Proteomic Analysis of Adipose Tissue Using Detergent-Free Protein Extraction by Pressure Cycling Technology (PCT) and High Resolution Tandem Mass Spectrometry

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1. Introduction

Proteomic analysis of adipose tissue is of great importance for studies of type 2 diabetes, obesity, cancer, and many other human disorders [1-4]. However, conventional protein solubilization methods applied to tissues with high lipid content tend to produce highly variable results, especially with respect to important hydrophobic proteins from mitochondria, ER, plasma membrane, and fat droplets. Abundant sample-derived lipids tend to sequester detergents into micelles, thus interfering with protein extraction. Therefore, substantially fewer reports of proteomic investigation of adipose tissue samples have been issued compared to other mammalian tissues.

This study investigated the use of alternating hydrostatic pressure (pressure cycling technology, or PCT) and a variety of organic solvents for the detergent-free disruption of cells, micelles, and membrane fragments, as well as for the increased efficiency of protein recovery from mouse adipose tissue samples as compared to conventional homogenization and dissolution techniques. Resulting protein extracts were analyzed by SDS-PAGE, 2D-electrophoresis, nanoflow HPLC, and high-resolution high-mass accuracy tandem mass spectrometry. As a result, a novel pressure cycling-assisted liquid-liquid extraction and fractionation method was developed to achieve nearly complete tissue dissolution and the fractionation of lipids and proteins into distinct liquid phases. In addition to the overall greater protein recovery with the detergent-free PCT method, several novel protein species were identified in the PCT extracts of adipose tissue, indicating that proteins may be under-represented in the extracts obtained using conventional methods.

Analysis of several genetically distinct model mouse lines has revealed certain trends in protein expression, which may be linked to disease progression, or which may serve as potential "druggable" targets. Lipid fractions resulting from the fractionation were obtained and stored for lipid profiling studies.

2. Materials and Methods

2.1 Sample Preparation by PCT

The goal of this study was to develop a reliable and reproducible method for protein extraction from adipose tissue to enable future proteomic investigations of a murine disease model. White adipose tissue samples (abdominal fat pads) from wild type (WT) and obese (ObOb +/+) animals were used. Approximately equal aliquots (100±15 mg) of adipose tissue from several individual animals were prepared.

Simultaneous sample homogenization and fractionation was carried out in specialized individual single-use 1.4 mL containers (PULSE Tubes) using alternating hydrostatic pressure generated in the Barocycler NEP3229 (Pressure BioSciences, West Bridgewater, MA,) for 20 cycles at room temperature **(Fig.1)**. Each cycle consisted of 20 s at 35,000 psi followed by 20 s at atmospheric pressure. A protease inhibitor cocktail (Sigma-Aldrich, St. Louis, MO) was added to the extraction reagent in each case. Following the pressure cycling treatment, sample tubes were removed from the Barocycler and were briefly centrifuged to promote complete separation of liquid phases. Unless otherwise noted, the top liquid layer from each tube was removed with a gel-loading pipette tip and stored for subsequent analysis. Each polar fraction was desolvated in a SpeedVac centrifugal concentrator (ThermoFisher Scientific) to approximately 5-10 µL, and was subsequently reconstituted in either 2x Laemmli SDS-PAGE buffer (4% SDS) or in 2D sample buffer (9M urea, 4% CHAPS) to provide compatibility with the desired downstream analysis method **(Fig.2).**

The extraction of "Controls" was performed directly with 2D sample buffer in two consecutive steps: 50 µL aliquots of each extraction procedure were removed for protein assay and SDS-PAGE analysis, and extracts from the first and second round were combined, reduced, and alkylated using TBP/acrylamide [5] and were then concentrated to the original sample volume in Amicon ULTRA-4 ultrafiltration devices (Millipore Corporation, Danvers, MA) (**Fig. 4A**).

Figure 1. (A)** The Barocycler NEP3229 facilitates the extraction of proteins from tissue samples with the use of alternating levels of high and ambient hydrostatic pressure. This instrument requires single-use, processing containers called PULSE Tubes **(B).

2.2 Electrophoresis, image analysis, and in-gel digestion

SDS PAGE was performed on 4-12% polyacrylamide gradient gels. Immobilized pH Gradient strips (pH 3-10) were hydrated with samples for 6 h, followed by IEF for 100,000 Volt-hours at 10,000V. All pre-cast electrophoresis supplies and Criterion vertical gel electrophoresis system were from Bio-Rad Laboratories, Hercules, CA, while the IsoelectrIQ2 integrated IEF instrument was from Proteome Systems, Woburn, MA. Gels were stained with colloidal CBB or SYPRO Ruby [6], scanned, and analyzed with PDQuest software to determine the presence of statistically significant, differentially extracted proteins. Selected gel spots or bands were excised and processed using conventional in-gel digestion protocol. Sequencing grade modified porcine trypsin (Promega) was used for digestion.

2.3 Protein identification by nano-LC FTICR tandem mass spectrometry

Protein digests (5-10 µL) were injected onto a C18 solid phase extraction trapping column (300 µm i.d. x 5 mm, Dionex, CA) and onto a 75 µm i.d. x 15 cm nano-LC reversed-phase self-packed fused silica column (stationary phase: Magic C18AQ, 3 µm, 100 Å; Michrom Bioresources, MA). Peptide separation was carried out using a linear gradient of acetonitrile in 0.1% FA and the eluent was introduced into the LTQ FTICR mass spectrometer (ThermoFisher Scientific) by nanoelectrospray. Data analysis was conducted on the Sorcerer (Sage-N) search engine using SEQUEST algorithm and GPMDB software. The search was performed against a combined "forward" and "reverse" FASTA DB. The balance between the reliability and sensitivity of protein identification data was set by adjusting the estimated false positive protein identification rate (FPPrR) to ≤1%. Duplicate peptide matches were purged on the basis of Xcorr to eliminate redundancy caused by homological proteins and isoforms. Similar proteins were listed with the protein entry of the highest score without adding redundancy into protein IDs lists of DTASelect and Protein Prophet outputs.

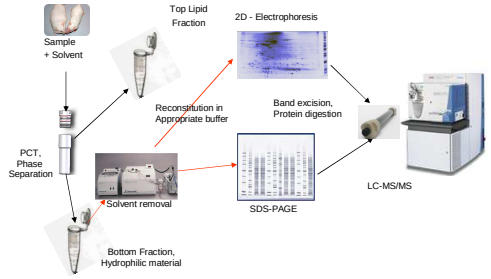


***Figure 2.** Adipose tissue: Lipid/Protein Fractionation Method. Alternating hydrostatic pressure is used to simultaneously homogenize the sample and promote partitioning of sample compounds between the poorly miscible liquid phases in a single disposable container. Resulting extract is directly compatible with LC-MS/MS applications and electrophoretic separation of protein.*

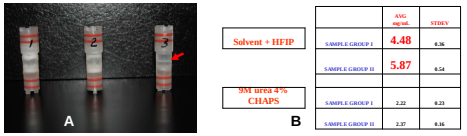


***Figure 3 A:** Detergent-free extraction of protein from adipose tissue. With current methods, high (up to 70% by weight) lipid content sequesters detergents into micelles, leaving the hydrophobic protein associated with the lipid mass, unless lipid mass is fully dissolved. Note the absence of residual fat tissue mass with the PCT detergent-free method (appears as white greasy residue) and a sharp phase boundary after the extraction in tube #3.*

***B:** Comparison of different extraction reagents. Protein concentration is determined by Bradford assay. Final volume of extract was 1.4 mL.*

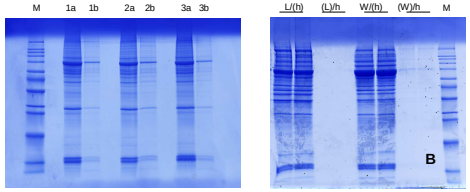
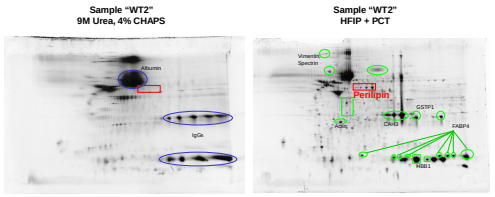


***Figure 4A:** Two consecutive protein extractions (lanes a and b) from adipose tissue samples with IEF sample buffer (9M urea, 4% CHAPS) – evidence of protein remaining in the mass of fat after the first extraction. Identical volumes of each extract are loaded onto each lane.*

***Figure 4B:** Simultaneous adipose tissue disruption, de-lipidation by hexane, and extraction by 2x Laemmli sample buffer (4% SDS) [L] or deionized water [W] using PCT. L/(h) – SDS fraction after SDS/hexane extraction; (L)/h – hexane fraction after SDS/hexane extraction, etc. M - molecular weight markers.*



* Protein loads are normalized to 200 µg per gel

***Figure 5:** Extraction of proteins from a 100 mg block of normal murine white adipose tissue. Comparison of conventional 2D sample extraction buffer and a tissue dissolution approach by PCT in Hexafluoroisopropanol-containing solvent system with the subsequent removal of the lipid fraction and solvent, followed by reconstitution in the 2D electrophoresis sample buffer. Extraction in the CHAPS-based buffer contains predominantly blood plasma proteins, while solvent-based extract appears to contain the entire proteome of the adipose tissue.*

Protein ID	Charge_Peptide Sequence	nsp adj prob
Lipid droplet-associated protein perilipin [*Mus musculus*]	3_ILHLTPAQAVSSTK	1
gi|26279005|gb|AAN77870.1|	2_EVTALPNPR	1
gi|26327331|dbj|BAC27409.1|	3_IASELKGTISTR	0.85
gi|28316726|ref|NP_783571.1|	2_LASGGADLALGSIEK	1
gi|42559472|sp|Q8CGN5|PLIN_MOUSE	2_ILHLTPAQAVSSTK	1
Probability Score: 1.00	2_VSTLANTLSR	0.95
Sequence Coverage: 28.11%	2_ETAEYAANTR	0.83
Number of Unique Peptide Matches: 7		

***Table 2.** Example of adipose-specific protein perilipin frequently under-represented in samples extracted by conventional methods. The 2D gel location of this protein is noted in **Figure 5**.*

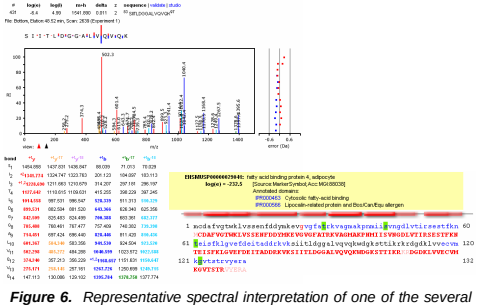


***Figure 6.** Representative spectral interpretation of one of the several isoforms of murine FABP aP2. Diversity of the aP2 gene products could be explained by post-translational modifications, possibly possessing regulatory role.*

3. Results and Discussion

Previously, removal of lipids from membrane preparations, which employed organic solvents and amphiphilic fluorinated alcohols (7,8), was carried out in series with cell disruption and fractionation using conventional methods. While increased recovery of protein from membrane preparations has been reported with these chemicals, the methods chosen were laborious and non-quantitative. We have developed a detergent–free tissue dissolution and fractionation technique based on liquid-liquid extraction, enhanced by alternating hydrostatic pressure, which promotes partitioning of analytes by transiently creating a metastable "hybrid" solvent at the interface between poorly miscible liquids. PCT instrumentation generates cycles of high and ambient hydrostatic pressure which have been shown to transiently alter the mutual solubility of poorly miscible solvents, resulting in a more efficient partitioning of sample constituents between liquid phases. Upon depressurization, appropriately chosen liquid phases separate, carrying their respective analytes based on their partitioning coefficients to corresponding solvents (**Fig. 3**). Several combinations of organic solvents, both non-polar and amphiphilic, along with aqueous buffers, were found to promote liquid-liquid partitioning. This new method offers direct compatibility with downstream separation methods such as electrophoresis, chromatography, and mass spectrometry. A remarkable difference in protein extraction efficiency was demonstrated with various solvent systems. While solvents, such as hexane and benzyl alcohol, allow de-lipidation of tissue concurrently with extraction by aqueous buffers **(Fig. 4B)**, several polar solvents, such as isopropanol, allow fractionation of proteins by their hydrophobicity through a stepwise extraction approach. Addition of amphiphilic solvents, *i.e.* HFIP, to some solvent compositions leads to a nearly complete tissue dissolution and phase separation **(Fig. 5).** By comparison, several proteins have been found to be significantly under-represented in proteomic profiles of adipose tissue obtained using conventional, detergent-based extraction techniques. Detailed analysis of these proteins and their post-translational modifications **(Fig. 6)** may provide crucial information on the regulation of fatty acid metabolism, which in turn may lead to the development of better methods for the prevention and treatment of Type II diabetes and obesity.

4. References

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