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New metallothionein assay in *Scrobicularia plana*: Heating effect and correlation with other biomarkers

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Quantification of total MTs by RP-HPLC-FD in non-heated bivalve extracts allows metal contamination assessment with high sensitivity and specificity.

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ABSTRACT

Metallothionein (MT) and other biomarker levels were measured in *Scrobicularia plana* clams to assess pollution of the Guadalquivir Estuary possibly affected by metals released from Aznalcóllar pyrite mine in 1998. After optimizing reagent concentrations for monobromobimane derivatization, MT levels were quantified by reversed-phase high-performance liquid chromatography coupled to fluorescence detection (RP-HPLC-FD) in heated or unheated digestive gland extracts and compared to those obtained by differential pulse polarography (DPP). MT content assayed by RP-HPLC-FD in unheated samples was higher than that obtained by DPP and correlated better with metals and anti-oxidant activities. MT assay by RP-HPLC-FD in unheated extracts would be preferable for assessing metal pollution, due to its greater sensitivity and specificity. In addition to MT induction, glyoxalase II inhibition was well correlated with metal contents. Our results suggest that metals at the estuary do not originate from Aznalcóllar spill, but from those carried along by the river and deposited at its concave bank.

1. Introduction

Transition metals convert O_2 into reactive oxygen species (ROS) that attack many biomolecules, such as fatty acids that generate malondialdehyde (MDA), a major oxidized by-product. Aerobic organisms have several defence lines from oxidative stress: primary anti-oxidant enzymes, such as catalase, detoxify ROS, while ancillary anti-oxidant enzymes, like 6-phosphogluconate dehydrogenase (6-PGDH), recycle $NADP^+$ to its reduced form, which is consumed by glutathione reductase (GRase) (Sies, 1986). In addition, glyoxalases (Glyox) I and II use GSH to detoxify α-oxoaldehydes, derived from cellular oxidative processes (Regoli et al., 1997) and glutathione-*S*-transferase (GST) conjugates GSH to electrophilic compounds. Some GSTs also detoxify reactive products derived from lipid peroxidation (Jensson et al., 1986).

Doñana National Park (DNP, SW Spain) is located north of Guadalquivir Estuary and was declared by UNESCO a "World Reserve of the Biosphere". It has been an area of concern since the tailings spill from Aznalcóllar pyrite mine (April 1998), which released large amounts of acid water and mud full of toxic metals (Fe, Zn, Pb, As, Cu, Cd, Ag, Ni, Mn, Cr) via Guadiamar stream, close to DNP (Achterberg et al., 1999; Grimalt et al., 1999). Some biological effects of this spill have been studied by our group in Algerian mice (*Mus spretus*) and clams (*Scrobicularia plana*). Mice living near Guadiamar stream showed greater anti-oxidant and detoxifying activities than controls (Ruiz-Laguna et al., 2001), and

Abbreviations: Bh, Bonanza harbour; BT, "Brazo de la Torre"; DNP, Doñana National Park; DTT, dithiothreitol; DPP, differential pulse polarography; EDTA, ethylenediaminetetraacetic acid; Glyox, glyoxalase; GRase, glutathione reductase; GSH, reduced glutathione; GSSG, oxidized glutathione; GST, glutathione-*S*-transferase; MDA, malondialdehyde; MT, metallothionein; mBBr, monobromobimane; PAGE, polyacrylamide gel electrophoresis; 6PGDH, 6-phosphogluconate dehydrogenase; PMSF, phenylmethylsulphonylfluoride; ROS, reactive oxygen species; RP-HPLC-FD, reversed-phase high-performance liquid chromatography coupled to fluorescence detection; SDS, sodium dodecyl sulphate; SRs, "San Rafael" salt works; TFA, trifluoroacetic acid; ww, wet weight.

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clams transplanted to the Guadalquivir Estuary displayed high metal uptake and oxidative stress, as revealed by higher levels of anti-oxidant, MT and GSH-related enzymes, MDA and over-oxidized glutathione status (Romero-Ruiz et al., 2003). The sedentary lifestyle, filter-feeding habits and metal-concentration ability of bivalves make them excellent bioindicators of metal exposure/effect in marine systems (Cosson, 2000; Hamza-Chaffai et al., 2000; Isani et al., 2000; Romero-Ruiz et al., 2003; Amiard et al., 2006).

Metallothioneins (MTs) are low M_r (~7 kDa) proteins with high Cys content and no aromatic or His residues. In accordance with their conservative character and ubiquity, they play crucial biological roles, including: (i) homeostasis of essential metals (Zn, Cu) (Kägi, 1991); (ii) detoxification of trace metals (Ag, Cd, Hg, Ni, Pb, Co); and (iii) protection from oxidative injury (Kägi, 1991; Viarengo et al., 2000; Kondoh et al., 2001; Haq et al., 2003; Erikson et al., 2004; Min et al., 2005). The inducible expression of MT genes by metals has resulted in their use as metal-pollution biomarkers (Cosson, 2000; Hamza-Chaffai et al., 2000; Dabrio et al., 2002; Haq et al., 2003; Amiard et al., 2006). Since an integrated view of environmental status requires the use of several biomarkers (Amiard et al., 2006), anti-oxidant defences and oxidative damage to biomolecules are used as sensitive biomarkers of metal pollutants, known to generate oxidative stress (Cajaraville et al., 2000; Rodríguez-Ortega et al., 2002; Romero-Ruiz et al., 2003; Funes et al., 2006).

A method for total MT assay by RP-HPLC coupled to fluorescence detection (RP-HPLC-FD) has been described in *Chamaelea gallina* clams by heating digestive gland extracts at 95 °C for 10 min (Alhama et al., 2006). MTs thiols are labelled with the fluorogenic reagent monobromobimane (mBBr) in the presence of EDTA, DTT and SDS. Heat-denaturation is also used in the MT assay by differential pulse polarography (DPP), the most popular MT-assay in bivalves (Thompson and Cosson, 1984; Cosson, 2000; Erk et al., 2002; Amiard et al., 2006). A comparative study of purification protocols has shown that the MT level diminishes after heating (Erk et al., 2002). Although there is a good correlation among different MT assays, concentrations diverge and are expressed in different units (Isani et al., 2000; Amiard et al., 2006). Thus, to compare MT contents in bivalve tissues with published levels, isolation and quantification procedures should be considered (Isani et al., 2000; Dabrio et al., 2002). The highest MT levels and metal responses are found in digestive glands compared to gills and other tissues (Cosson, 2000; Geffard et al., 2005; Amiard et al., 2006).

Here, in *S. plana*, we quantified the contents of MT and of biomarkers responsive to oxidative stress to assess the pollution status of the Guadalquivir Estuary (SW Spain). MT was assessed by RP-HPLC-FD in heat-treated and unheated extracts, and compared with those obtained by DPP. The relationships between MT content, metal levels, anti-oxidant enzymatic defences, and lipid oxidative damage were also studied.

2. Materials and methods

2.1. Materials, animals and sampling areas

Brilliant Blue G, D-lactoylglutathione, 5,5′-dithio-bis-2-nitrobenzoic acid, DTT, reduced (GSH) and oxidized (GSSG) glutathione, leupeptin, methylglyoxal, rabbit liver MT-I, SDS (95%), PMSF, 6-P-gluconate, $NADP^+$, Tricine, and M_r markers were from Sigma (Spain). Monobromobimane (mBBr) was from Fluka (Spain). H_2O_2, 1-Cl-2,4-dinitrobenzene, sodium dodecyl sulphate (SDS, >99%), ethylenediaminetetraacetic acid (EDTA), trifluoroacetic acid (TFA), thiobarbituric acid and Tris base were from Merck (Germany), and protein dye binding reagent from BioRad (Hercules, CA, USA). Other chemicals were reagent quality.

Clams were sampled on May, June and October 2003 at three sites on the DNP side of Guadalquivir Estuary (Fig. 1) and sent at 4 °C to the laboratory, where shells were washed and opened. For MT assay, digestive glands were excised, pooled and frozen at −80 °C, ground in a mortar with liquid N_2 and kept at −80 °C until extract preparation. For metal analysis, the whole soft tissues from 20 clams were frozen at −80 °C, ground in a mortar with liquid N_2, and kept at −80 °C until used.

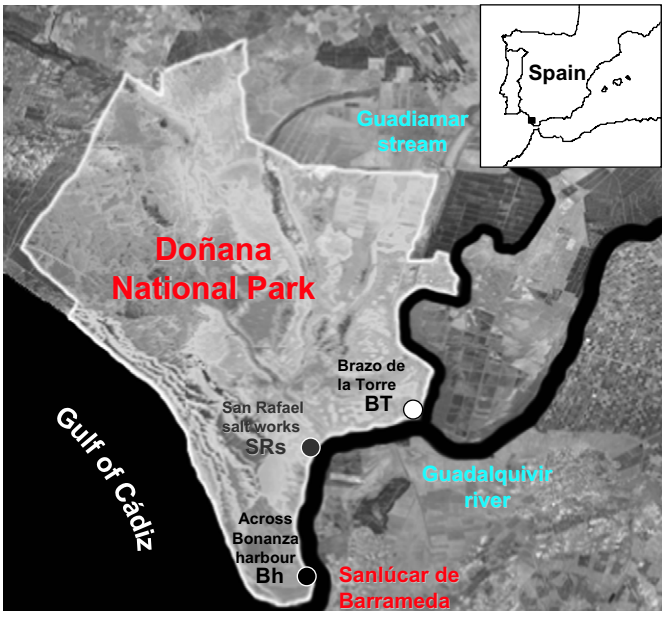

Fig. 1. Map of Doñana National Park (SW Spain) and sampling sites at Guadalquivir Estuary. *S. plana* specimens were collected at three sites: "Brazo de la Torre" (BT), "San Rafael" salt works (SRs), and across Bonanza harbour (Bh).

2.2. Metallothionein quantification by high-performance liquid chromatography

For extract preparation, ground digestive glands were disrupted in a T25 Ultraturrax (Janke & Kunkel, Germany) in 0.1 M Tris–HCl buffer (pH 9.5) with 1 mM DTT, 50 μM PMSF and 6 μM leupeptin at a ratio of 3 mL/g, and spun 20 min at 35 000 × *g*. Extracts were measured directly or after heating at 95 °C, 10 min, followed by centrifugation at 14 000 × *g*, 15 min. For reduction and denaturation, all samples were treated for 20 min at 70 °C with DTT, EDTA and SDS. For derivatization, mBBr was added and the mixture incubated for 15 min at room temperature away from light (Alhama et al., 2006). Final concentrations were DTT (12 mM), EDTA (2 mM), SDS (3%) and mBBr (12 mM) in 100 μL total volume.

Derivatized proteins in heated/unheated extracts (20 μL) were separated in a Supelcosil LC-18 column (0.46 × 25 cm, 5 μm particle, Supelco, PA, USA), as in *C. gallina* (Alhama et al., 2006). The HPLC system included a 7725i injection valve (Rheodyne, CA, USA), a 126 solvent module (Beckman, CA, USA) and a FP-2020 Plus Fluorescence Detector (Jasco, MD, USA) coupled on-line. Fluorescence of mBBr-labelled molecules was followed with excitation at 382 nm and emission at 470 nm (gain = 1). Peak areas were analyzed with the Beckman 32Karat Software (v. 7.0).

2.3. Metallothionein quantification by differential pulse polarography

Ground digestive glands were disrupted as indicated above in 0.1 M Tris–HCl buffer (pH 8.1) containing 1 mM DTT at a ratio of 3 mL/g, and spun for 1 h at 50 000 × *g*. The supernatants were diluted 10-fold with 0.9% NaCl, heated at 95 °C for 5 min and spun for 15 min at 10 000 × *g*.

DPP assay for MTs was made in a Methrom 746 VA Trace analyzer, in the heat-stable fraction by anodic stripped voltammetry in the Static Mercury Drop Electrode mode (Olafson, 1987) using rabbit liver MT-I as a reference for the calibration curve.

2.4. Electrophoresis

Two types of samples were analyzed: mBBr-labelled cell-free extracts (directly or heat-denatured) and samples after mBBr-labelling and purification by RP-HPLC-FD as described in Section 2.2. To this end, 40 μL of each type of extracts and 20 μL of rabbit liver MT-I were separated in 0.2 ml fractions; those containing fluorescent mBBr-MT were pooled, dried and freed from residual TFA as described (Alhama et al., 2006). The pellets were resuspended in 10 μL of 0.1 M Tris–HCl buffer (pH 9.5) containing 12 mM DTT, 2 mM EDTA and 3% SDS. For mBBr derivatization, M_r markers were dissolved in 86.6 μL deionized water, heated for 20 min at 70 °C, and labelled as shown in Section 2.2.

Then, 10 μL of sample and 10 μL of 2× sample buffer (90 mM Tris–HCl (pH 8.45), 8% SDS, 24% glycerol, 0.01% Brilliant Blue G and 10% β-mercaptoethanol) were mixed. Samples were denatured at 95 °C, 5 min, loaded, and separated in 13.5% Tricine SDS-PAGE gels (7 × 8 cm) at 30 mA/gel in a Mini-PROTEAN 3 cell, using as buffers 0.1 M Tris, 0.1 M Tricine (pH 8.2) and 0.1% SDS (cathode), and 0.2 M Tris–HCl (pH 8.9) (anode) (Schagger and von Jagow, 1987). Gels were washed with 50% methanol, 30 min; fluorescent protein bands were documented in a Gel Doc EQ system (Bio-Rad), and re-stained with Coomassie Blue R250.