

# Elevated hydrostatic pressure promotes protein recovery from formalin-fixed, paraffin-embedded tissue surrogates

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High-throughput proteomic studies on formalin-fixed, paraffin-embedded (FFPE) tissues have been hampered by inefficient methods to extract proteins from archival tissue and by an incomplete knowledge of formaldehyde-induced modifications to proteins. We previously reported a method for the formation of 'tissue surrogates' as a model to study formalin fixation, histochemical processing, and protein retrieval from FFPE tissues. In this study, we demonstrate the use of high hydrostatic pressure as a method for efficient protein recovery from FFPE tissue surrogates. Reversal of formaldehyde-induced protein adducts and crosslinks was observed when lysozyme tissue surrogates were extracted at 45 000 psi and 80–100°C in Tris buffers containing 2% sodium dodecyl sulfate and 0.2 M glycine at pH 4. These conditions also produced peptides resulting from acid-catalyzed aspartic acid cleavage. Additives such as trimethylamine *N*-oxide or copper (II) chloride decreased the total percentage of these aspartic acid cleavage products, while maintaining efficient reversal of intermolecular crosslinks in the FFPE tissue surrogates. Mass spectrometry analysis of the recovered lysozyme yielded 70% sequence coverage, correctly identified all formaldehyde-reactive amino acids, and demonstrated hydrolysis at all of the expected trypsin cleavage sites. This study demonstrates that elevated hydrostatic pressure treatment is a promising approach for improving the recovery of proteins from FFPE tissues for proteomic analysis.

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High-throughput proteomic profiling may be used to differentiate normal cells from cancer cells, to identify and define the use of biomarkers for specific cancers, and to characterize the clinical course of diseases. In many cases, malignant cells yield unique 'protein profiles' when total protein extracts from such cells are analyzed by two-dimensional (2D) gel electrophoresis or matrix-assisted laser desorption ionization (MALDI) mass spectrometry (MS) methods.<sup>1–5</sup> Such proteomic studies of cancer tissues have the potential to provide an important complement to the analysis of DNA and mRNA extracts from these tissues.<sup>6</sup> When fresh or frozen tissue is used for proteomic analyses, the results cannot be related directly to the clinical course of diseases. If routinely fixed and embedded archival tissues could be used for the standard proteomic methods such as

2D gel electrophoresis and MS, these powerful proteomic techniques could be used to both qualitatively and quantitatively analyze large numbers of tissues for which the clinical course has been established. However, analysis of archival formalin-fixed, paraffin-embedded (FFPE) tissues by high-throughput proteomic methods has been hampered by the adverse effects of formalin fixation.<sup>2</sup>

Metz *et al*<sup>7,8</sup> have identified three types of chemical modifications after treatment of proteins with formaldehyde. They are as follows: (a) methylol (hydroxymethyl) adducts, (b) Schiff's bases, and (c) stable methylene bridges. Formaldehyde can react with lysine, cysteine, arginine, tryptophan, histidine, and the N-terminal amine to form methylol adducts. The methylol adduct can subsequently undergo a dehydration reaction to form a Schiff's base, which

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is seen most frequently in lysine and tryptophan residues. Additionally, the protein N-terminal amine can be converted to a stable 4-imidazolidinone adduct<sup>8</sup> and a Mannich reaction can occur between adducted tyrosine and arginine residues in close spatial proximity.<sup>9</sup> Intramolecular protein crosslinks (methylene bridges) have been reported in both model peptides<sup>7</sup> and whole proteins, such as insulin.<sup>8</sup> Intermolecular crosslinks can form between formaldehyde-treated proteins and other macromolecules, such as nucleic acids.<sup>10</sup> These formaldehyde-induced reactions can complicate the extraction of proteins from FFPE tissues for proteomic analysis. However, if the adverse effects of formalin fixation could be overcome, the use of high-throughput proteomic methods to analyze existing archival FFPE tissues would help to develop knowledge of the molecular characteristics of cancer that can be translated into practical interventions for the diagnosis, treatment, and prevention of this disease.

Several proteomic studies using archival FFPE tissues have been reported in recent years. The majority of these studies employ protein extraction methods that are derived from heat-induced antigen retrieval techniques originally developed for immunohistochemistry.<sup>11–16</sup> For example, Shi *et al*<sup>12</sup> performed a comparative proteomic study using frozen and FFPE tissue sections from the same human renal cancer biopsy. In this study, protein extraction was performed by heating the tissue specimens in 10 mM Tris-HCl, 2% sodium dodecyl sulfate (SDS) buffer at 100°C for 30 min followed by incubation at 60°C for 2 h. Liquid chromatography (LC)-MS/MS identified 2404 and 3236 total proteins in the frozen and FFPE specimens, respectively. There were 1720 proteins common to both specimens, whereas 595 proteins (25%) were unique to the frozen tissue, and 1448 proteins (45%) were unique to the FFPE tissue. Similar studies have been reported by Hood *et al*,<sup>11</sup> Jiang *et al*,<sup>13</sup> Palmer-Toy *et al*,<sup>14</sup> and Guo *et al*.<sup>15</sup> A study by Crockett *et al*,<sup>17</sup> perhaps best illustrates the current state of our ability to use archival FFPE tissues for proteomic studies. LC/MS/MS was used to compare proteins identified in a fresh cell lysate to those from the same cells processed as an FFPE cell plug. A total of 263 common proteins were identified. However, 278 proteins (54%) identified in the fresh cell lysate were not seen in the FFPE cells, and 61 proteins (23%) identified in the FFPE cells were not seen in the fresh cell lysate. This result suggests incomplete, and possibly selective, protein recovery from the FFPE cells and misidentification of proteins, possibly due to the failure to completely reverse formaldehyde–protein modifications. Although the above results are encouraging, there are clearly a number of challenges that must be addressed to develop more efficient and reproducible methods for extracting proteins from archival FFPE tissue.

We have recently developed a procedure for the formation of a ‘tissue surrogate’ as a model system for studying protein recovery from archival FFPE tissues, which can be used to quickly evaluate the efficacy of tissue extraction protocols for

proteomic studies.<sup>18</sup> High concentrations of cytoplasmic proteins, such as lysozyme and ribonuclease A, are fixed with 10% neutral-buffered formalin. The resulting opaque gel is processed through graded alcohols, xylene, and then paraffin embedding according to the standard histological procedures. Tissue extraction protocols involving detergent and heating at elevated temperatures gave efficient protein extraction from the tissue surrogates, but they failed to reverse most formaldehyde-induced protein modifications. In this study, we report that elevated temperature together with elevated hydrostatic pressure facilitates both protein extraction and reversal of formaldehyde-induced protein modifications from an FFPE tissue surrogate.

## MATERIALS AND METHODS

Chicken egg white lysozyme, carbonic anhydrase, SDS, glycine, trifluoroacetic acid, trimethylamine *N*-oxide (TMAO), and Tris-HCl buffer were purchased from Sigma (St Louis, MO, USA). Copper (II) chloride was purchased from Riedel de Haen (Seelze, Germany). Aqueous 37% formaldehyde and xylenes were purchased from Fisher Scientific (Pittsburgh, PA, USA). Sequencing grade-modified trypsin was purchased from Promega (Madison, WI, USA). High-pressure liquid chromatography (HPLC) grade acetonitrile was obtained from EM Science (Darmstadt, Germany). Absolute ethanol was purchased from Pharmco-AAPER (Brookfield, IL, USA), and Paraplast tissue embedding medium was purchased from Oxford Labware (St Louis, MO, USA).

### Formation of Tissue Surrogates

The tissue surrogates were prepared as described previously.<sup>18</sup> Briefly, a solution of lysozyme or (1:1 w/w) lysozyme/carbonic anhydrase, at a total protein concentration of 150 mg/ml in deionized water was mixed with an equal volume of 20% phosphate-buffered formalin. An opaque gel was formed within 2 min, and the resulting surrogate was allowed to stand at room temperature in the presence of formaldehyde for at least 24 h to mimic normal tissue fixation procedures. Dehydration and paraffin embedding were then conducted according to the standard histological protocols.<sup>19</sup> The tissue surrogate was washed with distilled water and then dehydrated through a series of graded alcohols: 70% ethanol for 30 min, 85% ethanol for 30 min, 100% ethanol for 30 min, and a final 100% ethanol dehydration overnight. The tissue surrogate was then incubated through two changes of xylene, 30 min each, and was placed in hot liquid paraffin overnight. The FFPE tissue surrogates were stored at room temperature for up to 8 months prior to protein recovery.

### Deparaffinization and Recovery of Surrogates

Tissue surrogate aliquots (1.5 mg each) were transferred to 1.5 ml polypropylene microcentrifuge tubes and deparaffinized by removing the excess paraffin and incubating the surrogate in two changes of xylene for 10 min each. The

**Table 1 Effect of pressure, temperature, and buffer composition on the recovery of lysozyme from FFPE tissue surrogates**

Figure (lane)	Buffer	Pressure (psi)	Temperature/ time	% protein recovered	% monomer	% asp cleavages	% oligomers
<i>Effect of pressure</i>							
2a (1)	Tris+SDS, pH 4	14.7 <sup>a</sup>	80°C/2 h	60	20	None	80
2a (2)	Tris+SDS, pH 4 <sup>b</sup>	43 500	80°C/2 h	~100	42	58	None
<i>Effect of pH</i>							
2a (2)	Tris+SDS, pH 4 <sup>b</sup>	43 500	80°C/2 h	~100	42	58	None
2a (3)	Tris+SDS, pH 6 <sup>b</sup>	43 500	80°C/2 h	~100	31	None	69
2a (4)	Tris+SDS, pH 9 <sup>b</sup>	43 500	80°C/2 h	~100	32	None	68
<i>Effect of temperature and time</i>							
3a (1)	Tris+SDS, pH 4	45 000	100°C/2 h	~100	29	71	None
Not shown	Tris+SDS, pH 4	45 000	80°C/2 h	77	18	39	43
3a (2)	Tris+SDS, pH 4	45 000	80°C/18 h	~100	27	72	None
Not shown	Tris+SDS pH 4	45 000	65°C/2 h	21	31	20	49
3a (3)	Tris+SDS, pH 4	45 000	65°C/18 h	60	34	42	24
<i>Effect of additives</i>							
3b (1)	Tris+SDS+10 mM CuCl <sub>2</sub> , pH 4	45 000	100°C/2 h	~100	40	None	60
3b (2)	Tris+SDS+50 mM TMAO, pH 4	45 000	100°C/2 h	~100	35	65	None
3b (3)	Tris+SDS+20 mM TMAO +5 mM CuCl <sub>2</sub> , pH 4	45 000	100°C/2 h	~100	40	60	None
3b (4)	Tris+SDS+20 mM TMAO +5 mM CuCl <sub>2</sub> , pH 4	45 000	65°C/18 h	80	53	33	13
<i>Effect of storage conditions</i>							
(Stored for 8 months before recovery)							
Not shown	Tris+SDS, pH 4	45 000	100°C/2 h	~100	95	5	None

Lysozyme tissue surrogate samples (1.5 mg) that were histologically processed to paraffin embedding were rehydrated and resuspended in the indicated recovery buffer. Total protein in the supernatants was assessed spectrophotometrically following processing. Percentages of monomer, oligomers, and hydrolytic fragments were determined by integration of each lane of the indicated figure. Tris+SDS = 20–50 mM Tris-HCl, with 2% SDS.

<sup>a</sup>Indicates atmospheric pressure.

<sup>b</sup>Tissue surrogates processed by Barofold Inc. All other samples were processed using the pressure system shown in Figure 1.

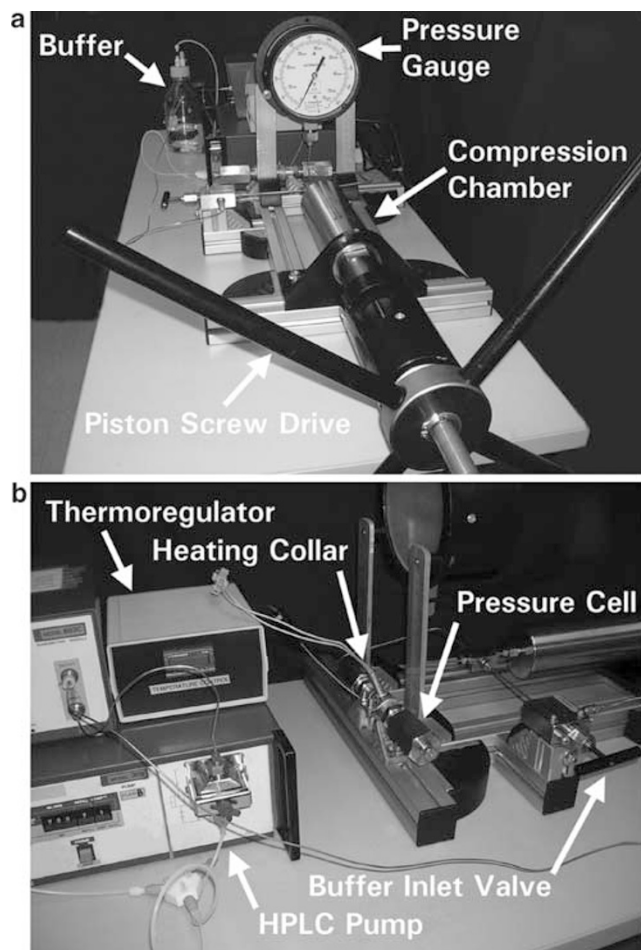
surrogates were then rehydrated through a series of graded alcohols for 10 min each: 100% ethanol, 100% ethanol, 85% ethanol, and 70% ethanol. The cleared surrogates were then incubated in distilled water for a minimum of 30 min.

The rehydrated tissue surrogates were resuspended in a panel of recovery buffers consisting of 20–50 mM Tris-HCl at pH 4, 6, or 9—with or without, 0.2 M glycine, 2% (w/v) SDS, 5–20 mM copper (II) chloride, or 20–50 mM TMAO. The concentration of Tris-HCl was increased to 50 mM in subsequent experiments to counteract the buffering capacity of the TMAO. The surrogates were then 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 (Fisher Scientific). The homogenized

surrogates were then heated in a water bath at 80°C for 2 h under ambient pressure, or they were processed for various times under elevated temperature and pressure as described in Table 1.

### Pressure Treatment of Tissue Surrogates

Our initial pressure-treated sample was prepared by Barofold Inc. (Boulder, CO, USA) on a fee-for-service basis. This sample was held at 80°C for 2 h under 3 kbar (43 500 psi) of pressure using a Barofold PreEMT E-150 instrument according to their standard protocol. Subsequent high-pressure experiments were conducted at 65–100°C under a pressure of 45 000 psi in a 2-ml capacity MS-1 stainless steel reaction vessel coupled to a manually operated HiP High Pressure Generator (High Pressure Equipment Company,



**Figure 1** The figure indicates high-pressure apparatus used for most of this study. (a) The piston screw drive, hydraulic compression chamber, pressure gauge, and buffer reservoir of the high-pressure apparatus are shown. (b) The pressure cell with its aluminum heating collar is shown. The temperature is regulated with an electronic thermo-regulator using feedback supplied by a J-type thermocouple positioned under the heating collar. An HPLC pump feeds the protein extraction buffer into the pressure cell through an inlet valve, which is turned off after injection of the buffer. The use of an HPLC pump to load the protein extraction buffer under pressure (typically 2000 psi) is optional.

Erie, PA, USA). The sample incubation temperature was regulated with a Eurotherm 2132 temperature controller (Leesburg, VA, USA) connected to an aluminum heating collar surrounding the reaction vessel. This pressure apparatus, shown in Figure 1, was fabricated to order by Applitech Corp. (Lancaster, PA, USA). An inline Gilson model 303 HPLC pump (Middleton, WI, USA) supplied the buffer to be pressurized.

### Analysis of Protein Composition

The protein concentrations of the solubilized fractions extracted from the lysozyme tissue surrogates were determined spectrophotometrically assuming that  $E^{1\%} = 26.9$  at 280 nm.<sup>20</sup> The protein composition of the solubilized

fractions was characterized by electrophoresis using 5–7  $\mu$ g of dithiothreitol-treated samples in the presence of 0.1% SDS. SDS-polyacrylamide gel electrophoresis (PAGE) was performed on precast NuPAGE Bis-Tris 4–12% gradient polyacrylamide gels (1  $\times$  80  $\times$  80 mm) using 2-(*N*-morpholino)ethanesulfonic acid-SDS running buffer at pH 7.3 (Invitrogen, Carlsbad, CA, USA). Molecular weight standards and the Coomassie blue-based colloidal staining kit were also purchased from Invitrogen. Gel images were documented using a Scanmaker i900 flat-bed scanner (Microtek, Carson, CA, USA) and annotated in Adobe Photoshop, version 7.1. The composition of individual gel lanes was analyzed and percentages were determined using Un-Scan-it Gel 6.1 analysis software (Silk Scientific Corp., Orem, UT, USA).

### Mass Spectrometry

Samples were received either lyophilized or in 40% acetonitrile and 60% aqueous 0.1% formic acid. After lyophilizing, samples were resolubilized in 15  $\mu$ l of 50 mM  $\text{NH}_4\text{HCO}_3$ , pH 7.9 for optimal tryptic digestion. Native, non-formaldehyde-fixed lysozyme was also analyzed. Sequencing-grade modified trypsin was added to each vial to give a final concentration of 0.012  $\mu$ g of trypsin per vial, and samples were digested overnight at 37°C. Tissue surrogate samples were analyzed by reverse-phase liquid chromatography (RPLC) coupled directly inline with a hybrid linear ion-trap Fourier transform mass spectrometer (Thermo Electron, San Jose, CA, USA) equipped with a nanoelectrospray ion source supplied by the manufacturer. Nanoflow RPLC was conducted with an Agilent 1100 nanoflow LC system (Palo Alto, CA, USA) using a 75  $\mu$ m (inner diameter)  $\times$  360  $\mu$ m (outer diameter)  $\times$  10 cm long in-house packed fused silica capillary column (Polymicro Technologies Inc., Phoenix, AZ, USA) with 5  $\mu$ m, 300 Å pore-size C18 media (Vydac, Hysperia, CA, USA). 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 5  $\mu$ l of sample, the column was washed for 30 min (at 0.5  $\mu$ l/min) with 2% B, and the peptides were then eluted (at 0.25  $\mu$ l/min) using a gradient as follows: 2–60% B over 100 min, 60–98% B over 20 min, and 98% B for 20 min. The column was re-equilibrated with 2% B for 30 min prior to subsequent sample loading using a flow rate of 0.5  $\mu$ l/min. The mass spectrometer was operated in a data-dependent mode where the seven most intense ions detected in each MS scan were selected for tandem MS in the linear ion trap. Normalized collision energy of 36% was employed for collision-induced dissociation along with a dynamic exclusion of 90 s to reduce redundant peptide selection. The electrospray ionization voltage and the heated capillary temperature were set at 1.6 kV and 160°C, respectively.

Raw MS/MS data were searched using BioWorks 3.2 (Sequest, Thermo Electron). Precursor ion tolerance was set to 0.08 Da and fragment ion tolerance was set to 0.5 Da. Only peptides possessing tryptic termini and exhibiting charge

state-dependent cross correlation (Xcorr) criteria of  $\geq 1.9$  for  $[M + H]^+1$  peptides,  $\geq 2.2$  for  $[M + 2H]^+2$  peptides, and  $\geq 3.5$  for  $[M + 3H]^+3$  peptides were considered legitimate identifications. The peptide searches were conducted allowing for up to two internal missed tryptic cleavage sites.

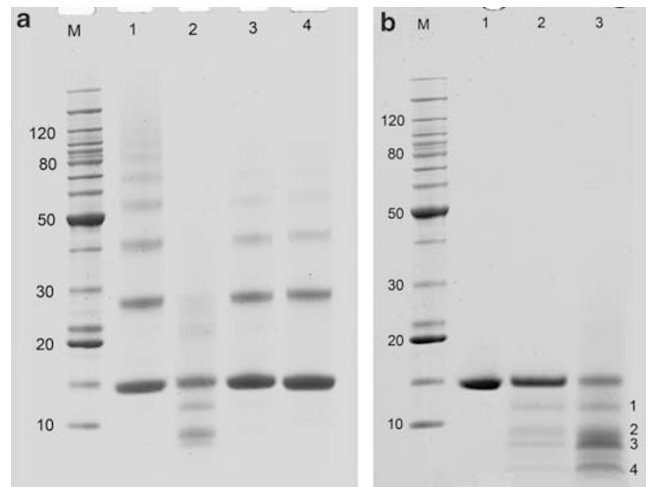
Analysis of the sample by MALDI-MS was performed using a 4700 Proteomics Analyzer (Applied Biosystems, Framingham, MA, USA). One microliter of tryptically digested lysozyme solution in 0.1% trifluoroacetic acid was mixed with 1  $\mu$ l of  $\alpha$ -cyano-4-hydroxycinnamic acid matrix (VWR International, West Chester, PA, USA) on a stainless steel plate and dried by evaporation at room temperature. The MS data were obtained using a laser intensity of 3400 kW with 2000 shots per sample. The mass peaks were extracted with Data Explorer 4.3 (Applied Biosystems) and searched using MASCOT peptide mass fingerprint software (Matrix Science, Boston, MA, USA) with a peptide mass tolerance of 100 p.p.m.

For the identification of aspartic acid cleavage sites, raw MS or MS/MS data were searched without enzyme constraint. Peptides possessing either two aspartic acid cleavage sites or one aspartic acid cleavage site and a tryptic cleavage site, and exhibiting acceptable Xcorr or MASCOT probability scores, as outlined above, were considered positive identifications.

## RESULTS

### The Effect of Elevated Hydrostatic Pressure on the Extraction of Protein from Lysozyme Tissue Surrogates

Samples of the lysozyme FFPE tissue surrogate (1.5 mg each) were rehydrated and resuspended in 20 mM Tris-HCl buffer, pH 4, containing 0.2 M glycine and 2% SDS. When heated at 80°C for 2 h at ambient pressure, approximately 60% of the total protein was extracted from the lysozyme surrogate (Table 1). These protein extraction conditions were chosen for their extraction efficiency based upon our previous tissue surrogate study.<sup>8</sup> However, when analyzed by SDS-PAGE, the total extract was highly crosslinked, with the total protein content corresponding to approximately 20% monomeric, 22% dimeric, 18% trimeric, 15% tetrameric, 12% pentameric, and 13% hexameric species (Figure 2a, lane 1; Table 1). In contrast, when the surrogate suspension was heated at 80°C for 2 h at elevated pressures (3000 bar or 43 500 psi), 100% of the protein was recovered in the soluble phase. In addition, complete reversal of the formaldehyde-induced intermolecular crosslinks was observed (Figure 2a, lane 2); 42% of total protein content corresponded to monomeric protein and only 1–2% of oligomeric protein (mostly dimer) was present. The remaining gel bands consisted of hydrolytic peptide fragments migrating below the monomer band. A similar electrophoretic pattern was observed for non-formaldehyde-treated lysozyme heated at 100°C for 1 h at ambient pressure (Figure 2b, lane 2). The gel profile of unheated, non-fixed lysozyme is shown in Figure 2b, lane 1 for reference. The hydrolytic peptide fragments have been shown to arise from thermally induced



**Figure 2** (a) The figure indicates the effect of pH and pressure on the reversal of formaldehyde-induced protein modifications. Lane M; molecular weight markers. SDS-PAGE of FFPE tissue surrogates extracted in 20 mM Tris-HCl, with 0.2 M glycine and 2% SDS at 80°C for 2 h at pH 4 at atmospheric pressure (lane 1); at pH 4 at 43 500 psi (lane 2); at pH 6 at 43 500 psi (lane 3); at pH 9 at 43 500 psi (lane 4). (b) Heating at pH 4 promotes hydrolysis of lysozyme at aspartic acid residues as revealed by SDS-PAGE. Lane M: molecular weight markers. Unheated lysozyme (lane 1); non-formaldehyde-treated lysozyme heated at 100°C for 1 h (lane 2); FFPE lysozyme tissue surrogate extracted at pH 4 at 45 000 psi and 80°C for 18 h (lane 3).

cleavage at aspartic acid residues.<sup>21–23</sup> Given the approximate mass of the four observed peptides, it is likely that the two intense bands found both in this specimen and in specimens heated at 80°C for 18 h at 45 000 psi (indicated in Figure 2b, lane 3, as bands 2 and 3) correspond to peptides that result from cleavage of aspartic acid residue 66, and that the two less intense bands (1 and 4) correspond to peptides that result from cleavage of aspartic acid residue 48, 52, or 87.

### The Effect of Buffer pH on the Extraction of Protein from Lysozyme Tissue Surrogates at Elevated Hydrostatic Pressure

We previously determined that when tissue surrogates were heated at 80°C for 2 h at ambient pressure, the protein extraction efficiency was dependent upon pH, with 60% of the total protein extracted at pH 4, 51% at pH 6, and 49% at pH 9.<sup>18</sup> In addition, the protein was observed to be highly crosslinked for all the three pH values.<sup>18</sup> Samples of the lysozyme FFPE tissue surrogate (1.5 mg each) were rehydrated and resuspended in 20 mM Tris-HCl buffer (pH 4, 6, or 9) containing 0.2 M glycine and 2% SDS. When the surrogate suspensions were heated at 80°C for 2 h at elevated pressures (43 500 psi), 100% of the protein was recovered in the soluble phase, regardless of pH (Table 1). However, complete reversal of the formaldehyde-induced intermolecular crosslinks was only seen at pH 4 (Figure 2a, lane 2). Protein oligomers accounted for 69% of the total extracted protein at pH 6 (Figure 2a, lane 3) and 68% at pH 9

(Figure 2a, lane 4). Thus, at elevated pressure, protein extraction efficiency is independent of pH, but reversal of formaldehyde-induced protein crosslinks is not.

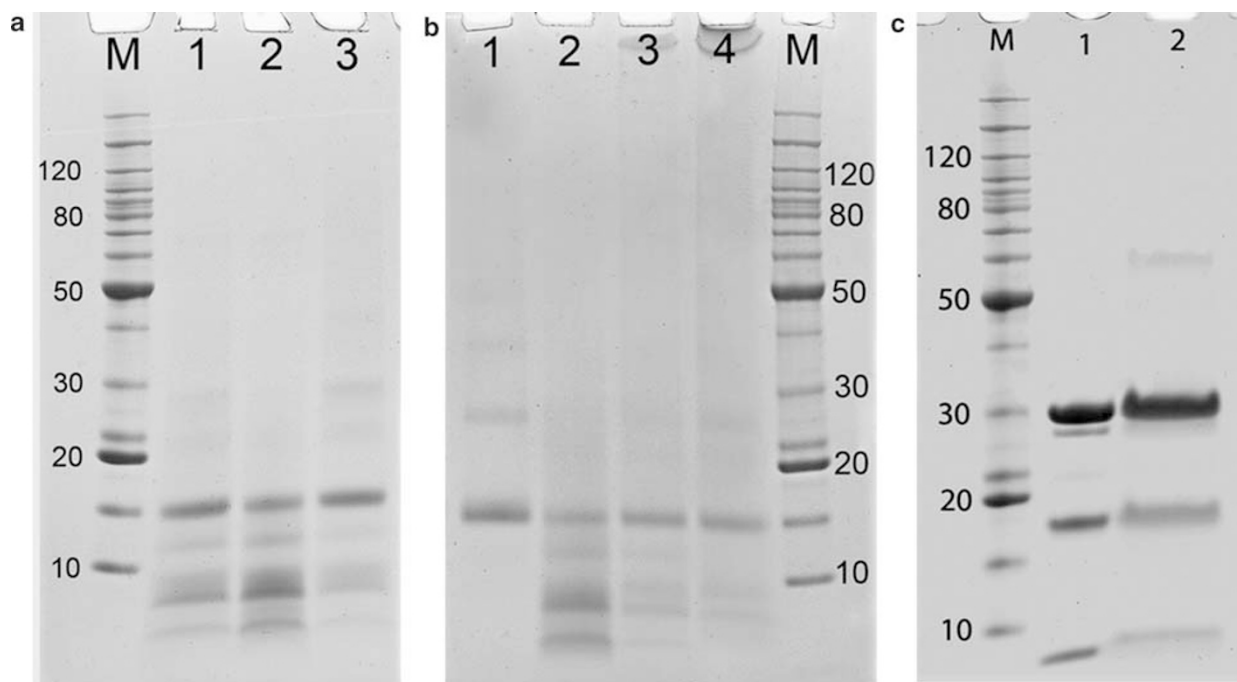
### The Effect of Temperature and Time on the Extraction of Protein from Lysozyme Tissue Surrogates at Elevated Hydrostatic Pressure

Samples of the lysozyme FFPE tissue surrogate (1.5 mg each) were rehydrated and resuspended in 20 mM Tris-HCl buffer, pH 4, containing 0.2 M glycine and 2% SDS. These suspensions were then processed under elevated pressure (45 000 psi) using protocols differing in incubation temperature and time. Experiments carried out at 45 000 psi were performed with the pressure apparatus shown in Figure 1. The increase in pressure to 45 000 psi from the experiments in the previous section was due to a difference in instrumentation and is not thermodynamically significant. As shown in Table 1, complete extraction of protein and reversal of formaldehyde-induced protein crosslinks was achieved by incubation at 100°C for 2 h (Figure 3a, lane 1) or 80°C for 18 h (Figure 3a, lane 2). This demonstrates the inverse relationship between incubation temperature and time for the efficient recovery of protein from the lysozyme tissue surrogate (Table 1). The efficiency of protein extraction by

high hydrostatic pressure did not appear to be affected by long-term storage of FFPE tissue surrogates. Up to 95% of monomeric lysozyme was recovered from lysozyme tissue surrogates stored in paraffin for up to 8 months (Table 1). Complete protein solubilization and reversal of formaldehyde-induced protein crosslinks was difficult to achieve at temperatures below ~75°C, even using long incubation times. For example, incubation for 18 h at 65°C solubilized only 60% of the protein, which retained a significant level of oligomerization (Figure 3a, lane 3).

### The Effect of Additives on the Extraction of Protein from Lysozyme Tissue Surrogates at Elevated Hydrostatic Pressure

Next, we investigated the effect of various additives for their ability to suppress the formation of aspartic acid cleavage peptides during protein extraction at elevated temperature and pressure. TMAO was chosen based upon its ability to promote structural integrity of the peptide–amide linkage at high temperatures,<sup>24</sup> and copper (II) chloride was chosen based upon its ability to form a complex involving the carbonyl oxygen of the amide linkage.<sup>25</sup> Samples of the lysozyme FFPE tissue surrogate (1.5 mg each) were rehydrated and resuspended in 20 mM Tris-HCl buffer, pH 4,



**Figure 3** The figure indicates the effect of temperature, buffer, and surrogate composition on the reversal of formaldehyde-induced protein modifications and hydrolysis at aspartic acid residues. All surrogates were processed in pH 4 buffer under 45 000 psi of pressure. **(a)** The effect of temperature is shown. Lane M: molecular weight markers. FFPE tissue surrogate extracted in 50 mM Tris-HCl, with 0.2 M glycine and 2% SDS at: 100°C for 2 h (lane 1); 80°C for 18 h (lane 2); or 65°C for 18 h (lane 3). **(b)** The effect of additives is shown. Tissue surrogates extracted at 100°C for 2 h in 50 mM Tris-HCl, with 2% SDS, and supplemented with 10 mM CuCl<sub>2</sub> (lane 1); 50 mM TMAO (lane 2); or 20 mM TMAO and 5 mM CuCl<sub>2</sub> (lane 3). Lane 4: Tissue surrogate extracted at 65°C for 18 h in 50 mM Tris-HCl, with 2% SDS, 20 mM TMAO and 5 mM CuCl<sub>2</sub>. Lane M: molecular weight marker. **(c)** Extraction of mixed tissue surrogate. (1:1 w/w) carbonic anhydrase/lysozyme solution before fixation (lane 1); mixed tissue surrogate after extraction in 50 mM Tris-HCl, with 2% SDS at 65°C for 18 h (lane 2). Lane M: molecular weight marker.

containing 0.2 M glycine, 2% SDS, plus the additives listed in Table 1. These suspensions were then incubated for 2 h at 100°C under an elevated pressure of 45 000 psi. Copper (II) chloride (10 mM) completely suppressed the formation of aspartic acid cleavages (Figure 3b, lane 1). However, this came at the expense of incomplete reversal of the formaldehyde-induced protein modifications. Treatment with 50 mM TMAO alone (Figure 3b, lane 2), or in combination with 5 mM copper (II) chloride (Figure 3b, lane 3), at 100°C for 2 h at 45 000 psi slightly suppressed the formation of aspartic acid cleavage peptides and increased the percentage of lysozyme monomer relative to an equivalent sample without either additive (Figure 3a, lane 1). Finally, the combination of 20 mM TMAO and 5 mM copper (II) chloride significantly suppressed the formation of aspartic acid cleavage products for a tissue surrogate suspension treated at 65°C for 18 h at 45 000 psi (Figure 3b, lane 4). However, the protein extraction efficiency was reduced to 80% and the extract consisted of 13% protein oligomers (mostly dimers).

### High-Pressure Extraction of a Mixed Tissue Surrogate

To investigate if high hydrostatic pressure promotes extraction of a representative complement of proteins from a multi-protein system, we investigated a previously described mixed tissue surrogate system.<sup>18</sup> A two-protein tissue surrogate composed of carbonic anhydrase/lysozyme (1:1, w/w) was treated at 45 000 psi for 18 h at 65°C (Figure 3c, lane 2; Table 2). The recovered solution contained 87% of the total surrogate protein, which was composed of 49% carbonic anhydrase monomer, 39% lysozyme monomer, and ~11% of a putative carbonic anhydrase–lysozyme dimer. The gel profile is almost identical to that of the unfixed protein control solution (Figure 3c, lane 1). When an identical mixed tissue surrogate was extracted at atmospheric pressure and 65°C, only 25% of the total protein was recovered, which consisted primarily of lysozyme, (see reference Fowler *et al*<sup>18</sup> and data not shown).

### MS of Protein Recovered under High Hydrostatic Pressure

The total protein extract from the lysozyme tissue surrogate heated at 80°C in Tris buffer, pH 4, at 43 500 psi (Baroflow sample; Figure 2a, lane 2) was digested with trypsin, and the resulting peptides were analyzed by MS. A total of 10 tryptic peptide fragments, representing >70% overall sequence coverage, were identified using RPLC-MS/MS and MALDI-MS (Table 3). Of the 10 fragments identified, 4 were identified by both MS methods. The lysozyme sequence coverage is also shown in Figure 4. The peptides identified by MS from the tissue surrogate are underlined, and the theoretical tryptic cleavage sites are indicated in red. All potential formaldehyde-reactive residues are indicated in green. There are two sequences (22–33 and 74–96) that were not observed. Two additional sequences that were not observed, spanning residues 1–5 and 113–116, would not provide tryptic peptides in the optimal molecular mass range for MS detection. When the MS data were analyzed allowing for cleavage at aspartic acid residues as well as arginine or lysine, three additional peptide fragments were identified (Table 4). All three identified fragments were a product of Asp-X cleavage at Asp 48 (34–48 and 49–61) or Asp 52 (53–61) All of the amino-acid residues that are known to react with formaldehyde to form adducts or crosslinks<sup>7</sup> were identified. Of these residues, only cysteine appears to be under-represented, with four of the seven residues residing in the two longer unobserved sequences. Thus, with the possible exception of cysteine, all of the other amino acid–formaldehyde adducts and crosslinks appear to have been reversed. Finally, cleavage was observed, or inferred, for all of the observable tryptic cleavage sites (lysine and arginine residues). This result is particularly notable as both residues react strongly with formaldehyde.<sup>7</sup> Tryptic digests of non-formaldehyde-treated lysozyme were also analyzed, with 10 fragments identified by LC MS/MS and MALDI-MS, representing >84% overall sequence coverage (Table 3). Of these, eight peptides were also found in the FFPE tissue surrogate samples. Two fragments (22–33 and 74–96) were

**Table 2 Recovery of a mixed tissue surrogate**

Surrogate composition	Pressure (psi)	Temperature/time	% total protein recovered	% lysozyme monomer	% carbonic anhydrase	% hetero dimer
1:1 lysozyme/carbonic anhydrase	45 000	65°C/18 h	87	39	49	12
1:1 lysozyme/carbonic anhydrase	14.7 <sup>a</sup>	100°C/20 min+65°C 2 h	81	84	14	2

Mixed tissue surrogate samples (1.5 mg) that were histologically processed to paraffin embedding were rehydrated and resuspended in 50 mM Tris–HCl, with 2% SDS, pH 4. Total protein in the supernatants was assessed spectrophotometrically following processing. Percentages of monomer and oligomers were determined by integration of each lane of the indicated figure.

<sup>a</sup>Indicates atmospheric pressure.

**Table 3** Lysozyme tryptic peptide fragments detected using MALDI and ESI-MS

Peptide	MALDI	Charge	Mascot	ESI	Charge	Xcorr
<i>(A) FFPE tissue surrogate</i>						
R.CELAAAMK.R	N	NA	NA	Y	2	2.321
R.HGLDNYSR.G	Y	1	$P < 0.05$	Y	2	2.681
K.FESNFNTQATNR.N	Y	1	$P < 0.05$	Y	2	3.395
R.NTDGSTDYGIQINSR.W	Y	1	$P < 0.05$	Y	2	4.321
R.WWCNDGR.T	Y	1	$P < 0.05$	N	NA	NA
R.TPGSR.N	Y	1	$P < 0.05$	N	NA	NA
K.KIVSDGNGMNAWVAWR.N	N	NA	NA	Y	2	2.337
K.IVSDGNGMNAWVAWR.N	N	NA	NA	Y	2	3.288
K.GTDVQAWIR.G	Y	1	$P < 0.05$	Y	2	2.646
R.GCR.L	Y	1	$P < 0.05$	N	NA	NA
<i>(B) Non-fixed lysozyme</i>						
R.HGLDNYSR.G	Y	1	$P < 0.05$	Y	2	2.755
R.GYSLGNMVAQK.F	Y	1	$P < 0.05$	N	NA	NA
K.FESNFNTQATNR.N	Y	1	$P < 0.05$	Y	2	3.664
R.NTDGSTDYGIQINSR.W	Y	1	$P < 0.05$	Y	2	4.208
R.WWCNDGR.T	Y	1	$P < 0.05$	N	NA	NA
R.TPGSR.N	Y	1	$P < 0.05$	N	NA	NA
R.NLCNIPCSALLSSDIT	Y	1	$P < 0.05$	N	NA	NA
ASVNCQAK.K						
K.KIVSDGNGMNAWVAWR.N	Y	1	$P < 0.05$	N	NA	NA
K.IVSDGNGMNAWVAWR.N	Y	1	$P < 0.05$	Y	2	3.437
K.GTDVQAWIR.G	Y	1	$P < 0.05$	Y	2	3.792

ESI, electrospray ionization; MALDI, matrix-assisted laser desorption ionization; MS, mass spectrometry; NA, not applicable.

(A) The FFPE lysozyme tissue surrogate sample was extracted in 20 mM Tris-HCl, pH 4, with 0.2 M glycine and 2% SDS at 80°C for 2 h at 43 500 psi. (B) In solution tryptic digest of non-formalin-treated, native lysozyme.

1 **KVFGR**CELAA **AMKR**HGLDNY **RGYSLGNMVC**  
31 **AAKFESNFNT** **QATNR**NTDGS **TDYGILQINS**  
61 **RWWCNDGR**TIP **GSRNLCNIPC** SALLSSDITA  
91 **SVNCAK**KIVS **DNGMNAWVA** **WRNRCKGTDV**

**Figure 4** The figure indicates the sequence of hen egg-white lysozyme. The peptides from the pressure extracted FFPE lysozyme tissue surrogate identified by MS are underlined; and the theoretical tryptic cleavage sites are indicated in red. All potential formaldehyde-reactive residues are indicated in green. The potential sites for aspartic acid hydrolysis are shown in blue.

identified in the non-formaldehyde-treated preparation, which were not observed in the FFPE tissue surrogate sample.

**Table 4** Aspartic acid cleavage products identified from a pressure-treated FFPE tissue surrogate

Peptide	M+H	Charge	Xcorr	DelCN
K.FESNFNTQATNRNTD.G	1758.76778	2	3.702	0.891
D.YGILQINSR.W	1063.58947	2	3.741	0.722
D.GSTDYGIQINSR.W	1423.71758	2	2.901	0.681

## DISCUSSION

In this study, we describe the efficient extraction of protein and the reversal of formaldehyde-induced protein adducts and crosslinks from a lysozyme FFPE tissue surrogate using heat treatment augmented by elevated hydrostatic pressure. Under elevated pressure, cavities in proteins become filled with water molecules, which leads to the hydration of the protein interior.<sup>26,27</sup> Hydration of the buried hydrophobic residues induces protein unfolding because the change in molar volume associated with this unfolding is negative.<sup>28</sup> Further, formaldehyde-induced protein modifications increase protein stability<sup>29</sup> and can raise the thermal denaturation temperature of fixed proteins to temperatures above 100°C.<sup>30</sup> Because the change in molar volume associated with unfolding is negative, the thermal transition temperature decreases with the increasing pressure,<sup>27,31</sup> thus counteracting the stabilizing effect of the formaldehyde modifications. Accordingly, there is a sound thermodynamic basis for hypothesizing that increased hydrostatic pressure, along with heat, will facilitate the extraction of proteins from FFPE tissues and promote the reversal of formaldehyde-induced protein adducts and crosslinks. The pressure instrument, as shown in Figure 1 is simple to use, does not require any proprietary reagents, and its cost (approximately US\$4000) makes it widely accessible to proteomic laboratories. This instrument uses a hydraulic screw pump to compress fluid to generate pressure. Since water is virtually noncompressible, in the event of a system failure, discharge forces are minimal and do not present a hazard to equipment or personnel. The pressure instrument is similar in operation and safety to an HPLC system.

An increase in hydrostatic pressure (from 14.7 to 43 500 psi) to augment heat treatment (80°C for 2 h) dramatically improved the protein extraction efficiency (from 60 to 100%) and the reversal of formaldehyde-induced protein modifications (from 20 to 100%) in a lysozyme tissue surrogate. Complete protein solubilization and reversal of formaldehyde modifications was observed for lysozyme tissue surrogates processed at 100°C for 2 h (45 000 psi) or 80°C for 2 h (43 500 psi). In a previous study, an incubation temperature of 120°C for 1 h at atmospheric pressure was required to produce the same level of protein recovery and formaldehyde reversal.<sup>18</sup> At temperatures below ~70°C at 45 000 psi, both the extent of protein extraction and the



reversal of formaldehyde modifications are significantly reduced as shown in Table 1. However, certain additives, such as TMAO and copper (II) chloride, may help to improve both protein extraction and reversal of formaldehyde-induced modifications. High hydrostatic pressures also markedly improved protein extraction in a mixed tissue surrogate over previously reported extractions at atmospheric pressure and significantly higher temperatures (100°C).<sup>18</sup> When a 1:1 (w/w) carbonic anhydrase/lysozyme tissue surrogate was extracted at 45 000 psi and 65°C in Tris-HCl, pH 4 with 2% SDS, the total surrogate solution was composed of 49% carbonic anhydrase monomer, 39% lysozyme monomer, and ~12% of a putative carbonic anhydrase-lysozyme dimer, similar to non-formaldehyde-treated protein solution (Figure 3c and Table 2). In previous studies, a mixed surrogate containing (1:1 w/w) carbonic anhydrase/lysozyme, was extracted at atmospheric pressure in Tris-HCl buffer, pH 4, with 2% SDS at 100°C for 20 min followed by 60°C for 2 h. Although 81% of the total protein was recovered, ~84% of this total corresponded to monomeric lysozyme, while monomeric carbonic anhydrase and a band of the correct size for a lysozyme/carbonic anhydrase heterodimer accounted for 14 and 2%, respectively.<sup>18</sup>

We plan to explore various combinations of pressure, incubation time, and buffer compositions to improve protein extraction and formaldehyde reversal at lower incubation temperatures. The ability to use elevated hydrostatic pressure to recover proteins from formaldehyde-treated specimens at lower temperatures is not trivial, as it increases the likelihood of being able to recover thermally sensitive proteins, and proteins with post-translational modifications, from FFPE tissues. Increased hydrostatic pressure may also prove to be of practical benefit in immunohistochemistry as a way to improve antigen retrieval.

Even at high hydrostatic pressures, elevated temperature is required to provide the energy necessary to reverse formaldehyde-induced protein adducts and crosslinks. Such heat treatment produces well-established protein modifications.<sup>21–23</sup> At pH 4, these modifications are deamidation of glutamine and asparagine residues, and  $n+1$  hydrolysis at aspartic acid residues.<sup>22</sup> The MS analysis of lysozyme recovered following treatment at 80°C and 43 500 psi suggests that deamidation is not a significant problem under these conditions. However, the hydrolysis products running below the lysozyme monomer in Figure 2b, lane 3, are probably peptide fragments produced by aspartic acid hydrolysis.<sup>21,23,32,33</sup> The mechanism for hydrolysis at aspartyl residues involves an anhydride or a cyclic imide intermediate, formed between the  $\alpha$ -carbonyl group and the amide group at either terminal through elimination of one water molecule. The Asp-X bond is most readily cleaved, but the X-Asp may also be cleaved. Thus, the resulting peptides may have at least one aspartyl residue at the point of cleavage, or they may lose the aspartyl residue by a double cleavage event.<sup>33</sup> Lysozyme contains seven aspartic acid residues (Figure 4). When the

LC-MS/MS data were analyzed allowing for aspartic acid as a potential cleavage site, fragments arising from cleavage of the aspartic acid  $n+1$  peptide bond at Asp 48 and 52 were identified (Table 4). Hydrolysis at either of these residues produces peptides of appropriate size for two of the fragments observed by SDS-PAGE (Figure 2b, lane 3, bands 1 and 4). Cleavage at Asp 66 or Asp 87 may be responsible for the remaining hydrolytic peptides, although these fragments were not identified by MS.

Model studies with the peptide hormone glucagon indicated that the rate of hydrolysis was strongly coupled to the ionization state of the aspartic acid side-chain carboxyl moiety.<sup>23</sup> With this mechanism in mind, several additives were investigated for their ability to abrogate aspartic acid hydrolysis, including the amide bond-stabilizing osmolyte TMAO and the amide complexing metal copper (II) chloride. Of these, a combination of 20 mM TMAO and 5 mM copper (II) chloride combined with an extraction temperature of 65°C for 18 h reduced the amount of hydrolytic fragments to 33% and gave almost quantitative recovery of lysozyme, with the dimeric protein band accounting for 13% of total protein (Figure 3b, lane 4). We hypothesize that copper may partially interfere with the formation of the anhydride intermediate; thus, preventing the peptide bond cleavage at aspartic acid residues, whereas TMAO may assist with protein disaggregation. It is interesting to note that conditions suppressing aspartic acid cleavage (such as copper (II) chloride and pH values >4) also reduce the reversal of protein formaldehyde modifications (Table 1). Aspartic acid cleavage may promote this reversal by facilitating the solubilization and hydration of fixed proteins by the production of smaller protein fragments. It is also possible that elevated hydrostatic pressure increases the kinetic rate of aspartic acid cleavage (compare Figure 2a, lanes 1 and 2). We plan to carry out experiments to determine if this is true.

Cleavage at aspartic acid residues does not present an obstacle to proteomic analysis since this chemical hydrolysis takes place at a well-defined location (the aspartic acid  $n+1$  amide bond). Thus, aspartic acid residues can simply be treated as possible cleavage sites in the bioinformatics analysis of the MS results. In fact, acid-catalyzed chemical cleavage at aspartic acid residues<sup>34</sup> followed by tryptic digestion of the partially degraded protein has been used in the proteomic identification of protease-resistant ribonuclease A by MALDI-MS.<sup>35</sup>

MS analysis identified 10 tryptic fragments, representing >70% overall sequence coverage (Table 3). Only two major tryptic fragments, from Gly 22 to Lys 33, and Asn 64 to Lys 86, were unaccounted for. An analysis of the relative distribution of amino acids known to react with formaldehyde throughout the lysozyme sequence did not indicate any residue bias in the unrepresented peptides (Figure 4). Since a number of peptides with four or more reactive amino-acid residues were identified by MS, there were no indications that any tryptic fragments were misidentified due to the presence

of formaldehyde adducts. A comparison of the peptides observed in the analysis of native and high-pressure FFPE lysozyme tissue surrogates by both MALDI and ESI-MS showed a high degree of overlap. Ten tryptic lysozyme peptides were identified within each sample. Eight of these peptides were in common, with each of the samples containing two uniquely identified peptides. The two peptides unique to the native lysozyme sample, GYSLGNMMVCAAK and LCNIPCSALLSSDITASVNC AK, both had very low signal intensity when the digested protein was analyzed using MALDI; therefore, it may not be surprising that they were not observed in the analysis of the FFPE sample.

In summary, we have used SDS-PAGE and MS to investigate the recovery of protein from tissue surrogates subjected to heat treatment augmented by elevated hydrostatic pressure. Our results demonstrate that treatment of the tissue surrogates at 80–100°C under elevated pressure (43 500–45 000 psi) yields quantitative solubilization of protein and near quantitative reversal of formaldehyde-induced protein adducts and crosslinks. This treatment, however, did lead to the formation of aspartic acid hydrolysis products that could be partially suppressed by TMAO and copper (II) chloride. Accordingly, this study demonstrates that elevated hydrostatic pressure treatment is a promising approach for improving the recovery of proteins from FFPE tissues for proteomic analysis. Future studies will entail extraction of tissue surrogates composed of up to 9 proteins of differing structural classes and pIs to determine the efficacy of our high-pressure protein de-modification protocols and the optimal conditions (such as buffer, detergent, and pH) required for efficient protein extraction. We also propose to conduct a proteomic comparison of fresh cell lysates and FFPE agarose-cell plugs using MCF7 (low Her2/neu expressing) and BT474 (high Her2/neu expressing) human breast cancer cells. Our ultimate goal is to develop high hydrostatic pressure as an improved method to extract proteins from archival FFPE tissues for the high-throughput proteomic analysis of disease.

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1. Chaurand P, Stoeckli M, Caprioli RM. Direct profiling of proteins in biological tissue sections by MALDI mass spectrometry. *Anal Chem* 1999;71:5263–5270.
2. Conti CJ, Larcher F, Chesner J, *et al*. Polyacrylamide gel electrophoresis and immunoblotting of proteins extracted from paraffin-embedded tissue sections. *J Histochem Cytochem* 1988;36:547–550.
3. de Noo ME, Deelder A, van der Werff M, *et al*. MALDI-TOF serum protein profiling for the detection of breast cancer. *Onkologie* 2006;29:501–506.
4. Cho WC, Cheng CH. Oncoproteomics: current trends and future perspectives. *Expert Rev Proteomics* 2007;4:401–410.
5. Neubauer H, Fehm T, Schutz C, *et al*. Proteomic expression profiling of breast cancer. *Recent Results Cancer Res* 2007;176:89–120.
6. Reymond MA, Schlegel W. Proteomics in cancer. *Adv Clin Chem* 2007;44:103–142.
7. Metz B, Kersten GFA, Hoogerhout P, *et al*. Identification of formaldehyde-induced modifications in proteins: reactions with model peptides. *J Biol Chem* 2004;279:6235–6243.
8. Metz B, Kersten GFA, Baart GJ, *et al*. Identification of formaldehyde-induced modifications in proteins: reactions with insulin. *Bioconjug Chem* 2006;17:815–822.
9. Sompuram SR, Vani K, Messana E, *et al*. A molecular mechanism of formalin fixation and antigen retrieval. *Am J Clin Pathol* 2004;121:190–199.
10. Masuda N, Ohnishi T, Kawamoto S, *et al*. Analysis of chemical modification of RNA from formalin-fixed samples and optimization of molecular biology applications for such samples. *Nucleic Acids Res* 1999;27:4436–4443.
11. Hood BL, Darfler MM, Guiel TG, *et al*. Proteomic analysis of formalin-fixed prostate cancer tissue. *Mol Cell Proteomics* 2005;4:1741–1753.
12. Shi S-R, Liu C, Balgley BM, *et al*. Protein extraction from formalin-fixed, paraffin-embedded tissue sections: quality evaluation by mass spectrometry. *J Histochem Cytochem* 2006;54:739–743.
13. Jiang X, Jiang X, Feng S, *et al*. Development of efficient protein extraction methods for shotgun proteome analysis of formalin-fixed tissues. *J Proteome Res* 2007;6:1038–1047.
14. Palmer-Toy DE, Krastins B, Sarracino DA, *et al*. Efficient method for the proteomic analysis of fixed and embedded tissues. *J Proteome Res* 2005;4:2404–2411.
15. Guo T, Wang W, Rudnick PA, *et al*. Proteome analysis of microdissected formalin-fixed and paraffin-embedded tissue specimens. *J Histochem Cytochem* 2007;55:763–772.
16. Hwang SI, Thumar J, Lundgren DH, *et al*. Direct cancer tissue proteomics: a method to identify candidate cancer biomarkers from formalin-fixed paraffin-embedded archival tissues. *Oncogene* 2006;26:65–76.
17. Crockett DK, Lin Z, Vaughn CP, *et al*. Identification of proteins from formalin-fixed paraffin-embedded cells by LC-MS/MS. *Lab Invest* 2005;85:1405–1415.
18. Fowler CB, Cunningham RE, O'Leary TJ, *et al*. 'Tissue surrogates' as a model for archival formalin-fixed paraffin-embedded tissues. *Lab Invest* 2007;87:836–846.
19. Bratthauer GL. Processing of tissue specimens. *Methods Mol Biol* 1999;115:77–84.
20. Pace CN, Vajdos F, Fee L, *et al*. How to measure and predict the molar absorption coefficient of a protein. *Protein Sci* 1995;4:2411–2423.
21. Tomizawa H, Yamada H, Imoto T. The mechanism of irreversible inactivation of lysozyme at pH 4 and 100 degrees C. *Biochemistry* 1994;33:13032–13037.
22. Zale SE, Klivanov AM. Why does ribonuclease irreversibly inactivate at high temperatures? *Biochemistry* 1986;25:5432–5444.
23. Joshi AB, Sawai M, Kearney WR, *et al*. Studies on the mechanism of aspartic acid cleavage and glutamine deamidation in the acidic degradation of glucagon. *J Pharm Sci* 2005;94:1912–1927.
24. Zou Q, Bennion BJ, Daggett V, *et al*. The molecular mechanism of stabilization of proteins by TMAO and its ability to counteract the effects of urea. *J Am Chem Soc* 2002;124:1192–1202.
25. Marciani DJ, Wilkie SD, Schwartz CL. Colorimetric determination of agarose-immobilized proteins by formation of copper-protein complexes. *Anal Biochem* 1983;128:130–137.
26. Refaee M, Tezuka T, Akasaka K, *et al*. Pressure-dependent changes in the solution structure of hen egg-white lysozyme. *J Mol Biol* 2003;327:857–865.
27. Frye KJ, Royer CA. Probing the contribution of internal cavities to the volume change of protein unfolding under pressure. *Protein Sci* 1998;7:2217–2222.
28. Kobashigawa Y, Sakurai M, Nitta K. Effect of hydrostatic pressure on unfolding of alpha-lactalbumin: volumetric equivalence of the molten globule and unfolded state. *Protein Sci* 1999;8:2765–2772.

29. Mason JT, O'Leary TJ. Effects of formaldehyde fixation on protein secondary structure: a calorimetric and infrared spectroscopic investigation. *J Histochem Cytochem* 1991;39:225–229.
30. Rait VK, O'Leary TJ, Mason JT. Modeling formalin fixation and antigen retrieval with bovine pancreatic ribonuclease A: I-structural and functional alterations. *Lab Invest* 2004;84:292–299.
31. Mason JT, O'Leary TJ. Effects of headgroup methylation and acyl chain length on the volume of melting of phosphatidylethanolamines. *Biophys J* 1990;58:277–281.
32. Ahern TJ, Klibanov AM. The mechanisms of irreversible enzyme inactivation at 100°C. *Science* 1985;228:1280–1284.
33. Li A, Sowder RC, Henderson LE, *et al*. Chemical cleavage at aspartyl residues for protein identification. *Anal Chem* 2001;73:5395–5402.
34. Inglis AS. Cleavage at aspartic acid. *Methods Enzymol* 1983;91:324–332.
35. de Koning LJ, Kasper PT, Back JW, *et al*. Computer-assisted mass spectrometric analysis of naturally occurring and artificially introduced cross-links in proteins and protein complexes. *Eur J Biochem* 2006;273:281–291.