Further Studies on the Analysis of DSP Toxin Profiles in Galician Mussels

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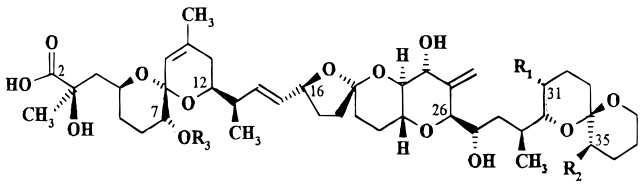
Further studies on mussel samples from Galicia, Spain, have revealed the presence of okadaic acid (OA), dinophysistoxin 2 (DTX2), and the fatty acid acyl esters of both of these toxins as the "DTX3" complex. Measurements were performed with an improved in situ method for the formation of 9-anthryldiazomethane (ADAM) derivatives followed by liquid chromatography with fluorescence detection. Base hydrolysis of DTX3 toxins gave free OA and DTX2, which were determined following ADAM derivatization. Results were confirmed by liquid chromatography/mass spectrometry analyses, and in most of the samples, free DTX2 was the most abundant toxin. However, the OA/DTX2 ratio in the DTX3 conjugated form was different, with OA being the most abundant in all cases. This difference could be due to different rates of metabolism of OA and DTX2 to the acyl esters or due to contamination of the shellfish by the two toxins at different points in time, resulting in less acyl ester formation for one toxin versus the other. The second possibility would be reasonable if two different source organisms were producing the toxins.

Keywords: *DSP toxins; okadaic acid; DTX2; DTX3; liquid chromatography; fluorescence; mass spectrometry*

INTRODUCTION

Diarrhetic shellfish poisoning (DSP) toxins have been recognized as a socioeconomic problem for many regions worldwide (Yasumoto et al., 1985). The Galician Rias, Spain, an area in which the shellfish industry is one of the main resources, has been significantly affected by DSP contamination over the past few years. The main toxins responsible for DSP are okadaic acid (OA), dinophysistoxins (DTX) 1 and 2 (Hu et al., 1992; Gago-Martínez et al., 1996a,b), and DTX3 (Figure 1), the latter being a complex mixture of 7-O-acyl esters of the first three formed as metabolites in shellfish (Marr et al., 1992). All of these cause gastrointestinal disorders, but the toxicological activity of greatest concern is the reported tumor promotion activities of OA and DTX1 (Suganuma et al., 1988).

The most common method for detecting DSP toxins is bioassay with mice (Yasumoto et al., 1985) or rats (Kat, 1983). Unfortunately, these assays suffer from many difficulties such as poor reproducibility, low sensitivity, and interferences from other lipophilic, endogenous compounds, such as fatty acids (Takagi et al., 1984). Several alternative methods have been developed for the detection of these toxins including immunoassays and other in vitro assays, but instrumental analysis methods are attractive because they offer the possibility of improvements in accuracy, precision, sensitivity, automation, and speed of determination. One method that is widely used is liquid chromatography (LC) with fluorometric detection of 9-anthryldiazomethane (ADAM) derivatives, initially proposed by Lee et al. (1987). Several laboratories have reported improvements to the method, such as the in situ method for preparation of the reagent (Yoshida et al., 1988; Quilliam et al., 1998) and better cleanup procedures (Quilliam, 1995; Stabell et al., 1991). LC combined with mass spectrometry (LC/MS) is another method that is gaining acceptance as a good quantitative and confirmatory method for toxins (Quilliam, 1995; Quilliam et al., 1989).



R_1	R_2	R_3	
CH_3	H	H	Okadaic acid (OA)
CH_3	CH_3	H	Dinophysistoxin-1 (DTX**1**)
H	CH_3	H	Dinophysistoxin-2 (DTX**2**)
H or CH_3		Acyl	Dinophysistoxin-3 (DTX**3**)

Figure 1. Chemical structure of DSP compounds.

In the present study, we have focused on optimization of the in situ ADAM LC/FLD method for the analysis of DSP components present in Galician mussel samples, including OA, DTX2, and the DTX3 complex. A comparison with LC/MS has also been performed to confirm the results, which provides a good proof of optimization and at the same time gives more complete information about the DSP toxins present in the samples.

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MATERIALS AND METHODS

Materials. Mussel samples were collected in December 1995 in different areas of Ría de Vigo and kindly provided by Socomgal S.A. (Galicia, Spain). All of the reagents and solvents were of analytical grade and were purchased from Sigma Chemical Co. (St. Louis, MO), Aldrich Chemical Co. (Milwaukee, WI), and Panreac Quimica S.A. (Barcelona, Spain). Instrument calibrations were performed using standard solutions of okadaic acid (OACS-1) provided by the Marine Analytical Chemistry Standard Program, National Research Council of Canada, Halifax. The hydrazone of 9-anthraldehyde was prepared according to the method of Nakaya et al. (1967) by reaction of 9-anthraldehyde and hydrazine monohydrate in ethanol. It may also be purchased from Lancaster Synthesis (Windham, NH). The in situ ADAM reagent was prepared by mixing 0.5 mL aliquots of three solutions: (A) 9-anthraldehyde hydrazone in tetrahydrofuran (THF) (35 mM); (B) *N*-chlorosuccinimide in THF (35 mM); and (C) quinuclidine in THF (70 mM). The reagent mixture was mixed for 1 min and allowed to stand at room temperature in the dark for 60 min before its use in derivatizations.

Safety Statement. Okadaic acid, DTX2, and related compounds are toxins responsible for diarrhetic shellfish poisoning, a severe gastrointestinal illness. Okadaic acid has also been reported to be a powerful tumor promoter. Gloves and eye protection should be worn when standards and contaminated materials are handled.

Sample Preparation and Derivatization. Digestive glands from shellfish samples were extracted with methanol/water (4:1), and crude extracts were partitioned with hexane and chloroform, as described previously (Quilliam, 1995), except that the hexane layer was saved to analyze for the presence of acyl esters (DTX3) which are partially solubilized in this phase. Residues from the evaporated hexane extract were hydrolyzed with 2 M NaOH/methanol (1:9) for 1 h at room temperature, and after the reaction was stopped with 2 M HCl, the reaction products were extracted with chloroform. Residues from the evaporated chloroform extracts were dissolved in methanol. Chloroform extracts from the initial partitioning were also hydrolyzed in some separate experiments to investigate the presence of acyl derivatives.

Derivatization was accomplished by transferring 35.0-μL aliquots of either sample extracts or calibration solutions into 1.5-mL amber vials and then adding 100-μL aliquots of ADAM reagent to each vial. The solutions were sonicated for 10 min in warm water (37 °C) and mantained at this temperature for 2 h in the dark. Afterward, reaction solutions were evaporated to dryness and taken through a silica gel column cleanup as described previously (Quilliam, 1995).

LC and LC/MS Analyses. LC/FLD analyses were performed using a Hewlett-Packard HP1050 LC equipped with a quaternary pumping system, an HP1046A fluorescence detector, HP ChemStation software, a Hewlett-Packard reversed-phase column (25 × 0.4 cm i.d.) packed with 5 mm Hypersil-C18, and an acetonitrile/water (85:15) mobile phase. LC/MS analyses were carried out using an HP1090M LC coupled to a Perkin-Elmer SCIEX API-III triple-quadrupole mass spectrometer equipped with a pneumatically assisted electrospray (ion-spray) ionization source. A Vydac column (25 cm × 2.1 mm i.d.) packed with 201TP ODS was used for LC/MS of OA and DTX2, whereas a 5 mm Vydac 214TP column was used for DTX3 analyses. LC/MS conditions were the same as those described previously (Quilliam, 1995).

RESULTS AND DISCUSION

Application of the Yoshida method (1988) for the in situ generation of the ADAM reagent to derivatization of the carboxyl function in DSP toxins revealed the formation of artifact products resulting in extra chromatographic peaks for each toxin. Using LC/MS, we have determined that these artifacts are due to base-catalyzed reactions between ethyl acetate, the solvent

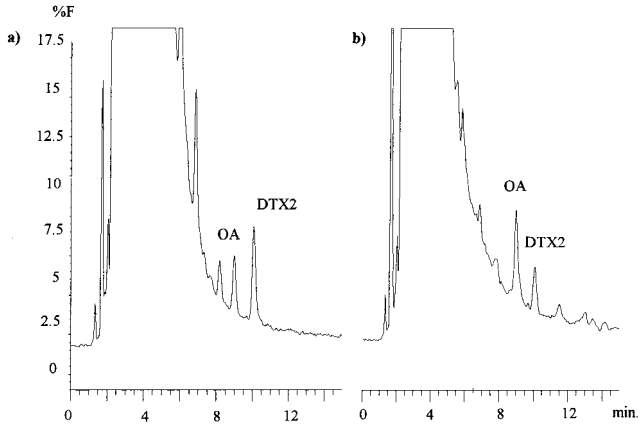

Figure 2. LC/FLD chromatograms of Galician mussel sample extracts: (a) conventional chloroform extract (0.035 g of tissue equiv derivatized); (b) hydrolysis products of DTX3 components extracted into the hexane phase (1 g of tissue equiv derivatized).

prescribed for the reagent system, and hydroxyl groups of the analyte. This resulted in the formation of O-acetylated ADAM derivatives.

Using a new formulation, with THF as solvent, it was possible to eliminate these artifact reactions. Various reaction parameters have also been rroptimized to ensure quantitative derivatization. The detailed studies are reported elsewhere (Quilliam et al., 1998).

Through the application of this improved in situ ADAM method, OA and DTX2 could be detected in 1996 Galician mussel samples with quite clean chromatograms as shown in Figure 2a.

LC/MS analyses confirmed the identities of both OA and DTX2 (Figure 2a). A medium-orifice voltage setting was used to induce some fragmentations, and selected ion monitoring was performed on the $[M + H]^+$, $[M + H - H_2O]^+$, and $[M + H - 2H_2O]^+$ ions of OA/DTX2 and DTX1. This supports our earlier finding of OA and DTX2 in 1993 Galician mussels (Gago-Martínez et al., 1996a,b).

The presence of DTX3 toxins was also investigated using hydrolysis reactions with sodium hydroxide, to convert acyl esters into the corresponding OA and DTX2. Reaction time and NaOH concentration were tested for maximum yield; optimum values were found to be close to those used previously (Marr et al., 1992). The presence of DTX3 was investigated in both hexane and chloroform phases by measuring the difference in OA and DTX2 concentrations before and after hydrolysis.

We have also found that most of the DTX3 compounds were present in the hexane phases (Figure 2b), as was reported by Fernández et al. (1996), although the total amount of these compounds is much lower than the amount of free OA and DTX2 present in the tissues (Table 1). These results were also confirmed by LC/MS analyses (Figure 3b). The peaks in the LC/MS mass chromatograms were identified as acyl esters of OA and DTX2 by matching their retention times with those of standards previously isolated from Irish mussels (Marr et al., 1992). The relative levels of OA and DTX2 esters are similar to those determined by LC/FLD analyses of hydrolyzed hexane extract.

The contribution of DTX3 to the DSP toxicity of chloroform extracts was difficult to measure directly with the ADAM LC/FLD method because it was necessary to measure a small change in OA and DTX2

Table 1. Toxin Concentrations in Mussel Samples Expressed as Micrograms per Gram of Digestive Gland[a]

sample	OA	acyl-OA	DTX2	acyl-DTX2
1	0.84 ± 0.02	0.09 ± 0.01	1.40 ± 0.02	0.04 ± 0.01
2	1.25 ± 0.01	0.06 ± 0.01	1.62 ± 0.01	0.03 ± 0.02
3	0.91 ± 0.02	0.05 ± 0.02	1.86 ± 0.01	0.04 ± 0.01
4	1.19 ± 0.01	0.09 ± 0.01	0.82 ± 0.02	0.03 ± 0.02
5	0.55 ± 0.03	0.05 ± 0.02	0.55 ± 0.03	0.03 ± 0.02
6	0.37 ± 0.04	0.03 ± 0.02	0.32 ± 0.02	0.01 ± 0.02
7	1.14 ± 0.01	0.07 ± 0.01	1.22 ± 0.01	0.02 ± 0.02
8	0.81 ± 0.02	0.05 ± 0.02	2.72 ± 0.01	0.04 ± 0.01

[a] Mean ± SD, $n = 3$. The OA and DTX levels were measured in the chloroform extract, whereas the acyl esters were measured in the hexane extract after hydrolysis to OA and DTX2.

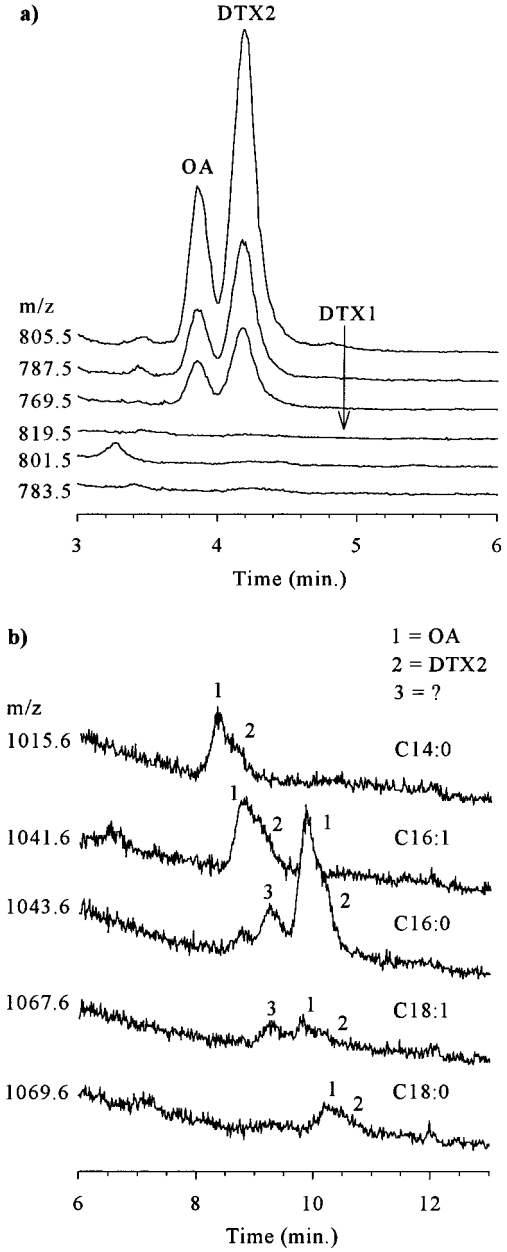

Figure 3. LC/MS chromatograms of Galician mussel sample extracts using (a) the Vydac 201TP-C18 column for OA and DTX2 analysis and (b) the Vydac 214TP-C4 column for DTX3 analysis.

concentrations between two extracts before and after hydrolysis, but the results obtained with the hydrolyzed chloroform extracts were a little higher than those obtained with the unhydrolyzed extracts (data not shown). This was not the case with the hexane extract, for which the measured OA and DTX2 in the hydrolyzed extracts came primarily from DTX3 compounds transferred to the hexane phase.

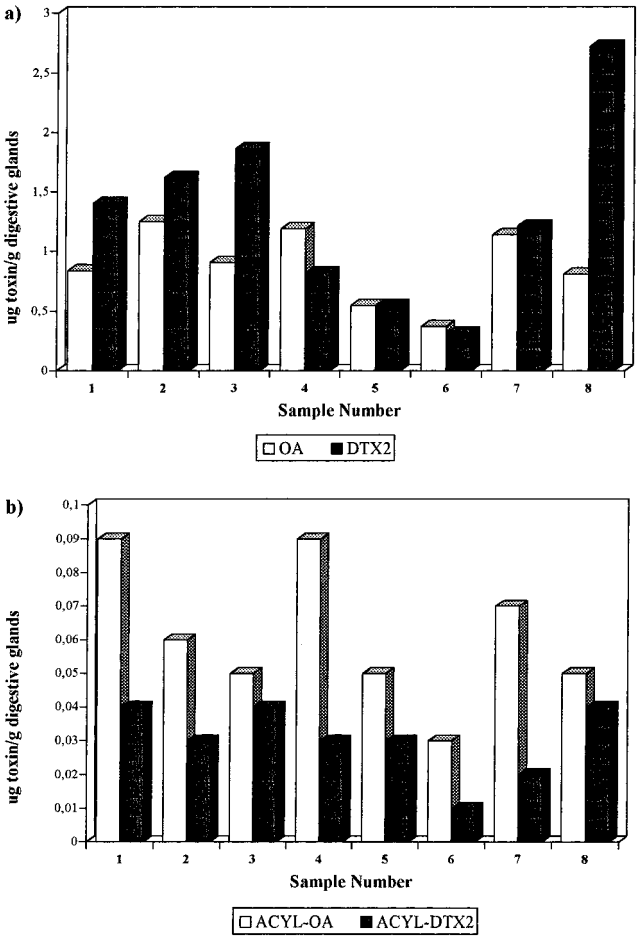

Figure 4. (a) OA/DTX2 ratio and (b) acyl-OA/acyl-DTX2 ratio.

A very interesting observation is that although in most of the samples free DTX2 was the most abundant toxin (Figure 4a), the ratio of the two toxins in the DTX3 conjugated form was different, with OA being the most abundant in all cases (Figure 4b). This difference could be due to different rates of metabolism of OA and DTX2 to the acyl esters or due to contamination of the shellfish by the two toxins at different points in time, resulting in less acyl ester formation for one toxin. The second possibility would be reasonable if two different source organisms were producing the toxins.

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