Pressure Cycling Technology (PCT) mediated sample preparation schemes for isolating proteins from the nematode *Caenorhabditis elegans*

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1. Introduction

The tough exterior cuticle of *Caenorhabditis elegans* makes this 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. In relatively mild physiological buffers, nematodes can withstand brief exposure to hydrostatic pressure up to 45,000 psi. We have developed several new Pressure Cycling Technology (PCT) mediated methods and reagents for extracting proteins from *C. elegans* nematodes in both denatured and native state for proteomic and biochemical analysis. The first method relies on chitinase, an enzyme that weakens the cuticle prior to pressure-mediated disruption. When Pressure Cycling Technology (PCT) was performed in physiological buffer optimal for chitinase activity, biologically active proteins were recovered. The second method utilizes the ProteoSolve-CE Lysis Reagent, which effectively disrupts the collagen-rich basement membrane beneath the cuticle. This facilitates the expulsion of nematode tissues from the cuticle under high pressure, leaving only a cuticle "ghost" behind, and exemplifies the synergistic effects of using optimized chemistries in combination with high pressure. The last method relies on the mechanical disruption of frozen nematodes followed by PCT-driven dissolution of proteins, resulting in a high yields of proteins in their native conformation.

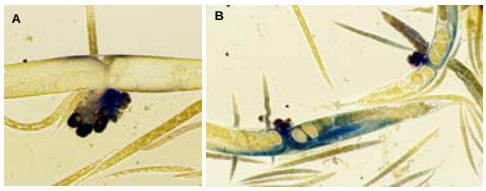


Figure 1. *Detachment of basement membranes from the dorsal cuticle and expulsion of embryos from live hermaphrodites at high pressure in physiological buffer. PCT was performed at (A) 35,000 psi and (B) 45,000 psi without optimization. (An optimized protocol is described below.) Trypan Blue permeability was observed in 15.5% ± 2.1% and 33.3% ± 5.1% of counted nematodes (n ≥ 200), respectively. All micrographs at 100X magnification.*

2. Extraction of native proteins in physiological buffers

2.1 The challenge

Frequently, downstream analyses require 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 immunoprecipitation methods and prohibitive of direct analysis by mass spectrometry. However, without such stringent reagents, total protein yields may be diminished 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 due to protein aggregation and precipitation (**Figure 4B and C**). Higher protein yields in physiological buffers were obtained using an optimized PCT method described herein.

The resilience of *C. elegans* is attributed to the chitinous exoskeletal cuticle which imparts chemical and pressure resistance. 2.2% of the culture survived 20 pressure cycles at 20,000 psi in physiological buffer. At higher pressures, all worms were killed following 20 or more pressure cycles, but as indicated by Trypan Blue permeability, measurable damage to the cuticle was observed in less than half of the worms (**Figure 1**). An LD50 of 7,400 psi is estimated (**Figure 7**).

2.2 Enzymatic hydrolysis of the chitin exoskeleton

Chitinase from *Trichoderma viridae* or *Streptomyces griseus* was used at 0.1 units per milligram of nematode mass. Collagenase from *Clostridium histolyticum* was used at 5.8 units per milligram of nematode mass. Samples were incubated with each enzyme for two hours at 37°C.

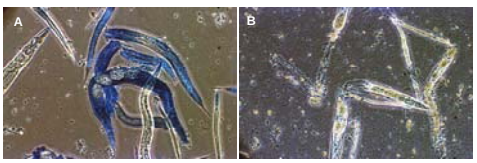


Figure 2. *Phase contrast microscopy of nematodes (A) incubated two hours with Trichoderma chitinase (0.1 U/mg) then followed by (B) subjecting the chitinase treated worms to 60 cycles of PCT at 45,000 psi. Nematodes were Trypan blue stained to demonstrate permeability of the cuticle.*

Total disruption of nematode cuticles using the ProteoSolve-CE Reagent

The ProteoSolve-CE Lysis Reagent was developed for the effective disruption of *C. elegans* and other recalcitrant biological samples. ProteoSolve-CE Lysis Reagent facilitates the extensive disruption of nematode cuticles resulting in as much as one order of magnitude greater protein yields than from conventional lysis reagents. ProteoSolve-CE is a urea-based reagent that uses a proprietary detergents and reducing agent to maximize total protein yields from cells, tissues, and small organisms and is recommended for use in PCT-mediated sample preparation schemes.

Figure 5. *Residual cuticle "ghosts" following total disruption of nematodes by PCT in ProteoSolve CE Lysis Reagent at 35,000 psi maximum pressure. Total detachment of the basement membrane from the cuticle allows PCT to work analogously to "squeezing toothpaste completely from the tube".*

Nematodes were stained with Trypan Blue and cuticle damaged evaluated microscopically within 10 minutes of staining. Trypan blue staining was observed in 5.7% untreated nematodes and in 92.3% of chitinase treated nematodes prior to PCT. 100% disruption of cuticles was observed following 60 pressure cycles at 45,000 psi maximum pressure (**Figure 2B**). Protein yields were increased 24.3% over unpressurized controls. A major disadvantage of using chitinase is that commercial preparations of this enzyme contribute much extraneous protein, and in one experiment, 79.4% ± 0.3% of the total protein recovered was determined to be of *Trichoderma* origin. Collagenase treatment did not significantly increase protein yields.

2.3 Rapid freeze-thaw cycles

Rapid freeze-thaw cycles were performed directly in PULSE Tubes (**Figure 3**). Samples were flash frozen on dry ice for 5 minutes, followed by thawing at 30 C. Eight freeze-thaw cycles were performed within one hour, with samples reserved after 0, 1, 2, 4, and 8 cycles. Eight freeze-thaw cycles followed by PCT for 60 cycles at 35,000 psi yield nearly four times more protein than unfrozen control. Trypan Blue permeability was increased from 6.3% to 24.1%.

2.4 Optimized PCT method for extraction of native proteins in physiological buffer

An optimized method in which frozen nematodes were mechanically processed directly in the PULSE Tube prior to PCT maximized protein yields under non-denaturing conditions. Live nematodes were harvested by washing the surfaces of culture plates. Worms were centrifuged and the resulting pellets were washed two times to remove growth media. A "worm paste" was produced by collecting the liive worms on a 0.8 micron filter under low vacuum. For PCT, samples of nematode paste (40-50 mg) were weighed into tared PULSE Tubes, frozen in liquid nitrogen and ground using the PCT Shredder device (Pressure BioSciences, South Easton, MA) followed by addition of 1.4 mL of 50 mM KPO_4 pH 7.4 containing protease inhibitors. PCT conditions were as described below.

The optimized PCT protocol in which frozen nematode pellets are ground inside the PULSE Tube yielded six times more protein than standard PCT in mild physiological buffer. (Both performed for 40 cycles at 35,000 psi.) **Figure 4A** shows the mechanical disruption of the cuticles prior to PCT resulting in a nearly 8-fold increase in Trypan Blue permeability over standard PCT, based on counts of the few remaining nematodes following PCT, from 10.7% 0.1% to 77.7% ± 0.5%.

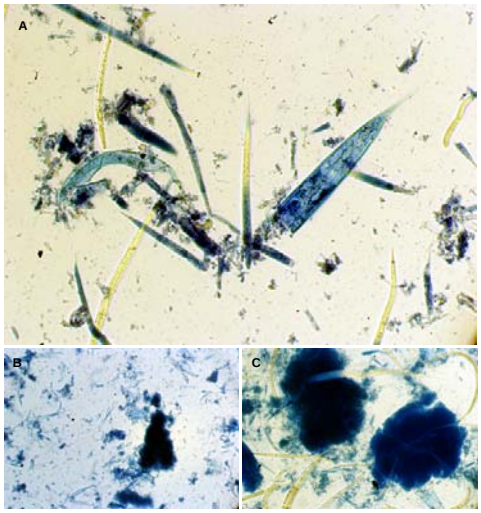


Figure 4. ***(A)*** *Optimized PCT method for the extraction of native proteins under non-denaturing conditions in which live worms are frozen and macerated directly in the PULSE Tube followed by 40 pressure cycles at 35,000 psi. Protein yields were six times greater than standard PCT. Protein yields were slightly less at 45,000 psi, presumably because proteins begin to denature and aggregate at this pressure.* ***(B)*** *Protein aggregates formed after 3X 10 seconds bead beating with 0.7 mm zirconium beads.* ***(C)*** *Even larger protein aggregates and numerous undisrupted nematode larvae following 4X 10 seconds sonication. Trypan Blue permeability was observed in only 55% of the visually intact nematodes. Buffer was 50 mM K_3PO_4 pH 7.4.*

3. Use of strong denaturants for the extraction of proteins

The analysis of protein primary structures including methods such as SDS PAGE, 2DGE, and mass spectrometric analyses of peptides from tryptic digests of proteins following electrophoretic separation permits the use of powerful denaturants and reducing agents without interference. While denaturing, the use of detergents or organic solvents greatly improves the recovery of extremely hydrophobic proteins that might otherwise be excluded from the analysis.

The ProteoSolve-CE Lysis Reagent was developed specifically to maximize protein yields from *C. elegans* (**Figure 5**), but has also been shown to be effective for a wide range of recalcitrant samples. Used in combination with PCT, the high detergency and reducing capacity of this reagent effectively disrupts collagen-rich basement membranes of the nematodes such that internal components are extruded from cuticles under pressure. Protein recoveries were nearly an order of magnitude greater than in physiological buffers, while solubility of hydrophobic proteins is maintained for downstream IEF and 2DGE applications.

Table 1: Protein yields and Trypan Blue permeability of *C. elegans* using PCT-mediated methods.

method	protein assay (mg/mL)	Trypan Blue permeability (%)	comments
negative buffer control	0.053 ± 0.007	3.3	*nondenaturing*
standard PCT	0.118 ± 0.047	10.6	*nondenaturing*
chitinase, PCT	0.184 ± 0.002	-	*nondenaturing, contamination from chitinase source*
freeze-thaw, PCT	0.150 ± 0.015	24.1	*nondenaturing*
PCT Shredder	0.654 ± 0.011	77.8	*nondenaturing*
ProteoSOLVE CE Reagent	1.042 ± 0.018	-	*strongly denaturing*

4. Synchronization of *C. elegans* cultures

Unless synchronized, C. elegans cultures exist as a continuum of embryonic, larval (L1 through L4), and adult hermaphroditic stage nematodes. Thus, synchronization of cultures becomes necessary to study specific larval stages of the organism. This necessarily involves the complete eradication of all larval and adult stage nematodes in a manner that only viable embryos are produced. Typically, this is done chemically by incubating the heterogeneous population in alkaline hypochlorite solution (140 mM NaOCl, 250 mM KOH) for the destruction and dissolution of nematodes to release viable embryos. The embryos are then recovered centrifugally, but must be copiously washed to remove all traces of hypochlorite before they can be used to produce synchronized cultures.

Dead nematodes produced by PCT at 30,000 or 40,000 psi were used to seed new cultures with apparent synchronization. Microscopic assessment of the pellets, including Trypan Blue permeability assay, confirmed 100% mortality of both larval and adult nematodes, and regrowth on new plates evidences the viability of residual embryos. However, at 60,000 psi, no regrowth was observed suggesting that at this higher pressure, embryos are effectively destroyed. The proposed method reduces the procedural complexity of chemical methods and eliminates the possibility of carrying over harsh chemicals detrimental to the downstream.

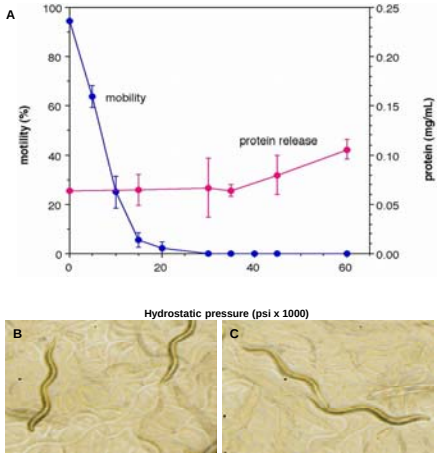


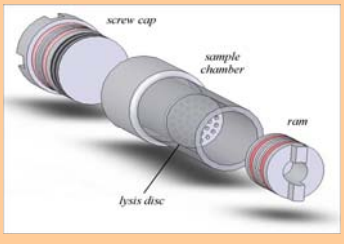
Figure 7. *(A) Survival rate of C. elegans (based on nematode motility) and synchronized cultures produced following total eradication of live nematodes by (B) PCT at 30,000 psi or (C) 40,000 psi.*

5. Summary

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. For the extraction of proteins from *C. elegans* under non-denaturing conditions, pretreatment with chitinase increased protein yields 24.3% when followed by PCT. However, chitinase contributes much extraneous protein from the enzyme source organism. The best results were obtained using the PCT Shredder, in which frozen nematode pellets are ground inside the PULSE Tube yielded six times more protein than standard PCT in non-denaturing buffers.

The highest protein yields were obtained under denaturing conditions using the ProteoSolve-CE Lysis Reagent

Pressure Cycling Technology (PCT)



PCT uses alternating cycles of hydrostatic pressure for the effective disruption of cells and tissues. Rapid cycling between high and low pressure has been demonstrated to be more disruptive than sustained high pressure. Unless otherwise specified, *C. elegans* were processed for 20 pressure cycles. Each cycle consisted of 10 seconds at maximum pressure (0-60,000 psi), followed by rapid return to atmospheric pressure and hold for 10 seconds. Total elapsed time at maximum pressure was 200 seconds. Total sample processing time was 7 minutes. The Barocycler NEP-3229 or NEP-2320 instruments, PULSE Tubes FT500 and FT500-ND, and the ProteoSolve-CE Lysis Reagent Kit were from Pressure BioSciences (South Easton, MA, USA).

Figure 3. *Exploded view showing the components of the PULSE Tube FT-500. Under high pressure, the ram forces tissue and fluid through the perforated lysis disc. Upon return to ambient pressure, the ram retracts pulling in solvent from the other chamber.*