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Protein phosphatase inhibition assays for okadaic acid detection in shellfish: Matrix effects, applicability and comparison with LC–MS/MS analysis

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ABSTRACT

The applicability of the protein phosphatase inhibition assay (PPIA) to the determination of okadaic acid (OA) and its acyl derivatives in shellfish samples has been investigated, using a recombinant PP2A and a commercial one. Mediterranean mussel, wedge clam, Pacific oyster and flat oyster have been chosen as model species. Shellfish matrix loading limits for the PPIA have been established, according to the shellfish species and the enzyme source. A synergistic inhibitory effect has been observed in the presence of OA and shellfish matrix, which has been overcome by the application of a correction factor (0.48). Finally, Mediterranean mussel samples obtained from Ría de Arousa during a DSP closure associated to *Dinophysis acuminata*, determined as positive by the mouse bioassay, have been analysed with the PPIAs. The OA equivalent contents provided by the PPIAs correlate satisfactorily with those obtained by liquid chromatography–tandem mass spectrometry (LC–MS/MS).

1. Introduction

Okadaic acid (OA) and the dinophysistoxins (DTXs) derivatives are lipophilic marine toxins produced by microalgae of the *Dinophysis* and *Prorocentrum* genera (Steidinger, 1993), usually found in shellfish. The consumption of shellfish contaminated by OA and some DTXs causes diarrheic shellfish poisoning (DSP), human illness characterised by gastro-intestinal symptoms including diarrhoea, nausea and vomiting (Yasumoto and Murata, 1993).

Diarrheic lipophilic toxins are known to inhibit the activity of several serine/threonine protein phosphatases (PPs) (Bialojan and Takai, 1988). These toxins bind to PP in the hydrophobic region near to the active site, blocking their activity. As a consequence, hyperphosphorylation of the proteins that control sodium secretion by intestinal cells and of cytoskeletal or junctional moieties that regulate solute permeability is favoured, causing a sodium release and a subsequent passive loss of fluids, responsible for the diarrheic symptoms.

DSP toxic episodes are recurrent in coastal waters of European countries. Specifically in Spain, Galicia and Catalonia are very affected regions (Reguera et al., 2012). In order to protect public health, the Commission Regulation (EC) No 853/2004 has established a maximum permitted level (MPL) of 160 μg of OA equivalents/kg in bivalve molluscs. Until recently, the official control method was the mouse bioassay (MBA) (Yasumoto et al., 1978). This method has been successful for the management of shellfish controls because it gives an indication of the total toxicity of a sample. However, because of its lack of specificity, and controversy regarding its application (reference), a European Commission regulation (EC No. 15/2011) has recently approved the use of a liquid chromatography–tandem mass spectrometry (LC–MS/MS) method as the reference method for the detection of lipophilic toxins in live bivalve molluscs. In Europe this new regulation has been applied since 1st July 2011, although the MBA can still be used until 31st December 2014. This Commission Regulation also allows a series of methods, such as other chromatographic techniques with appropriate detection, immunoassays and functional (e.g. phosphatase inhibition) assays, as alternatives or supplementary to the LC–MS/MS method, provided that either alone or combined they can detect the required analogues, that they fulfil the corresponding method performance criteria, and that their implementation provides an equivalent level of public health protection.

Based on the OA mechanism of action, protein phosphatase inhibition assays (PPIAs) for the determination of DSP toxins have been developed. Although radioactivity (Honkanen et al., 1996) and fluorescence detection has been used (Tsuchiya et al., 1997; Vieytes et al., 1997; Mountfort et al., 1999; Leira et al., 2000;

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Mountfort et al., 2001; González et al., 2002), the colorimetric detection method has received more acceptance (Tubaro et al., 1996; Della Loggia et al., 1999; Campàs and Marty, 2007; Albano et al., 2009; Caillaud et al., 2010; Cañete et al., 2010).

This functional method has gained acceptance because of its advantages in terms of simplicity, multiple sample analysis, sensitivity and limit of detection (LOD), and short analysis time. As a consequence, researchers are trying to improve and refine PPIAs from different perspectives. Undesirable shellfish and microalgae matrix effects have been diminished by the use of sample purification strategies, such as a previous chromatographic fractionation protocol (Caillaud et al., 2010; Cañete et al., 2010). The use of recombinant enzymes (Ikehara et al., 2010), some of them with improved sensitivity (Zhang et al., 1994), has also been exploited. The establishment of toxicity equivalent factors (TEFs) in alternative methods to MBA for marine toxin detection is necessary to guarantee consumer protection in monitoring programmes, as they allow a better estimation of the toxic potential of a mixture of toxins with different potency (Botana et al., 2010). With regards to this, the inhibitory potencies of different OA derivatives should be investigated. Nevertheless, in this work OA has been taken as reference compound of the diarrheic lipophilic toxins group.

The development of a PPIA using a recombinant PP2A catalytic subunit has already been described in a previous work, where the genetically-engineered enzyme was compared with a commercial one in terms of activity, stability and inhibition by OA (Garibo et al., 2012). In the present work, an exhaustive evaluation of the matrix loading effect on the PP2A activity was performed in order to apply the developed PPIA to the determination of OA and its acyl derivatives in shellfish samples. Results have also been compared with those obtained with a commercial PP2A. Mediterranean mussel, wedge clam, Pacific oyster and flat oyster have been chosen as model shellfish species. Samples determined as negative by the MBA for DSP toxins (toxin content lower than 160 μg OA eq/kg) and by LC–MS/MS analysis (diarrheic lipophilic toxin content lower than the limit of quantification, LOQ = 30 μg OA/kg shellfish meat) have been used for the study of the matrix effects. Once the matrix loading limits were established, the PPIA was applied to the analysis of naturally-contaminated mussels and the results were compared to those obtained by LC–MS/MS analysis.

2. Materials and methods

2.1. Reagents and materials

Certified Reference Material of okadaic acid (CRM-OA) in methanol was purchased from the Institute for Marine Biosciences of the National Research Council (Halifax, Canada). The genetically-engineered PP2A catalytic subunit was produced by Gene to Protein (GTP) Technology (Toulouse, France) and contains a hexa-His tail at the C-terminus. A commercial protein phosphatase 2A (PP2A), isolated as the heterodimer of 60 kDa and 36 kDa subunits from human red blood cells, was obtained from Upstate Biotechnology (NY, USA). The activity of the stock solutions was between 766 and 1364 U/mL for GTP Technology and 5660 U/mL for Upstate Biotechnology, 1 U being defined as the amount of enzyme required to hydrolyze of 1 nmol *p*-nitrophenyl phosphate (*p*-NPP) in one min at room temperature. Components of buffers and *p*-NPP were purchased from Sigma (Tres Cantos, España). For LC–MS/MS analysis, gradient-grade methanol, formic acid and hyper-grade acetonitrile were purchased from Merck (Darmstadt, Germany). Ammonium formate (≥99.995%), sodium hydroxide pellets (≥99%) and hydrochloric acid 37% for analysis were purchased from Sigma–Aldrich (St. Louis, MO, USA), Riedel-de Haën (Seelze, Germany) and Panreac (Barcelona, Spain), respectively. All solutions were prepared using Milli-Q grade water obtained from a Millipore purification system (Bedford, USA).

2.2. Shellfish and phytoplankton samples

Three negative Mediterranean mussel (*Mytilus galloprovincialis*) samples according to LC–MS/MS analysis were obtained from Ebro Delta, Catalonia (NW Mediterranean, Spain) in August, September and December 2008. Two negative wedge clam (*Donax trunculus*) samples according to MBA for DSP toxins and LC–MS/MS analysis were obtained from the shellfish monitoring programme of Catalonia (Ebro Delta and Vilanova i la Geltrú) in October and November 2009. Three negative Pacific oyster (*Crassostrea gigas*) samples according to MBA for DSP toxins and LC–MS/MS analysis were obtained from the shellfish monitoring programme of Catalonia (Ebro Delta) in January and February 2010. Four negative flat oysters (*Ostrea edulis*) were used, two of them provided by *Ostres de la Badia* (Santa Pola, Alicante, SW Mediterranean, Spain) in January and June 2010, according to LC–MS/MS analysis, and the other two obtained from the shellfish monitoring programme of Catalonia (Ebro Delta) in December 2009 and January 2010, negative according to MBA for DSP toxins and LC–MS/MS analysis.

Twelve positive Mediterranean mussel (*M. galloprovincialis*) samples according to MBA for DSP toxins (Yasumoto et al., 1978) and to LC–MS/MS analysis were provided by *Amegrove* (O Grove, Spain) from Galicia (NE Atlantic Ocean, Spain) in August 2010. These samples were obtained from 4 different rafts (C1, C2, C3, C4) at 1, 5 and 10 m depth during a DSP closure in Ría de Arousa, inforced between July 2010 and November 2010 by INTECMAR (INTECMAR, 2010). The samples were available because of the Amegrove's own-checks on biotoxins. Integrated phytoplankton samples (0–15 m) were collected from stations A0 (42°28′54″N, 08°57′48″W), at the entrance of the Ría de Arousa, and A8 (42°29′48″N, 08°55′36″W), close to the rafts, using a PVC hose and preserved with Lugol's solution. The Utermöhl method was used for phytoplankton identification and quantification (Utermöhl, 1958): 25 mL of sample were settled during 12 h. The chamber was examined for quantification of *Dinophysis* species and total phytoplankton.

2.3. Lipophilic toxins extraction

Crude shellfish extracts were prepared by extracting 2 g (out of 100 g) of shellfish homogenate with 9 mL of MeOH for 2 min at 17,500 rpm with an Ultra-Turrax® T25 Digital by IKA® from Rose Scientific Ltd. (Alberta, Canada). Extracts were centrifuged at 3000 rpm for 10 min in a Jouan centrifuge at room temperature. Supernatants were removed. A second extraction was performed by the addition of 5 mL of MeOH to the solid residue and 3-min agitation with a vortex. After centrifugation under the same conditions, the two supernatants were joined and passed through a 0.2-μm cut-off Whatman nylon membrane filter (Brentford, United Kingdom). Samples were directly injected into the LC–MS/MS system. For samples to be tested with the PPIA, extracts were evaporated in a Speed VAC concentrator (Organomation Associates, Inc., Berlin, USA) under nitrogen at room temperature, and the residues were resuspended in a buffer solution containing 30 mM Tris–HCl, 20 mM $MgCl_2$, pH 8.4.

2.4. Sample hydrolysis

The protocol for the hydrolysis of lipophilic toxins in mussels was based on that of Mountfort et al., 2001. Briefly, 125 μL of NaOH at 2.5 N were added to 1.25 mL of extract and homogenised for 10 s with a vortex. Extracts were then incubated at 76 °C for 40 min in a