Robust pre-analytical sample preparation process preserves the accuracy and fidelity of protein phosphorylation states

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1. Abstract

Controlled pre-analytical sample processing and preservation are synonymous with reliable proteomics analysis, particularly in respect to labile molecules such as phosphoproteins. Rapid inactivation of proteases, kinases, and phosphatases is necessary to ensure that an accurate representation of the proteome and phosphoproteome is attained from tissue biospecimens. For example, phospho-AKT has a half-life of 20 minutes and is not accurately represented in tissue samples if phosphatases are not inhibited and the phosphorylation state preserved immediately upon collection. Hence, reliable phosphoprotein analysis can only be derived from tissues that are stabilized within seconds of procurement. Flash-freezing fresh tissue samples remains the best available method for stabilizing tissue biospecimens for proteome studies. Chemical inhibitors are effective at inhibiting proteases, kinases, and phosphatases in solution, but are slow to inhibit activity in whole tissues (i.e. diffusion limited). More recently, rapid heat inactivation has been described; however, while effective at inhibiting kinase and phosphatase activity, the entire sample is denatured and proteins are rendered relatively insoluble adversely limiting biomarker detection. Additionally, with heat inactivation, before proteins are completely denatured, enzyme activity is actually increased. Processing frozen tissue samples has traditionally been limited by the methodologies for solubilizing the frozen tissue for analysis while preserving the in vivo state. A simple, robust, and integrated pre-analytical sample processing workflow for flash-freezing and efficiently processing tissue samples to extract proteins and labile molecules is described. Comparative studies with conventional homogenization techniques demonstrate the benefits and efficacy of this workflow for phosphoprotein analysis.

2. Introduction

There are 518 known kinases and possibly over 1000 phosphatases that regulate cellular activity. The addition or elimination of a phosphate at one or multiple sites on a protein alters its net charge, conformation, and regulates functionality by rapidly oscillating between functional and nonfunctional states. Isoelectric focusing is particularly sensitive for simultaneously detecting changes in the phosphorylation state of multiple proteins.

Protein phosphorylation state, at any given moment, is the net result of the opposing activities of kinases and phophatases, which occurs very rapidly. As examples, one molecule of tissue non-specific alkaline phosphatase (TNSALP) can convert 971 molecules of substrate per second [28], and a turnover rate of over 2,000 molecules per second has been reported for human intestinal phosphatase [29]. Hence, it is extremely difficult to obtain a phosphoprotein profile that accurately reflects the in vivo state.

3. Materials and Methods

2.1 Materials

Adaptive Focused Acoustics (AFA) S220 instrument, Kapton tissue tubes, t-PREP sample processing devices, and CryoPrep were from Covaris (Woburn, MA, USA). The Bio-Rad PROTEAN i12 multichannel IEF device and Criterion electrophoresis cells were from Bio-Rad Laboratories (Hercules, CA, USA).

2.2 Sample preparation

Frozen murine heart muscle (12.1 +/- 1.4 mg, n = 5) in the t-PREP was cryofractured on dry ice using the CryoPREP. 250 uL of Covaris Protein Extraction Buffer N supplemented with HALT Protease Inhibitors (Thermo-Scientific, USA), 5 mM EDTA, and Phosphatase Inhibitors (Cell Signaling Technologies, Beverly, MA, USA) and processed by AFA at 70W peak incident power (PIP), 10% duty cycle, 200 cycles per burst for 120 seconds. Replicate samples (n = 5) were processed in a dounce homogenizer from Kimble-Kontes (VWR Scientific, Bridgeport, NJ, USA). Following tissue disruption, samples were centrifuged at 16,000 RCF for 10 minutes and the supernatant reserved for analysis. Pellets acquired from dounce homogenization were resuspended in 130 uL of fresh buffer and subjected to AFA.

Protein phosphorylation state was studied in murine liver frozen in liquid nitrogen at 1, 3, 7, 15, 41, and 90 minutes after resection.

2.3 Protein assay

Protein concentrations of supernatants was determined by QuickStart Bradford assay (Bio-Rad Laboratories, Hercules, CA, USA).

2.4 Antibodies

The PathScan MultiTarget Sandwich ELISA from Cell Signaling Technologies (Beverly, MA, USA) included S6 ribosomal protein, pS6 (Ser235/236), AKT1, pAKT1 (Ser473) and pAKT (Thr308). Anti-histone H2B was from Millipore (Danvers, MA, USA).

2.5 Two dimensional electrophoresis (2DE)

Protein concentrations were normalized to 1 mg/mL and ReadyStrip IPGs pH 3-10 or 3-10NL were hydrated with 200 uL of each sample for four hours. IEF was performed for 80 kVh in the PROTEAN i12 multichannel IEF device followed by IPG equilibration and second dimension PAGE in 8-16% Tris-HCl gels (Bio-Rad Laboratories, Hercules, CA, USA). Pro-Q Diamond phosphoprotein stain and SYPRO Ruby total protein stain were from Invitrogen (Carlsbad, CA, USA).

2.6 Gel image analysis

Images analysis was performed using PDQuest software (Bio-Rad Laboratories, Hercules, CA, USA).

*BioRad Laboratories, Hercules, CA, USA.

4. Results

FIGURE 1

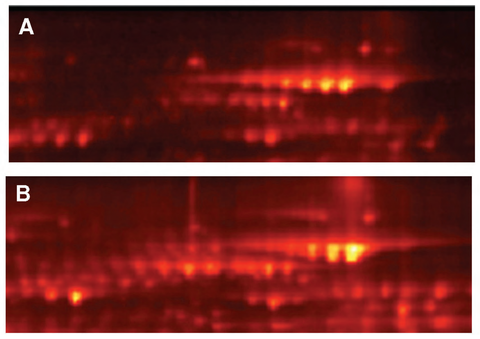

FIGURE 1 . Example of rapid change in protein phosphorylation state shown for mouse liver cryostabilized in liquid nitrogen within (A) 1 min or (B) 15 min of tissue resection. Samples were collected, frozen, and cryofractured in Covaris Kapton tissueTUBEs.

FIGURE 2

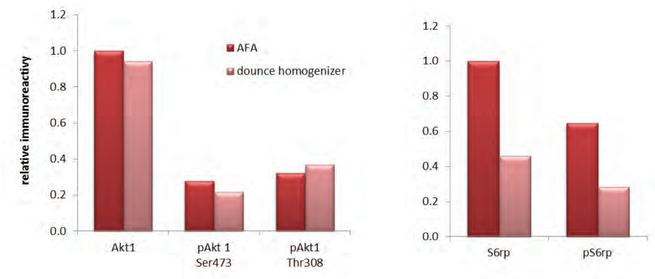

FIGURE 2. Phosphorylation states of AKT and ribosomal protein S6 in AFA or dounce homogenizer samples. Immunoassay using phosphosite-specific antibodies in the PathScan Multi-Target Sandwich ELISA. Sample application was normalized to total protein.

FIGURE 3

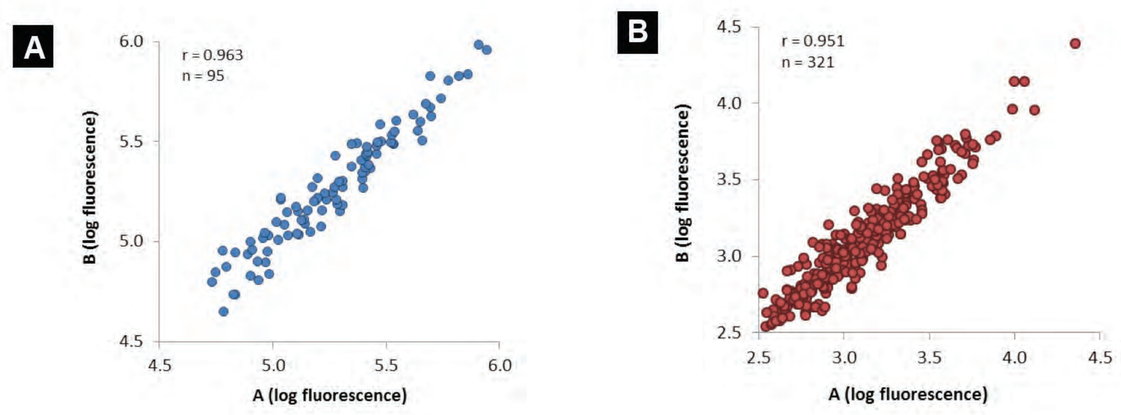

FIGURE 3. Reproducibility of 2DE. Spot fluorescence of (A) Pro-Q Diamond phosphostaining and (B) SYPRO Ruby total protein staining in replicate gels.

FIGURE 4

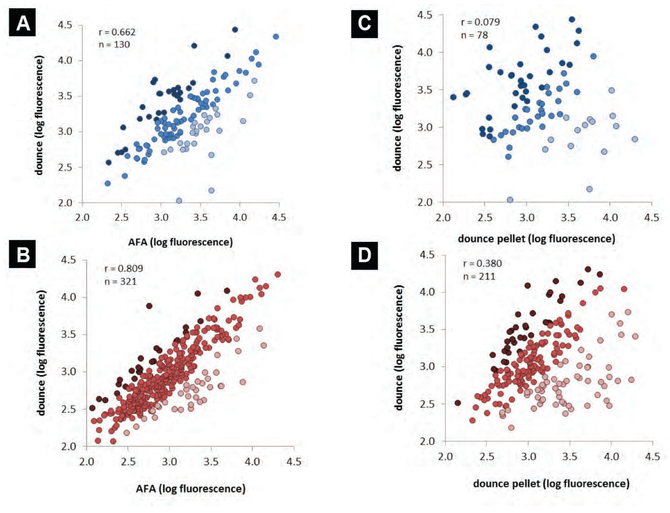

FIGURE 4. **(A)** 26 phosphoproteins were isolated at two-fold or greater concentration in AFA samples (light blue). 26 phoshoproteins were isolated at two-fold or greater concentration in dounce homogenates (dark blue). **(B)** Comparison of proteins isolated by dounce and from the dounce pellet subsequently solubilized by AFA. **(C)** 32 proteins exhibited two-fold or greater increase in AFA samples (light red). 26 proteins exhibited two-fold or greater increase in dounce homogenates (dark red). **(D)** 59 proteins increased two-fold in dounce pellet solubilized by AFA (light red).

5. Conclusion

- Not only did AFA extract more total protein than Dounce homogenizer, it extracted different proteins.
- 2DGE identified 29 unique phosphoproteins from murine cardiac tissue not isolated in Dounce homogenates.
- Proteins not isolated by Dounce homogenization were recovered when Dounce pellets were later solubilized by AFA.
- Results suggest a strong bias of Dounce homogenizer towards cytosolic proteins. If organelles are not effectively disrupted, organellar proteins may not be adequately protected by protease and phosphatase inhibitors.