Proteomic Analysis of Individual *Daphnia microcrustaceans*

Daphnia are parthogenic microcrustacea belonging to the family Daphniidae. Under normal environmental conditions, *Daphnia* populations are exclusively female and reproduction is clonal. However, in response to adverse environmental stimuli, sexual reproduction is induced, enabling genetic recombination and allowing rapid adaptive response. Sexual daphnids produce resting eggs, termed ephippia, which can remain viable for centuries. Thus, the analyses of Daphnids grown from epipphia isolated from layers of lake or stream sediment could potentially provide a chronology of environmental changes over several decades. Therefore, it is important to derive sufficient protein from a single Daphnia for phenotypic analyses. Standard sample preparation methods are inadequate to provide sufficient protein from a single Daphnia. However, the Pressure Cycling Technology Sample Processing System (PCT SPS) proved to be an excellent method for the extraction of proteins when followed by ultrafiltrative exchange to remove non-proteinaceous components from the homogenate. Proteins derived from PCT and then analyzed on two-dimensional gel electrophoresis (2DGE) was capable of resolving differences between asexual and sexual phenotype from solitary *Daphnia magna*. For the smaller *Daphnia pulex*, 2DGE resolved 904 ± 7 protein spots from a single organism, and 1,267 ± 3 protein spots from a pool of five organisms. These data suggest the feasibility of using 2DGE for following phenotypic response to environmental stimuli such as hepatotoxin contamination during cyanobacterial blooms.

Materials and Methods

D. pulex cultures

Cultures were maintained in 8 L of modified COMBO media [1] at a density of 30 individuals/L. Daphnids were cultured at 20º ± 1ºC under 16:8 hours light:dark photoperiod of low intensity. Cultures were fed daily with 1mg C/L of the green algae *Ankistrodesmus falcatus* obtained from The Culture Collection of Algae (University of Texas, Austin, TX, USA). Daphnid gut contents were minimized by allowing the microcrustaceans to feed on copolymer microspheres of 4.3 micron mean diameter (Duke Scientific, Fremont, CA, USA) for one hr prior to harvesting. Microspheres were fed at a concentration equal to the number of algal cells previously supplied. *D. pulex* were harvested by filtration through 250 um Nitex mesh (Sefar America, Depew, NY, USA).

D. magna cultures

D. magna starter cultures were obtained from Sachs Systems Aquaculture (St. Augustine FL, USA). Stabilized cultures were maintained in 8 L of 25% mineralized water (Vermont Spring Water Company, Brattleboro, VT, USA) at a density of 60-120 individuals/L. Daphnids were cultured at 22º ± 1ºC under constant illumination with standard fluorescent bulbs. Cultures were maintained at pH 7.0-7.4 by the addition of 100 g/L crushed coral (Tideline, Inglewood, CA, USA) supplied in nylon bags. Starter cultures were fed daily with 1 mL/L of *Nanochloropsis* microalgae liquid concentrate (Reed Maricultures, Campbell, CA, USA) for the first four weeks, followed by 0.1 mL/L thereafter.

The average mass of adult *D. magna* was 1.37 ± 0.46 mg fully hydrated and 0.23 ± 0.06 mg when dehydrated (n = 64) indicating a 90.6% water content.

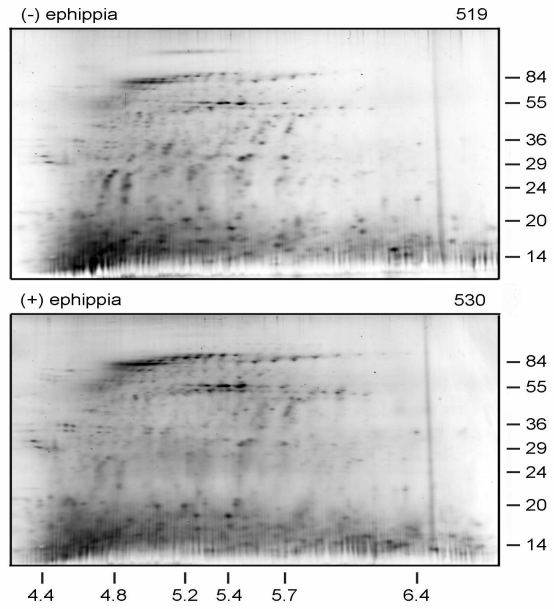

Figure 1. *Phenotypic differences in D. magna (+) or (-) ephippia displayed by 2DGE. A single organism of each phenotype was processed by PCT for each analysis. The number of protein spots in each gel is indicated (upper right). Protein molecular weight and isoelectric point (pI) are estimated (ordinate and abscissa, respectively). Estimates of pI assume linearity of the IPG.*

Pressure Cycling Technology (PCT)

Daphnids collected in PULSE Tubes were suspended in 500 uL of 7M urea, 2M thiourea, and 4% CHAPS (IEF reagent) supplemented with 100 mM dithiothreitol (DTT) and protease inhibitor cocktail (Sigma Aldrich Chemicals, St. Louis, MO). An additional 900 µL of mineral oil was added to meet the minimum volume requirement of the PULSE Tube (Pressure BioSciences, West Bridgewater, MA, USA). Samples were then subjected to 60 pressure cycles in a NEP3229 Barocycler (Pressure BioSciences, West Bridgewater, MA, USA). Each pressure cycle consisted of 10 seconds at 35,000 psi followed by rapid depressurization and a hold for 2 seconds at atmospheric pressure. Mineral oil was removed from the sample prior to any further analyses.

IEF and 2DGE

Proteins were reduced and alkylated directly in the ultrafiltration devices as previously described [2]. Dried immobilized pH 4-7 gradients (Bio-Rad, Hercules, CA, USA) were hydrated for six hours with 200 uL of each sample. Isoelectric focusing (IEF) and 2DGE was performed as described [3]. Gels were stained with SilverQuest Silver Stain Kit (Invitrogen, Carlsbad, CA, USA). Images were analyzed using PDQuest™ Version 7.1 software (Bio-Rad, Hercules, CA, USA). After background subtraction and spot matching, the total spot count was determined for each gel. Protein spot density peaks were detected and counted.

LC-Tandem MS protein identification

Protein bands were cut from the gel, destained in Farmer's reagent, and treated with trypsin (5 µL of 20 ng/µL trypsin in 50 mM ammonium bicarbonate) and incubated overnight at RT. The peptides that were formed were extracted from the polyacrylamide, evaporated to near dryness, and reconstituted in 30 µL of 1% acetic acid. The LC-MS system was a Finnigan LTQ ion trap mass spectrometer system. The HPLC column was a self-packed 9 cm x 75 µm ID Phenomenex Jupiter C18 reversed-phase capillary chromatography column. Two microliter volumes were injected and the peptides eluted from the column by linear acetonitrile gradient at a flow rate of 0.2 µL/min. The MS system used a data-dependent multitask capability that acquires a full scan mass spectra to survey the column eluate followed by 3 to 5 product ion spectra to determine amino acid sequence in successive scans. This mode of analysis produces approximately 2500 collisionally induced dissociation (CID) spectra. Data were analyzed by using all CID spectra collected in the experiment to search the NCBI non-redundant database with the search program Mascot. Each identified protein was verified by manual interpretation of at least two spectra.

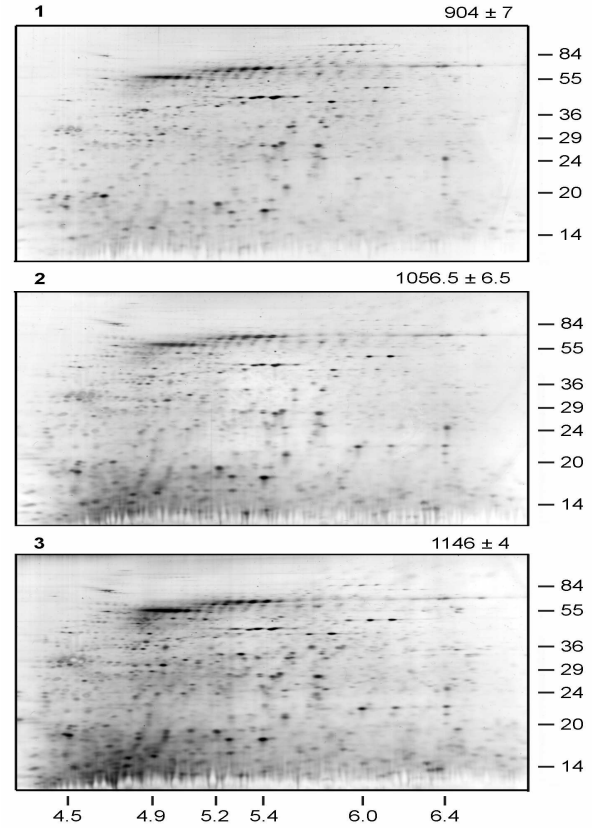

Figure 2. Representative silver stained 2D gels of 1, 2, or 3 individual D. pulex organisms. The number of protein spots (mean ± SD) from duplicate gels are indicated.

Results and Discussion

Image analysis of 2D gels

Figure 1 shows a silver stain of 2D gels revealed 519 and 530 protein spots from single unepphipiated and epphipiated *D. magna* organisms. Image analysis comparing the two phenotypes detected 60 mismatched proteins. These data demonstrate the feasibility of using 2DGE for following phenotypic response to environmental stimuli. For the smaller *Daphnia pulex*, 2DGE resolved 904 ± 7 protein spots from a single organism, and 1,267 ± 3 protein spots from a pool of five organisms. Figure 2 shows 2DGE of 1, 2, or 3 individual *D. pulex*. Figure 3 shows the number of proteins detected as a function of the number of organisms. In duplicate gels, a low coefficient of variation (CV) indicated the high degree of reproducibility.

Status of protein identification

Two overlapping approaches to identify the proteins were used in these experiments. Cutting proteins directly from the gel proved to be the best way to identify selected proteins. For silver stained bands, the LC-tandem MS identification had a 50% success rate, so this approach has some practical limits. Therefore, a second approach, in which parallel gels are run with higher protein loads, specifically for the identification experiment, was also employed.

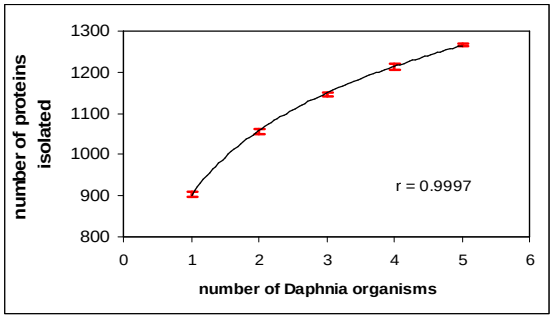

Figure 3. The number of protein spots detected by silver staining as a function of the number of D. pulex and the reproducibility of 2DGE.

Conclusion

The fast, efficient, and accurate release of proteins from cells and tissues is a critically important initial step in most analytical processes, and this is essential for reliable proteomic analyses. 2DGE can be an accurate representation of a proteome only if the entire protein constituency of cells is recovered during the sample preparation process. Previously, PCT has been shown to release high molecular weight proteins associated with the chitin present in exoskeleton [4]. Here PCT is shown to effectively induce the lysis of Daphnia. In these experiments, two-dimensional arrays of the *D. pulex* and *magna* proteomes were extracted from single organisms by PCT. Downstream proteomic analyses, including the identification of proteins and their post-translational modifications will increase the understanding of how this species undergoes natural alterations in protein expression relating to reproduction and genetic recombination during adverse changes in the environment.

References

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