Analysis of Diarrhetic Shellfish Toxins in Mussels from the Adriatic Coast of Italy

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Diarrhetic shellfish toxins in mussels collected from the Adriatic coast of Italy were analyzed by both mouse bioassay and fluorometric high performance liquid chromatography (HPLC). Of eleven specimens tested, one each from Trieste, Rimini, and Ancona proved to be toxic in the mouse bioassay. In each case, okadaic acid was confirmed as the major toxin by HPLC analysis, but in addition to okadaic acid, unprecedented toxins having cytotoxic activity were found to be present in a Rimini sample.

Key words: DSP, Italy, Adriatic Sea, HPLC, okadaic acid, new toxins, mussel, diarrhetic shellfish toxin

Incidents of diarrhetic shellfish poisoning (DSP) have dramatically increased in both frequency and geographical distribution in recent years.¹⁾ In addition to the health risks, the economic damage caused by frequent closures of farms poses a serious problem to local communities. To alleviate these health and economic problems, monitoring programs on shellfish toxicity are being implemented in many places using bioassays with mice.²⁾ Besides the ethical problem of using an animal bioassay, the method has a drawback of not distinguishing three different groups of toxins implicated in DSP (DS-toxins, Fig. 1); namely, the okadaic acid (OA) group, the pectenotoxin group (PTXs), and yessotoxin

(YTX).³⁾ As the toxins differ not only in chemical structure but also in toxicological effects, elucidation of toxin profiles by instrumental analyses is indispensable for evaluation of the health risks. Of these toxins, OA and dinophysistoxin-1 (DTX1) are considered to be more important than others because of their potent diarrheogenicity^{4,5)} and tumor promoting activity.⁶⁾ Both OA and DTX1 can be determined by fluorometric high performance liquid chromatography (HPLC).⁷⁾

In the Adriatic Sea, DSP outbreaks have been known only recently.^{8–10)} Toxicity was tested chiefly by mouse bioassays or by an ELISA method, necessitating further confirmation by HPLC analyses. In the present paper we report confirmation of OA in mussels from Trieste, Rimini, and Ancona, and occurrence of unprecedented toxins in one specimen from Rimini.

Materials and Methods

Materials

Mussels *Mytilus falloprovincialis* were collected in 1990 at Trieste, Goro, Rimini, Ancona, and Pescara (Fig. 2). Dates of the collection are shown in Table 1. The hepatopancreas which comprised 13 to 17% of the whole meat was removed from other tissues. Ten grams of the hepatopancreas was used for analysis.

Mouse Bioassays

Lethal potencies of acetone extracts of hepatopancreas were determined by a mouse bioassay method.²⁾ The method expresses toxicity by a mouse unit (MU), which is defined as the amount of a toxin, or toxins, to kill a male mouse of ddY strain of 20 g body weight in 24 h. The amounts of OA, DTX1, and PTX6 equivalent to one MU are 4.0, 3.2, and 10.0 µg, respectively.³⁾

HPLC Analysis

Toxins were esterified with 9-anthryldiazomethane (ADAM) as described previously.⁷⁾ The resultant fluorescent esters were chromatographed first on a Develosil ODS-7 column (4.6 × 250 mm) with acetonitrile–methanol–water (8:1:1). Fluorescent peaks whose retention times agreed with or were close to those of the reference toxins were collected from the outlet of the fluoromonitor and re-chromatographed on a second column (Capcell pak CN SG120, 4.6 × 250 mm) with acetonitrile–water (53:47) for further confirmation.

Fig. 1. Structures of diarrhetic shellfish toxins.

Tests for PTXs and YTX

The possible occurrence of PTXs and YTX in the Rimini sample was tested by a method previously described for the purification of DS-toxins.¹¹⁾ Methanol extracts of the hepatopancreas were first separated into neutral and acidic fractions on an alumina column. Each fraction, after being tested by mouse bioassays, was further purified by columns of silica gel (Merck) and then C_{18} bonded silic (ODS-Q3, Fuji gel) chromatography. The toxic fraction thus obtained was tested for the presence of YTX by thin layer chromatography (TLC) on a silica gel 60 plate (Merck) with chloroform-methanol-water (50:10:1).¹¹⁾

Purification of New Toxins

Lyophilized powder (13.8 g) prepared from the hepatopancreas of mussels collected at Rimini on July 9, 1990 was extracted with methanol, and the toxic extract purified over columns of alumina, silica gel, and ODS-Q3, as described previously.¹¹⁾ Final purification was carried out on a Develosil ODS-5 column (Nomura Chemicals) with methanol-0.1% acetic acid (8:2). The flow rate was kept at 1.0 ml/min. Four toxic fractions found to be lethal to mice were collected. Individual toxic fractions were further purified on the Develosil ODS-5 column under the same conditions. Elution of toxins was monitored by mouse bioassays during the early stages of purification and with a UV-detector (at 205 nm) at the final stage.

Fig. 2. Sites of mussel collection.

Table 1. Results of mouse bioassays and fluorometric HPLC analyses of mussels collected from the Adriatic Coasts of Italy in 1990

Sampling sites	Date	Bioassay results (MU/g)	OA by HPLC* $\mu\text{g/g}$ (MU/g)
Trieste	July 27	0.5–1.0	3.2 (0.8)
Goro	June 11	<0.5	ND
	July 26	<0.5	ND
	Sep. 3	<0.5	ND
Rimini	June 9	2.0–3.0	3.3 (0.8)
	July 24	<0.5	ND
Ancona	June 19	<0.5	ND
	July 13	0.5–1.0	1.4 (0.4)
	Sep. 4	<0.5	ND
Pescara	May 15	<0.5	ND
	July 23	<0.5	ND

DTX1 was not detected in all samples.

* ND denotes not detected.

Cytotoxicity Tests

Test samples were prepared by suspending 2 μg of individual toxin in 1 ml of a culture medium (PRMI-FBS-antibiotics, 10:1:0.1, Flow Laboratories Co.). Murine leukemia cells (P388) were grown in the same culture medium at 37°C in an atmosphere enriched in CO_2 (5%), and cell density was adjusted to $5 \times 10^5/\text{ml}$. A 5 mg/ml solution of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide (MTT, Dojin Co.) was prepared by dissolving MTT in a phosphate buffer (7.5 mM, pH 7.1). For bioassay 96 well plates were seeded with 4.5×10^4 cells per well in 90 μl of growth medium. A 10 μl aliquot of the individual test sample was then added to the cell suspension in a well, and the mixture was incubated for 48 h at 37°C. After adding the MTT solution (10 μl) to each well, cells were kept for another 4 h at 37°C for detection of cytotoxicity. Black precipitate of formazan generated by cellular reduction of MTT was separated from the overlying medium by pipetting out the medium and then dissolved with 150 μl of isopropanol containing 0.04 N HCl. The absorbance of the formazan solution was read with a microplate reader at 490 nm. Three parallel tests were run. Control experiments were run without adding the test samples. Cytotoxicity was evaluated as the decrease of formation of formazan.¹²⁾

Results and Discussion

The results of mouse bioassays and fluorometric HPLC analysis are shown in Table 1. Of the eleven samples tested, three showed toxicity levels above 0.5 MU/g, indicating their inadequacy as food. Occurrence of OA in these three samples was confirmed by HPLC analysis on two columns. Neither DTX1 nor PTX6 was detected by HPLC analysis. Neutral fractions obtained by treatment over alumina did not show any mouse lethality, ruling out the presence of PTXs 1 through 4. A significant discrepancy was observed in the Rimini sample between toxicity levels determined by mouse assay and by HPLC analysis. Mice injected with an acidic fraction of the Rimini sample quickly developed signs of illness and died within 4 h with severe convulsions before death. Although these signs resembled those of YTX, TLC comparison ruled out the presence of this toxin, and suggested the occurrence of a new toxin or toxins. Purification by reversed-phase HPLC showed four peaks eluting prior to that of OA, indicating their higher polarity than OA (Fig. 3). All four fractions were cytotoxic against P388 murine leukemia cells, having $\text{IC}_{50\text{s}} < 0.2 \mu\text{g}/\text{ml}$. Formazan formation was reduced to approximately one third of that of the control (Fig. 4). Because of the extremely small amounts available, further characterization of the toxins was unsuccessful. They did not give fluorescent esters by the treatment with ADAM. Thus, the

Fig. 3. Chromatograms of four cytotoxic fractions (1–4) from the Rimini sample and reference okadaic acid (OA).

Column, Develosil ODS-5 (4.6×250 mm); mobile phase, MeOH-0.1% HOAC (8:2); flow rate, 1.0 ml/min; monitor, 205 nm.

Fig. 4. Cytotoxicity of four fractions from the Rimini sample.

Test solution was applied to cells in the presence of MTT, and cytotoxicity was observed as the decrease of formazan formation. Tests were run in triplicate.

possibility of contaminant OA in these fractions was ruled out.

The present study confirmed that OA is the major DS-toxin in mussels from the Adriatic Sea. This result agrees with our previous finding that OA is the principal toxin in mussels collected from the Atlantic coast of Europe and Scandinavian waters¹³⁾ and agrees with a recent report on identification of OA in Italian mussels.¹⁰⁾ This is the first time, however, to observe discrepancy between mouse assay and HPLC results and to explain the discrepancy as due to new toxins having cytotoxic activity. As OA and its derivatives also have potent cytotoxicity,⁴⁾ cell assay methods using MTT can be an effective means for monitoring these toxins. None of the new toxins corresponded with DTX2, a new member of DS-toxins reported to have been eluted after OA in the HPLC analysis.¹⁴⁾ Neither were they identical in chromatographic properties with other known members of DSP. The source and toxicological properties of these new toxins should be elucidated in future studies. Another interesting aspect of the present study is the absence of pectenotoxins in the mussels tested. Apparently, *Dinophysis fortii* occurring in the Adriatic Sea⁸⁾ differs from the Japanese strain which produces pectenotoxin-2, along with dinophysistoxin-1 and/or okadaic acid.¹⁵⁾ The cause of the intraspecies variation is an intriguing problem in the biosynthesis of polyether toxins.

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