Elevated Pressure Improves the Extraction and Identification of Proteins Recovered from Formalin-Fixed, Paraffin-Embedded Tissue Surrogates

Carol B Fowler[1], Cedric D Moore[2], Timothy J O'Leary,[3] and Jeffrey T Mason[1]

[1]Baltimore VA Medical Center, Baltimore, MD, USA; [2] Johns Hopkins University, Baltimore, MD, USA; [3] Veterans Health Administration, Washington, DC, USA

OVERVIEW

- We demonstrate the utility of elevated hydrostatic pressure for improved protein recovery from formalin-fixed paraffin-embedded (FFPE) tissue.
- As a model for FFPE tissue, a multi-protein FFPE tissue surrogate comprised of lysozyme, carbonic anhydrase, ribonuclease A, bovine serum albumin, and myoglobin (55:15:15:10:5 wt%) was developed.
- Mass spectrometry of the FFPE tissue surrogates retrieved under elevated pressure showed that both the low and high-abundance proteins were identified with sequence coverage comparable to that of the surrogate mixture prior to formaldehyde treatment.
- In contrast, non-pressure-extracted tissue surrogate samples yielded few positive and many false peptide identifications.

Introduction

Proteomic studies of formalin-fixed paraffin-embedded (FFPE) tissues are frustrated by the inability to extract proteins from archival tissue in a form suitable for analysis by 2-D gel electrophoresis or mass spectrometry.

This inability arises from the difficulty of reversing formaldehyde-induced protein adducts and cross-links within FFPE tissues. Three types of formaldehyde-induced chemical modifications have been identified in proteins and model peptides: (a) methylol (hydroxymethyl) adducts, (b) Schiff's bases, and (c) stable methylene bridges [1,2].

The majority of the proteomic studies on FFPE tissues employ tissue extraction methods that are derived from heat-induced antigen retrieval (AR) methods originally developed for immunohistochemistry. Our studies with model FFPE tissue surrogates [3], and formaldehyde-fixed proteins [4] showed that these AR-based methods did not completely reverse formaldehyde-induced protein cross-links.

There is a sound thermodynamic basis for hypothesizing that increased hydrostatic pressure, along with heat, will facilitate the extraction of proteins from FFPE tissues. Under elevated pressure, cavities in proteins become filled with water molecules, which leads to the hydration of the protein interior, thus inducing protein unfolding [5].

We report the improved extraction of proteins from FFPE tissue surrogates using a combination of heat and elevated hydrostatic pressure. Protein identity, sequence coverage, and false identification rates were evaluated by liquid chromatography-MS (LC/MS). We also investigated the effect of pressure on the rate of reversal of formaldehyde-induced protein adducts and cross-links and on the size of the protein aggregates recovered from the tissue surrogates. The results of these studies provide insight into the mechanism of pressure-enhanced protein recovery from FFPE tissues [6].

METHODS

Formation of FFPE Tissue Surrogates: The FFPE tissue surrogates were prepared as described previously (3). Briefly, aliquots of a 150 mg/mL solution (total protein) consisting of lysozyme, or lysozyme, carbonic anhydrates, ribonuclease A, BSA, and myoglobin (55:15:15:10:5 w/w, from Sigma) were mixed with an equal volume of 20% phosphate-buffered formalin (Thermo Fisher Scientific). An opaque gel formed within 2 minutes, and the tissue surrogate was allowed to fix overnight. Dehydration through graded alcohols and xylene, and paraffin-embedding were carried out according to standard laboratory procedures.

Deparaffinization and Recovery of Multi-protein Tissue Surrogates 10 µm sections of the FFPE tissue surrogates were deparaffinized by incubating the sections through two changes of xylene for 10 min each. The sections were rehydrated through a series of graded alcohols for 10 min each: 2 changes of 100% ethanol, 85% ethanol, and 70% ethanol, and then incubated in distilled water for a minimum of 30 min. For routine protein recovery, 6-8 of the rehydrated FFPE liver sections and tissue surrogate sections were resuspended in 6 mL of 50 mM Tris-HCl at pH 4 or 8, with 2% (w/v) SDS. The samples were homogenized with a disposable pellet pestle (Kontes Scientific, Vineland, NJ, USA), followed by two 10 s cycles of sonication on ice using a Sonic Dismembrator, model 550, fitted with a 0.125 inch tapered microtip (Thermo Fisher Scientific). The homogenized FFPE samples were split in half and incubated at 100 °C for 30 min followed by 80°C for 2 h at either atmospheric pressure (14.7 psi) or 40,000 psi as previously described [6]. Extracts were separated by SDS-PAGE.

Effect of Pressure on Aggregate Size and formaldehyde adduct reversal Lysozyme tissue surrogates were heated at 100°C for 2 h in 50 mM Tris-HCl, pH 4, with 2% SDS and 0.2 M glycine at the following pressures: 14.7, 2,500, 5,000, 10,000, 15,000, 20,000, 30,000, 40,000, or 50,000 psi. Triplicate samples processed at each pressure were diluted 1:10 in PBS, pH 7.4, and the size of the recovered lysozyme protein aggregates were measured by dynamic light scattering using a NICOMP model 370 particle sizer (Particle Sizing Systems, Santa Barbara, CA, USA). RNase A (2 mg/mL) in phosphate-buffered saline, pH 7.4 (PBS), was treated with an equal volume of 20% formalin in PBS for 1 hour and the excess formaldehyde was removed by dialysis. The fixed RNase A solutions were incubated under pressures ranging from 14.7 to 40,000 psi for 3.5 h at either 55°C or 65 °C.

Mass Spectrometry Samples(15 µg each) were washed three times with 50 mM NH4HCO3, pH 7.9, using an Amicon Ultra 3K centrifugal filter (Millipore, Billerica, MA, USA). Acetonitrile was added to a final concentration of 20%, and the samples were denatured at 50°C for 1 h in the presence of 20 mM DTT, then alkylated with 10 mM IAA for 1 h at room temperature. A solution of the surrogate proteins prior to treatment with formaldehyde (native, unfixed mixture) was also analyzed. Sequencing-grade modified trypsin was added to each vial to a final concentration of 0.75 µg/mL, and the samples were digested overnight at 37°C. Samples were analyzed by reversed-phase liquid chromatography (RPLC) coupled directly in-line with an Agilent 6340 ion trap mass spectrometer (Palo Alto, CA, USA). Microflow RPLC was conducted with an Agilent 1100 LC system using a 0.3 mm (inner diameter) x 15 cm long Zorbax 300 Stable Bond C8 column (Agilent). A binary gradient consisting of 0.1% formic acid in water (A) and 0.1% formic acid in acetonitrile (B) was used as the mobile phase. After injecting 8 µl (4.5 µg) of sample, the column was washed for 10 min (at 10 µl/min) with 2% B, and the peptides were then eluted (at 10 µl/min) using the following gradient: 2–70% B over 136 min, 70–95% B over 1 min, and 95% B for 15 min. The mass spectrometer was operated in a data-dependent mode where the three most intense ions detected in each MS scan were selected for tandem MS (MS/MS) in the linear ion trap. The drying gas temperature was 300ºC, and normalized collision energy of 1.3 V was employed for collision-induced dissociation along with a dynamic exclusion of 30 s to reduce redundant peptide selection.

Raw MS/MS data were analyzed with Spectrum Mill Proteomics Work Bench (Agilent) using a UniProtKB/Swiss-Prot combined database containing 517,802 protein sequences (www.expasy.org). Precursor ion tolerance was set to 2.5 Da and fragment ion tolerance was set to 0.75 Da. Only peptides possessing tryptic termini and exhibiting a score of ≥ 10.5, and a scored peak intensity of ≥ 70%, were considered legitimate identifications. The peptide searches were conducted allowing for up to two internal missed tryptic cleavage sites.

RESULTS

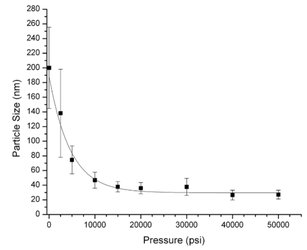


Figure 1. Effect of elevated pressure on aggregate size. Lysozyme tissue surrogates were incubated at pressures ranging from atmospheric pressure (14.7 psi) to 50,000 psi. The average particle size of the solubilized protein was measured by dynamic light scattering to determine the degree of protein aggregation.

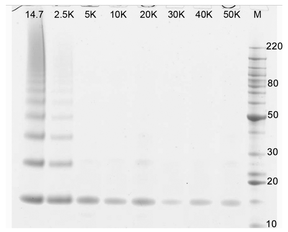


Figure 2. Effect of elevated pressure and temperature on the reversal of intermolecular protein cross-links in lysozyme FFPE tissue surrogates. The tissue surrogates from figure 1 were separated by 1-D SDS-PAGE. Pressure values are shown at the top of each gel lane.

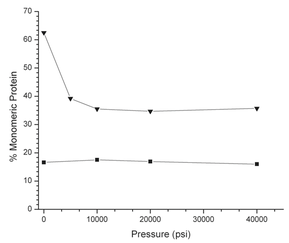


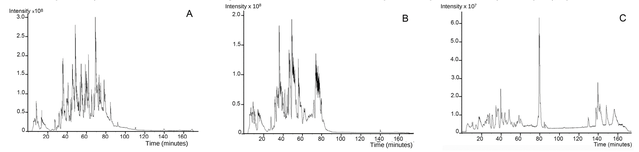
Figure 3. Effect of elevated pressure on the rate of cross-link reversal. Percent monomeric protein recovered. Aliquots of formalin fixed RNase A were incubated at 14.7 – 40,000 psi for 3.5 hours at either 55°C (squares) or 65°C (triangles), and separated by SDS-PAGE. The gel bands were integrated to determine the percentage of monomeric protein at each pressure.

RESULTS

Table 1. LC/MS analysis for a 5-protein FFPE tissue surrogate extracted under atmospheric or elevated hydrostatic pressure.

Condition	% False peptide IDs	Lysozyme		Carbonic Anhydrase		RNAse A		BSA		Myoglobin	
		Peptide hits	% Sequence Coverage	Peptide hits	% Sequence Coverage	Peptide hits	% Sequence Coverage	Peptide hits	% Sequence Coverage	Peptide hits	% Sequence Coverage
Native protein mixture	3.3 ± 0.6	67/10	66%	25/10	56%	10/6	63%	34/23	54%	6/5	38%
FFPE; pH 4, 40 Kpsi	7.8 ± 1.5	26/8	69%	9/7	36%	12/5	59%	21/12	26%	3/3	28%
FFPE; pH 4, 14.7 psi	42 ± 4.0	4/1	15%	n.d		1/1	7%	n.d		n.d	
FFPE; pH 8, 40 Kpsi	5.7 ± 1.1	75/7	57%	12/7	30%	11/5	71%	23/15	29%	3/2	16%
FFPE; pH 8, 14.7 psi	100	n.d		n.d		n.d		n.d		n.d	

Figure 4. Quality comparison of MS profiles of native protein mixture and tissue surrogate extracts. A) native, unfixed tissue surrogate mixture; B) FFPE tissue surrogate retrieved at 40,000 psi; C) FFPE tissue surrogate retrieved at atmospheric pressure (14.7 psi), pH 4.



CONCLUSIONS

Extracting FFPE Tissue surrogate with Elevated Pressure and Heat:

•Improves total protein recovery by 4-fold.

•Both the low and high-abundance proteins were identified by LC/MS with sequence coverage comparable to that of the native, unfixed protein mixture

•The false protein identification rates by MS for the pressure extracted multi-protein surrogate samples were comparable to the native protein mixture. The false identification rate for the non-pressure extracted tissue surrogates was 42% to 100%.

• There was a rapid decrease in protein aggregate size with increasing hydrostatic pressure (Fig. 1). Recovery of monomeric protein, as shown by SDS-PAGE (Fig. 2), indicates that the decrease in aggregate size corresponded to the reversal of formaldehyde-induced protein cross-links.

•These results suggest that elevated hydrostatic pressures improves the recovery of proteins from FFPE tissue surrogates by hydrating and promoting solubilization of the protein aggregates, allowing for the subsequent reversal (by hydrolysis) of formaldehyde-induced protein adducts and cross-links.

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