Isolation of Mitochondria from Rat Lung Using *The PCT Shredder* and Pressure Cycling Technology (PCT)

Introduction

Isolation of intact mitochondria from human and animal tissue is crucial for studies in aging, diabetes and cancer. High quality functional mitochondrial isolates are also important for drug screening studies [1]. Mitochondria isolation from solid tissue is usually performed by labor-intensive homogenizer-based methods [2] that require extensive operator experience. To facilitate efficient and reproducible mitochondria preparation, we have developed a semi-automated method to release mitochondria from solid fibrous tissues, such as lung, using *The PCT Shredder* for tissue homogenization. This method can be combined with a subsequent treatment using pressure cycling technology (PCT) to further increase yield, if desired.

The PCT Shredder

The PCT Shredder is designed to quickly and gently grind fibrous samples such as plant leaves and animal muscle tissue. Since shredding is performed in a disposable sample container (Figure 1), the likelihood of cross contamination with other samples is very low. In addition, shredding for 10-20 seconds generates relatively little heat and thus does not increase protein denaturation or damage delicate organelles.

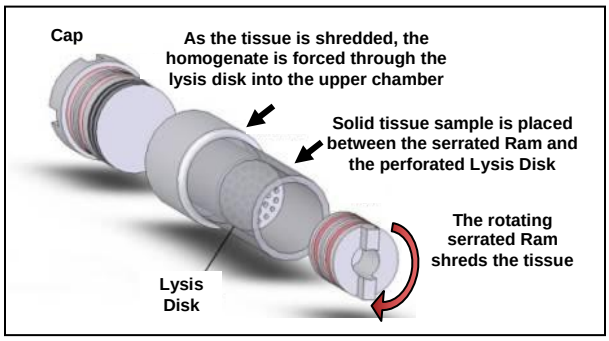

Figure 1. The FT500-S Shredder PULSE Tube As the tissue is shredded, the Shredder Ram of the PULSE Tube forces the homogenate through the holes of the Lysis Disk and into the upper compartment. This method reduces the risk of sample over-homogenization and mitochondrial damage.

Pressure Cycling Technology (PCT) - optional

PCT destabilizes molecular interactions by rapidly and repeatedly raising and lowering pressure in the reaction vessel from ambient to high pressures (up to 45,000 psi [310 MPa, 3000 Atm]).

High hydrostatic pressure acts preferentially on the more compressible constituents of the sample, leading to destabilization of molecular interactions, but not of covalent bonds. At lower pressures, e.g. 10,000 - 20,000 psi, PCT can lyse most cells and release their intracellular contents, including intact organelles. This method has been shown to be relatively gentle, and has been used to isolate mitochondria from cell culture [3].

Methods

Mitochondria-enriched fractions were prepared from frozen/thawed adult rat lung tissue. All steps were performed on ice or at 4˚C. Since the sample preparation was aimed at proteomic, rather than functional, mitochondrial assays, BSA was omitted from the isolation buffer to prevent BSA carry-over into the final samples.

Partially thawed tissue (0.6-0.9 g) was minced in buffer N2 (250 mM sucrose, 10 mM HEPES, pH 7.4) [4] or MMIB (120 mM KCl, 20mM HEPES, 5 mM $MgCl_2$, 1 mM EGTA, pH adjusted to 7.4 with KOH) [5] to remove residual blood.

Control tissue samples were transferred to a 50 mL glass homogenizer with a Teflon pestle and homogenized gently by hand. The homogenate was transferred to a centrifuge tube and the homogenizer was rinsed with additional buffer to remove remaining tissue. All subsequent steps were carried out as described below except the control was not subjected to *The PCT Shredder* or PCT treatment.

For tissue disruption with *The PCT Shredder*, each sample of minced and rinsed tissue was split into three FT500-S PULSE Tubes (0.2 - 0.3g of tissue per tube with 0.5 mL of buffer) and shredded for 10 seconds. The metal Shredder stand was pre-chilled to compensate for any heat that might be generated during shredding. Subsequently, the volume of each shredded sample was brought up to 1.4 mL per PULSE Tube with additional isolation buffer (N2 or MMIB, as appropriate). The samples (3 PULSE Tubes per sample) were either pressurized in a Barocycler at 10,000, 20,000 or 30,000 psi for 5 cycles at 4˚C, or were not subjected to pressurization (*Shredder* alone samples). Each cycle consisted of 20 seconds at high pressure and 5 seconds at atmospheric pressure.

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After PCT or control treatment, the contents of the 3 PULSE Tubes were pooled back together, and the samples were centrifuged for 8 minutes at 1000 x g. Any remaining lung tissue debris was carefully removed (in some cases a sample was spun a second time to remove remaining tissue) and the supernatant was transferred to a clean centrifuge tube. Removal of tissue debris was found to be easier from samples that had been pressurized at 20,000 psi; this may have occurred if the tissue was less buoyant due to the air sacs collapsing during pressure cycling. Samples that were pressurized to only 10,000 psi, or control tissue that was not pressurized, did not pellet easily.

The mitochondria-enriched fraction was pelleted from the supernatant by centrifugation for 8 minutes at 14,000 x g. The resulting pellet was gently suspended in 1.5 mL of fresh isolation buffer (MMIB or N2). The suspension was transferred to a 1.5 mL microfuge tube and was centrifuged a second time at 14,000 x g. The final pellet was suspended in 50 µL of isolation buffer by gentle homogenization with a small plastic pestle.

Results and Discussion

Here we describe a novel method for preparation of mitochondria-enriched fractions from frozen rat lung tissue suitable for proteomic applications. We demonstrate that mitochondria, extracted using *The PCT Shredder* with or without subsequent PCT treatment, are intact and can be used for proteomic studies and other applications equally as well as conventional methods.

Mitochondria were efficiently extracted when tissue was homogenized by a ten second disruption in *The PCT Shredder*. Yield was significantly increased when shredding was followed by PCT treatment for 5 cycles at 20,000 psi (Figure 2). The yield of mitochondria was consistent whether the isolation was performed in salt-based buffer (MMIB) or in sucrose-based buffer (N2). Additional cycles of pressure (20,000 psi for 15 cycles) did not improve yield.

In Figure 3, 2D PAGE results indicate that the protein profiles of control mitochondria samples, isolated solely by a homogenizer, are essentially the same as the test samples prepared using *The PCT Shredder* with or without subsequent PCT treatment.

Coomassie blue-stained SDS-PAGE gels and Western blots (Figure 4) confirm that mitochondrial samples isolated by a homogenizer are essentially the same as samples prepared using *The PCT Shredder* with or without pressure cycling, indicating that this combination of methods can be used to safely, easily and reproducibly generate high quality mitochondrial preparations.

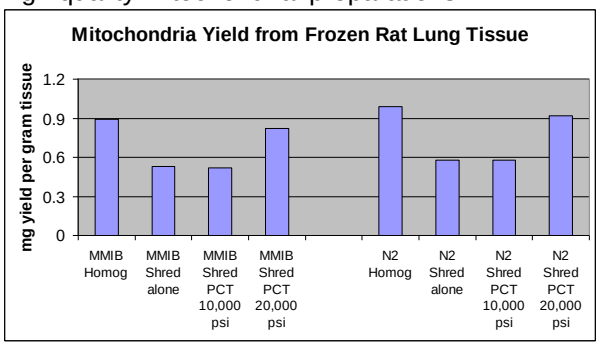

Figure 2. Yield of Mitochondria
Mitochondria were prepared in either MMIB or N2 buffer. Tissue was homogenized using a homogenizer, *The PCT Shredder*, or *The PCT Shredder* followed by PCT at 10,000 or 20,000 psi for 5 cycles.

In addition, Western blots confirm that the mitochondria preparations are enriched for protein markers for all three mitochondrial compartments, providing further evidence that the mitochondria are intact (Figure 4). The presence of GAPDH in all the preparations is likely due to carryover of cellular cytosolic material, which could be further reduced by additional washing of the mitochondrial pellet. The presence of actin in the mitochondrial pellet is expected, as it has been reported to be associated with mitochondria [6].

Two different buffers (N2 and MMIB) were used in this application to demonstrate that *The PCT Shredder* and PCT treatment can be used with different types of mitochondria isolation reagents for compatibility with various downstream methods.

Conclusion

We have demonstrated a convenient isolation method for intact mitochondria from frozen rat lung tissue using *The PCT Shredder* for initial tissue disruption, with or without subsequent PCT treatment. The mitochondria, isolated using this novel method, are comparable to controls and can be used for proteomic and other studies.

Similar methods have been used with fresh rat kidney and muscle tissue to extract functional

mitochondria [7]. Respiration assays of mitochondria extracted from freshly harvested tissue with and without pressure cycling confirmed that exposure to hydrostatic pressure of 10,000-20,000 psi does not damage mitochondria. As expected, mitochondria exposed to 30,000 psi are severely damaged, as indicated by very low respiratory control ratios.

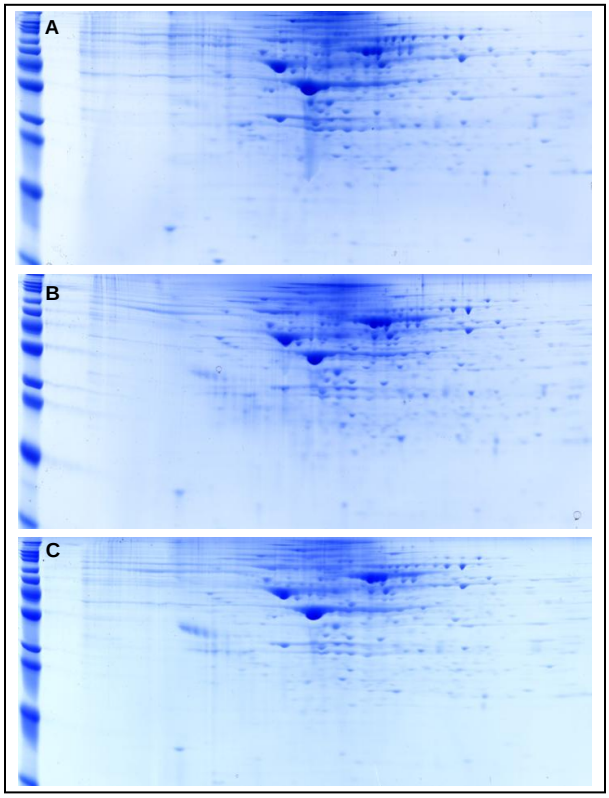

Figure 3. Total Protein Profile
Mitochondria prepared using a homogenizer (A); *The PCT Shredder* (B); or *The PCT Shredder* followed by PCT treatment at 10,000 psi (C) exhibited essentially the same protein pattern by 2D PAGE (125 µg per gel). Molecular weight markers are shown on the left of each gel.

Traditional manual homogenization methods for isolation of functional mitochondria from tissues rely heavily on operator training, experience and skill. Without considerable training, common mistakes, such as tissue over-homogenization, can result in damaged mitochondria, and thus might cause highly variable results. This simple, but effective, method significantly reduces the likelihood of sample over-homogenization. In addition, we demonstrated that overall yield of mitochondria from rat lung tissue can be increased if initial processing in *The PCT Shredder* is followed by a brief PCT treatment above 10,000 psi. This *Shredder*-based method, with or without subsequent PCT treatment, is simple, easy to master, and eliminates many of the problems of traditional manual mitochondria preparation methods.

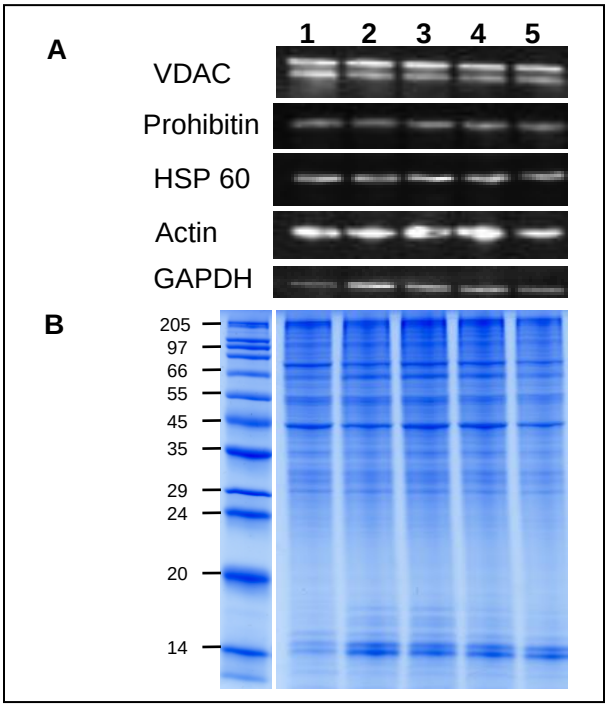

Figure 4. SDS-PAGE and Western Blot Analysis of Mitochondria Preparations in N2 Buffer Samples are as follows: 1. Homogenizer control; 2. Shredder alone; 3. Shredder with PCT at 10,000 psi for 5 cycles; 4. Shredder with PCT at 20,000 psi for 5 cycles; 5. Shredder with PCT at 20,000 psi for 15 cycles. **A**: Western blots were probed with antibodies to VDAC (outer mitochondria membrane), Prohibitin (inner mitochondria membrane), HSP60 (mitochondrial matrix), actin and GAPDH (cytosolic protein). **B**: SDS-PAGE was run on 8-16% tris-glycine gels and stained with Coomassie blue. Protein load was adjusted to 8 µg per lane. Molecular weight markers are shown on the left.

References

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