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An ecotoxicoproteomic approach (SELDI-TOF mass spectrometry) to biomarker discovery in crab exposed to pollutants under laboratory conditions

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Abstract

Ciphergen ProteinChip Technology™ is a proteomic tool, used for the discovery of new and sensitive biomarkers. This approach was used to evaluate the protein profile of crabs exposed to various pollutants. Two different exposure experiments were performed: spider crabs (*Hyas araneus*) were exposed for 3 weeks to diallyl phatalate (DAP), bisphenol A (BisA) and polybrominated diphenyl ether (PBDE-47), while shore crabs (*Carcinus maeanas*) were exposed to crude oil, crude oil spiked with alkylphenols (APs) and 4-nonylphenol (NP). Gender and species-related protein pattern alterations were observed and compared to controls. Results showed different responses to pollutants by the two species. Major disruption in protein peak expression was observed in samples exposed to mixtures of pollutants, i.e. oil spiked with APs. Compared to shore crab, spider crab species showed a lower degree of response in terms of number of altered protein peaks following exposure. In general, female individuals of both species showed a larger number of significantly altered proteins compared to males. Data analysis by non-metric multi-dimensional scaling (MDS) was performed. Bi-dimesional-MDS plots revealed a good separation of groups for both spider and shore crabs. In some cases, a good discrimination can also be observed between the two genders within each treatment.

Results highlight the potential of crabs as sentinel organisms for the aquatic environment. The results indicate that SELDI-ToF technology is a powerful tool to discover protein expression signatures for different pollutants and sex dependent responses.

Keywords: Proteomics; SELDI-ToF; Biomarkers; Pollutants; Crab; Crude oil; Alkylphenols; Nonylphenol; Diallyl phatalate; Bisphenol A; Flame retardant

1. Introduction

Since deleterious effects on aquatic and terrestrial wildlife have been reported worldwide (Depledge and Billinghurst, 1999; Guillette, 2000), novel approaches based on monitoring effects rather than detecting and quantifying pollutants are needed. The use of biological markers or biomarkers has been proposed as a sensitive "early warning" tool for biological effect measurement in environmental quality assessment (Cajarville et al., 2000).

Variations in specific protein expression are commonly used as indicators of pollutant exposure (Stagg, 1998). Nevertheless, limitation of most single-molecule biomarkers is the lack of sensitivity to mixed pollutants, making risk assessment analysis related to chemicals much more uncertain. Protein analysis may provide a specific "fingerprint" directly associated with either exposure to or effect of certain classes of chemicals. The focus is now on the evaluation of the effects of contaminants by measuring the unbalance of specific gene, protein or metabolite expressions in exposed organisms. Rapid progress of "-omics" technologies during the last years has revolutionized biology, providing a clear increase of knowledge and development of new methods for measuring families of cellular molecules, such as genes, mRNA, proteins and intermediary metabolites. Genomics, proteomics and metabolomics are complementary approaches that all contribute unique information. The word proteome (PROTEin complement expressed by a genOME), introduced by Wilkins et al. (1996), defines the complete set of proteins expressed and modified following their expression by a genome. Applied to environmental toxicology, proteomics may be used to identify chemical-specific protein expression signatures (PES) that in some cases provide useful molecular descriptions of the cell or tissue state. Since this approach

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involves measuring changes in hundreds of proteins simultaneously it provides multiple endpoints. A multi-endpoint analysis is robust against external factors (such as age, season or abiotic factors) other than the given stressor.

Recently, environmental proteomics has been applied to laboratory and field studies in marine organisms, such as fish and molluscs (Shrader et al., 2001; Bradley et al., 2002; Rodriguez-Ortega et al., 2003; Knigge et al., 2004; Bjørnstad et al., 2006; Larsen et al., 2006).

The most commonly used method for proteomics analysis is based on the separation of proteins by two-dimensional gel electrophoresis (2DE) and their subsequent identification by mass spectrometry (MS) or tandem mass spectrometry (MS/MS) (Gorg et al., 2000). This approach has some limitations: gel reproducibility requires great technical skill and a limited capacity of analysis for low abundance proteins has been observed (Rodland, 2004). Some of the limitations with 2DE can be overcome by alternative means of detecting proteins (Caputo et al., 2003, 2005).

In this study, Surface Enhanced Laser Desorption/Ionization (SELDI) Technology (ProteinChip™ Array System, Ciphergen Biosystems, Fremont, CA, USA) was utilised. This approach combines two powerful techniques: chromatography and mass spectrometry, and involves the use of chemical or biochemical surfaces to select proteins. The ProteinChip™ approach revealed several advantages: (i) high-throughput screening, (ii) versatility, (iii) ease of use, (iv) speed and (v) comparative low cost. Furthermore, this technology allows the simultaneous and semi-quantitative detection of femtomole amounts of proteins with a molecular mass precision within 0.2% of the true mass, as reported by Merchant and Weinberger (2000).

The overall aim of this work was to investigate the potential of the ProteinChip™ Array system as a tool for PES surveys and biomarker discovery in two species of crabs: shore crabs (*Carcinus maeanas*) and spider crabs (*Hyas araneus*). Crabs were selected as sentinel organisms because they are ubiquitous, easy to collect and classify and easy to keep under experimental conditions (Stentiford and Feist, 2005; Brown et al., 2004). Flame retardants, phthalates, surfactants, oil related compounds and Bis A were chosen for this study because strong indications of effects on the health status of aquatic organisms have been reported (Ying et al., 2002; de Boer et al., 2003; Aas et al., 2000; Sundt et al., 2006).

2. Material and methods

CM10 and Q10 ProteinChips™, the molecular weight calibration standard kit C100-0002, C100-0001 and sinapinic acid (SPA) were purchased from Ciphergen Biosystem (Fremont, CA, USA). 4-Nonylphenol (NP), bisphenol A (Bis A), diallyl phthalate (DAP) and tertabromodiphenylether (PBDE-47) of purity >99%, DL-dithiothreitol (DTT), trifluoroacetic acid (TFA), Trizma HCl, potassium phosphate (KH_2PO_4), Triton-X 100 and protease inhibitor cocktail (P2714) were supplied by Sigma–Aldrich. Acetone and acetonitrile (ACN) were HPLC ultra-gradient grade from Romil (Dublin, Ireland). Mass spectra were recorded using a Ciphergen PBS II ProteinChip™ array reader, a linear laser desorption/ionization-time-of-flight mass spectrometer with time lag-focusing (Merchant and Weinberger, 2000). Instrumental tasks and raw data interpretation were run using Ciphergen ProteinChip™ software 3.1.

2.1. Exposure experiment

The exposure experiment is described in detail in Sundt et al. (2006). Briefly, spider crabs were exposed continuously for three weeks to nominal concentrations of 30 ppb DAP, 50 ppb of Bis A and 5 ppb of PBDE-47.

Shore crabs were exposed to 0.5 ppm crude oil, 0.5 ppm crude oil spiked with 0.1 ppm APs and 30 ppb NP for 3 weeks.

2.2. Sample collection and protein extraction

After exposure, 32 crabs from each group were sacrificed and the gender recorded. In all groups, the gender ratio was close to 1:1. Hepatopancreas tissue was quickly dissected, snap frozen in liquid nitrogen, and stored at −80 °C prior to further analysis. The tissues were homogenized in ice-cold K-phosphate buffer (50 mM, pH 7.4) containing 2.5% NaCl and 10 mM DTT. The protease inhibitor cocktail contained: 4-(2-aminoethhyl)benzenesulfonyl fluoride hydrocloride (2 mM), trans-epoxysuccinyl-L-leucylamido(4-guanidino)butane (14 mM), 1-ethylenediamine-*N*,*N*,*N*′-tetracetic acid (1 mM), bestatin (130 μM), leupeptin (1 μM) and aprotinin (0.3 μM) and it was added to the homogenates at a ratio of 1:4 (v/v). Samples were centrifuged for 1 h at 100,000 × *g*. The cytosolic fractions were then loaded into each well of two Q10 arrays to check intra sample reproducibility. Coefficient of variance (CV) was calculated for 150 peaks in a range between 2 and 150 kDa before mass and intensity normalization of spectra. CV varied between 0.4 and 3.1% in sample 1 and between 0.9 and 12.3% in sample 2. The array-to-array reproducibility was satisfactory (see Fig. 1).

Two different chip types were tested; the surface of CM 10 chips has a carboxylate group matrix, giving them the property of binding semi-positively charged peptides, while the Q10 chips bind low p*I* proteins, owing to the positively charged quaternary ammonium groups on the chip surface (Issaq et al., 2003).

Q10 chips were selected for the analysis, in combination with a Tris–HCl binding buffer (50 mM Tris–HCl, 0.1% Triton-X 100, pH 9) since they gave the best performance.

In order to standardize the loading of proteins onto the chip surface, the total protein content of the extracts was determined using the Bradford method (Bradford, 1976). Clear spectra with good peak resolution were obtained with 0.3 mg/ml protein loading concentration.

Each ProteinChip™ "spot" was prepared with 250 μl of binding buffer and then 150 μl of diluted sample was applied to each spot and incubated overnight at +4 °C. Arrays were then washed three times with 150 μl of binding buffer without Triton-X 100 and then washed again with distilled water to remove interferences and weakly bound proteins. The chip surfaces were dried before energy absorbing molecules were added.