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Detection of 13,19-didesmethyl C spirolide by fluorescence polarization using *Torpedo* electrocyte membranes

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ABSTRACT

Fluorescence polarization (FP) is a powerful tool for studying molecular interactions by monitoring changes in the apparent size of fluorescent molecules. In this paper, a previously described fluorescence polarization assay was used to detect 13,19-didesmethyl C spirolide. The assay is based on the competition of cyclic imine marine biotoxins with α-bungarotoxin for binding to nicotinic acetylcholine receptor-enriched membranes of *Torpedo marmorata*. The 13,19-didesmethyl C spirolide was detected in buffer and mussel matrix. The sensitivity of the assay for the 13,19-didesmethyl C spirolide and the 13-desmethyl C spirolide was similar. After an acetone/chloroform extraction of spiked mussel meat, the average recovery rate of 13,19-didesmethyl C spirolide was 77.7 ± 1.9%. The quantification range for this toxin in mussel was 40–200 μg/kg of shellfish meat. This assay can be used to detect the spirolides 13,19-didesmethyl C spirolide and 13-desmethyl C spirolide, in shellfish as a screening assay.

The spirolides are macrocyclic phycotoxins belonging to the cyclic imines group, which also includes gymnodimines, pinnatoxins, pteriatoxins, prorocentrolides, and spiro-prorocentrimine [1]. The spirolides were first discovered in extracts of digestive glands of mussels (*Mytilus edulis*) and scallops (*Placopecten magellancicus*) from the Atlantic coast of Nova Scotia, Canada, in the early 1990s [2]. Since then, these compounds have been detected in shellfish and/or plankton in Canada, Denmark, Norway, Adriatic Sea, France, Ireland, Scotland, New Zealand, and Spain among other locations [3–9]. Fourteen members of the spirolide family have been isolated and structurally characterized [2,3,5,10–13]. The dinoflagellate *Alexandrium ostenfeldii* has been identified as the producing organism of spirolides [14]. The spirolides E and F are nontoxic products of enzymatic hydrolysis in shellfish [10].

Although human illness has never been directly linked to these compounds and no regulatory limits have been established for their presence in seafood, the spirolides are highly toxic to rodents, causing false positives in lipophilic toxin detection by mouse bioassay. In initial studies with mice, oral and intraperitoneal LD_{50} values of a spirolide mixture were determined to be approximately 1 mg/kg and 40 μg/kg, respectively [15]. However, recent studies show a higher toxicity if pure spirolides are used [16]. The mode of action of these phycotoxins has not been fully elucidated, although nicotinic and muscarinic acetylcholine receptors seem to be involved in spirolide toxicity [15,17,18].

To detect spirolides in shellfish, we developed a fluorescence polarization assay based on the ability of these toxins to inhibit the interaction between fluorescent α-bungarotoxin (α-BTX)[1] and the nicotinic acetylcholine receptor (nAChR) [19]. Fluorescence polarization (FP) is a spectroscopic technique specially used to study molecular interactions [20,21]. It is based on the excitation of a fluorescent molecule with plane-polarized light and the measurement of the polarization degree of the emitted light [22]. FP is proportional to the rotational relaxation time, which is related to the molecular volume. Small fluorescent molecules rotate quickly and yield low FP values. When a bigger molecule binds to them, large complexes rotate slowly and exhibit higher FP values. This FP assay offers several advantages versus the methods currently used to determine the presence of spirolides in a sample (the mouse bioassay and liquid chromatography–mass spectrometry-based detection techniques [3,4,11]). The FP method does not entail legal or ethical problems

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[1] *Abbreviations used*: nAChR, nicotinic acetylcholine receptor; BSA, bovine serum albumin; α-BTX, α-bungarotoxin; FP, fluorescence polarization; PBS, phosphate-buffered saline; SPX, spirolide.

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related to the use of laboratory animals and standards of all toxic analogues would not be needed to evaluate sample toxicity, although until now only detection of the 13-desmethyl C spirolide and gymnodimine had been demonstrated.

The aim of this work is to study the feasibility of using this fluorescence polarization assay to detect another analogue of the cyclic imine family, the 13,19-didesmethyl C spirolide, in shellfish extracts.

Materials and methods

Materials

Alexa Fluor 488 α-BTX was purchased from Molecular Probes (Eugene, OR, USA). 13-Desmethyl C spirolide and 13,19-didesmethyl C spirolide were obtained from CIFGA (Lugo, Spain). Bovine serum albumin (BSA) and Tween 20 were from Sigma Chemical Co. (Madrid, Spain). Acetone, chloroform, *n*-hexane, methanol, ethanol (96%), sodium chloride, sodium phosphates, and hydrochloric acid were from reagent grade commercial sources. Phosphate-buffered saline solution (PBS) was 130 mM NaCl, 1.5 mM NaH_2PO_4, 8.5 mM Na_2HPO_4, pH 7.0. PBS-BT solution was PBS supplemented with 0.1% w/v BSA and 0.1% v/v Tween 20. Amicon Ultra-4, PLGC Ultracel-PL Membrane, 10-kDa MWCO filter was from Millipore Corporation (Bedford, MA, USA). Toxin free mussel (*Mytilus galloprovincialis*) was purchased from the market in Lugo, Spain, in August 2008. The *Torpedo marmorata* were obtained from the Marine Station in Arcachon (France) and kept in artificial seawater for a few weeks in the aquarium of the CNRS-animal house in Gif sur Yvette. Electrocyte membranes rich in nAChRs from *T. marmorata* electric tissue were purified according to procedures previously described [19].

Fluorescence polarization measurement

Fluorescence intensity and fluorescence polarization were measured in a Synergy 4 Multi-Mode Microplate Reader from Biotek Instruments (Winnoski, VT, USA), using a xenon flash lamp as light source. Both measurements were obtained using 485/20 nm excitation and 528/25 nm emission filters with a 510-nm cutoff dichroic mirror. After optimization, the gain was set at a value of 42. The autofluorescence of the *Torpedo* membranes and the spirolides did not interfere with the detection method.

FP (in millipolarization units, mP) was calculated by the equation

$$\mathrm{mP} = 1000\left[\frac{I_V - GI_H}{I_V + GI_H}\right],$$

where I_V is the fluorescence intensity measured with vertical polarization excitation filters and vertical polarization emission filters (called parallel intensity), I_H is the fluorescence intensity measured with vertical polarization excitation filters and horizontal polarization emission filters (called perpendicular intensity), and G is a correction factor that accounts for the optical components of the instrument that affect the light beam depending on its polarization plane [23]. The experimental value obtained for G under our conditions was 1.23. Fluorescence polarization values were used for further calculations to maintain uniformity with previously published papers related to this technique; however, the results were the same using fluorescence anisotropy values.

Fluorescence polarization competition assay

A fluorescence polarization assay was developed using the ability of spirolides to inhibit the interaction between fluorescent α-BTX and nAChR from *Torpedo* membranes [19]. The problem solution was incubated with nAChR-enriched membrane of *Torpedo*'s electric organ for 2 h. For this purpose 40 μl of the problem solution were added to 40 μl of a 1:40 dilution of the nAChR stock (2.7 mg/ml of protein) in a 96-well plate (black, flat-bottom plates, TermoLabsystems, Franklin, MA, USA). The contents of the wells were mixed by shaking at 90 rpm for 1 min before the 2-h incubation. Finally, 80 μl of 40 nM Alexa Fluor 488-α-BTX was added to the well and shaken again for 1 min. Fluorescence polarization was measured 30 min after the addition of fluorescent α-BTX. All the incubations took place in the dark at room temperature. The concentrations of Alexa Fluor 488-α-BTX and receptor were optimized by choosing the conditions that provided the best FP range in our detector. To correct for background, vertical and horizontal fluorescence values from control wells in which fluorescent α-BTX was omitted were respectively subtracted from vertical and horizontal fluorescence values of fluorophore-containing wells, obtaining the I_V and I_H values used in the equation above. The percentage of α-BTX-binding response under certain conditions was calculated as

$$\%\ \mathrm{BR} = \frac{(\mathrm{mP_i} - \mathrm{mP_{min}})}{(\mathrm{mP_{max}} - \mathrm{mP_{min}})} \times 100,$$

where % BR is the percentage of α-BTX-binding response, $\mathrm{mP_i}$ is the mP value for a given condition, $\mathrm{mP_{max}}$ is the mP value obtained for control wells containing the nAChR-enriched membranes plus Alexa Fluor 488-α-BTX, $\mathrm{mP_{min}}$ is the mP value obtained for control wells containing only Alexa Fluor 488-α-BTX.

Shellfish sample preparation for the fluorescence polarization assay

Sample preparation was performed as previously described [19]. One hundred grams of mussel meat (whole body) was homogenized with a blender, divided in 10-g aliquots, and stored at −20 °C until use. The extraction procedure consisted in the addition of 4 ml acetone to 1 g of shellfish homogenate. The mixture was vortexed for 10 s and roller-mixed for 15 min at room temperature. After centrifugation at 3500*g* for 10 min at 4 °C, the supernatant was saved and the pellet was reextracted twice with acetone, as described. The combined supernatants were evaporated and the resulting residue was dissolved in 4 ml of water. The aqueous extract was partitioned against 4 ml of hexane for 30 min. The hexane was then discarded and the water was extracted three times with chloroform (1:1.5 v/v) for 1 h. Chloroform layers were pooled together and evaporated. The residue was reconstituted with 1 ml ethanol:PBS (2:3 v/v) and filtered through a 10-kDa MWCO filter (Millipore). Then, an equal volume of PBS with 0.2% BSA and 0.2% Tween 20 was added. These extracts were analyzed by the fluorescence polarization competition assay on the same day the extraction was started. The mussel homogenates were also extracted with methanol and analyzed by LC–MS for the presence of GYM, 13-desmethyl C spirolide, and 13,19-didesmethyl C spirolide following a previously published protocol [24] to find out if the shellfish was naturally contaminated by an amount of these toxins that would be detected by the fluorescence polarization method and interfere with the calibration curve. There was no detectable amount of GYM or 13,19-didesmethyl C spirolide from natural contamination in these mussel samples when they were analyzed by LC–MS. However, 13-desmethyl C spirolide was present at a level 2.1 times lower than the detection limit of this fluorescence polarization assay, close to the detection limit of the LC–MS method.

Data analysis

All experiments were carried out at least three times and in every experiment each condition was tested by triplicate. The