Use of a Strong Cation Exchange Resin Column for the Study of Paralytic Shellfish Poisons

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The behavior of paralytic shellfish poisons (PSP's) in crude extracts on a strong cation exchange resin column was studied by using fluorescence generated by oxidation with hydrogen peroxide to locate the toxins. The sulfated 11-hydroxysaxitoxins (GTX_2 and GTX_3) were retained by the resin and eluted with pH 5 sodium acetate buffer, the GTX_2 slightly ahead of GTX_3. The saxitoxin (STX) was tightly bound and not eluted until HCl was applied to the column. The method appears to be useful for the detection of small quantities of the STX family of toxins (STX, GTX_2, and GTX_3) in crude extracts. Runs with toxic soft-shelled clam, scallop, and crab extracts showed the presence of STX and its sulfated 11-hydroxy derivatives.

The occurrence of a multiplicity of paralytic shellfish poisons (PSP's) produced by *Gonyaulax* species makes it desirable to be able to readily separate, identify, and determine the individual components. Evans (1970), following the procedure of Schantz et al. (1957) for the purification of saxitoxin (STX) from toxic shellfish, found that most of the toxicity of a crude 0.1 N HCl extract of whole mussels which had been exposed to a bloom of *Gonyaulax tamarensis* was not retained on a weak cation exchange resin column (Amberlite CG-50, Na form) when developed with 1 M acetic acid adjusted to pH 4 with saturated sodium acetate. STX is retained by the column and is eluted with 0.5 M acetic acid (Schantz et al., 1957). Shimizu et al. (1975), using acidified 80% ethanol extracts of the hepatopancreas of *G. tamarensis* exposed *Mya arenaria*, found that the toxins were retained on a weak cation exchange resin column (Bio-Rex 70, H form) and that they were eluted with a gradient of dilute acetic acid. Boyer et al. (1979) applied crude acidified 80% ethanol extracts of toxic scallop hepatopancreas directly onto a weak cation exchange resin column (Amberlite CG-50, H form). The toxicity was retained and was eluted by a 0.1 M acetic acid to 0.1 N HCl gradient. Retention of some of the less basic toxins in a crude pH 2 extract was also obtained by Hall et al. (1979) on a weak cation exchange resin column (Amberlite IRP-64, Na form). These were eluted with 1 M pH 5.2 sodium acetate buffer.

Gel filtration resins (Bio-Gel P-2; Sephadex G-15) have also been applied to the separation of the *G. tamarensis* toxins, but the resolution achieved by these resins appears to be less than that achieved by the weak cation exchange resins (Shimizu et al., 1975; Buckley et al., 1976).

In view of the weak nature of the binding of some of the *Gonyaulax* toxins to a weak cation exchange resin column, especially from crude extracts, we became interested in studying the behavior of these toxins on a strong sulfonic acid type cation exchange resin, where stronger retentions might be expected. In previous work we reported on the similarities between the toxins of the blue-green alga *Aphanizomenon flos-aquae* and the *Gonyaulax* toxins (Alam et al., 1978) and have shown the applicability of a strong cation exchange resin column for the separation of the *Aphanizomenon* toxins (Ikawa et al., 1981).

Toxins were detected and measured by following the fluorescence generated when the toxins are treated with alkaline hydrogen peroxide (Bates and Rapoport, 1975). Although the major toxins found in toxic New England shellfish become fluorescent under these conditions (Buckley et al., 1976), some of the toxins, such as neosaxitoxin and the sulfates of its 11-hydroxy derivatives (GTX_1 and GTX_4), show less or no significant fluorescence when treated and read under the conditions used for the detection of STX (Buckley et al., 1978). Despite this limitation of the fluorescence method in its present form, we thought that useful toxin profiles might be realized from crude extracts. These profiles might aid in the identification and determination of the STX family of toxins (STX, GTX_2, and GTX_3) and for the identification of the PSP-causative organisms on a chemotaxonomic basis.

EXPERIMENTAL SECTION

Materials. Toxic soft-shelled clams, *M. arenaria*, were collected during the 1972 New England Red Tide and stored in the frozen state. Recent bioassays showed that their toxicity had not changed drastically. Nontoxic samples were obtained from the local markets.

GTX_2 and GTX_3 had been isolated by Buckley et al. (1976) from the 1972 toxic *M. arenaria* as major toxins H and L, respectively, and kept frozen. These toxins are the 11-hydroxy epimers of STX (Shimizu et al., 1976) which have been shown to occur as their 11-hydroxy sulfate esters (Boyer et al., 1979). STX was obtained through the courtesy of Dr. E. J. Schantz, Food Research Institute, University of Wisconsin–Madison.

Scallop toxin was extracted from frozen hepatopancreas of toxic *Pectinopectin yessoensis* collected from Ofunato Bay, Iwate Prefecture, Japan, in August 1979 and partially purified by adsorption on activated charcoal followed by chromatography on Bio-Gel P-2 (Noguchi et al., 1981a,b). Partially purified crab toxin was obtained by a similar procedure from toxic *Zosimus aeneus* collected at Ishigaki Island, Okinawa Prefecture, Japan, in June 1980.

***Mya arenaria* Extraction Procedure.** Both toxic and nontoxic *M. arenaria* were extracted by the extraction procedure used in the standard mouse bioassay; i.e., 100 g of drained shellfish meat was homogenized in a blender with 100 mL of 0.1 N HCl, and the mixture was gently boiled for 5 min, cooled to room temperature, brought to pH 4–4.5 with HCl or NaOH, if necessary, and brought to a volume of 200 mL with water. The mixture was cen-

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0021-8561/82/1430-0526$01.25/0

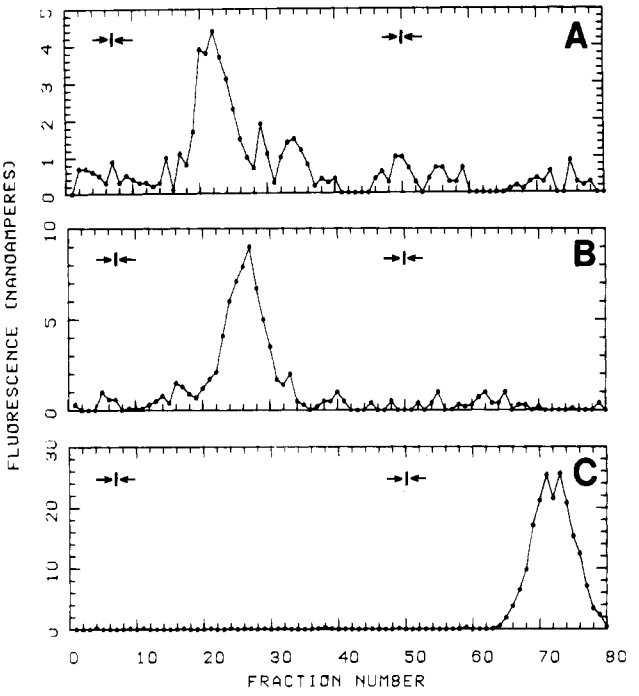

Figure 1. Elution profiles of *Gonyaulax* toxins. Tubes 1–7, 0.01 M pH 5 sodium acetate buffer; tubes 8–50, 0.1 M pH 5 sodium acetate buffer; tubes 51–80, 0.75 M HCl. (A) ca. 20 mouse units (MU) of GTX_2; (B) ca. 20 MU of GTX_3; (C) ca. 125 MU of STX.

trifuged and 2 mL of the supernate or a smaller aliquot diluted to 2 mL was applied to the column. The extraction procedure could be scaled down.

AG-50 Column Procedure. AG 50-X4, 200–400 mesh (hydrogen form) (Bio-Rad Laboratories, Richmond, CA), was converted to the sodium form by adding 1 N NaOH to the resin in water until the pH was higher than 10. The resin was washed with distilled water until the pH dropped to 8.0–8.5 and then converted back to the hydrogen form by adding 1 N HCl until the pH dropped below 4. The resin was again washed with water until the pH rose above 5 and then converted back to the sodium form by adding 1 N NaOH until the pH rose above 10. After a final rinsing with distilled water to a pH of 8.0–8.5, the resin was equilibrated with 0.01 M sodium acetate buffer at the pH of the run. The resin was packed in a disposable Pasteur pipet to form a 0.6 × 7.5 cm (2 mL) column and equilibrated with 50 mL of 0.01 M acetate buffer. Two milliliters of extract at a pH of 4–4.5 was applied to the column and developed as follows, 5-mL fractions being collected. Tubes 1–7 were collected by using 0.01 M sodium acetate buffer, tubes 8–50 with 0.1 M sodium acetate buffer of the same pH, and tubes 51–80 with 0.75 M HCl. Two 2-mL aliquots of each fraction were pipetted out, and 2 mL of 1 N NaOH was added to each aliquot. A total of 0.15 mL of 1% hydrogen peroxide was added to each sample tube and 0.15 mL of water to each corresponding blank tube. The tubes were allowed to stand at room temperature in the dark for 40 min, then 0.2 mL of glacial acetic acid was added to each tube, and the fluorescence was read in a Farrand MK-1 spectrofluorometer with the excitation frequency at 330 nm and a 5-nm slit width and the emission frequency at 380 nm and a 10-nm slit width.

RESULTS AND DISCUSSION

The elution profiles of the purified *Gonyaulax* toxins at pH 5 are shown in Figure 1. The GTX_2 and GTX_3 were retained but eluted early, the GTX_2 slightly ahead of GTX_3. As expected, the STX was most tightly bound and was eluted only with HCl. Figure 2 shows the results with

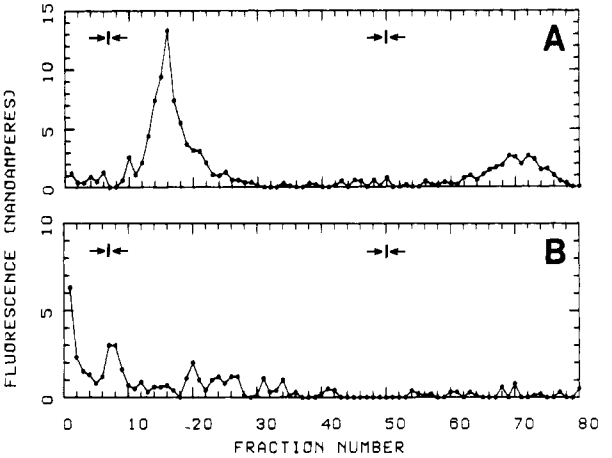

Figure 2. Elution profiles of crude *Mya arenaria* extracts. (A) Extract of ca. 0.5 g of toxic wet meat (ca. 25 MU); (B) extract of ca. 1.0 g of nontoxic wet meat. Same elution regimen as Figure 1.

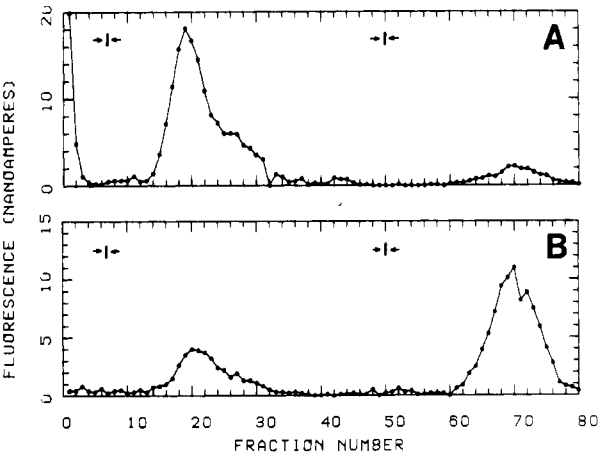

Figure 3. Elution profiles of partially purified scallop and crab toxins. (A) ca. 112 MU of scallop toxin; (B) ca. 67 MU of crab toxin. Same elution regimen as Figure 1.

toxic *M. arenaria* extract. No separation of GTX_2 and GTX_3 was achieved, but the later STX peak is clearly visible. Nontoxic *Mya* extract did not exhibit any significant fluorescence peaks (Figure 2). The GTX_2/GTX_3 peak in the extract appeared earlier than in the case of the pure toxins. This may be due, at least in part, to differences in salt concentrations, because increasing the ionic strength of buffers did show a tendency to hasten elution. Reducing the pH by using a 0.1 M pH 4 acetate buffer caused retardation in the elution of GTX_2 and GTX_3 to the point where GTX_3 trailed GTX_2 sufficiently that a partial separation was seen. However, the behavior at pH 4 was unpredictable and sometimes the elution was delayed to the point where the toxins did not appear until the first HCl tube. Because of this difficulty at pH 4, runs at pH 5 were adopted, even though the resolution between GTX_2 and GTX_3 was only partial.

Figure 3 shows the results obtained with partially purified scallop and crab toxin preparations obtained from Japanese sources. The main components of the scallop toxin preparation were GTX_1 (12%), GTX_2 + GTX_3 (>50%), STX (7%), and unidentified toxins (32%) (T. Noguchi et al., 1981, unpublished data). The present results also indicate the preponderance of GTX_2 + GTX_3 over STX. The main components of the crab toxin preparation were STX and neo-STX, with the GTX toxins being but minor components (Koyama et al., 1981; Yasumoto et al., 1981). The present results also show the

relatively high levels of STX as compared to the GTX's, but the present procedure did not detect neo-STX.

Although the toxicity of the scallops from Ofunato Bay in the northern part of Honshu Island was not associated with any visible dinoflagellate blooms, the responsible organism appeared to be *Gonyaulax* spp. (referred to as a *Protogonyaulax* spp.), possibly *tamarensis* (Ueda et al., 1981). The presence of a large proportion of the sulfated derivatives of 11-hydroxysaxitoxin indicated by the fluorescence profile would confirm the causative agent as a *Gonyaulax*. The presence of a relatively small sulfated 11-hydroxysaxitoxin peak in the crab toxin profile may also suggest a *Gonyaulax* origin. Shimizu and Yoshioka (1981) showed that, on incubation, these derivatives decreased and saxitoxin increased. This may account for their presence in relatively low amounts. Since these crabs were collected in the tropical waters of the southern-most islands of Japan, *Gonyaulax* has not been a suspect organism because of its apparent preference for colder waters.

The procedure in its present form thus offers a convenient method by which very small quantities of the STX family of PSP's (STX, GTX_2, and GTX_3) may be separated and identified in crude shellfish extracts. It would be highly desirable to be able to also separate and identify the neo-STX family of toxins (neo-STX, GTX_1, and GTX_4) by a sensitive fluorometric procedure rather than having to rely upon mouse toxicity tests, which require larger amounts of toxin and are more inconvenient to carry out.

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Received for review August 14, 1981. Accepted December 14, 1981. This work was supported in part by Hatch Project 205 and by National Science Foundation Grant DAR 80-03523 (to J.J.S. and M.I.). Scientific Contribution No. 1115 from the New Hampshire Agricultural Experiment Station.

Operator Exposure Measurements during Application of the Herbicide Diallate

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Measurements of dermal and inhalation exposure of field operators were made during application of the herbicide diallate preemergent to sugar beets. Each operation—tank fill, application, and incorporation—was measured individually in order to assess its relative contribution to the total exposure value. Inhalation exposure was measured by trapping the herbicide on polyurethane foam plugs while sampling the air around the operator's face. Dermal deposition, which was determined by attaching gauze pads to the operator's clothing and cotton gloves on the hands, was the main contributor to the total exposure. Dermal deposition on the hands during tank-fill operations exceeded all other dermal values by about 200-fold. The use of closed-system chemical transfer and neoprene gloves during tank fills reduced total exposure to diallate by about 2 orders of magnitude.

Diallate [*S*-(2,3-dichloroallyl) diisopropylthiocarbamate; the active ingredient in Avadex herbicide] is a preemergent, soil-incorporated herbicide marketed by Monsanto Co. for the control of wild oats in sugar beets and other crops. It has been estimated that yield and production losses due to wild oats exceed $300 million annually, with half of these losses occurring in North Dakota (USDA, 1977).

On May 31, 1977, the EPA issued an RPAR (Rebuttable Presumption Against Reregistration) notice on diallate for suspected oncogenicity and neurotoxic effects. Since diallate residues have not been detected in raw agricultural commodities (tolerances for "negligible residues" at the

Monsanto Agricultural Products Co., Research Department, Environmental Science Section, St. Louis, Missouri 63166.

0021-8561/82/1430-0528$01.25/0