Isolation and Properties of Gonyautoxin-5, an Extremely Low-toxic Component of Paralytic Shellfish Poison

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Gonyautoxin-5 (GTX₅) was isolated from the toxic specimens of the oyster *Crassostrea gigas* collected from Senzaki Bay, Yamaguchi Prefecture. The digestive glands were extracted with acidic ethanol and the extract was applied successively to activated charcoal treatment and three types of chromatography: on Bio-Gel P-2, Amberlite CG-50, and Bio-Rex 70. The GTX₅ preparation thus obtained was homogeneous in both thin-layer chromatography and cellulose acetate membrane electrophoresis. The specific toxicity was found to be 280 MU/mg, a value which is much lower than that of gonyautoxin-1, gonyautoxin-2, or saxitoxin (STX). However, the toxicity of the preparation was increased five times by mild acid-hydrolysis. In the hydrolyzate, STX was found together with two unidentified components. The GTX₅ contained approximately one mole of sulfate per mole, and showed a PMR spectrum which is closely similar to that of STX.

These results suggest that GTX₅ possesses the structure of carbamoyl-*N*-sulfo-saxitoxin.

Chromatographic or electrophoretic patterns of paralytic shellfish poison (PSP) differ from each other, depending on the species of causative plankton, the species of bivalves infested, and their environments.¹⁾

It is well known that PSP is composed of a number of gonyautoxin (GTX) and saxitoxin (STX) components. Gonyautoxin-5 (GTX₅) was first found in *Protogonyaulax catenella* which bloomed in Owase Bay, Mie Prefecture, in 1975 and tentatively designated JGX₁.²⁾ The same toxin was also detected in the bivalves infested there.²⁾ Subsequently, GTX₅ was also detected in the *P. tamarensis* culture,³⁾ the mussel *Mytilus* sp. collected from Oda Bay, Kagawa Prefecture,⁴⁾ the scallop *Placopecten magellanicus* from the Bay of Fundy,⁵⁾ and the oyster *Crassostrea gigas* from Senzaki Bay, Yamaguchi Prefecture.⁶⁾

As far as the observations in Japan are concerned, GTX₅ seems to be produced specifically by *P. catenella* inhabiting the western part of Japan Proper. This, together with some other facts,⁷⁾ let us speculate that GTX₅ could be a precursor of other PSP components. One of the authors⁴⁾ reported preliminarily the isolation and characterization of GTX₅, indicating its specifically

low toxicity. No other papers are available on this toxin.

The present paper deals with isolation of GTX₅ from the toxic oysters collected from Senzaki Bay, and with the structure and some properties of the toxin.

Material and Methods

Material

Cultured specimens of the oyster *Crassostrea gigas* were harvested from Senzaki Bay, Yamaguchi Prefecture, in January, 1980. About 10 kg of the edible part was immediately frozen, transported to the Laboratory of Marine Biochemistry, Univ. of Tokyo, and kept frozen below -20°C for about one year. The frozen oysters were partially thawed, and the digestive glands (1.8 kg) were removed and used for isolation of GTX₅.

Isolation of GTX₅

The digestive glands were homogenized with almost the same volume of 1% acetic acid in 80% ethanol, using an Ultra-Turrax homogenizer and centrifuged at 5000 rpm for 10 min. For the residue, the above operations were repeated four

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more times. The supernatants were combined, concentrated under reduced pressure, and defatted with chloroform. The aqueous solution (*ca.* 2 l) was adjusted to pH 5 with 1 N NaOH, and to it was added 2 l of water-washed activated charcoal. After being washed with water repeatedly, the charcoal was developed with 1% acetic acid in 20% ethanol. Toxic fractions eluted were combined, concentrated, adjusted to pH 5.5, and applied to a Bio-Gel P-2 column (5 × 35 cm). The column was repeatedly washed with 4 l of water. The toxin was eluted with 0.15 N acetic acid, and the toxic fractions freeze-dried. The yellow powder obtained was dissolved in a small amount of water and applied to a column of Amberlite CG-50 (H⁺ form, 1.5 × 95 cm). The column was first washed with water, followed by 0.1 and 0.5 N acetic acid, and then 0.1 N HCl. Most toxin was eluted with 300 ml of water and 330 ml of 0.5 N acetic acid (Fig. 1). Three toxin peaks designated CG-A, -B, and -C appeared: Peak CG-A eluted with water was composed of unbound toxins. Peaks CG-B and -C which were eluted with 0.5 N acetic acid were composed of GTX group.

Peak CG-B (GTX group) was repeatedly chromatographed on a column of Bio-Rex 70 (H⁺ form, 0.8 × 95 cm). In the first run, GTX₅ was eluted with 0.1 N acetic acid, and in the second and third runs a linear gradient of acetic acid from 0 to 0.02 N and from 0 to 0.01 N, respectively. The third run made it possible to isolate the GTX₅, as described in *Results and Discussion*.

GTX₁⁹⁾ and GTX₂⁹⁾ containing some GTX₅ were isolated from the scallop as reported previously and used as reference standards. An authentic specimen of STX which was kindly given by Dr. CAMPBELL, U.S. Public Health Service, was also used.

Identification of GTX₅

GTX₅ was identified by *R_f* value in thin-layer chromatography (TLC) and by *R_m* value in electrophoresis. TLC was conducted on 5 × 20 cm silica gel precoated plates (Merck) with two solvent systems [pyridine: ethylacetate: water: acetic acid (75: 25: 30: 15) and *tert*-butanol: acetic acid: water (2: 1: 1)]. Electrophoresis was performed on 5 × 18 cm cellulose acetate strips (Chemetron) in 0.08 M Tris-HCl buffer pH 8.7, at 0.8 mA/cm for 30 min. The plates or strips were sprayed with 1% hydrogen peroxide and heated at 110°C for 10 min, and then toxins detected under UV light at 365 nm.

Toxicity

Toxicity was determined by a mouse assay for PSP.¹⁰⁾

Degradation of GTX₅

To the purified GTX₅ (0.5 mg) was added 4 ml of 0.01 N HCl and the solution divided into two equal portions. One of them was evaporated to dryness under reduced pressure at 40°C, and the other portion was subjected to 4 repetitions of the evaporation-dissolution procedure. After completion of those treatments, each sample was analyzed by TLC, electrophoresis, or mouse assay.

¹H-NMR Spectrum

About 2 milligrams of the purified GTX₅ was dissolved in 0.3 ml of D₂O or H₂O and put in a test tube. Tetramethylsilane (TMS) was used as an external standard. The GTX₅ was dissolved in D₂O, and analyzed for ¹H-NMR spectra at 15, 30, 60, and 180 min, using a JEOL JNM FX-400 spectrometer at 400 MHz. Then the GTX₅ solution was freeze-dried and the resultant powder was dissolved in H₂O and similarly analyzed.

Determination of Sulfate

The GTX₅ (100 μg) was hydrolyzed at 100°C for 24 h with 25% formic acid or 6 N HCl, and determined for sulfate by the method of ANTONOPOULOS.¹¹⁾

Results and Discussion

Isolation of GTX₅

Toxins (90,000 MU) were extracted with the acidic ethanol from 1.8 kg of the digestive gland. From the extract, GTX₅ was purified and isolated by means of activated charcoal treatment, and chromatography on Bio-Gel P-2, Amberlite CG-50 and Bio-Rex 70. The progress in purification

Table 1. Purification of GTX₅ from toxic oyster digestive glands

Steps	Total toxicity (MU)	Specific toxicity of GTX ₅ (MU/mg)
1% AcOH-80% EtOH extract	90,000	
Treatment with activated charcoal	38,000	
Chromatography on:		
Bio-Gel P-2	30,000	
Amberlite CG-50	8,000	
1st Bio-Rex 70	4,100	11.6
2nd Bio-Rex 70	2,600	107
3rd Bio-Rex 70	1,120	280

Fig. 1. Elution profile of toxins from an Amberlite CG-50 (H^+ form) column. A total toxicity of 30,000 MU was applied to the column.

Fig. 2. Elution profile of peak CG-B (Fig. 1) in the first Bio-Rex 70 column chromatography (upper), and electrophoresis of some fractions (lower).

tion of this toxin is summarized in Table 1. The elution profile of toxins from the Amberlite CG-50 column is shown in Fig. 1. Herein, peak CG-A (10,000 MU) was first eluted, followed by peaks CG-B (8000 MU) and CG-C (750 MU).

Electrophoretic analyses of the fractions belonging to those peaks demonstrated that frs. 116-124 (peak CG-B) showed one spot with an R_f value similar to that of GTX₅.²⁾ Therefore, those fractions (peak CG-B) were combined and further purified by chromatography on Bio-Rex 70 (H^+), resulting in separation into peak 1-A (500 MU),

peak 1-B (2600 MU) and peak 1-C (4100 MU), as shown in Fig. 2. Frs. 136 and 138 of peak 1-C showed a band whose R_m was comparable to that of GTX₅, 0.28.²⁾ Then, frs. 134-138 were combined and subjected to the second column chromatography on Bio-Rex 70 (H^+). Two peaks 2-A and 2-B appeared (Fig. 3). Frs. 102-110 of peak 2-B showed a single spot whose R_f value was similar to that of GTX₅.²⁾ These frs. (2600 MU) were combined and submitted to the third chromatographic run. As shown in Fig. 4, each portion of peak 3-A (1120 MU) showed a

Fig. 3. Elution profile of peak 1-C (Fig. 2) in the 2nd Bio-Rex 70 column chromatography (upper), and electrophoresis of some fractions (lower).

Fig. 4. Elution profile of peak 2-b (Fig. 3) in the 3rd Bio-Rex 70 column chromatography (upper), and electrophoresis of some fractions (lower).

single spot with an *R_f* value corresponding to GTX₅, while each fraction of peak 3-B (400 MU) showed an additional spot. Fractions of peak 3-A were combined, freeze-dried, and assayed.

Purity of GTX₅

The results of TLC and electrophoresis of the GTX₅ preparation thus isolated are shown in Figs. 5 and 6.

As shown in Fig. 5, the GTX₅ gave a single spot with the *R_f* value 0.25 in the solvent system of *tert*-butanol: acetic acid: water (2: 1: 1) and with the *R_f* value 0.41 in that of pyridine:

ethylacetate: water: acetic acid (75: 25: 30: 15). On the other hand, the GTX₅ showed again a single spot (*R_m*=0.22) in electrophoresis (Fig. 6). These results indicated that our GTX₅ preparation was highly purified.

Specific Toxicity of GTX₅

The value of 280 MU/mg was obtained, which is much less than that of STX, GTX₁, or GTX₂ (4200–5500 MU/mg).⁷⁻⁹⁾

Degradation of GTX₅

When the GTX₅ was dissolved in 0.01 N HCl

Fig. 5. Thin-layer chromatography of purified GTX₅. Fig. 6. Electrophoresis of purified GTX₅.

and concentrated, it was partially degraded (Fig. 7). However, the whole mixture showed 1.5 times the original toxicity. The toxicity was raised about 5 times when the concentration-dissolution operation was repeated. As seen in Fig. 7, the whole mixture showed STX and two unidentified components, in addition to some still remaining GTX₅. Essentially the same results were obtained when the degraded mixture of GTX₅ was analyzed chromatographically (Fig. 8).

Fig. 7. Electrophoresis of purified GTX₅ before and after acid treatment.

- [A]: Immediately after dissolving in water.
 [B]: After dissolving in 0.01 N HCl and concentrating under reduced pressure.
 [C]: After repeating 4 times the concentration-dissolution operation.

¹H-NMR Spectrum of GTX₅

¹H-NMR spectra of the GTX₅ in D₂O and H₂O are shown in Fig. 9. Chemical shifts of protons in the GTX₅ are summarized in Table 2. The chemical shifts agree with those of STX except that those of protons adjacent to carbon-13 are slightly downfield, compared with those of STX.

As seen in spectra (a)-(d) of Fig. 9, the protons

adjacent to carbon-11 were gradually deuterated and disappeared 180 min after being dissolved in D_2O . This phenomenon was also observed in STX¹²⁾ and neosaxitoxin.¹³⁾ The signal of the proton bonding to carbon-11 is clear from the NMR spectrum of the GTX₅ dissolved in H_2O (refer to spectrum (e) in Fig. 9).

It was postulated from the above NMR data that the GTX₅ has the same skeleton as that of STX except for the carbamoyl branch bonding to carbon-6.

Fig. 8. Thin-layer chromatography of purified GTX₅ before and after acid treatment. Refer to Fig. 7 for the detail of sample [C].

Table 2. Comparison of 1H -NMR data of purified GTX₅ and STX

H (Carbon No.)	GTX ₅ ^{*1}	STX ¹⁵⁾
H (5)	4.37 d (1.0)	4.33 d (1.3)
H (6)	3.51 ddd (1.0, 5.9, 9.3)	3.47 m (1.3, 6, 9)
H (10)	3.25 d (9.9)	3.20 ddd (8, 10, 11)
	3.44 d (9.9)	3.45 ddd (2.5, 10, 11)
H (11)	2.00 m ^{*2}	2.00 m
H (13)	3.76 dd (5.9, 11.5)	3.65 dd (6, 12)
	4.04 dd (9.3, 11.5)	3.88 dd (9, 12)

*1 1H -NMR of GTX₅ was measured 180 min after being dissolved in D_2O .

*2 Chemical shift of H(11) in GTX₅ was measured in H_2O or soon after being dissolved in D_2O .

Sulfate in GTX₅

One hundred micrograms of GTX₅ released about 25 μg sulfate when digested with formic acid or HCl under the conditions described before. It follows that the GTX₅ contained 1.0 mole sulfate per mole, assuming that it has a molecular weight of 378.

Structure of GTX₅

From the above results, it was confirmed that our GTX₅ possesses one mole sulfate per mole and easily liberates the sulfate on mild acid-hydrolysis, transforming itself into STX and some other components. The 1H -NMR spectrum agreed well with that of STX except the slight differences

Fig. 9. 1H -NMR spectra of purified GTX₅.

The spectra (a)-(d) were measured 15, 30, 60 and 180 min after dissolution in D_2O , and that of (e) measured in H_2O .

Fig. 10. Structure of GTX_5 .

in chemical shift of the protons bonding to carbon-13.

These facts strongly suggest that GTX_5 is STX sulfate and the sulfate is located at the carbamate amino group adjacent to carbon-13 in Fig. 10, and not at the carbon-11 as in GTX_1 ⁸⁾ or GTX_2 .⁹⁾

Incidentally, we observed during the course of study that the GTX_5 did not yield its epimer. Then, we propose the structure of GTX_5 as shown in Fig. 10. In this connection, KOBAYASHI and SHIMIZU¹⁴⁾ have suggested recently that one of the two sulfate groups contained in GTX_5 is located at the same position as in GTX_5 .

GTX_5 was for the first time isolated in a highly pure state and its structure was proposed as described above. The specific toxicity, 280 MU/mg, is much lower than those of STX, GTX_1 and GTX_2 . However, it significantly increased its toxicity when treated with dilute HCl. The electrophoretic and chromatographic analyses on the hydrolyzate demonstrated the presence of some STX. This fact may suggest that GTX_5 is a precursor of STX in the toxic *Protogonyaulax* plankton or bivalves infested and is converted into STX by the enzymic or nonenzymic action in the cell. The studies along these lines are now in progress.

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