Detection of differential protein expression between individual *Daphnia* by Pressure Cycling Technology (PCT) and two-dimensional gel electrophoresis

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ABSTRACT

Understanding how an organism responds to its environment on the molecular level is paramount to advancing the field of individualized molecular medicine and requires a model system with both well-established ecological and molecular resources. *Daphnia* have long been used in ecological studies and now, with the development of vast molecular resources, including a complete genome sequence, stand as one of the few model systems with both components in place. Under ideal environmental conditions, *Daphnia* populations are exclusively female and reproduction is parthenogenic. However, in response to environmental stress, the appearance of males and the shift to sexual reproduction facilitates genetic recombination. Using pressure cycling technology (PCT) in combination with two-dimensional gel electrophoresis (2DGE), we were able to reliably detect differences in protein expression from individual *Daphnia* of distinct genotypes and from individuals exhibiting distinct phenotypes. We conclude that this highly-sensitive method of protein extraction and detection, reveals differences in protein expression that are biologically meaningful and is an important step in understanding individual variation and how that variation matters in the context of the natural environment.

INTRODUCTION

Understanding and predicting how individual organisms respond at the molecular level to environmental change will provide new insight into the evolution of complex biological systems. This insight will lead to the development of new predictive models of host-pathogen interactions, environmental stress and community dynamics as a function of environment and genotype/phenotype (National Science Board, 2000), advancing the field of individualized molecular medicine.

Long recognized as a model for ecological research, the freshwater crustacean *Daphnia* is rapidly maturing into a powerful model for understanding basic biological processes. A common resident of lakes and ponds, *Daphnia* has been the subject of over a century of study in the areas of rapid environmental response, physiology, nutrition, predation, parasitology, toxicology and behavior. The reproductive cycle of *Daphnia* is ideal for experimental genetics. Generation time in the laboratory rivals that of almost all other model eukaryotic systems, reaching maturity within 5-10 days. Under favorable environmental conditions, *Daphnia* reproduce through parthenogenesis, allowing the conservation of genetic lines. Sexual reproduction is induced by environmental changes allowing the production of inbred or outbred lineages. The sexually produced diapausing eggs, termed ephippia, can be stored viably for considerable periods of time. Moreover, they have been hatched from lake sediments up to a century old (Limburg and Weider, 2002; Hairston and others, 2001) allowing tracking of genetic changes over ecological and evolutionary time scales.

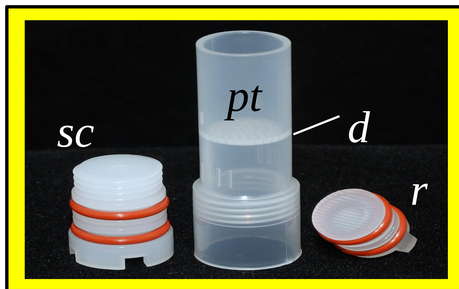
Pressure Cycling Technology (PCT) has been shown to be effective for isolating proteins, nucleic acids, lipids, and small molecules from a wide range of cells, tissues , and small organisms. PCT uses alternating cycles of hydrostatic pressure to efficiently disrupt cells and tissues. Rapid cycling between high and low pressure has been demonstrated to be more disruptive than sustained high pressure.

Our goal was to demonstrate that it is possible to conduct a comparative analysis of protein expression and to analyze biologically relevant variation from an individual *Daphnia*. Using pressure cycling technology (PCT) for sample preparation and two-dimensional gel electrophoresis (2DGE), we were able to detect individual variation from single *Daphnia* of distinct genotypes (Iinb1 vs. Xinb3) and exhibiting distinct phenotypic differences (parthenogenic vs. sexual)

MATERIALS AND METHODS

Daphnia cultures

Daphnia clones were maintained in 8 L of COMBO media (Kilham and others, 1998) at a density of 30 individuals/L. Cultures were maintained at 20º ± 1ºC under a 16:8 hours light:dark photoperiod of low intensity. Cultures were fed daily with 1mg Carbon/L of the green algae *Ankistrodesmus falcatus* obtained from UTEX, The Culture Collection of Algae at The University of Texas (Austin, TX, USA). Prior to PCT, 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 hour prior to harvesting. Microspheres were fed at a concentration equal to the number of algal cells previously supplied. *Daphnia* were harvested by filtration through 250 um Nitex mesh (Sefar America, Depew, NY, USA). Live *Daphnia* were retained on the perforated lysis disc (0.6 mm pore size) of the PULSE Tube and excess water was removed. The PULSE Tubes were assembled and stored promptly at -40º C. Average mass of adult *D. magna* was 1.37± 0.46 mg fully hydrated and 0.23 ± 0.06 mg when dehydrated (n = 64). Average mass of adult *Daphnia pulex* was 115.8 µg ± 8.3 µg fully hydrated and 52.85 µg ± 10.60 µg dehydrated (n = 50).



Components of PCT Shredder Pulse Tube . The screw cap (sc) is threaded into the base of the pulse tube (pt). The sample and appropriate lysis buffer are placed on the perforated lysis disc (d). The ram (r), is installed in the pulse tube. Under high pressure, the ram forces tissue and fluid through the lysis disc. Upon return to atmospheric pressure, the ram retracts pulling sample through the lysis disc. Pressure cycling effectively disrupts the sample.

Pressure Cycling Technology (PCT)

PULSE Tubes, ProteoSOLVE SB Kits, and the Barocycler 3229 were from Pressure BioSciences (South Easton, MA). Individual *Daphnia* were transferred to PULSE Tubes and suspended in 500 µL of IEF Reagent from the ProteoSOLVE SB Kit supplemented with protease inhibitors. PCT was performed for 60 cycles at 35,000 psi maximum pressure. Following PCT, each PULSE tube was coupled to an Ultrafee-CL centrifugal filtration device with a 5-micron pore size (Milipore Corporation, Danvers, MA) and evacuated by centrifugation for 1 minute at 1000 RCF. The PULSE tube was removed and centrifugation continued for 4 minutes at 4000 RCF.

Reduction, alkylation, and ultrafiltration

Samples were transferred to ULTRA-4 ultrafiltration devices with 10 kDa MWCO (Millipore Corporation, Danvers, MA). Centrifugation assisted the ultrafiltration and the samples were exchanged with fresh UTC until the final DTT concentration was 10 mM. Reduction and alkylation of the samples were performed directly in the ultrafiltration devices using 5 mM tributylphosphine and 50 mM acrylamide as described (Smejkal and others, 2006a).

IEF and 2DGE

Two-hundred µL of each sample was placed onto individual wells in IPG rehydration trays from Proteome Systems (Woburn, MA). Bio-Rad ReadyStrip IPG strips with a pH range of 4-7 (Hercules, CA) were placed onto each sample, and the tray was placed into a humidifying ziploc bag. Rehydration occurred over six hours until all the sample was visibly absorbed by the strip. At the termination of rehydration, strips were placed into isoelectric focusing trays and ran at 10,000 volts (maximum voltage) for 110,000 accumulative volt-hours. Strips were equilibrated, then placed onto Criterion Tris-HCl 8-16% IPG+1 gels (Bio-Rad Laboratories, Hercules, CA) and ran at 120 V and 60 mA/gel.

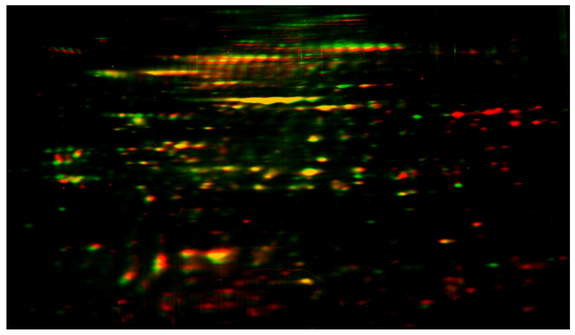
Digital image analyses

The 24-bit images were analyzed using PDQuest™ software (Bio-Rad, v.7.1). Background was subtracted and protein spot density peaks were detected and counted. A reference pattern was constructed from one of the individual gels to which each of the gels in the matchset was matched. Numerous proteins that were uniformly expressed in all patterns were used as landmarks to facilitate rapid gel matching. After matching, the total spot count was determined in each gel.

Results and Discussion

Protein variation between individual D. magna of distinct genotypes

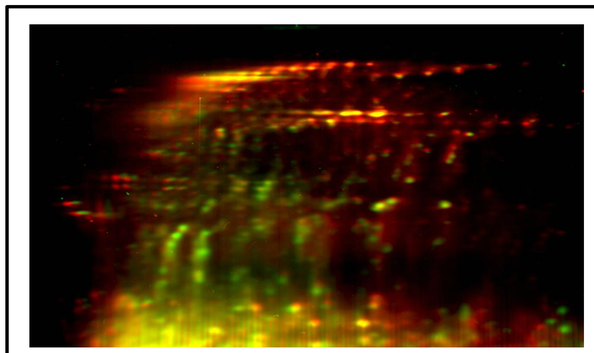
Our goal was to demonstrate that PCT and 2DGE are effective methods of sample preparation to detect differences in protein expression between individual *Daphnia* of distinct genotypes. Individual *D. magna* from Iinb1 and Xinb3 genotypes were isolated, proteins extracted and analyzed in quadruplicate by 2DGE as describe above. Silver staining detected an average of 687 ± 11 protein spots from the Xinb3 gels and 692 ± 14 protein spots from the Iinb1 gels. After normalization of the gel images based on total intensity, 679 spots were matched between the two gel images. A total of 136 spots showed a two-fold or greater difference in spot intensity. 79 of these were more abundant in Xinb3 and 57 were more abundant in the Iinb1.



Digitally enhanced, superimposed gel image of individual *Daphnia magna* of distinct genotypes. Red represents spots unique to Iinb1, green represents spots unique to Xinb3, yellow represents spots common to both genotypes.

Protein variation between individual D. magna of distinct genotypes

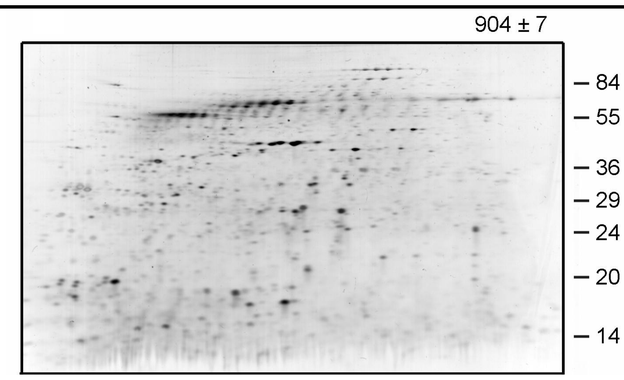
We were also able to demonstrate that differences in protein expression could be detected between individual *Daphnia* with distinct phenotypes. Individual *D. magna*, with and without ephippia, were isolated, proteins extracted and subjected to 2DGE as described above. Silver staining detected 524.5 ± 7.8 protein spots in 2D gels produced from single *D. magna* . After normalization of the gel images based on total intensity, 386 spots were matched between the two gel images. A total of 84 spots showed a three-fold or greater difference in spot intensity. Fifty-five of these were more abundant in the parthenogenic (no ephippia) animal, while 29 were more abundant in the sexual animal. In addition, eleven protein spots were unique to the parthenogenic phenotype, while 49 protein spots were unique to the sexual phenotype. This demonstrates the feasibility of 2DGE and image analysis for the differentiation of *Daphnia* phenotypes isolated in the field as indicators of environmental variables. Recent studies with parthenogenic and sexual *Daphnia carinata* were able to identify several proteins that were differentially expressed between the two pheontypes by 2DGE, however 100's of animals were used (Zhang and others, 2006). It is interesting to note that using single animals, we discovered similar patterns of up-regulation in the parthenogenic vs. the sexual phenotype.



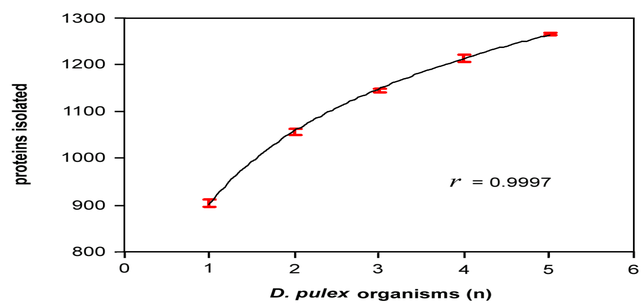
Digitally enhanced, superimposed gel image of individual *Daphnia magna* of distinct phenotypes. Red represent spots unique to the sexual *D. magna,* green represents spots unique to parthenogenic *D. magna,* yellow represents spots common to both phenotypes.

Extending approach to single D. pulex

As *D. magna* is the largest of the *Daphnia* genus, we used PCT to extract protein from single *D. pulex* to demonstrate that our technique would be feasible with smaller samples. Even when using the much smaller *Daphnia pulex,* we were able to detect 904 ± 7 spots from a single individual. It is reasonable to expect that PCT and 2DGE would reveal differences between single *D. pulex* with phenotypic or genotypic differences. As an indication of reproducibility of our method, we ran duplicate 2DGE of PCT extracted proteins from 1, 2, 3, 4 and 5 *Daphnia pulex*. We were able to detect 1267 ± 3 spots from 5 *Daphnia* pulex. The low standard deviation indicates that PCT and 2DGE provide an efficient and highly reproducible method of sample preparation and protein detection.



Representative gel of single *Daphnia pulex*. Silver staining revealed 904 ± 7 spots.



Graph indicating the number of spots revealed after silver staining of up to 5 *Daphnia pulex*. The low standard deviation indicates that PCT coupled with 2DEGE is an effective and reliable method of protein isolation and detection.

Conclusions

PCT, which rapidly cycles pressure, has been shown to be an effective means for isolating proteins from a variety of microorganisms, as well as many difficult to lyse samples such as *Caenorhabditis elegans* (Smejkal and others, 2007; Geiser and others, 2002; Smejkal and others, 2006b). PCT facilitated extraction of proteins from single *Daphnia magna* with distinct phenotypes (sexual vs. parthenogenic) and with distinct genotypes (Iinb1vs Xinb3) and from single *Daphnia pulex*. 2DGE and silver staining revealed that protein expression differences between individual *Daphnia* can be detected. We predict that the proteins from a single *Daphnia* 2D gel, while biased towards the most abundant proteins, represent a functionally diverse set of proteins. The highly sensitive technique of using PCT to extract proteins, coupled with 2DGE represents an important step to a greater understanding of individual variation of gene and protein expression, how individuals interact with their environment on a molecular level and is critical to advancing the field of individualized molecular medicine.

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