Toxins of the *Gonyaulax* sp. and Infested Bivalves in Owase Bay

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The algal specimens, mussels and short-necked clams were collected during or immediately after the outbreak of *Gonyaulax* sp. at Owase Bay in 1975. Extraction and purification of the toxins were carried out according to the method used for the isolation of the toxins in softshell clams and cultured *G. tamarensis*. By a combination of Sephadex G-15, Bio-Gel P-2 and Bio-Rex 70 column chromatography, six toxins were isolated from each specimen. Of these, five were identified as saxitoxin, GTX₁, GTX₁', GTX₂, and GTX₃, respectively, which were found in *G. tamarensis*. The major toxin JGX1 was found to be a new one.

In January, 1975, the occurrence of a *Gonyaulax* sp. bloom was reported at Owase Bay, Mie Prefecture, Japan. In the preceding paper the biological nature of the bloom and a preliminary study of the toxins were reported.¹⁾ This paper will report the purification and identification of the toxins in the dinoflagellate, mussels and short-necked clams according to the method used for the isolation of the toxins in softshell clams *Mya arenaria* and cultured *G. tamarensis* cells.²⁾

Materials and Methods

The collection and preservation of the dinoflagellate specimen, short-necked clams *Tapes (Amygdala) japonica* and mussels *Mytilus* sp. were described in the preceding report.¹⁾ Extraction was carried out from 200 g each of the shucked short-necked clams and mussels with 400 ml of 80% ethanol adjusted to pH 2.0 with HCl. The extract was concentrated and washed with chloroform. Three hundred milliliters of condensed dinoflagellate suspension, which was kept frozen after being adjusted to pH 2.0, was thawed and centrifuged. The supernatant was adjusted to pH 4.2 with 1 N NaOH, evaporated to dryness, and then extracted twice with 5 ml of 80% ethanol.

Sephadex G-15 (Pharmacia Fine Chemicals) and Bio-Gel P-2 (minus 400 mesh, Bio Rad Laboratories) columns were prepared under water and eluted with water followed by 0.05 N acetic acid. Prior to chromatography, the sample solution was adjusted to pH 6.5 with 0.1 N NaOH.

High speed liquid chromatography (HSLC) was carried out using a Model Milroyal

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D constant flow pump (Milton Roy Co.) and glass columns which were prepared by packing Bio-Rex 70 (acidic form, minus 400 mesh, Bio Rad Laboratories). The toxins were eluted with a linear gradient of acetic acid from 0 to 0.03 N and then with 0.02 N HCl.

Purified toxins were compared with *G. tamarensis* toxins²⁾ on Silica Gel GF (Analtech, Inc.) and Silica Gel 60 (EM Laboratories, Inc.) precoated plates with solvent systems of *t*-butanol:acetic acid:water (2:1:1) and pyridine:ethylacetate:water:acetic acid (75:25:30:15), respectively. Electrophoresis (PE) was carried out on cellulose acetate strips (Shleicher & Schuell, Inc., No. 2500) in 0.083 N Tris-HCl buffer pH 8.7, at 200 V (constant) and 0.2 mA/cm for 1 to 3 hr. Toxins were detected by spraying with 1% hydrogen peroxide and heating at 120°C for 15 min. Fluorescent spots were viewed under UV light (350 nm).

Toxicity assays were done according to the USPHS method originally described by Sommer and Meyer³⁾ using the Death-Time/Dose curve reported in the previous work.²⁾

Results

Purification of mussel toxins The mussel extract, which showed a total toxicity of 7,000 MU, was fractionated into two toxic fractions (the major part, 5,000 MU, eluted with water; and the minor part, 1,200 MU, eluted with acetic acid), by Sephadex G-15 chromatography (column size: 2.65×100 cm).

The major toxic fraction was chromatographed on a Bio-Gel P-2 column (2.5×40 cm). The elution pattern of toxins from the Bio-Gel P-2 column is shown in Fig. 1a. Out of the total 3,300 MU charged on the column, 1,100 MU were eluted with water and 1,900 MU with 0.05 N acetic acid. The rest of toxicity was found in the water elute. The toxins eluted with acetic acid from Sephadex G-15 column were rechromatographed on a Bio-Gel P-2 column (1.0×40 cm). All the toxin was bound well on the column in water and elution with 0.05 N acetic acid afforded 670 MU of a mixture of toxins.

Toxic fractions eluted with acetic acid from the both Bio-Gel P-2 columns were combined and fractionated by HSLC using a Bio-Rex 70 column (0.5×60 cm). The major part of toxins (1,500 MU) was eluted with acetic acid and separated from the minor part (290 MU) which was later eluted with 0.02 N HCl as a single peak. The former fraction was rechromatographed on a longer column (0.6×100 cm). The elution pattern of these toxins is shown in Fig. 2a. The presence of five toxins, tentatively named JGX1-5, in order of elution, were observed by TLC and PE. The three peaks shown in Fig. 2a corresponded to the major toxins JGX1, JGX3, and JGX5. In addition, two minor toxins were found to overlap the main peaks; JGX2 was eluted concomitantly with JGX1 and JGX3, while JGX4 was eluted JGX3 and JGX5. Another toxin (JGX6)

Fig. 1. Bio-Gel P-2 column chromatography.

(a) Mussel toxin (3,300 MU) and (b) *Gonyaulax* sp. toxin (4,800 MU) were chromatographed on the 2.5×40 cm column at a flow rate of 0.6 ml/min.

was also isolated by rechromatography of the HCl eluate from the first Bio-Rex 70 column.

Purification of short-necked clam toxins The extract from the short-necked clams having an activity of 3,500 MU was subjected to Sephadex G-15 column chromatography. Since only a portion of the toxicity was retained on the gel, probably due to the interference of a large amount of coexisting substances, the Sephadex filtration was repeated and a total of 2,200 MU of the bound toxins was obtained by 0.05 M acetic acid elution.

Sephadex G-15 purified toxins (700 MU) were reappplied onto a Bio-Gel P-2 column (1.0×40 cm). All the toxins were found in the acetic acid eluate. This fraction was found to contain six components identical to those found in mussels and dinoflagellate as analyzed by TLC and electrophoresis.

Purification of *Gonyaulax* sp. toxins The extract from the bloom (4,800 MU) was directly applied onto a Bio-Gel P-2 column (2.5×40 cm). The elution pattern is shown in Fig. 1b. The combined acetic acid eluates (3,700 MU) were rechromatographed on a smaller Bio-Gel column (1.0×40 cm), and 3,000 MU of toxins were recovered. This preparation was purified further by HSLC using a Bio-Rex 70 column (0.6×100 cm). The elution pattern with acetic acid was identical to that of the mussel toxins (Fig. 2b).

Fig. 2. Bio-Rex 70 column chromatography.

(a) Mussel toxin (1,090 MU) and (b) *Gonyaulax* sp. toxin (3,000 MU) were chromatographed on the 0.6×100 cm column at a flow rate of 1 ml/min. The dotted line indicates the concentration of acetic acid.

Furthermore, on TLC and PE, the same five toxins (JGX1–5) appeared at the same elution volume as did the mussel toxins. In the fraction eluted later with 0.02 N HCl the toxin corresponding to JGX6 (100 MU) was also recognized.

Table 1. Chromatography and electrophoretic behavior of isolated toxins

Toxins	Elution order from Bio-Rex 70 column	TLC		PE Rm ^{*3}	Toxins Identified
		Rf ^{*1}	Rf ^{*2}		
JGX1	1	0.61	0.52	0.28	—
JGX2	2	0.81	0.65	0	GTX ₁ '
JGX3	3	0.90	0.70	0.16	GTX ₁
JGX4	4	0.69	0.61	0.28	GTX ₈
JGX5	5	0.81	0.65	0.56	GTX ₂
JGX6	6	0.62	0.51	1.00	saxitoxin

*¹ Silica Gel 60: pyridine, ethylacetate, water, acetic acid (75: 25: 30: 15).

*² Silica Gel GF: t-butanol, acetic acid, water (2: 1: 1).

*³ Relative mobility on cellulose acetate strip in Tris-HCl buffer pH 8.7, at 200 V (constant) and 0.2 mA/cm for 1 hr to that of saxitoxin.

Thin-layer chromatography and paper electrophoresis The results of TLC and PE of the toxins from mussels and *Gonyaulax* sp. are summarized in Table 1. JGX2, JGX3, JGX4, JGX5 and JGX6 showed the same mobilities as GTX₁'*, GTX₁, GTX₃, GTX₂ and saxitoxin, respectively. JGX2 (GTX₁') and JGX5 (GTX₂) have close R_f values on TLC in both solvent systems, but they were clearly distinguishable from each other by PE.

Discussion

The purification methods used for *G. tamarensis* toxins were also effective for this study and the previous finding that the major toxins in short-necked clams were different from saxitoxin, the sole toxin reported in *G. catenella* was confirmed. Although the organism was tentatively identified as *G. catenella*,¹⁾ it was revealed that the composition of toxins in the *Gonyaulax* sp. bloom at Owase Bay is similar to that of *G. tamarensis*. From the behavior of the toxins on HPLC, TLC and PE, we conclude that five toxins (JGX 2-6) are identical to those found in *G. tamarensis*. However, there were two points of difference between the paralytic shellfish poisoning toxin in Japan and New England. The former contained a significant amount of an additional toxin (JGX1), and also the relative concentration of GTX₁ in the mussel and *Gonyaulax* sp. toxins from Japan was far greater than that in *G. tamarensis*.

In the column chromatography on Sephadex G-15 and Bio-Gel P-2, the crude toxin was fractionated into unbound and bound toxins. The results of previous studies^{4,5)} on *G. tamarensis* toxins suggest that a large amount of salts or other contaminants in the extract partially prevented the adsorption of toxins onto both gels and caused the apparent fractionation in the column chromatography. Nevertheless, the possibility that toxin(s) other than JGX1-6 exist in the unbound portion is not excluded, because toxins in the water eluted fraction could not be characterized directly by TLC and PE due to a large amount of residues in the fractions.

After rechromatography on Bio-Rex 70, all the toxic fractions appeared as almost invisible residues. A determination of specific toxicities was not achieved due to the scarcity of the compounds.

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* This toxin appears just before GTX₁ in Bio-Rex 70 column chromatography. However, the toxicity of this compound was not confirmed before (See the legend in Fig. 2 of the previous report)²⁾

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