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Profiles of fatty acids and 7-*O*-acyl okadaic acid esters in bivalves: Can bacteria be involved in acyl esterification of okadaic acid?

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ABSTRACT

The presence of 7-*O*-acyl okadaic acid (OA) esters was studied by LC–MS in the digestive glands of blue mussel (*Mytilus galloprovincialis*) and common cockle (*Cerastoderma edule*) from Albufeira lagoon, located 20 km south of Lisbon. The profile of free and total fatty acids (FA) was analysed using a similar LC separation with a reversed phase C8 column and mass spectrometry detection. In mussel the free FA profile was reflected in the FA esterified to OA, being palmitic acid for instance the most abundant in both cases. In cockle, 7-*O*-acyl esters with palmitic acid were almost absent and esters with a C16:0 isomer were dominant, followed by esters with C15:1 and C15:0. The cockle free FA profile was similar to mussel, and in accordance with literature findings in bivalves. After hydrolysis, a major difference in the FA profile occurred in both species, presenting a high percentage of a C16:0 isomer. The isomer found in general lipids and bound to OA seemed to be related, presenting similar relative retention times (RRT) to C16:0, differing from expected RRT of monomethyl-branched isomers (iso- or anteiso-). A tentative identification was made with the multimethyl-branched isoprenoid, 4,8,12-trimethyltridecanoic acid (TMTD). TMTD is a product of phytol degradation. This was also suspected when the proportion of this compound in relation to palmitic acid was reduced *in vivo* in mussels fed a chlorophyll-free diet. Extensive esterification of OA by, among others, phytol-degrading bacteria is discussed as a plausible hypothesis in cockle, but not in mussel, due to the relatively high specific proportion of odd-numbered and branched FA.

1. Introduction

The okadaites include okadaic acid (OA), the dinophysistoxins (DTX1, DTX2) and several esters at C1 or C7 of these three parent toxins (Quilliam, 2003). These compounds are involved in the human syndrome known as diarrhetic shellfish poisoning (DSP). The transfer of toxins to humans occurs mainly through bivalves that have predated upon microalgae of the genus *Dinophysis*. After predation upon toxic *Dinophysis* spp., in a variety of bivalve species okadaite's esters at C7 with fatty acids (FA) are largely dominant over free okadaites (Fig. 1) (reviewed in Vale, 2007). In Portuguese bivalves a few exceptions are known, in particular the blue mussel, and often the clam *Donax trunculus*. These two species retain free toxins longer than the esters, causing long periods of harvest restriction for public health protection (Vale et al., 2008). Detailed knowledge of the profiles of these acyl esters might help the understanding of this discrepancy in toxin dynamics.

Despite the advance in techniques such as liquid chromatography coupled to mass spectrometry (LC–MS) detection, detailed studies of profiles of acyl esters in bivalves are still scarce (Marr et al., 1992; Quilliam et al., 2003; Vale, 2006; Torgersen et al., 2008a,b; Suzuki et al., 1999, 2005). Some of them have focused mainly in describing the most common FA combined with OA in blue mussels, scallops and more recently in oysters.

The first detailed study on Portuguese bivalves covered only some FA from the C16- and C18-series in *Mytilus* spp. and *Donax* spp. (Quilliam et al., 2003). An intriguing peak eluting earlier than palmitoyl-OA was not understood at the time. On the second detailed study on Portuguese bivalves, covering a larger variety of commercial species, some intriguing results were found and discussed (Vale, 2006). Several odd-numbered and a possibly branched chain FA conjugated with OA and DTX2 were tentatively identified for the first time. An apparent species-specific relationship was noticed in the profile of these odd-br-FA. Blue mussels presented the lowest ratio, followed by common cockle, razors and clams. These FA are not produced by eukariotes but are commonly seen as bacterial markers (Harvey and Macko, 1997; Ivanova et al., 2000).

The fatty acid composition of marine organisms reflects to some extent the seasonal FA pattern of their food sources (Bradshaw et al., 1991; Pazos et al., 2003; Taylor and Savage, 2006). Besides existing as free fatty acids (FFA), FA are commonly incorporated in several lipids classes, such as phospholipids and acylglicerols. Studies relating the general FA pool and the okadaite's esters pool are inexistent. From the literature available a high contribution of odd-br-FA to the FA pool of common coastal bivalves is not common, and would have been

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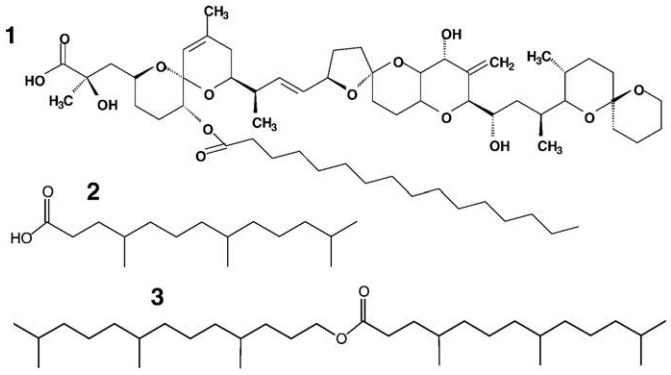

Fig. 1. Molecular structure of: **1**—palmitoyl-OA, **2**—TMTD acid, **3**—wax ester of TMTD.

noticed. Unfortunately, several of the recently published studies on FA profiles of bivalves lack the study of odd and/or branched FA.

An example where unusual profiles of acyl esters of OA were recently found in common cockle will be presented in order to steer the question of the possible bacterial involvement in okadaite's metabolization within bivalves. LC–MS analysis of FA was simultaneously implemented for a double purpose: to help in identification of okadaite's acyl esters through comparative chromatographic relationships and to compare the profile of FA in bivalves with those found in acyl esters. The findings will be reported with the aim of discussing to what extent profiles of okadaite's acyl esters reflect profiles of FA in general lipids.

2. Material and methods

2.1. Reagents and standards

Reagents were of analytical grade, except for acetonitrile (ACN) that was LC grade. Water was purified using a Milli-Q 185 Plus system (Millipore, Bedford, MA, USA). Standard solution from OA was purchased from the Certified Reference Materials Program (CRMP) of the Institute for Marine Biosciences, National Research Council (Halifax, NS, Canada). DTX1 was available from Calbiochem (San Diego, CA, USA). Standard solution of 13-methylpentadecanoic acid methyl ester (ante-iso C16:0) was no. M-3539 from Sigma-Aldrich (Sintra, Portugal). The free form was obtained after alkaline hydrolysis.

2.2. Sample collection and preparation

Bivalve samples were collected as part of the regular coastal Portuguese monitoring for marine biotoxins. Digestive glands (DG) of specimens were dissected from 10 individuals (or more depending on species size), in order to obtain a pool of at least 1 g of glands. Homogenisation was performed at 20,000 rpm in a Polytron PT3100 (Kinematica).

2.3. Bivalve artificial feeding trials

Mussels were placed in aerated 5 L jars and were fed daily *ad libitum* with *Chlorella marina* produced in-house. Control mussels were fed *ad libitum* with commercial powder nutritional yeast. DG were dissected fresh for FA analysis.

2.4. Okadaite's extraction

Okadaites extraction was modified from Vale (2006). A portion (1.0 g) of DG homogenate was vortex mixed for 1 min with methanol (5 mL) and centrifuged at 600*g* for 10 min. For analysis of acyl esters, 1 mL of supernatant was diluted with 2 mL of water, and applied to a 500-mg SPE C18 cartridge (Varian, Bond Elut, n° 12102028) pre-conditioned with methanol and water, followed by washing with 2.5 mL aqueous 50% MeOH. It was eluted with 3 mL of methanol with 0.1% acetic acid and 0.1% water. The first 0.5 mL was rejected, and the remaining 2.5 mL collected and vacuum dried at 50 °C (RapidVap, Labconco, Kansas City, MO, USA). A 120-mg silica-gel cartridge (Waters, Sep-Pak Light, n° 23537) was pre-conditioned with 2 mL hexane. The dried residue was dissolved in 1 mL dichloromethane and applied on the cartridge, followed by washing with 4 mL dichloromethane. Toxins were eluted with 4 mL dichloromethane/methanol (4:1, v/v). The eluate was vacuum dried and dissolved in 200 μL methanol.

For determination of free and total OA only the C18 clean-up was used. For determination of total OA a prior alkaline hydrolysis step was done by mixing 1 mL of the supernatant obtained above with 200 μL 2.5 M NaOH and heating at 70 °C for 40 min. in a BT1 Block Thermostat (Grant Instruments (Cambridge) Ltd., Royston, UK). After neutralization with 210 μL 2.5 M HCl, MeOH concentration was reduced with 2 mL of water as above before transferring to the SPE cartridge.

2.5. Fatty acid extraction

For rapid screening purposes an extraction based in the methanolic supernatant prepared in Section 2.4 was used. For FFA, a 200-μL aliquot was acidified with 55 μL of 0.1 M HCl, and extracted with 2.0 mL of *n*-hexane. After vortex mixing and centrifugation the hexane layer was transferred to autosampler vials.

For releasing bound FA, the same procedure optimised for OA acyl esters was used (Rodrigues and Vale, 2009): a 200-μL aliquot was hydrolysed with 50 μL of 2.5 M NaOH and incubated at 70 °C for 40 min in a Block Thermostat. After neutralization with 55 μL of 2.5 M HCl, it was extracted with 2.0 mL of *n*-hexane. After vortex mixing and centrifugation the hexane layer was transferred to autosampler vials.

2.6. LC–MS for okadaites and FAs

Analyses were performed on a LC–MS system from Hewlett-Packard 1100-Series (Palo Alto, CA, USA), consisting of an in-line degasser, quaternary pump, autosampler, column oven and the 1946A single-quadrupole mass detector. Separation was carried out isocratically on a 50×2 mm column packed with 3 μm Hypersil-BDS-C8 (Thermo Electron Corporation, Waltham, MA, USA), protected by a 10×2 mm guard cartridge packed with the same material. The mobile phase consisted of an 84:16 acetonitrile/water (v/v) solution, both containing 0.05% acetic acid. The flow rate was 200 μL/min, the injection volume was 5-μL and column temperature was maintained at 30 °C. The LC flow was introduced into the ESI interface without any splitting. The spray capillary voltage on the ESI interface was maintained at −4.0 kV and the nebulizer pressure at 25 psig. High-purity nitrogen, obtained through an N_2-Generator (Dominick-Hunter, Durham, England), was used as a drying gas at 10 L/min and heated to 350 °C. The fragmentor was kept at 180 V. Selected ion monitoring (SIM) was performed for the $[M\text{-}H]^-$ ions.

Analysis of FA was based on the same equipment and conditions described above. The mobile phase described by Lacaze et al. (2007) was replaced by a weakly acidic phase for best ionisation yields (formic acid is inadequate for negative ion mode of okadaites and FAs in this equipment). The mobile phase consisted of 65:35 acetonitrile/water (v/v) mixture, both containing 0.05 % acetic acid. Flow rate was 200 μL/min, injection volume was 0.3 μL and column temperature was maintained at 30 °C. Mass spectrometer parameters were optimised for oleic acid: fragmentor at 120 V, spray capillary voltage at −4.5 kV, drying gas at 300 °C and nebulizer pressure at 25 psig. SIM was performed for negative ions.

3. Results and discussion

The first toxic samples from the 2009 monitoring were found at Albufeira lagoon, during a *D. acuminata* bloom (T. Moita, personal communication). Blue mussel (*Mytilus galloprovincialis*) grown in rafts and common cockle (*Cerastoderma edule*) picked in natural