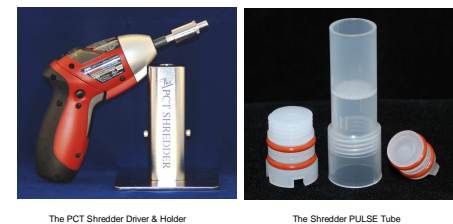


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1. ABSTRACT

The purpose of these experiments was to develop an improved method to lyse the nematode *Caenorhabditis elegans* for proteomic studies. The tough exterior cuticle of *Caenorhabditis elegans* makes the nematode resilient to lysis. This resilience to lysis impedes proteomic analyses. Proteomic analyses are even more difficult when preservation of the native conformation and biological activity of proteins are desired, thus prohibiting the use of denaturing chaotropes or detergents to enhance lysis. In the course of our studies, we determined that in physiological buffers, these nematodes are so resilient that they can even withstand hydrostatic pressure cycling technology (PCT) up to 20,000 psi with a 2.3% survival rate. However, by combining pressure and nondenaturing buffer with pre-processing using PBI's new *PCT Shredder*, it is possible to achieve nearly total disruption of the nematodes and maximal protein yields. In these experiments, the nematodes were mixed with silicate (SiC) abrasive and frozen directly in a Shredder PULSE Tube™, a specialized container used in both *The PCT Shredder* and subsequent high pressure treatment in a PBI Barocycler. To achieve optimum lysis, the frozen sample-abrasive mix was first ground with *The PCT Shredder*. The sample was then subjected to PCT. Damage to cuticles was evaluated by Trypan blue permeability. By comparison to other processing methods, *The PCT Shredder* in combination with PCT disrupted virtually all nematodes in a heterogeneous culture, whereas when processed by sonication, some larval stage nematodes remained undamaged. Furthermore, temperature fluctuations during processing by thermostated bead beating and sonication resulted in highly variable protein recoveries when compared to the PCT method of processing. In addition, large protein aggregates were observed under a microscope in bead beating and sonication preparations, but were not present in *The PCT Shredder* preparations. Moreover, protein denaturation and precipitation was observed in bead beating, resulting in the gradual loss of soluble protein over repetitive cycles; this did not occur with *The PCT Shredder* and PCT.



2. INTRODUCTION

The tough exterior cuticle of *Caenorhabditis elegans* makes the nematode very resistant to lysis and impedes proteomic and glycoproteomic analyses. So resilient is this organism, that specimens sent into space were the only survivors of the disastrous crash of the space shuttle Columbia [1]. Our experiments showed that in relatively mild physiological buffers, nematodes can withstand brief exposure to hydrostatic pressure up to 45,000 psi. Live worms were observed after 20 pressure cycles in which the pressure was sustained at 20,000 psi for 20 seconds during each cycle. After 40 cycles, 100% of the worms were killed, but negligible disruption of the cuticle was observed by microscopy. Damage to adult hermaphrodites evaluated by Trypan Blue staining was minimal, and led to the expulsion of embryos and some detachment of basement membranes from the cuticle, while dauer stage larvae were more resistant to high pressure. The poor disruption observed by microscopy correlated with minimal protein recovery.

Frequently, downstream analyses requires the preservation of molecular conformation and biological activity of proteins, as well as the stabilization of protein complexes. Chaotropes and detergents alter the native state of proteins and are incompatible with affinity chromatography, ELISA, and

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immunoprecipitation methods and prohibitive of direct analysis by mass spectrometry. However, without such stringent reagents, total protein yields may be curtailed by an order of magnitude and may also be biased towards hydrophilic proteins. Even when thermostated, temperature fluctuations during bead beating and sonication affects reproducibility and can result in losses from protein aggregation and precipitation [2]. Higher protein yields in physiological buffers were obtained using *The PCT Shredder*.

3. MATERIAL AND METHODS

3.1 Nematode cultures

Heterogeneous *C. elegans* N2 wild type populations (larval through adult hermaphroditic stages) were collected by washing the surfaces of 2% agarose cultures twice with 50 mM K₂PO₄ pH 7.2. The washes were combined and the biomass was pelleted centrifugally. The pellet was additionally washed to remove residual *Escherichia coli* and the live nematodes were concentrated in an Ultrafree-CL centrifugal filtration device.

3.2 Pressure Enhanced Processing (PrEP) Kits

The PCT Shredder Kit includes Shredder PULSE Tubes, cordless Shredder Driver, and spring-tensioned Shredder Holder with pressure indicator. The CE PrEP Kit includes the ProteoSOLVE CE Lysis Reagent, ion-exchange resin, ProteoSOLVE Reducing Reagent, and low protein binding abrasive particles. Both kits are available from Pressure BioSciences (South Easton, MA).

3.3 Frozen Abrasive Shredder Technique (FAST)

Fifty milligrams of live worm paste, 100 mg of abrasive particles, and 50 uL of protease inhibitor cocktail (Sigma-Aldrich, St. Louis, MO) were added to the ram side of the PULSE Tube. (As much as 250 mg of paste and 250 mg abrasive can be processed in a single PULSE Tube.)

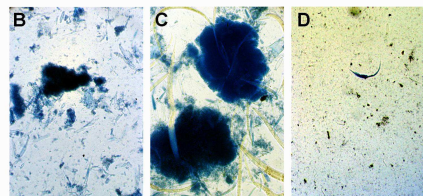
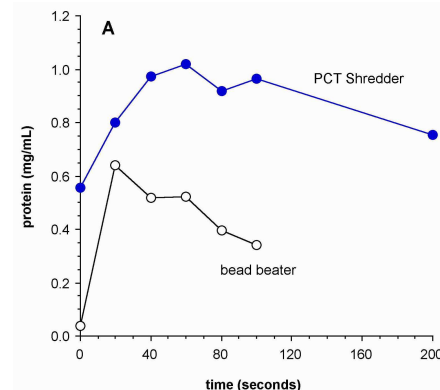


Figure 2. (A) Comparative protein yields following sequential 20 second cycles of either PCT or bead beating. In physiological buffers, the gradual denaturation, aggregation, and precipitative loss of proteins was observed in bead beating preparations. **(B)** Protein aggregates produced from 3X 10 seconds bead beating. **(C)** Aggregates produced from 3X 10 seconds sonication. Trypan Blue permeability was observed in only 55% of the visually intact nematodes. **(D)** Complete disruption of nematodes without the formation of aggregates in PCT Shredder preparation.

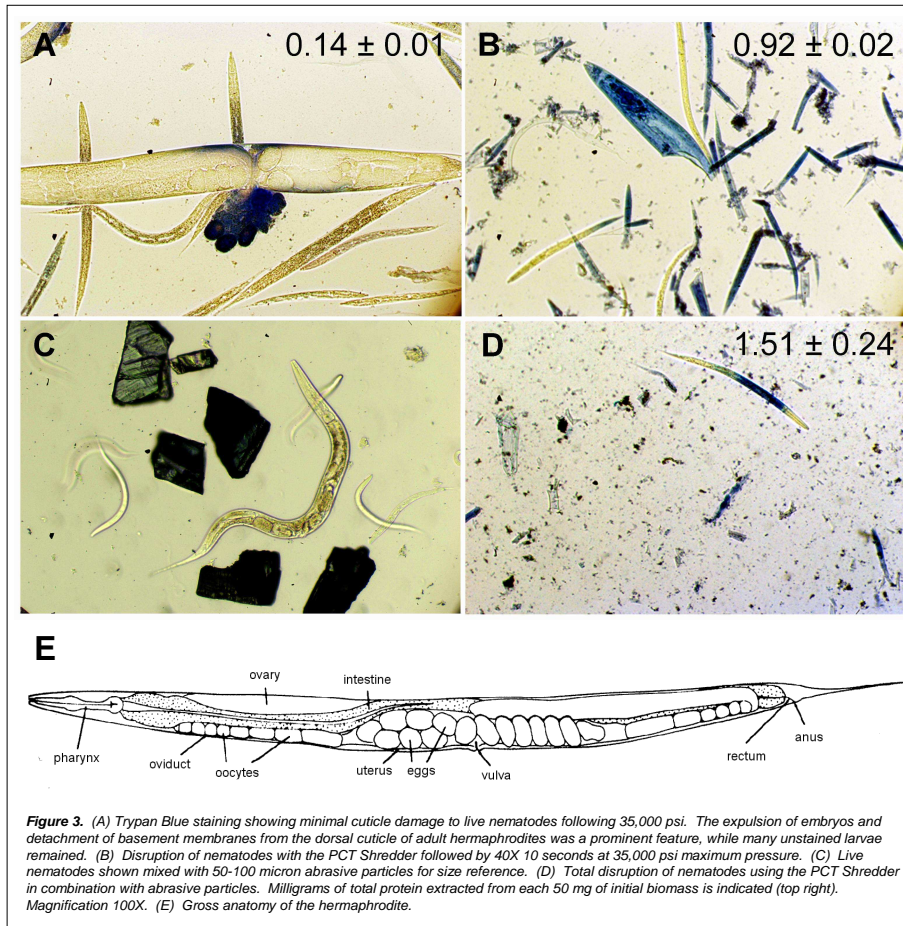


Figure 3. (A) Trypan Blue staining showing minimal cuticle damage to live nematodes following 35,000 psi. The expulsion of embryos and detachment of basement membranes from the dorsal cuticle of adult hermaphrodites was a prominent feature, while many unstained larvae remained. **(B)** Disruption of nematodes with the PCT Shredder followed by 40X 10 seconds at 35,000 psi maximum pressure. **(C)** Live nematodes shown mixed with 50-100 micron abrasive particles for size reference. **(D)** Total disruption of nematodes using the PCT Shredder in combination with abrasive particles. Milligrams of total protein extracted from each 50 mg of initial biomass is indicated (top right). Magnification 100X. **(E)** Gross anatomy of the hermaphrodite.

A serrated ram was inserted and the completed assembly was vortexed, then flash frozen on dry ice for 5-10 minutes. The PULSE Tube was then engaged in the Shredder Holder and the frozen sample was rotationally ground with the Shredder Driver until the entire sample was expressed through the perforations of the stationary disc.

3.3 Pressure Cycling Technology (PCT)

1300 uL of 50 mM K₂PO₄ buffer was added to each PULSE Tube and vortexed. 100 uL of the suspension was reserved as negative control. PCT was performed in the Barocycler NEP 3229, typically for 20-60 cycles at 35,000 or 45,000 psi maximum pressure. Following PCT, the PULSE Tubes were evacuated. Abrasive particles and any nematode debris were pelleted centrifugally at 10,000 RCF for 10 minutes. The supernatants were reserved for protein assay and the pellets were examined microscopically following Trypan Blue staining.

4. RESULTS AND DISCUSSION

In physiological buffers, nematodes can withstand high pressure as evidenced by Trypan Blue permeability of only 10-14% of nematode cuticles following 20 cycles at 35,000 psi maximum pressure. Adult hermaphrodites were selectively destroyed at this pressure, while larvae were more resilient (Figure 3A).

The PCT Shredder disrupted all nematodes including dauer stage larvae (Figure 3B). In physiological buffers, protein yields were six times greater with *The PCT Shredder* than with high pressure alone, and an order magnitude higher when *The PCT Shredder* was used in combination with freezing and abrasives (Figure 3C and 3D). Protein yields were more than doubled when the chaotropic ProteoSOLVE CE Lysis Reagent and ProteoSOLVE Reducing Reagent provided in the CE PrEP kit were used (not shown).

By comparison, temperature fluctuations during thermostated bead beating resulted in highly variable protein recoveries. Further, large protein aggregates observed microscopically in bead beating preparations were not observed in *The PCT Shredder* preparations (Figure 2). Moreover, protein denaturation was observed in bead beating preparations, resulting in the gradual loss of soluble protein with each successive cycle.

5. REFERENCES:

[1] Szewczyk, N.J. *et al.* (2005). *Astrobiology*, 5, 690-705.
 [2] Smejkal, G.B. *et al.* (2008). *HUPO 7th Annual World Congress*, Amsterdam, August 16-20, 2008.