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Subcellular distribution of okadaic acid in the digestive gland of *Mytilus galloprovincialis*: First evidences of lipoprotein binding to okadaic acid

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ABSTRACT

The subcellular distribution of okadaic acid, the main diarrhetic shellfish poisoning (DSP) toxin, in the cells of the digestive gland of the mussel *Mytilus galloprovincialis* was studied. By means of differential centrifugation, ultrafiltration and extraction with methanol, it was found that most okadaic acid was stored in the cytosol. Notwithstanding only a small proportion of the total toxin was found to be in free form, being most of it bound to a soluble cellular compound with a molecular mass which ranged from 30 to 300 kDa. A series of fractionations of samples digested with a protease, a lipase, and amylase suggested that the component to which okadaic acid is bound is a high density lipoprotein. A new fractionation after digestion with a protein lipase additionally supports the previous conclusion.

1. Introduction

Molluscan bivalves exploitation is an important activity around the world. The appearance (existence) of toxic episodes due to any kind of toxins (specially diarrhetic shellfish poisoning (DSP)), involves serious economic and social repercussions for fisheries and aquaculture. DSP, even when is one of the less dangerous toxicity due to its low morbidity, is the one with most impact, through market closures in areas as Galicia which is one of the top five mussels producers of the world.

DSP episodes are directly linked with some species of *Dinophysis*: *Dinophysis fortii* (Della Loggia et al., 1993; Yasumoto et al., 1980), *Dinophysis acuta* (Reguera et al., 1990; Edler and Hageltorn, 1990), *Dinophysis acuminata* (Kat, 1983; Lassus et al., 1985), *Dinophysis sacculus* (Giacobbe et al., 2000), and with *Prorocentrum lima* (Lawrence et al., 1998). The typical syndrome includes diarrhoea, vomiting and nausea. DSP toxins are nearly exclusively accumulated in the digestive gland of the mussels (Blanco et al., 2007), and though, okadaic acid (OA) is the main toxin (Murata et al., 1982), two dinophysistoxins (DTX 1 and DTX 2) and the esters of the main toxins with fatty acids, can also produce this toxicity. The structure of OA and DTXs differs in the number or position of the methyl group. All these toxins are lipophilic compounds and their solubility in organic solvents (dichloromethane, methanol, acetone or chloroform) is in general high but variable between compounds. These toxins act inhibiting serine/threonine protein phosphatases (PP1 and specially PP2A), enzymes which are responsible for catalyze the protein dephosphorylation process (Cembella et al., 1995).

Minimizing the impact of this kind of episodes on aquaculture requires minimizing the ingestion or accelerating the elimination of these toxins. Some results have been obtained within the first approach (Desbiens and Cembella, 1993; Franzosini et al., 2000; Jørgensen et al., 2008) but limited success has been obtained with the second (Blanco et al., 1999; Croci et al., 1994; Poletti et al., 1996; Svensson, 2003).

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Finding new and more effective ways of accelerate toxin elimination requires the knowledge of the accumulation/elimination mechanisms.

In a previous study on the elimination of okadaic acid from the digestive gland of the mussel *Mytilus galloprovincialis*, Blanco et al. (1999), found that it followed a kinetics that was well described by a two-compartment model. This suggested that the toxin was present in two forms in the digestive gland, one of them being more difficult to eliminate than the other. There were some possible explanations for this differential behavior of the toxins. One was the varying ability of the different parts of the digestive gland to eliminate toxin, which has been discarded in a previous study of our team (Rossignoli and Blanco, 2008), and the other is the differential binding of the toxin to cellular components. In this sense, Sugiyama et al. (2007), isolated okadaic acid from crude extract of the marine sponge *Halichondria okadai* and two okadaic acid binding proteins were characterized: OABP1 with approximately 37 kDa molecular weight in sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis and OABP2 consisted of three 22 kDa proteins (OABP2.1, OABP2.2 and OABP2.3) revealed by HPLC analysis. OABP2 was not detected in the sponge *Halichondria japonica* or the dinoflagellate *Prorocentrum lima*, so they postulated that probably OABP2 might be involved in detoxifying okadaic acid. Also recently, Nzoughet et al. (2008), identified by isoelectric focusing, SEC and SDS-PAGE, a target protein for azaspiracid (AZA) in mussels with 45 kDa molecular weight and that was close to ovalbumin. This protein was also present in blank material but at substantially lower levels providing evidence to support the hypothesis that a protein is expressed in greater abundance in shellfish due to AZA contamination (Nzoughet et al., 2008).

The approach used was to check the localization of okadaic acid in the subcellular fractions of the digestive gland, and to identify the group of substances which bound the okadaic acid by means of selective enzymatic hydrolysis.

2. Material and methods

2.1. Biological material and reagents

Contaminated mussels (*M. galloprovincialis*) containing mainly OA, DTX 2 and acyl derivates were obtained from a culture raft in Galicia after a *D. acuminata* outbreak. All mussels were stored at −20 °C prior to extraction.

OA reference solution OA-b was obtained from IMB – NRC, Canada. Water was purified through a MilliQ-gradient system, fed with an Elix-10, both from Millipore. Acetonitrile and methanol of HPLC-grade were obtained from Rathburn and Labscan, respectively. Protease inhibitor cocktail (P2714), Pronase E from *Streptomyces griseus* (P5147), Pancreatic porcine lipase (L3126), Amyloglucosidase from *Aspergillus niger* (A1602) and Lipoprotein lipase from *Pseudomonas* sp. (L9656) were obtained from Sigma–Aldrich.

2.2. Experiment 1. Subcellular fractionation with protease inhibitor cocktail

Digestive glands of 20 mussels were dissected, cut into small pieces of *ca.* 2 × 2 mm and homogenized for 3 min at 11,000 rpm (Ultraturrax IKA) with a buffer (1:5, w/v) which contained TRIS 0.1 M pH 7.8, ammonium formate 0.15 M and protease inhibitor cocktail (AEBSF 2 mM, EDTA 1 mM, Bestatine 130 μM, E-64 14 μM, Leupeptin 1 μM, Aprotinin 0.3 μM) (Huber et al., 2003).

A 600 μL aliquot of the homogenized tissue was taken as initial sample and the OA it contained was extracted with aqueous methanol 80% in a proportion of 4 mL MeOH/g of homogenate. The extract was clarified by centrifugation for 20 min at 48,000 × *g* (Beckman Avanti J-25).

The remaining homogenate was centrifuged at 45,000 × *g* for 1 h in order to separate soluble (or cytosolic) from precipitable fractions (Fig. 1). The state of the okadaic acid in the cytosolic fraction (supernatant) was examined by means of chromatographic analysis of the unprocessed supernatant, the MeOH 80% extract, and the fraction ultrafiltrated through an Ultrafree-MC of 10 kDa of nominal pore size (Millipore) and extracted with MeOH 80% (1:5, v/v), in order to obtain estimates of the free, total and bound to large biomolecules or very small organules that could be present in the cytosolic fraction, respectively.

2.3. Experiment 2. Fractionation using ultrafilters with different pore

In a second experiment, performed in order to identify the size fraction of the substances/organules to which a large portion of the OA was bound, the cytosolic fraction obtained as in the first experiment was ultrafiltered using Pall Corporation NanoSEP OMEGA (10, 30 and 300 kDa) and Pall Corporation MicroSEP (1000 kDa) filters. The toxin was extracted with 80% methanol (1:5, v/v) from each of the obtained filtrates. The extracts were clarified by centrifugation for 20 min. at 48,000 × *g* and analysed by HPLC–MS.

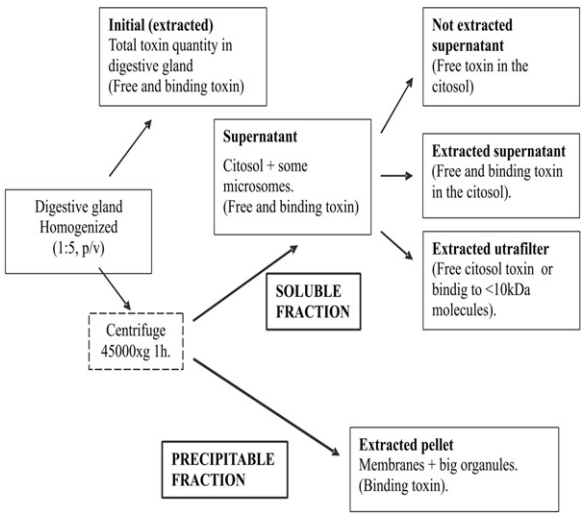

Fig. 1. Subcellular fractionation diagram.