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Peroxisomal proteomics: Biomonitoring in mussels after the *Prestige*'s oil spill

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ABSTRACT

Peroxisomal proteomics was applied to assess possible biological effects after the *Prestige*'s oil spill. Mussels were sampled in July 2004 and 2005 in four stations in the NW (closest to the spill) and NE coasts of the Iberian Peninsula. Principal components analysis (PCA) suggested differences in protein expression among stations and sampling years. Several proteins were putatively identified by mass spectrometry and immunolocalization. PC1 separated the NW stations in 2004 from the rest of the stations and sampling years mainly due to up-regulation of peroxisomal β-oxidation proteins and PMP70. PC3 separated the NE stations, based on up-regulation of the antioxidant enzyme catalase in 2004 compared to 2005. PC4 separated the stations in the NE and the NW. This work shows that environmental proteomics, together with multivariate data analysis, could provide information to interpret the effects of oil spills at cellular level in mussels.

1. Introduction

The accident of the oil tanker *Prestige* transporting 77,000 tones of heavy fuel–oil (M-100) in Galician waters on the NW coast of Spain in November 2002 led to one of the largest spills in the maritime history. In the following months, around 60,000 tons of fuel–oil were spilled over an area of 30,000 km^2 and oil slicks drifted from Galician waters onto the Cantabrian shelf, affecting to approximately 2600 km of the N and NE Iberian coastline up to Brittany. The unique characteristics of this spill regarding the type and volume of spilled oil, duration and large extension of the affected coast have attracted great attention from the scientific community.

In general, prolonged monitoring of the affected area is essential to evaluate the possible effects on the biota as well as recovery of the area's environmental quality. The use of mussels as bioindicators of exposure to pollution and as sentinels of pollution effects is widely applied and recommended (Bayne, 1976; O'Connor and Lauenstein, 2006). These organisms are sessile and filter feeders, and can accumulate in their tissues contaminants from the water column. Historically, mussels have been utilized to evaluate the biological consequences of oil spills, such as after the accidents of the Exxon Valdez in Alaska (Thomas et al., 1999) and Aegean Sea in NW Spain (Porte et al., 2000). In the case of the *Prestige* oil spill, health status of mussels sampled along the northern coast of the Iberian Peninsula was determined using a battery of biomarkers of exposure and effects (Marigomez et al., 2006; Orbea et al., 2006). The digestive gland in mussels is in charge of the intracellular digestion of nutrients as well as pollutants. Moreover, the digestive gland is a pollution-respondent organ for which many biomarkers have been developed and applied. And peroxisome proliferation measured in mussel digestive glands has already been introduced as a possible biomarker of exposure to various marine pollutants including polycyclic aromatic hydrocarbons (PAHs) (Cajaraville et al., 2003).

New methods that perform global analyses of proteins are especially powerful to provide molecular signatures that could overcome the traditional disadvantages of single parameter biomarkers. Proteomics-based methods could provide a more comprehensive view of toxicity due to the cascade of alterations triggered by pollution exposure (Aardema and MacGregor, 2002). These methodologies have seldom been applied to large biomonitoring programs for two main reasons: lack of historical data, and utilization of not highly reproducible, robust, and affordable methods. In line, several methods have recently been developed (Mi et al., 2005; Bjornstad et al., 2006; Amelina et al., 2007). In the last years, proteomics has been applied for the screening of protein expression signatures (PES) of exposure to model pollutants (Shepard and Bradley, 2000; Apraiz et al., 2006; Mi et al., 2007) and in field experiments (Knigge et al., 2004; Mi et al., 2005; Bjornstad et al., 2006; Amelina et al., 2007).

Petroleum-based products are the major source of energy for industry and daily life. Transport of petroleum across the world is frequent, and consequently, the potential for oil spills and their devastating effects in marine ecosystems is significant. Applying peroxisomal proteomics in a laboratory experiment, we have obtained a robust protein profile of exposure to different crude oil

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mixtures that indicates this methodology could be used to measure the effects of oil exposure in marine pollution assessments (Mi et al., 2007). Results from another approach, namely surface-enhanced laser desorption/ionization–time of flight mass spectrometry (SELDI–TOF MS) indicated that exposure to an oil mixture had a higher significant effect than oil alone on protein expression in mussels (Bjornstad et al., 2006). In the assessment of oil derivatives in field experiments, the PES showed specific protein inductions or suppressions associated with the sampling station (Knigge et al., 2004).

In order to assess the biological impact caused by the *Prestige*'s oil spill we have applied peroxisomal proteomics in mussels *Mytilus galloprovincialis*, collected from four different stations along the NW and NE Iberian coasts in July 2004 and 2005. We monitored variations in the peroxisomal proteome from the different sampling stations. The quantitative proteomics data and the multivariate statistical analysis mapped differences in expression levels of several proteins involved in xenobiotic metabolism and other cellular functions in the studied regions. These proteins were identified by immunolocalization and MS. On the basis of these results, proteomics techniques could provide PES to assess the biological impact and/or recovery of mussels after an oil spill based on differences in protein expression.

2. Methods

2.1. Sample collection

Mussels, *M. galloprovincialis*, 3.5–4.5 cm shell length, were collected from two stations in the NW coast of the Iberian Peninsula: Sao Bartolomeu do Mar (from now on referred as Sao Bartolomeu) (41°34′36″N, 8°48′2″W) and Aguiño (42°31′13″N, 9°0′36″W), and two stations in the NE coast: Mundaka (43°24′16″N, 2°41′43″W) and Hondarribia (43°22′40″N, 1°47′24″W) in July 2004 and July 2005 (sampling map in Fig. A1). Sao Bartolomeu in Portugal was selected as a location not affected by the *Prestige* oil spill although chemical analysis indicated it was polluted with PAHs (Bartolome, 2007). Aguiño in Galicia was heavily impacted by the oil spill whereas Mundaka and Hondarribia in the Basque Country were moderately affected. In all four stations levels of PAHs decreased significantly from 2004 to 2005 (Bartolome, 2007). In each sampling station, 50 mussels were collected in the lowest low tide and, when possible, from under the water. Mussels' digestive glands were immediately dissected out and frozen in liquid nitrogen. Frozen samples were stored in the laboratory at −80 °C until required for analysis.

2.2. Cell fractionation and isolation of peroxisome enriched fractions

Five to six grams glands (or 50 glands) were homogenized per station and year. Homogenization of minced tissue and subcellular fractionation by differential centrifugation followed by density gradient centrifugation in iodixanol was performed according to an established method (Ghosh and Hajra, 1986) with a few modifications outlined in the Appendix. The activities of the following marker enzymes were measured in the different main fractions across the fractionation procedure: catalase for peroxisomes, succinate dehydrogenase for mitochondria and acid phosphatase for lysosomes (Graham and Higgens, 1993). Protein concentration was determined according to Bradford (1976).

2.3. Protein extraction and 2-DE PAGE

Proteins were precipitated at −20 °C by 10% trichloroacetic acid (TCA) and 0.07% β-mercaptoethanol in cold acetone. The protein precipitate was washed twice with 1 mL acetone containing 0.07% β-mercaptoethanol and dried at room temperature. Precipitated proteins were solubilized in a solubilization buffer modified from Rabilloud (1998) (specified in the Appendix). Afterwards, samples were first alkalized with 30 mM IAA for 1 h in darkness, then mixed with a rehydration solution (specified in the Appendix), and 200 μg soluble protein per replicate were applied onto 11 cm IPG strips, pH 3–10 non-linear (Bio-Rad Inc., Hercules, CA, USA). Four microliters of 2-DE markers (p*I* range 7.6–3.8, Mw range 17,000–89,000) were added (Sigma, St. Louis, MO, USA). Three to four experimental replicates were obtained per station and year, the number of replicates being dependent on the amount of protein available after solubilization (see Appendix). Isoelectric focusing was performed on a Protean IEF Cell (Bio-Rad) at 20 °C using the following program: passive rehydration for 15 h, rapid voltage slope in all the steps, step 1: 250 V for 15 min, step 2: 8000 V for 2.5 h and step 3: at 8000 V until 45,000 V h were reached. Immobilized IPG strips were reduced (1% DTT) and then alkalized (4% IAA) in equilibration buffer (see Appendix). The second dimension, or SDS–PAGE, was carried out on homogeneous 11 cm 12.5% Criterion Tris–HCl Precast Gels (Bio-Rad), at 160 V using a Criterium™ Dodeca Cell (Bio-Rad).

2.4. Image acquisition and analysis

Protein spots in the gels were visualized by the CBB G-250 stain (Molloy et al., 1999), and gel images were obtained using a UMAX Image Scanner (GE Healthcare, UK). Data was analyzed using Image Master 2D Platinum 6.0 (GE Healthcare). Image analysis included spot detection, quantification and normalization, and matching. Errors in automatic matching were manually corrected when needed. The volume of each spot from each gel was calculated and normalized to the total gel spot volume in order to correct it for differences in gel staining. The new value was a percentage of the total volume of all spots in the gel.

2.5. Statistical analysis

Volume % (vol.%) values for all protein spots were evaluated for significant differences among groups using the statistical program MINITAB 15 (Minitab Inc., State College, PA, USA). Briefly, data was first normalized, then the inter-replicate variability was reduced by filtering data using the coefficient of variation (CV) values, and finally the Principal Components Analysis (PCA) was applied to reduce the dimensionality in the data, as follows.

Vol.% data was first normalized by: Nvol.% = Ln (vol.% + 1), where Nvol.% is the normalized vol.%. This transformation improves the linearity of our data and this is important for increasing the efficiency in the PCA (Quinn and Keough, 2002).

In order to obtain the spots with the least inter-replicate variability, coefficients of variation (CV) were calculated for each spot in each station and year. The average CV for each spot was calculated taking into account only the groups with CV > 0 in order to avoid biasing the averaging. Spots with CV < 40% (134 spots) were taken into the following analysis. In the case of spot absence in one of the gels, the group mean was input. When there was only one value per group, that value was deleted.

Differences in Nvol.% data were set by PCA. PCA was applied to first, reduce the number of variables (in our case spots) that can summarize the total amount of information in the analyzed objects (in our case gels) to a set of principal components, and second, to reveal similarity patterns among the stations that could be scaled in a multidimensional space using the principal component scores for the objects. Principal components were extracted from a correlation matrix composed by 134 variables. The principal components are linear combinations of the original variables and are