Confirmation of Paralytic Shellfish Toxins in the Dinoflagellate *Pyrodinium bahamense* var. *compressa* and Bivalves in Palau*¹

Takako HARADA*², Yasukatsu OSHIMA*², Hisao KAMIYA*³,
and Takeshi YASUMOTO*²

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Pyrodinium bahamense var. *compressa* collected in Palau was confirmed to contain paralytic shellfish poisons at a high level of 1.5×10^{-4} MU/cell. The presence of gonyautoxin V (GTX₅), neosaxitoxin (neoSTX), saxitoxin (STX), and two unidentified toxins coded tentatively as PBT₁ and PBT₂ were confirmed but gonyautoxins I-IV were undetectable, in contrast to *Protogonyaulax* spp. Molluscs were found to be highly infested by the toxins. The following toxins were identified: GTX₅, neoSTX, STX, and PBT₁ in *Spondylus butleri*; GTX₅, STX, and PBT₁ in *Tridacna crocea*; GTX₅, neoSTX, STX, and PBT₁ in *Septifer bilocularis*. In these bivalves, however, the proportion of GTX₅ and neoSTX was decreased while that of STX was increased. The population of *P. bahamense* var. *compressa* was relatively high inside Arumizu Bay, Koror, but was low outside the bay.

Paralytic shellfish poisons (PSP) in bivalves and in the causative dinoflagellates *Protogonyaulax* (= *Gonyaulax*) spp. have been well investigated in the cold and temperate zones of the northern hemisphere. On the contrary there have been only a few reports on PSP in tropical or subtropical waters. PSP occurring in tropical areas may be classified into two categories by the type of the toxin progenitor. One includes the toxic crabs and marine snails living on coral reefs in which saxitoxin and its related toxins were detected as reported in our previous reports.^{1,2)} Judging from their surface grazing feeding habit and the absence of dinoflagellate bloom in the areas, the primary source of the toxin is assumed not to be a planktonic dinoflagellate but is a benthic organism of unknown nature. The other includes toxic bivalves which caused paralytic poisoning in Papua New Guinea and Sabah.³⁻⁷⁾ Although the poisoning was similar in symptomatology to the paralytic shellfish poisoning occurring in the northern waters, there has been no chemical evidence that saxitoxin and its analogues were responsible for the poisoning. *Pyrodinium bahamense*, which was recently amended by STEIDINGER *et al.*⁸⁾ as *Pyrodinium bahamense* var. *compressa*, was connected with the poisoning.⁷⁾

The toxins, however, were again left unclarified. On the other hand one of the authors⁹⁾ detected in Palauan shellfishes at least 5 components of PSP, though with some ambiguity about their identification. This finding led us to undertake a full investigation of toxin profiles of the shellfishes and a search for the toxin progenitor at the islands.

The present paper deals with the confirmation of the presence of saxitoxin and related toxins in Palauan shellfishes and *P. bahamense* var. *compressa*. The regional distribution of the toxic dinoflagellate and the shellfishes was also surveyed to provide information to the local public health officials.

Materials and Methods

Measurement of the Dinoflagellate Population

Population of *Pyrodinium bahamense* var. *compressa* (Fig. 1) were measured at 19 stations around Koror and Babelthuap Islands as shown in Fig. 2 on May 18-20, 1981. A plankton net (20 cm diameter, NXXX-25, 34 μ m) was towed vertically from the near bottom to the surface and the plankton hauls were suspended in 100 ml of sea water. The number of *Pyrodinium* in 1 ml of the suspension was counted under microscope.

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*² Faculty of Agriculture, Tohoku University, Tsutsumi-dori, Sendai, Miyagi 980, Japan (原田孝子・大島泰克・安元 健: 東北大学農学部).

*³ Faculty of Fisheries Science, Kitasato University, Sanriku, Iwate 022-01, Japan (神谷久男: 北里大学水産学部).

The vertical distribution was investigated at Tsukikagetan (St. 1) which is a small indentation inside Arumizu Bay and where the dinoflagellate was most densely populated. Two substations (St. 1a and 1b) were set in Tsukikagetan: St. 1a in the middle and St. 1b at the western side. The organism in the sea water samples was counted directly.

Specimens for Toxicity Study

Planktons collected by vertical tows at St. 1 were concentrated by filtration through membrane filter (Millipore SCWP, 8 μ m) or sedimentation by gravity. More than 97% of these samples were comprised by *P. bahamense* var. *compressa* as shown in Fig. 1b. Other contaminant species were *Ceratium furca*, *Fragilidium* sp., *Protoperidinium pentagonum*, *Leptocylindrus danicus*, *Bactriastrum varians*, *Chaetoceros curvisetus*, *Skeletonema costatum*, and *Nitzschia* sp. Shellfishes collected at Arumizu Bay in December 1980 and May 1981 consisted of bivalves (*Spondylus butleri*, *Tridacna crocea*, *Septifer bilocularis*, *Saxostrea*

mordax, *Lopha cristagalli*, *Modiolus* sp., *Barbatia* sp.) and the gastropod (*Tectus* sp.). Sampling site and date are shown in Table 2 with the results of the toxicity tests. All the specimens were kept frozen at -20 – 25°C until used. To determine the anatomical distribution of the toxin, 6 specimens of *S. butleri* were dissected immediately after catch into the visceral part, adductor muscles, mantles and gills.

Assay Method

The dinoflagellate specimens were suspended in 75% ethanol acidified to pH 3.5 with hydrochloric acid, homogenized with Ultra-Turrax homogenizer and then centrifuged at 3000 rpm for 15 min. The sediments were extracted further twice in the same procedures. The combined extracts were freed from the solvent under *vacuo* and dissolved in 0.03 N acetic acid for mouse assay. Except the specimens for anatomical distribution of the toxins, shellfish toxicity was measured on the whole tissues. The standard assay method for paralytic shellfish poison¹⁰⁾ was adopted using male mice of ddY strain weighing about 20 g each.

Toxin Profiles Analysis

The following specimens were subjected to toxin profile analysis: 3.0×10^7 cells of *P. bahamense* var. *compressa* collected in December 1980 and 6.4×10^8 cells of the same organism collected in May 1981, and three bivalves collected in December 1980, *Spondylus butleri* (74 g, 13,000 MU), *Tridacna crocea* (445 g, 2,300 MU) and *Septifer bilocularis* (74 g, 3,500 MU). Toxins were extracted with 75% ethanol (pH 3.8) three times. The extracts were concentrated under reduced pressure and washed with chloroform. The

Fig. 1. Scanning electron micrographs of *Pyrodinium bahamense* var. *compressa* (a) and plankton specimen tested for the toxicity (b).

Fig. 2. Maps of sampling stations. Palau Islands (a) and enlarged map around Koror Island (b).

aqueous phase was freed from chloroform under *vacuo* and adjusted to pH 5.5 with a dilute sodium hydroxide solution. The extracts were treated successively with activated charcoal, Bio-Gel P-2, and Bio-Rex 70 columns as described in the previous reports.^{1,2} The elution pattern of the toxin was drawn by testing the toxicity of each 5 ml fraction of the eluate from the Bio-Rex 70 column.

Identity of the toxins was confirmed by TLC and electrophoresis. TLC was performed on precoated Silica gel 60 plates (Merck) with pyridine-ethyl acetate-acetic acid-water (75:35:15:30) as the solvent. Electrophoresis was carried out on cellulose acetate strips (Separax, Fuji Photo Film Co.) in 0.08 M Tris-hydrochloric acid buffer of pH 8.7 at 0.56 mA/cm for 18 min. Gonyautoxins and neosaxitoxin prepared from *Protogonyaulax tamarensis* and saxitoxin from F.D.A. were used as the standards. Toxins were detected under UV light (365 nm) after spraying 1% hydroperoxide and heating at 120°C for 5 min.

Results

Regional Distribution of the Dinoflagellate

The results of the survey are shown in Table 1. The distribution of the organism was confined in Arumizu Bay and its adjacent areas (St. 1-9) and the densest population was observed in the innermost part (east side) of the bay. During the two weeks survey, discoloration of sea water was sighted only once at St. 1 when the cell density reached to 450 cells/ml at the surface. Usually the dinoflagellate formed a patchy concentrate in the middle layer and was visible as a turbid layer by submarine observation. The typical vertical distribution of *P. bahamense* var. *compressa* at St. 1 is also shown in Table 1.

Toxicity of the Dinoflagellate and Shellfishes

As shown in Table 2, the *Pyrodinium* samples gave high toxic scores. No apparent difference was observed in the toxic levels between December and May samples.

Toxicities of shellfishes are also summarized in Table 2. Specimens of *S. butleri* and *T. crocea* collected in May showed far higher toxicities than those collected in December at the same station. The different toxic levels of *S. butleri* from different stations of Arumizu Bay reflect the variation in the distribution of *Pyrodinium*. The specimens from St. 1 gave the highest toxicity of 1100 MU/g, as expected from the high population

Table 1. Regional and vertical distribution of *Pyrodinium bahamense* var. *compressa*

Regional distribution		Vertical distribution	
St. No.	Density (cells/ml)	Depth (m)	Density (cells/ml)
1	30.5	Sub-St. 1a	
2	10.1	0	2
3	26.7	5	44
4	10.0	10	95
5	8.3	15	8
6	1.6		
7	0.3	Sub-St. 1b	
8	0.3	0	32
9	0.05	2.5	125
10-19	ND	5.0	79

Table 2. Toxicities of the dinoflagellate and molluscs in Palau

Specimens*1	Toxicities*2
<i>P. bahamense</i> var. <i>compressa</i>	
Specimen collected in Dec. 1980	1.5×10^{-4}
Specimen collected in May 1981	1.4×10^{-4}
Shellfishes collected in Dec. 1980	
<i>Spondylus butleri</i>	310
<i>Tridacna crocea</i>	5.2
<i>Septifer bilocularis</i>	48
Shellfishes collected in May 1981	
<i>Spondylus butleri</i>	1100
<i>Spondylus butleri</i> (St. 2)	520
" " (St. 3)	410
" " (St. 5)	300
<i>Tridacna crocea</i>	96
<i>Lopha cristagalli</i>	130
<i>Saxostrea mordax</i>	210
<i>Modiolus</i> sp.	160
<i>Barbatia</i> sp.	140
<i>Tectus</i> sp.	5.3

*1 All the specimens without note in parentheses were collected at St. 1.

*2 Toxicity of the dinoflagellate was expressed in MU/cell and that of shellfish in MU/g.

Table 3. Anatomical distribution of the toxin in *Spondylus butleri*

Tissue	Weight (%)	Toxicity (MU/g)	Toxicity (%)
Adductor muscle	24.3	42	0.9
Mantle	18.1	34	0.6
Viscera	48.0	2300	98.0
Gill	9.6	57	0.5

density of the dinoflagellate. The difference in toxicity among shellfishes collected at the same place indicates that the ability of toxin accumulation varies greatly from species to species. Interestingly, the marine snail *Tectus* sp. was found

Table 4. Toxin composition of *Pyrodinium* and shellfishes

Species	PBT ₂	GTX ₅	GTX ₃	neoSTX	PBT ₁	STX
<i>P. bahamense</i> var. <i>compressa</i>	+	18	—	31	5	46
<i>Spondylus butleri</i>	—	1	—	18	10	71
<i>Tridacna crocea</i>	—	5	—	+	33	62
<i>Septifer bilocularis</i>	—	—	+	+	+	97

* The composition is expressed in % based on the toxicity.
(+): trace, (—): not detected.

Fig. 3. Elution patterns of the toxins of *Pyrodinium bahamense* var. *compressa* (a) and bivalves (b-d) from Bio-Rex 70 column.

to be toxic, though weakly, in addition to the bivalves. As shown in Table 3, the anatomical study indicates that the 98% of the toxicity is

Table 5. Chromatographic and electrophoretic properties of toxins

Toxin	Bio-Rex 70* ₁	TLC (R _f)	Electrophoresis (R _m * ₂)
PBT ₂	1	0.57	0.26
GTX ₅	2	0.54	0.50
GTX ₃	3	0.67	0.51
neoSTX	4	0.67	0.70
PBT ₁	5	0.50	0.70
STX	6	0.60	1.00

*₁ Elution order from the column.

*₂ Relative mobility to STX (1.00).

contained in the visceral part.

Analysis of the Toxin

The toxin profiles of the dinoflagellate and shellfishes are shown in Fig. 3 and Table 4, respectively. Chromatographic data of each toxin on Bio-Rex 70 column, TLC, and electrophoresis are summarized in Table 5. In *Pyrodinium* specimen collected in December (Fig. 3a), neosaxitoxin (neoSTX) and STX comprised 78% of the toxicity and gonyautoxin V (GTX₅) and a novel component tentatively coded as PBT₁ the rest of it. The *Pyrodinium* sample collected in May showed almost identical toxin profile but the use of a large quantity of the sample for analysis enabled us to detect an additional component (less than 0.5%) coded as PBT₂ appearing just before GTX₅ from Bio-Rex 70 column. The toxins contained in *S. butleri* and *T. crocea* were the same with those of *Pyrodinium* but the proportion of PBT₁ and STX was increased while that of GTX₅ and neoSTX was remarkably reduced. In *S. bilocularis*, GTX₃ was detected instead of GTX₅.

Discussion

The present study provides a firm evidence that paralytic shellfish poisoning in the tropical water is caused by the same type of toxins as involved in the poisoning in the north and *P. bahamense* var. *compressa* is the primary source of the toxins.

This is the first time that production of PSP is unambiguously established in a dinoflagellate other than *Protogonyaulax* spp. The toxin profile of *P. bahamense* var. *compressa*, however, is distinctly different from those of *Protogonyaulax* spp. occurring in U.S.A. and Japan by the absence of GTX₁₋₄ and the presence of PBT_{1,2} and a large amount of GTX₅. The toxicity level of *P. bahamense* var. *compressa* on the basis of MU/cell is higher than that of our cultured *P. tamarensis*¹¹⁾ but is comparable to that of wild specimens of Ofunato Bay in Japan.¹²⁾ Culturing experiment to confirm the toxin production by *P. bahamense* var. *compressa* is now under way. The discrepancy in toxin profiles between the shellfishes and the dinoflagellate may be explainable by the bioconversion of the toxins in the shellfishes.

As to the assignment of PBT₁ and PBT₂, the unavailability of reference toxins prevented us from drawing a final conclusion. However, PBT₂ seems to be assignable to GTX₆ since its chromatographic and electrophoretic behaviors are comparable with the reported values of GTX₆ found in Alaskan mussels.¹³⁾ With regard to PBT₁ there seems no previous report on a component with the identical properties. So far only two components have been known to be eluted from Bio-Rex 70 column between neoSTX and STX: one is GTX₇ found in the deep sea scallop in Atlantic¹⁴⁾ and the other is the turban shell toxin (TST) found in marine snails of Okinawa.¹⁾ However, PBT₁ is distinguishable from GTX₇ by its lower R_m value and from TST by its lower R_f and R_m values. Thus it is highly probable that PBT₁ is a new toxin.

From the food hygienic point of view it is very important that people are well informed about the high toxicity of shellfishes in Arumizu Bay. The highest toxicity of 1100 MU/g detected in *S. butleri* is exceptionally high even compared with the PSP contaminated shellfishes in U.S.A., Canada, and Japan. Our samples of two different seasons, May and December, indicate that *Pyrodinium* is always present in Arumizu Bay and the shellfishes maintain toxicity throughout the year. It should be also born in mind that even the marine snails such as *Tectus* sp. can be toxic in some part of the bay.

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