Breaking the Tough Cuticle of *Caenorhabditis elegans* Using Pressure Cycling Technology and Optimized Lysis Reagents: Giving the Worms a Perm

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Abstract

The tough exterior cuticle of *Caenorhabditis elegans* makes the nematode resilient to lysis and impedes proteomic and glycoproteomic analyses. The cuticle is comprised largely of chitin and of collagen that is enriched in N- and C-terminal cysteines, most of which are presumably involved in disulfide linkages. The use of Pressure Cycling Technology (PCT) with nematode suspensions supplemented with high concentrations of reducing agent facilitated the effective rupture of the cuticle and the release of proteins. PCT uses alternating cycles of high and low pressure to efficiently extract proteins, nucleic acids, and small molecules from cells, tissues, and entire organisms. Nematode suspensions were placed in specially designed PULSE Tubes and subjected to alternating cycles of 40,000 PSI and ambient pressure. The suspensions were subjected to 10 or 20 pressure cycles, each cycle consisting of 30 seconds at 40,000 PSI followed by 10 seconds at ambient pressure. PCT performed for 10 and 20 cycles yielded 8.8% and 10.9% more total protein, respectively, than was derived by bead mill oscillation as determined by Bradford assay. The chemical degradation of the cuticle involved the use of dithiothreitol (DTT) at high concentrations. These experiments suggest that DTT promotes more aggressive lysis of the cuticle via disulfide reduction than tributylphosphine (TBP). This may be due to the instability of TBP, which rapidly oxidizes to Bu3PO in aqueous solutions.

Introduction

No other multicellular organism has been studied more extensively at the cellular and molecular levels than the nematode *Caenhoribditis elegans* [1]. However, the tough exterior cuticle makes the nematode resilient to lysis and impedes proteomic and glycoproteomic analyses (*Figure 1*). The cuticle is comprised largely of chitin and of a collagen that is particularly rich in *N*- and *C*-terminal cysteines, most of which are involved in disulfide linkages. Enzymes such as chitinase or collagenase could be used to weaken the cuticle. However, unless these enzymes are immobilized, they would contribute exogenous protein that would be present in downstream analyses. Further, enzymatically induced cell lysis has been shown to be biased towards the recovery of cytoplasmic and membrane proteins and other hydrophobic proteins, which results in a misrepresentation of the proteome in the lysate [2]. Inspired by earlier chemists who developed the "permanent wave" in which the cuticle of human hair is first softened in a chemical process that reduces protein disulfides, the ProteoSOLVE Lysis Reagent was designed specifically for maximal disruption of the *C. elegans* cuticle and increased protein yield. The reagent contains a proprietary detergent and reducing agent. Both the type and the concentration of reducing agent were optimized to maximize the efficiency of nematode cuticle disruption.

Use of the alternative reducing agent, tributylphosphine (TBP), was less effective than ProteoSolve when high disulfide concentrations are present. This is likely related to the instability of TBP, which oxidizes within minutes to Bu3PO [3]. When used in combination with PCT, ProteoSOLVE Lysis Reagent produced higher protein yields than other disruption methods.

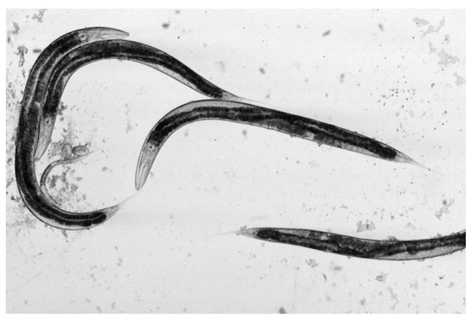

Figure 1. Micrograph of *C. elegans* nematodes showing recalcitrant cuticle.

Pressure Cycling Technology (PCT)

PCT uses alternating cycles of high and low pressure to induce cell lysis. Cell suspensions or tissues are placed in specially designed single-use PULSE Tubes (*Figure 2*) and are subjected to alternating cycles of high (up to 35,000 PSI) and ambient pressure in a pressure-generating instrument (Barocycler Models NEP2017 or NEP3229). Maximum and minimum pressures, the time sustained at each pressure level, and the number of cycles is defined using a computer or programmable logic controller interface. The Barocycler instrument reaction chambers are temperature controlled using a peripheral circulating water bath. Safety features in the PCT system design significantly reduce risk of exposure to the researcher to pathogens [4].

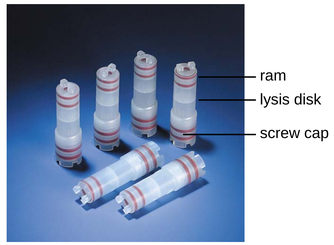

Figure 2. PULSE Tubes FT500 can hold 20 – 500 mg of solid sample or 1.2 – 1.5 mL of liquid sample. The sealed PULSE Tubes are immersed in pressurization fluid in the two reaction chambers of the Barocycler NEP3229 and are then subjected to cycled pressure. The recovered volume of PCT prepared samples is between 0.8 and 1.4 mL. Samples were placed on the lysis disk in the PT and subjected to 10 pressure cycles in the Barocycler instrument. The combination of physical passage through the PT lysis disk, rapid pressure changes, and other bio-physical mechanisms effectively induce cell lysis.

Figure 3. The Barocycler NEP3229 can raise pressure from ambient to 35 kpsi (235 MPa) in less than 5 seconds and ramp down pressure from its maximum to ambient instantaneously. Three PULSE Tubes can be processed simultaneously. The process temperature can be controlled using an external circulating water bath, allowing experiments to be performed between 4 and 37°C.

Methods

Fifty milligrams of lyophilized *C. elegans* were suspended in 1.4 mL ProteoSOLVE Lysis Reagent in each PULSE Tube. Fourteen microliters of water-soluble protease inhibitor cocktail (Sigma, St. Louis, MO, USA) was added and pressure cycling was performed for 10 or 20 cycles. Each cycle consisted of 40 seconds at 35,000 PSI (235 MPa), followed by 10 seconds at ambient pressure. Alternatively, 1.4 of the *C. elegans* suspension, 14 mL protease inhibitor cocktail, and 100 mg of 1 mm glass beads (BioSpec, Madison, WI, USA) was added to a standard Eppendorf tube and processed for five 15 second cycles at 2,400 oscillations per minute on a BioSpec Mini-Beadbeater. The sample was placed on ice between cycles. As a negative control, 1.4 mL of the same nematode suspension was incubated 30 minutes at 25º C without mechanical agitation. The lysates were centrifuged 10 minutes at 10,000 RCF to pellet cellular debris. The supernatants were alkylated for two hours following the addition of 40 mM Tris and 200 mM acrylamide monomer. Protein concentrations were determined with the Bradford Reagent (BioRad, Hercules, CA, USA). The supernatants were then ultrafiltered and exchanged into 7M urea, 2M thiourea, and 65 mM CHAPS.

IEF was performed as described by Smejkal *et al.* [2]. Immobilized pH gradients (IPGs), ProteomIQ Equilibration Reagent, and ProteomIQ Blue Polyacrylamide Gel Stain were from Proteome Systems (Woburn, MA, USA). Following IEF, the IPGs were equilibrated twice for ten minutes in ProteomIQ Equilibration Reagent that was supplemented with an additional 0.5% SDS. Second dimension PAGE was performed on 12% polyacrylamide gels. Gels were stained with the ProteomIQ Blue reagent.

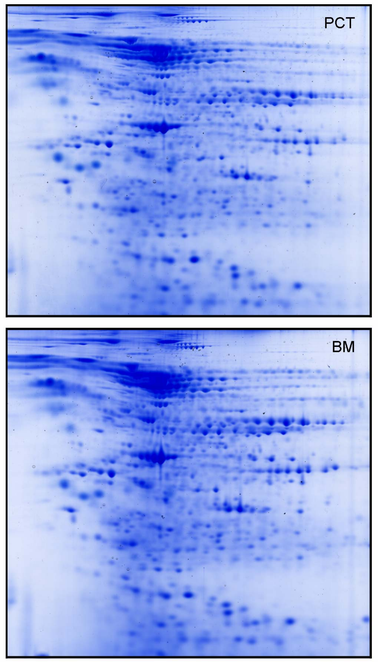

Figure 4 . 2DGE of C. elegans lysates derived from PCT (top) or bead mill oscillation (bottom). IPG pH 3-10 was hydrated with 0.2 mL of each lysate diluted 1:6.

Results and Discussion

ProteoSOLVE, when used in combination with PCT, produced maximal protein yields, PCT performed for 10 and 20 cycles yielded 8.8% and 10.9% more total protein than was derived by bead mill oscillation as determined by Bradford assay (Table 1). The bead mill lysate yielded a similar amount of protein, as the negative control in which there was no mechanical agitation. This implies that *C. elegans* resistance to disruption is perhaps overstated, particularly when reagents that exploit structural characteristics of the cuticle are used to weaken the integument.

Table 1. Protein concentration of PCT and bead mill lysates

method	total protein recovered*	% increase
negative control	6.94	-
bead mill	6.91	-
PCT, 10 cycles	7.52	8.8
PCT, 20 cycles	7.66	10.9

*milligrams protein recovered from 50 mg lyophilized *C. elegans* dry weight.

Summary

The PCT Sample Preparation System (PCT SPS) Extraction Method:

- Employs a new approach for the extraction of biomolecules from cells and tissues, including difficult-to-lyse materials, such as nematodes with a strong exoskeleton
- Functions by different mechanisms to release biomolecules compared to other commonly used mechanical and chemical extraction methods
- Is rapid, safe and reproducible
- Is fully contained to minimize cross contamination and the exposure of operators to toxic or infectious agents
- Is capable of releasing proteins small molecules, and nucleic acids into buffers suitable for downstream purification or direct analysis

References

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