from two copepods infecting the grass puffer *Takifugu niphobles*: TTX attracting the parasites?

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Abstract

In May 2002, two parasitic copepods, *Pseudocaligus fugu* and *Taeniacanthus* sp., were collected from the body surface and gill of the grass puffer *Takifugu niphobles*, respectively, in Takehara city, Hiroshima Prefecture, faced with Seto Inland Sea located in the western part of Japan. To them was added 5 ml of 0.1% acetic acid, then the suspension was subjected to ultrasonic disruption with an ultrasonicator for 10 min. The resulting mixture was heated in a boiling water bath for 10 min, and then centrifuged. The supernatant was concentrated under reduced pressure, and loaded on to a Sep-Pak plus C18 Environmental Cartridge (Waters). The unbound fraction was analyzed by HPLC and gas chromatography–mass spectrometry (GC–MS) for tetrodotoxin (TTX). It was rather unexpectedly revealed from these results that this fraction was comprised of TTX and its analogues. As far as we know, this is the first record to show the existence of TTX in the copepods. In addition, 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. 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 high host-specificity of *P. fugu* on the toxic puffer and the present bioassay of its skin mucus suggest a possibility that TTXs may attract the parasite.
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1. Introduction

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Tetrodotoxin (TTX), known as puffer fish toxins, is one of the most potent nonpeptidic neurotoxin

because of its frequent involvement in fatal food poisoning, its unique chemical structure, and its specific action of blocking sodium channels of excitable membranes (Colquhon et al., 1972; Evans 1972; Narahashi 2001). The toxin derives its name from the pufferfish family *Tetraodontidae*, but past studies have revealed its wide distribution in both terrestrial and marine animal kingdoms such as vertebrate species inclusive of pufferfish, goby, newt and frog, and invertebrates of octopus, gastropod mollusk, crab, starfish, and nemertean and turbellarian (Ali et al., 1990; Hwang et al., 1994; Mebs et al., 1995; Mahmud et al., 1999; Asakawa et al., 2000, 2003; Mebs, 2001; Miyazawa and Noguchi, 2001; Hanifin et al., 2002). There is no phylogenic relationship among these TTX-containing animals. In many species of the family *Tetraodontidae* such as the grass puffer *Takifugu niphobles*, TTX are also well known to be contained in the skin, skin mucus except internal organs. The external body surface plays a very important role in the prevention of pathogen invasion, especially in fish, which continuously exposed to an aquatic environment. In addition to the mechanical barrier of scales, the skin surface of fish is protected by the secretion of mucus. Fish skin mucus contains many molecules that may act as defense factors (Ingram, 1980; Alexander and Ingram, 1992). However, the biological adaptive values of the presence of TTX in these organisms are not evidently clarified. In the recent studies on toxic puffers, as possible physiological roles of TTX except a defense agent, functions as sex pheromone (Matsumura, 1995) and enhancing substances of the immune system are reported (Arakawa, 2002). On the other hand, it is also known that there are a number of ectoparasites infecting toxic puffers (Ogawa, 1991, Hirazawa et al., 2001, Okabe, 2003). Ikeda et al. (2006) have first revealed with an immunoenzymatic technique that TTX is accumulated in the whole parts of an ectoparasitic copepod *Pseudocaligus fugu* infecting the grass puffer *T. niphobles* except for its female reproductive system and eggs, suggesting that TTX might have been accumulated by their feeding on the skin and mucus of the host.

These circumstances prompted us to investigate TTX and its derivatives in parasitic copepod attaching to the grass puffer. The present paper reports to confirm the existence of them in two parasitic copepods, *P. fugu* and *Taeniacanthus* sp., attaching to the host *T. niphobles* collected from Seto Inland Sea, the western part of Japan in May 2002, using high performance liquid chromatography (HPLC)-fluorometric system and gas chromatography–mass spectrometry (GC–MS). In addition, relationships between the number of these two parasitic copepods infecting *T. niphobles* and the toxicity of the skin mucus of *T. niphobles* are also investigated.

2. Materials and methods

2.1. Parasitic copepods and skin mucus

In May 2002, specimens of the grass puffer *T. niphobles* were collected by fishing in front of the Takehara Marine Science Station, Setouchi Field Science Center in the Seto Inland Sea, Hiroshima University, Hiroshima Prefecture, Japan (Fig. 1). With fine forceps, all live parasitic copepods *P. fugu* (preadults and adults) and *Taeniacanthus* sp. (copepodids and adults) were carefully picked up from the body surface and gill of the host, respectively. Live copepods were kept in glass beakers filled with filtered (Whatman GF/C) seawater at 20 °C for 2 or 3 days to remove contamination of organization and mucus of the host and gut contents of the parasites. After that copepods were kept frozen at −20 °C in a freezer.

The host *T. niphobles* were subjected to a light wiping of the whole body surface with gauzes (designated "handling stimulus"). The wiped gauzes were kept frozen at −20 °C in a freezer.

2.2. Assay of toxicity

Toxicity of the skin mucus on the body surface of *T. niphobles* was estimated by the official Japanese method for TTX (Kawabata, 1978). The toxicity was expressed in mouse unit (MU), in which one MU was defined here as the amount of toxin, which killed 19–21 g ddY strain male mice in 30 min after intraperitoneal administration.

2.3. Purification of toxin

Collected specimens of parasitic copepods *P. fugu* and *Taeniacanthus* sp. weighed 0.042 g ($n = 30$) and 0.027 g ($n = 50$), respectively, on a wet basis. To them was added to 5 ml of 0.1% acetic acid, then the suspension was subjected to ultrasonic disruption with an ultrasonicator for 10 min. The resulting mixture was heated in a boiling water bath for 10 min, cooled to room temperature, and then
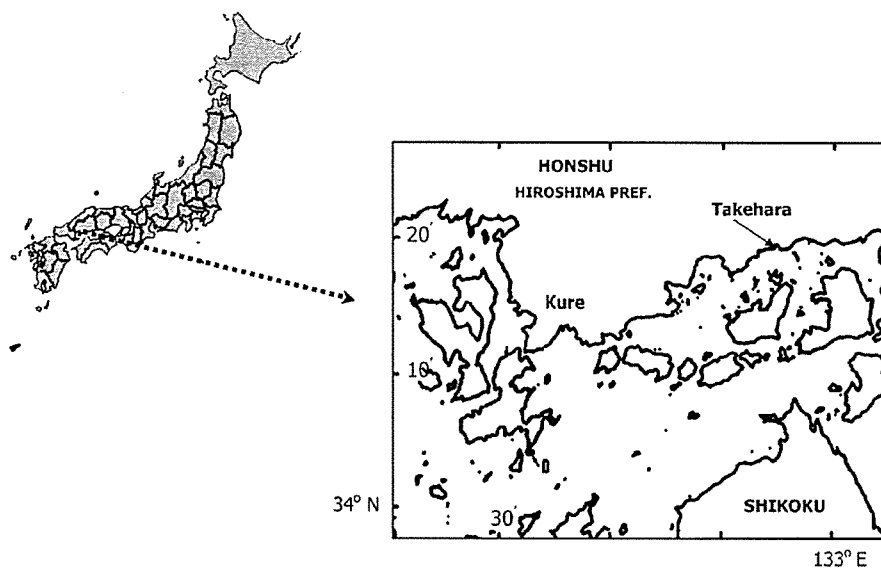
Fig. 1. Map showing the sampling location of Takaehara in Hiroshima Prefecture, Japan.

centrifuged at 3000 × g for 10 min. The supernatant was concentrated under reduced pressure, and loaded on to a Sep-Pak plus C18 Environmental Cartridge (Waters). The unbound portion was collected and concentrated to dryness. The resulting solid was dissolved in a small amount of water, and injected into the HPLC-fluorometric system for the analysis of TTX and its related toxins (Asakawa et al., 2000, 2003). The wiped gauzes were ultra-sonicated in 10 ml of 0.1 ml of acetic acid for 3 min and centrifuged at 3000 × g for 10 min. The supernatant was concentrated under reduced pressure and filtered with an ultra-filtration kit (Ultra-free C3, Millipore), whose cut-off limit was 3000 Da. The obtained extracts were described as the "secreted toxin solution", or simply the "secretion", and then analyzed for the same HPLC system mentioned above.

As authentic toxins, a mixture of TTX and anhydrotetrodotoxin (anhTTX) was prepared from the ovaries of puffer T. vermicularis essentially by the method of Goto et al. (1965). It contained also several percent of 4-epiTTX.

2.4. Gas chromatography–mass spectrometry

A small amount of the extract from the parasitic copepods and authentic TTX were degraded with alkali. The degradation product was trimethylsily-lated, and then analyzed for 2-amino-6-hydroxy-methyl-8-hydroxyquninazoline (C9 base) by GC–MS according to the previously described

procedure (Narita et al., 1987; Asakawa et al., 2000, 2003). Prepared trimethylsilyl (TMS) derivatives were submitted to a gas chromatograph (Hewlett Packard, HP-5890-II) equipped with a mass spectrometer (AutoSpec, Micromass Inc., UK). A column (φ0.25 × 250 cm) of UB-5 (GL Sci., Japan) was used and the temperature was raised from 180 to 250 °C at a rate of 5 °C/min. The ionization voltage was 70 eV and the ion source temperature was kept at 200 °C. Scanning was carried out in the mass range of m/z 40–600 at 3 s interval.

3. Results and discussion

The HPLC patterns of the TTX group contained in 0.1% acetic acid extracts from the two species of parasitic copepods and mucus of the puffer fish are shown in Fig. 2. The analysis of skin mucus of T. niphobles clearly revealed three peaks containing of TTX, 4-epiTTX and anhTTX (Fig. 2B) in comparison with the standard TTXs (Fig. 2A). The analysis of the extract of P. fugu revealed four peaks, of which three could be identified clearly as TTX, 4-epiTTX and anhTTX. The fourth component was not identified (Fig. 2C). In the HPLC analysis of the extract of Taeniacanthus sp., TTX was clearly detected along with the same retention time of the standard TTX (Fig. 2D). The ion-monitored mass chromatograms of TMS derivatives of alkali-hydrolyzed extracts of the two parasitic copepods P. fugu and Taeniacanthus sp. are