Proteomic Analysis of Murine Adipose Tissue Using Pressure Cycling Technology and High Resolution Tandem Mass Spectrometry

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1. Introduction

Proteomic analysis of adipose tissue is considered as highly valuable for studies of type 2 diabetes, obesity, cancer and other human disorders [1,2]. 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 and plasma membrane. Abundant sample-derived lipids tend to sequester detergents into micelles, thus interfering with protein extraction. Therefore, reports of proteomic investigation of adipose tissue samples have been greatly outnumbered compared to those of other mammalian tissues. The protein extraction procedures from adipose tissue samples described to date are quite diverse and sometimes confusing. Drastic improvement of protein recovery and standardization of sample preparation methods is needed to enable generation of consistent adipose proteome results between laboratories.

This study investigated the use of alternating hydrostatic pressure, Pressure Cycling Technology (PCT), for disruption of cells, micelles and membrane fragments to boost the efficiency of protein recovery from mouse adipose tissue samples as compared to the conventional homogenization techniques. Resulting protein extracts were analyzed by 2D-electrophoresis, nanoflow HPLC and high resolution high mass accuracy tandem mass spectrometry. In addition to the overall higher protein recovery by the novel PCT method, several novel protein species were identified in the PCT extracts of adipose tissue.

Comparative proteomic analysis of adipose tissue isolated from several genetically different model animal lines has been performed to reveal differences in protein expression, which may be used to study mechanisms of obesity and associated disorders such as type 2 diabetes and nonalcoholic fatty liver disease.

Convenience and reproducibility of this novel extraction method, as well as possible mechanisms of the pressure cycling impact on protein recovery are also discussed in this presentation.

2 Materials and Methods

2.1 Sample Preparation

The goal of this study was to develop a reliable and reproducible protein extraction method from murine adipose tissue to enable future proteomic investigations of murine disease models. Adipose tissue samples from three genetically distinct mouse lines were used in this study: mice with homozygous null mutations in two adipocyte/macrophage fatty acid binding proteins, FABP4 and FABP5 (also known as aP2 and mal1) (aP2-mal1**−/−**) [3, 4] were compared to the genetically obese (obob) and wild type animals. Approximately equal aliquots (100±15 mg) of adipose tissue from several individual animals representing genetically distinct mouse lines were prepared. 2D-electrophoresis buffer (9M urea, 4% CHAPS) was used to facilitate direct compatibility with the respective downstream separation method. Each sample was extracted in 1.3 mL of corresponding extraction buffer, containing protease inhibitor cocktail (Sigma-Aldrich, St. Louis, MO). Extraction was conducted in two consecutive steps, resulting protein extracts were combined for the final analysis. Unless otherwise noted, 50 µL aliquots of each extraction were removed for protein assay and SDS-PAGE analysis, then extracts from the first and second round were combined, reduced, alkylated using TBP/acrylamide [5] and concentrated to the original sample volume as described below.

2.2 Pressure Cycling Technology (PCT)

The aliquots of adipose tissue were transferred to PULSE Tubes and processed in the appropriate extraction buffer using Barocycler NEP 3229 (Pressure BioSciences, West Bridgewater, MA, **Figure 1**) for 20 cycles at room temperature. Each cycle consisted of 20 s at 35,000 psi followed by 20 s at atmospheric pressure. All samples were processed in a pressure cycling instrument using identical instrument parameters. The supernatants were removed and the remaining fat residues were subjected to an additional 20 cycles in fresh reagent.

Figure 1. *(**A**) The Barocycler NEP 3229 facilitates extraction of proteins from tissue samples by means of alternating hydrostatic pressure. This instrument uses single-use sample containers termed PULSE Tubes **(B).***

2.3 Sample Concentration and Removal of Interfering Substances

Extracted protein samples were transferred to ULTRA-4 ultrafiltration devices (Millipore Corporation, Danvers, MA) and reduced and alkylated as previously described [5]. Alternatively, samples were precipitated by acetone following reduction and alkylation to remove excess reagents and detergents incompatible with the chosen downstream separation method.

2.4 Electrophoresis and image analysis

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 IsoelectrIQ[2] 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 statistically significant differentially expressed proteins among animals from different lines.

2.5 Protein identification by nano-LC FTICR tandem mass spectrometry

Protein digests (5-10 µL) were injected onto a C18 solid phase extraction (SPE or "trapping") column (300 µm i.d. x 5 mm, Dionex, CA) and 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); column: PicoFrit, 15 µm i.d. pulled tip through the 10-port Valco valve. Samples were desalted on a "trapping" column, eluted onto and separated on the nano-LC column with linear gradient of acetonitrile in 0.1% FA and the eluent was introduced into the LTQ FTICR mass spectrometer (ThermoElectron) by nanoelectrospray. The .raw files were converted to .dta or . files using Bioworks 3.2 (Thermo) or ReAdW (ISB, WA) software. The .dta (.mzXML) files were submitted through the local network to the database (DB) search on both the high power high throughput searching engine Sorcerer (Sage-N) using SEQUEST algorithm and GPMDB software. The search was performed against a combined "forward" and "reverse" FASTA DB. Met oxidation and Cys alkylation were set as differential modifications. No static modifications or differential posttranslational modifications were employed at the stage of global protein profiling if not needed. Peptide mass tolerance equal to 10 ppm and fragment ion mass tolerance equal to 0.8 amu were used for all searches. Monoisotopic mass type, fully tryptic peptide termini and up to two missed cleavages were used in all searches. The SEQUEST output (.out files) was analyzed using DTASelect software (provided by Dr. J. Yates, Scripps) and Peptide/Protein (P/P) Prophet. 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%. Filtering parameters of DTASelect and Peptide Prophet software were developed insensitive to a dataset and resulted in a specified FPPrR for each search output. 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.

3. Results and Discussion

Extraction of proteins from lipid-rich media is complicated by the great excess of the lipid material over the technically feasible amount of detergent in the solubilization buffer. Under conventional homogenization conditions lipids sequester most of the available detergents into stable micelles, thus leaving cellular proteins embedded in the unsolubilized lipid mass. Our prior comparisons of PCT extraction of protein from adipose tissue with conventional methods, such as pulverization under liquid nitrogen, has demonstrated that cyclic hydrostatic pressure destroys micelles containing fat, proteins and detergents and facilitate the phase separation of lipids and aqueous buffer resulting in improved recovery of both cytosolic and membrane proteins [7]. While SDS is known as one of the strongest detergents for this purpose, it is incompatible with 2D electrophoresis. Moreover, if non-ionic detergents are employed for extraction, salt and reducing agents are commonly removed by acetone precipitation. However, we have found that acetone appears to diminish advantages of protein extraction from fat tissue, presumably because hydrophobic proteins are carried away by the solvent (**Table 1 and Figure 2**).

1st Extraction	1.06±0.31%
2nd Extraction	0.77±0.06%
Combined extracts	1.83±0.30%
UF 10 MWCO	1.12±0.16%
Acetone precipitation	0.41±0.14%

Table 1. *Protein recovery (percent of initial tissue weight) from Murine adipose tissue samples using two consecutive PCT extraction steps in the IEF sample buffer and two alternative sample clean-up methods.*

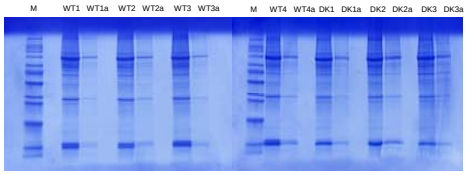

Figure 2. *Sequential protein extraction with IEF sample buffer – evidence of protein left in the mass of fat after first extraction*

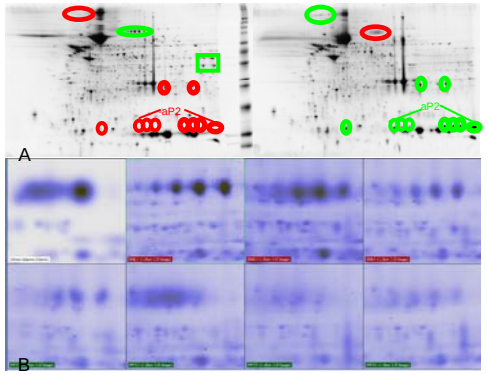

Figure 3. A. *Highly reproducible differences in the proteome of* aP2-mal1**−/−** *double-knockout mouse line compared to the wild type. All isoforms of aP2 gene product are clearly absent in all double knockout animals. Several proteins are consistently overexpressed, a possible compensatory reaction on the lack of two normally abundant FABP.* ***B*** *– individual gel replicas.*

Analysis of gel-separated proteins by nano-LC FT-ICR tandem mass spectrometry revealed several endogenous post-translationally modified isoforms of fatty acid binding proteins (Figures 5, 6) and identified a number of other protein species differentially expressed in the aP2-mal1**−/−** mutant mice as compared to a wild type and genetically obese animals. Several proteins appear to be over-expressed in the mutant animals, possibly as a result of a compensatory up-regulation due to the lack of FABP activity. Detailed analysis of these proteins and their post-translational modifications may provide crucial information for understanding of regulation of fatty acid metabolism and possible ways to prevent the occurrence of Type II diabetes and obesity.

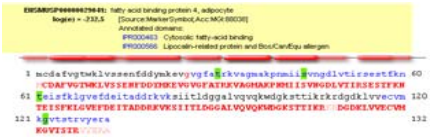

Figure 5. *Sequence coverage obtained for one of the isoforms of aP2 protein.*

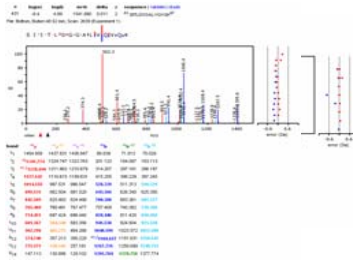

Figure 6. *Representative spectra of several isoforms of FABP aP2. Diversity of the aP2 gene products could be explained by post-translational modifications, possibly possessing regulatory role.*

4. References

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