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Protein markers of algal toxin contamination in shellfish

Giuseppe Ronzitti [a], Anna Milandri [b], Giampiero Scortichini [c], Roberto Poletti [b], Gian Paolo Rossini [a,*]

[a] Università di Modena e Reggio Emilia, Dipartimento di Scienze Biomediche, Via G. Campi 287, I-41100 Modena, Italy
[b] Centro Ricerche Marine, Via A. Vespucci 2, I-47042 Cesenatico, Italy
[c] Istituto Zooprofilattico Sperimentale dell'Abruzzo e del Molise "G. Caporale", Via Campo Boario, I-64100 Teramo, Italy

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ABSTRACT

Filter-feeding bivalve molluscs are often contaminated by algal toxins. We have probed whether proteomic analysis of extracts from the digestive gland (DG) of mussels could be employed to identify biomarkers of contamination due to okadaic acid-group toxins. The protein extracts were obtained from 18 separate mussel samples and were analyzed by two-dimensional gel electrophoresis. When samples were divided into four different classes based on the content of OA-group toxins in the starting material, we found that two proteins varied as a function of OA contamination. By BLAST analysis, the two proteins were identified as a component of photosystem II and a subunit of NADH dehydrogenase. The analysis of peptide homologies showed that the peptide of photosystem II we detected in extracts from the DG of mussels contaminated by OA-group toxins is identical to its counterpart in *Dinophysis* algae, which are the producers of this group of toxins. We concluded that proteomic analysis can be used for the detection and identification of biomarkers of biotoxin contamination in shellfish, including both proteins expressed by the toxin producers and components that participate to the tissue response to the exogenous bioactive contaminant.

The detection of toxic substances contaminating food matrices is usually performed by instrumental methods that allow separation, identification and quantification of relevant analytes. Classical analytical approaches employed for the detection of agents that display biological activity and pose a threat to human health are being extended to include functional methods. These methods are based on the property of bioactive, toxicologically relevant, analytes to interact with a living system, where they trigger selective responses that are detected by biomarkers of toxin activity in the model system (Rossini, 2005).

Many toxic compounds are produced by some algal species and have been found to be accumulated in seafood, such as shellfish and fish (Hallegraeff, 2004). Because of the risks that seafood contamination by algal biotoxins pose to consumers, procedures are set for the monitoring of biotoxins in materials destined to human consumption (Andersen et al., 2004; European Commission, 2004, 2005).

The complexity of monitoring algal biotoxin contamination in seafood stems from some features of the phenomenon. For instance, the contamination of seafood can be due to different toxin groups, and to multiple analogues of each class of compounds that can contribute to total toxicity of the contaminated material (Yasumoto et al., 1985; Sidari et al., 1995; MacKenzie et al., 2002). Instrumental methods of biotoxin detection demand that individual compounds are detected and quantified, and a variety of standards are then needed to properly perform the analysis of contaminated materials.

These factors have practical implications, because availability of appropriate standards and reference materials for several groups of toxins to be used for analytical

* Corresponding author. Tel.: +39 059 205 5388; fax: +39 059 205 5410.
E-mail address: rossini.gianpaolo@unimore.it (G.P. Rossini).

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purposes has been problematic in the field of marine biotoxins.

Under these circumstances, the use of functional methods for biotoxin detection has some advantages, because these procedures discriminate only between biologically active and inactive compounds, thereby providing evaluations of the overall content of toxic compounds contaminating the materials of interest (Rossini, 2005).

So far, the detection of algal toxin contamination using functional methods has been performed by analysis of responses triggered by relevant toxins in biological systems, mostly represented by cultured cell lines (Gallacher and Birkbeck, 1992; Manger et al., 1993; García-Rodríguez et al., 1998; Pierotti et al., 2003).

A different analytical setting, however, could include the direct analysis of contaminated biological materials for the presence of biomarkers of toxin activity *in situ*.

In that analytical setting, biomarker identification would be supported by high throughput analytical methods, including proteomic tools. We have then set out this study, to probe whether proteomic analysis could be employed to identify biomarkers of algal biotoxin contamination in seafood.

In this investigation, we have probed the existence of biomarkers of contamination due to okadaic acid (OA) and related dinophysistoxins (OA-group toxins) in the digestive gland (DG) of mussels. That toxin was chosen because consumption of shellfish contaminated by OA is the cause of diarrhoeic shellfish poisoning, displaying gastrointestinal symptoms in humans and other animals (Tubaro et al., 2008). Mussels have been chosen because they are filter-feeding edible bivalve molluscs whose recurrent contamination by OA has been reported in many Countries (Hallegraeff, 2004), and the use of DG as the starting material for the preparation of samples used in this study is fully justified by recognition that it represents the organ accumulating most of contaminating OA-group toxins (Míguez et al., 1998).

In this study we show that proteomic analysis of extracts from the DG of mussels allows the detection and identification of biomarkers of OA contamination.

1. Materials and methods

1.1. Materials

Okadaic acid, pectenotoxin-2 (PTX-2) and 13-desmethyl-spirolide C (13-DesMeC) standards were purchased from the National Research Council of Canada (Halifax, Canada). Yessotoxin standard was purchased from the Institute of Environmental Science & Research Limited (Wellington Science Center, New Zealand).

Chemicals used to carry out two-dimensional electrophoresis were from Bio-Rad. All other reagents were of analytical grade or best.

1.2. Preparation of mussel homogenates

Mussels of various sizes were collected in off-shore farms in the four areas indicated in Fig. 1, and were stored up to 1 day at 4 °C before further processing. Digestive glands were dissected from mussels, pooled, and 60 g were homogenized in Ultra-Turrax. The homogenates were divided into aliquots to prepare the extracts used for paired analysis of the protein profiles and liposoluble toxins in mussel DG.

1.3. Toxin detection by LC-MS

Accurately weighed 2 g aliquots of DG homogenates were extracted with 18 ml of 90% methanol:water (90:10 v/v). The extract was then centrifuged and 2 ml of the supernatant shaken with 5 mL hexane. After filtration 1 ml of the lower layer was transferred to an autosampler vial for chemical analysis. For determination of the ester forms of OA, dinophysistoxin-1 and dinophysistoxin-2, 1 ml of the centrifuged crude extract (prior to hexane wash) was subjected to the alkaline hydrolysis procedure by Mountfort et al. (2001). The contents of OA, dinophysistoxin-1 and dinophysistoxin-2 detected in the extracts subjected to the alkaline hydrolysis were added to obtain the total levels of OA-group toxins in our samples.

Chemical analyses were carried out by LC-MS using a 1200L triple quadrupole mass spectrometer (Varian Inc., Walnut Creek, CA, USA). Lipophilic toxins were separated with a 5 μm Sunfire C18, 150 × 2.1 mm column (Waters Corporation, Milford, MA, USA), at 30 °C. The mobile phase consisted of two components: 13% methanol (A) and 90% methanol (B), both containing 50 mM formic acid and 4 mM ammonium hydroxide. The flow rate was 0.2 ml/min. A gradient elution was programmed.

Multiple reaction monitoring experiments were carried out in negative ion mode in order to investigate the presence of the following toxins in mussel samples: OA and dinophysistoxin-2 (m/z 803.5 > 255.0); dinophysistoxin-1 (m/z 817.5 > 255.0); PTX-2 (m/z 876.5 > 823.5); YTX (m/z 1141.5 > 1061.5); homoyessotoxin (m/z 1155.5 > 1075.5); desMeC spirolide (m/z 692.5 > 444.0 and m/z 692.5 > 164.0).

1.4. Preparation of extracts for proteomic analysis

Aliquots (4 g) of DG homogenates were rapidly resuspended with 10 ml of acetone and subjected to vigorous mixing by vortexing for 15 s. The homogenates were then centrifuged for 10 min at 800*g* and the supernatants were collected. The precipitate was then washed twice by resuspension with 10 ml of acetone and sonication (2 × 10 s bursts at an output of 10 W) and the suspension was next centrifuged for 10 min at 800*g*. The precipitate obtained from this centrifugation was then washed three times by resuspension with 10 ml of acetone 60% in water, sonication and centrifugation at 800*g* for 10 min, as described above. Finally the washed pellet was extracted with 10 ml of 10% acetone in water. The supernatants of the previous centrifugations were pooled and the proteins present in this extract were precipitated with 10% trichloroacetic acid, were recovered by low speed centrifugation, and the protein pellet was finally solubilized with IEF buffer (8 M Urea 2%, CHAPS 0.2%, Biolyte Ampholite pH 3–10, 50 mM DTT). The solution was then clarified by centrifugation for 10 min at 16 000*g*, and the supernatant of this centrifugation