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Proteomic discovery of biomarkers of metal contamination in Sydney Rock oysters (*Saccostrea glomerata*)

Emma L. Thompson[a,b,*], Daisy A. Taylor[a,b], Sham V. Nair[c], Gavin Birch[d], Paul A. Haynes[e], David A. Raftos[a,b]

[a] *Department of Biological Sciences, Macquarie University, NSW 2109, Australia*
[b] *Sydney Institute of Marine Science, Chowder Bay, NSW 2088, Australia*
[c] *Department of Biological Sciences, Macquarie University, NSW 2109, Australia*
[d] *Department of Geochemistry, University of Sydney, NSW 2006, Australia*
[e] *Department of Chemistry and Biomolecular Sciences, Macquarie University, NSW 2109, Australia*

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ABSTRACT

In the current study we examined the effects of metal contamination on the protein complement of Sydney Rock oysters. *Saccostrea glomerata* were exposed for 4 days to three environmentally relevant concentrations (100 μg/l, 50 μg/l and 5 μg/l) of cadmium, copper, lead and zinc. Protein abundances in oyster haemolymph from metal-exposed oysters were compared to those from non-exposed controls using two-dimensional electrophoresis to display differentially expressed proteins. Differentially expressed proteins were subsequently identified using tandem mass spectrometry (LC–MS/MS), to assign their putative biological functions. Unique sets of differentially expressed proteins were affected by each metal, in addition to proteins that were affected by more than one metal. The proteins identified included some that are commonly associated with environmental monitoring, such as HSP 70, and other novel proteins not previously considered as candidates for molecular biomonitoring. The most common biological functions of proteins were associated with stress response, cytoskeletal activity and protein synthesis.

1. Introduction

Metal contamination from anthropogenic sources poses a significant threat to the biological sustainability of coastal waterways around the world (Li et al., 2000). A variety of potentially toxic metals enter coastal waterways via urban and industrial processes. These include copper (Cu) from electrical products and agricultural fertilizers; zinc (Zn) from car tyres and pharmaceuticals; and cadmium (Cd) from household batteries (Birch and Taylor, 1999; Snowdon and Birch, 2004). In Australia, metal contamination in the marine environment poses a particularly significant threat because over 86% of the population lives in heavy urbanised areas within 3 km of coastal rivers, estuaries or embayments (Zann, 1996). Persistent and ongoing metal contamination has led government regulatory authorities to seek effective methods to monitor contamination at both the geochemical and biological levels.

Chemical analyses of sediments provide fine-scale contaminant mapping of waterways (Birch and Taylor, 1999; Scanes and Roach, 1999) which allows historical and current day levels of contaminants to be mapped. However, such geochemical analyses do not give an accurate assessment of the potential effects of metal contamination on the biota that inhabit coastal waterways, nor on the possible impacts on their ecosystems (Gray, 1992).

Ecotoxicological techniques that assess mortality or morbidity among key indicator species provide some information about the potential biological impacts of metal contamination (Sanders et al., 1998; Stark, 1998). However, these types of ecotoxicological tools are generally endpoint assays that identify effects only at acute levels of toxicity (Newton and Bartsch, 2007; Roman et al., 2007). They do not provide information about the cellular and physiological processes affected by metal contamination at sub-lethal concentrations that might affect ecosystem function without resulting in mortality or morbidity.

In contrast, biomarkers can be measured from cellular to ecosystem level. The use of molecular biomarkers has the potential

Abbreviations: Cd/$CdCl_2$, cadmium chloride; Cu/$CuCl_2$, copper chloride; Pb/$PbCl_2$, lead chloride; Zn/$ZnCl_2$, zinc chloride; 2-DE, two-dimensional electrophoresis; CHAPS, 3-[(3-cholamidopropyl)dimethyl-ammonio]-1-propanesulfonate; DTT, DT dithiothreitol; IAA, iodoacetamide; IEF, isoelectric focussing; SDS, sodium dodecyl sulfate; MS, mass spectrometry; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; HSP, heat shock protein.

* Corresponding author at: Department of Biological Sciences, Macquarie University, NSW 2109, Australia. Tel.: +61 9850 8213; fax: +61 9850 8245.
E-mail address: emma.thompson@mq.edu.au (E.L. Thompson).

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to identify cellular effects of metal contamination at sub-lethal levels. For instance, metallothionein and heat shock protein (HSP) levels have been used in field studies as indicators of sub-lethal Cd and Cu contamination in the crab *Carcinus maenas* (Pedersen and Lundebye, 1996), and as indicators for Cd, Cu and Zn stress in the freshwater mussel *Pyganodon grandis* (Giguère et al., 2003). Changes in the expression of molecular biomarkers reflect an organism's initial response to environmental perturbation. They reveal biochemical or physiological effects on organisms and thus show a depth of biological information on the impact of contaminants (van der Oost et al., 1996).

Despite its increasing adoption in weight-of-evidence approaches to environmental monitoring, the effectiveness of molecular biomonitoring is limited by the restricted suites of available biomarkers. Most of the currently used biomarkers are generic molecules that are thought to have similar potentialities across a broad range of species. These include molecules such as HSPs that act as molecular chaperones during cellular stress responses, and metallothioneins, which are a family of cysteine-rich proteins that interact with a range of physiological and xenobiotic metals. Even though the currently used molecules represent effective biomarkers over a range of species, it is likely that there are other tissue- or species-specific genes and proteins that may be more effective molecular biomonitoring tools. A species relevant to the study area also makes the study ecologically relevant (Wu et al., 2005).

In this study we used two-dimensional protein gel electrophoresis (2-DE) to discover novel protein biomarkers of metal contamination in Sydney Rock oysters (*Saccostrea glomerata*) that may be more sensitive or specific than the currently limited range of molecular biomarkers used in other species. *Saccostrea glomerata* is a ubiquitous and ecologically relevant species in NSW estuaries, and is the focus of a major aquaculture industry. Bivalve molluscs have been used extensively in biomonitoring due to their efficiency in bio-accumulating contaminants and their ability to show time-and dose-dependent relationships to contaminant exposure (Tanabe et al., 2000; Chase et al., 2001; Gillikin et al., 2005). Their ability to bio-accumulate contaminants quickly and depurate them slowly makes them an ideal test species for this study (Brown and McPherson, 1992; Hardiman and Pearson, 1995; Scanes, 1997; Scanes and Roach, 1999).

Proteomic approaches are powerful tools for biomarker discovery in environmental science (Viant et al., 2002; Jonsson et al., 2006). Large numbers of proteins can be analysed simultaneously, potentially providing complex protein expression signatures for individual chemicals and synergistic exposures (Bradley et al., 2002; Rodríguez-Ortega et al., 2003; Apraiz et al., 2006). This allows subtle environmental changes to be detected at low levels and provides quantitative information on the responses of individual proteins and underlying biological activities of these proteins (Nesatyy and Suter, 2007). Such approaches have already been used previously to detect biomarkers for contaminant assessment, in the mussel, *Mytilus edulis* (Apraiz et al., 2006), the oyster, *Crassostrea virginica* (Cruz-Rodríguez and Chu, 2002), and the clam, *Chamaelea gallina* (Rodríguez-Ortega et al., 2003).

In this study we identified proteins in Sydney Rock oyster haemolymph that are differentially expressed in oysters that had been exposed to a range of environmentally relevant concentrations of Cd, Cu, Pb and Zn under controlled laboratory conditions. The data are analysed in an effort to identify new biomarkers that can discriminate between different types of metal contamination over a broad range of doses.

2. Materials and methods

2.1. Oyster acclimation and exposure to metals

Eighteen month to 2 year old Sydney Rock oysters were purchased from Aquaculture Enterprises (Eden, NSW, Australia). Oysters were placed in 12 × 25 l aquaria at the Sydney Institute of Marine Science (Chowder Bay, Sydney, Australia) with each aquarium containing 7 oysters. They were left to acclimate to aquarium conditions for 10 days prior to metal exposures. Complete water changes were performed daily throughout the duration of the experiment with water taken directly from Chowder Bay, and oysters were fed every 4 days with M-1 bivalve food (Aquasonic, Wauchope, NSW, Australia). Water quality parameters (temperature and salinity) were recorded daily. Prior to the start of the exposure trials, water samples were taken from Chowder Bay and the levels of a suite of metals, polychlorinated biphenyls (PCBs), and poly-aromatic hydrocarbons (PAHs) were assessed by the Australian Government National Measurement Institute (Pymble, NSW, Australia).

Following the 10 day acclimation period, oysters were exposed for 4 days to 100 μg/l (three aquaria), 50 μg/l (three aquaria) and 5 μg/l (three aquaria) of $CdCl_2$. The remaining three aquaria were used as controls with no added metal. The experiment was repeated three times using $CuCl_2$, $PbCl_2$ then $ZnCl_2$. Stock solutions of metals were dissolved in seawater before being added to aquaria and water containing metals was changed daily.

2.2. Protein extraction

After exposure to metals, oysters were shucked and 500 μl of haemolymph was harvested from the pericardial cavity using a micropipette. The haemolymph was immediately mixed with 1.3 ml of Tri-reagent LS (Sigma–Aldrich). RNA was removed by adding 100 μl of bromochloropropane for 15 min followed by centrifugation for 15 min at 12,000 × *g* (4 °C) and removal of the colourless aqueous phase. DNA was then removed by adding 300 μl of 100% ethanol for 3 min followed by centrifugation at 2,000 × *g* for 5 min (4 °C), after which the DNA pellet was discarded. Finally, proteins were precipitated by adding 3× volumes of ice cold acetone, standing samples at room temperature for 10 min, followed by centrifugation for 10 min at 12,000 × *g* (4 °C). The protein pellets were washed by incubation four times in 1 ml 0.3 M guanidine hydrochloride in 95% ethanol (V:V) for 10 min per wash, followed by centrifugation at 8,000 × *g* for 5 min (4 °C) and removal of the supernatant. The resulting protein pellets were then air dried at room temperature before re-suspension in 50 μl re-hydration buffer (7 M urea, 2 M thiourea, 4% 3-[(3-cholamidopropyl)dimethyl-ammonio]-1-propanesulfonate (CHAPS); 50 mM dithiothreitol (DTT)).

2.3. Protein quantification and pooling of samples

The concentrations of proteins in the re-suspended pellets were quantified using Amersham 2-DE Quant Kits according to manufacturer's instructions (GE Healthcare, Buckinghamshire, UK). Briefly, 2 μl of each sample was added to each three wells of a 96-well microtiter plate. Ten microlitres of Cu solution, 40 μl of Milli Q water and 100 μl of colour reagent was also added to each well and the plate was left to incubate at room temperature for 20 min. Absorbance was measured at 490 nm on a microplate reader and protein concentrations were interpolated from a standard curve generated with bovine serum albumin. Haemolymph from five randomly selected oysters in each aquarium were pooled to give a total of three replicates per metal concentration (each replicate