PRESENCE OF FOUR TOXINS IN RED TIDE INFESTED CLAMS AND CULTURED GONYAULAX TAMARENSIS CELLS

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Summary: Four toxins have been isolated by Sephadex G-15 and high pressure ion exchange chromatography from the soft shell clams, Mya arenaria, which were collected during 1972 and 1974 red tide outbreaks on the New England coast. One of the toxins was saxitoxin and the rest seem to be new toxins. The same toxins were isolated from the extract of cultured Gonyaulax tamarensis.

Introduction: Dinoflagellate blooms caused by Gonyaulax tamarensis have been responsible for the toxicity in various shellfish on the North Atlantic coasts of Canada, United States, and Great Britain. In the past the occurrence of the blooms have been rather sporadic and limited in scale, but massive outbreaks in 1972 and 1974 along the northeastern coast of New England created serious health and economic problems.

The nature of the east coast shellfish poisoning is similar to the West Coast paralytic shellfish poisoning (PSP) caused by Gonyaulax catenella, and all the sanitary measures are based on the West Coast PSP. However, the identity of the toxin(s) has been the subject of discussion over the past ten years. Schantz (1) first reported that the isolation method for saxitoxin was not applicable to the East Coast PSP, and Evans (2) reported the similar observation with the PSP in Great Britain. Recently, however, Ghazarossian, et al. (3) have been able to isolate only saxitoxin

from the scallops infested with G. tamarensis and stored in dilute HCl for many years. Shimizu, et al. (4,5) and Buckley, et al (6) also reported the identification of saxitoxin, and in addition, the presence of another major toxin in the G. tamarensis infested soft shell clams.

Now we report the separation of the major toxin into three closely related compounds and present evidence of the existence of at least four toxins in the original intact G. tamarensis cells as well as in clams.

Experimental Procedure: Toxic clams, Mya arenaria, were collected on the North Shore of Cape Ann, Massachusetts, during the September, 1972, and September, 1974, red tide outbreaks. The dissected hepatopancreases, which retained 80% of the total toxicity, were used for extraction. The G. tamarensis originating from the 1972 New England outbreak was cultured in enriched seawater at 10 ± 2^{o} under regulated fluorescent light. The cells were harvested at peak density (ca. 28 days) by centrifugation and used for extraction.

Both the hepatopancreases and cells were extracted with 80% ethanol adjusted to pH 2.0 with HCl. The extract was concentrated and washed with $CHCl_3$. Sephadex G-15 and Bio-Gel P-2 columns were prepared under H_2O and eluted with H_2O and 0.025-0.05 N acetic acid.

High speed liquid chromatography (HSLC) columns were prepared by packing Bio-Rex 70 (acidic form, minus 400 mesh granular) or Amberlite CG-50 (acidic form, 200-400 mesh granular). Chromatography was generally carried out at ca. 40 psi using a Milton Roy Model Milroyal D or Fluid Metering RP-G constant flow pump.

Toxicity assays were done according to the USPHS method originally described by Sommer and Meyer (7). The Death-Time/Dose curve used

in this work was prepared using partially purified toxin from the above-mentioned clams (4).

Results: It was discovered that the majority of the toxicity was retained on Sephadex G-15 at around pH 5.0 and eluted with dilute acetic acid and effectively separated from the rest of the material. In the elution, saxitoxin preceded the rest of the toxins (Fig. 1a). Polyacryl amide gel, Bio-Gel P-2, worked similarly but with the reverse elution pattern, saxitoxin following the rest of the toxins (Fig. 1b). In both cases only

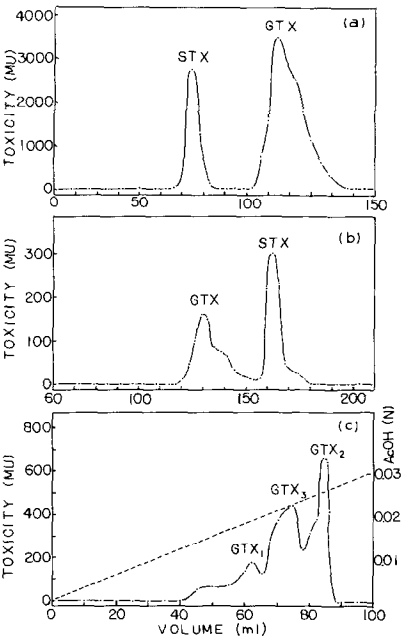

Fig. 1. (a) Separation of GTX and STX by Sephadex G-15. The column (60 x 2.0 cm, void volume 30 ml) was eluted with 20 ml of water followed by 0.025 N acetic acid. (b) Separation of GTX and STX by Bio-Gel P-2. The column (60 x 1.0 cm, void volume 20 ml) was eluted with 0.01 N acetic acid. (c) Separation of GTX_1, GTX_2, and GTX_3 by Bio-Rex 70 (acidic form). The column (60 x 0.5 cm) was eluted with a linear gradient of acetic acid at 0.25 ml/min. The dotted line indicates the concentration of acetic acid.

slight separation of the new toxins was observed. Examination of the fraction showed that it was a mixture of three components, which we tentatively named GTX_1, GTX_2 and GTX_3. For further separation high speed liquid chromatography proved to be very effective (Fig. 1c).

The toxicity peaks shown in Fig. 1c correspond to the spots on the thin-layer chromatography as indicated (Fig. 2). The order on tlc and chromatography elution was reversed with GTX_2 and GTX_3. GTX_2 seemed to be the largest in quantity and toxicity and was obtained in sufficient amount to be used for spectroscopic and toxicity studies. The IR spectrum (Fig. 3) shows a quite different pattern from that of saxitoxin. Although it was difficult to determine the exact toxicity due to the extreme hygroscopicity and scarcity, a toxicity of ca. 2000 mouse units per mg., about half that of saxitoxin, was observed. Other toxins showed similar ranges of toxicity in preliminary assays.

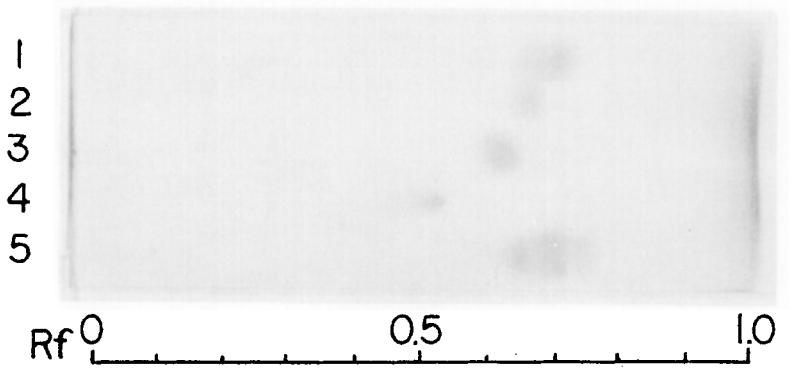

Fig. 2. Thin-layer chromatography of GTX and STX. The following samples were developed with a solvent system of t-butanol, acetic acid, water (2:1:1) on Silica Gel GF (Analabs, Inc.). 1; GTX_1, 2; GTX_2, 3; GTX_3, 4: STX, 5; mixture of four toxins. GTX_1 was accompanied by a small amount of an additional spot, it is not known if this spot also represents toxicity.

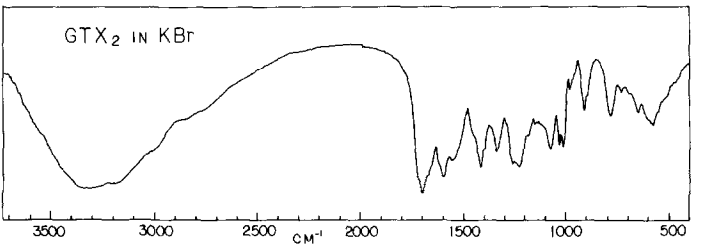

Fig. 3. Infrared spectrum of GTX_2. It was taken with Perkin-Elmer Model 521 equipped with a beam-condenser in a KBr micropellet.

Discussion: It has been suspected that some of the inconsistent results observed with the G. tamarensis toxins are due to the instability of some of the toxins under the conditions used in the isolation procedure.

Long-term storage of the extract in a strongly acidic medium apparently destroys all the toxins except saxitoxin.

Also, we reported that the use of ion-exchange resin with sodium acetate buffer in the initial stage of isolation led to the destruction of some of the toxins because of the high pH reached during concentration of the toxic fraction (5).

Conditions which may result in the destruction or transformation of the toxins were avoided as much as possible in this experiment. Aqueous alcohol was chosen for the extraction to avoid possible enzyme actions during homogenization. Purification was achieved solely by means of Sephadex filtration and ion-exchange chromatography using very dilute acetic acid. We did not observe any change in the number of spots during the purification process. The toxins seemed to be reasonably stable under weakly acidic conditions. We also found that the amount of saxitoxin in clams decreases substantially upon storage while the other toxins remain fairly well.

This observation may be significant from the viewpoint of sanitary control.

The three new toxins seem to be very closely related; they gave almost the same Rf values which are completely different from that of saxitoxin in various tlc systems. All gave greenish yellow coloration with fluorescence with H_2SO_4 while saxitoxin gave a blue color. It was observed that upon storage in a neutral medium GTX_2 and GTX_3 slowly undergo equilibration to give mixtures of both compounds. The nature of the structural change associated with this is not known. Tetrodotoxin gives almost similar coloration and Rf values, but careful comparison denied its identity with any of the new toxins.

Structure elucidation study is underway in collaboration with Prof. Koji Nakanishi at Columbia University.

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