Stop activity and preserve the molecular integrity of tissue samples by heat stabilization

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Background

Tissue sampling leads to major disturbance of the tissue homeostasis. Proteolytic and other protein-modifying enzymes rapidly change the composition of protein, peptides and their post-translational modifications (PTMs)[1]. Subsequent analytical results reflect a mix of in vivo proteome and ex vivo degradation products and display increased inter-sample variation. This may be misleading when drawing conclusions since vital information about the 'pre-sampling' state may be destroyed or distorted. Enzyme inactivation and standardization of sample handling can eliminate this problem.

In this study, a heat stabilization system, utilizing controlled conductive heating to generate rapid, homogenous thermal denaturation of enzymes and thereby stop degradation in different kinds of tissue, has been used. The heat-stabilized samples were compared to snap-frozen samples and, in time study manner, compared with different post-mortem intervals. Phosphorylation states and levels of endogenous peptides were examined using Western blot, RPPA and MALDI imaging.

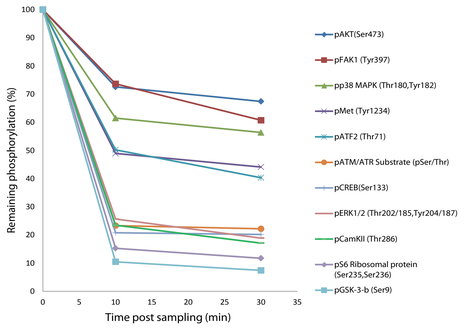

Figure 1. Ratio of remaining phosphorylations in snap-frozen samples of mouse brain striatum over 30 min in room temperature post sampling. Samples were analyzed using RPPA. Phosphorylation states chage drastically over a relative short period of time.

Figure 4. MALDI Imaging of full-length PEP-19 and metabolites in snap-frozen vs. heat-stabilized brain tissue samples.

The neuropeptide PEP-19 (m/z 6715) is detected at higher levels in the heat-stabilized brain than in snap-frozen brain (A) indicating preservation. Further proof of prevention of port-mortem degradation is the two well-known fragments of PEP-19 (m/z 1755 and m/z 1393) that are only found in snap-frozen tissue (B and C). This suggests extensive degradation of the peptide during sample preparation and emphasizes the need for heat stabilization of the tissue in order to avoid such changes.

Method

To demonstrate changes in phosphorylation states post sampling:
Mouse brain striatum samples were extracted using a denaturing extraction buffer, at a buffer to sample ratio of 10:1. Samples were kept frozen until the buffer was added and then immediately homogenized using 3x15 seconds of rod sonication (60%). Samples were kept in room temperature for 0, 10 and 30 min to follow the post sampling changes in phosphorylation states. Protein concentrations were measured using "Coomassie Plus – The Better Bradford". Samples were analyzed using RPPA (Zeptosens).

To demonstrate preservation of phosphorylation states using heat stabilization:
Western blots were run on proteins after room temperature incubation for different lengths of time and compared between snap frozen and heat stabilized.

To demonstrate preservation of endogenous peptides using heat stabilization:
Fresh rat brain tissue was either heat-stabilized or snap frozen on dry ice. Samples were embedded in 2.5% CMC before cryosectioning. Sections were transferred using a fine artist brush to pre-chilled slides. Sections were thaw-mounted from one side and continuously across the section. Mounted sections were kept frozen and dried using desiccant silica gel. Sections were washed in 70 and 100% EtOH and analyzed using MALDI imaging.

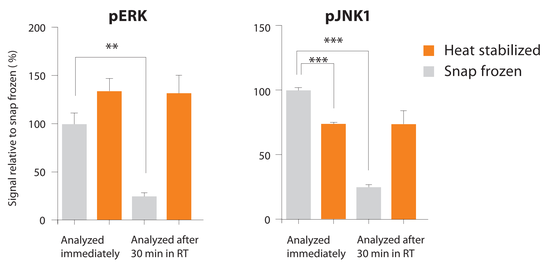

Figure 2. Data extracted from Preserving protein profiles in tissue samples: Differing outcomes with and without heat stabilization[1].

Quantitation of phosphoproteins in hippocampal lysates. Heat-stabilized and snap-frozen samples were analyzed immediately or after 30 min at room temperature. Snap-frozen samples show loss of phosphorylations while heat-stabilized samples retains phosphorylation states.

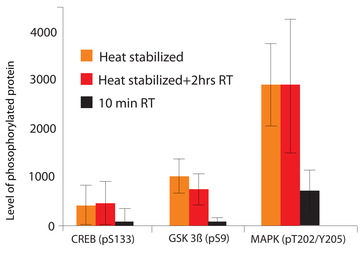

Figure 3. Levels of phosphorylated CREB (Ser 133), GSK3ß (Ser 9)and MAPK (Thr202/Tyr205) in samples of mouse brain cortex . Tissue samples were prepared as follows:
- Heat stabilized (orange): brain tissue heat stabilized immediately post-dissection
- Heat stabilized + 2hrs RT (red): brain tissue heat stabilized immediately post-dissection then left for 2 hours at room temperature prior to homogenization
- 10 min RT (black): brain tissue heat stabilized 10 minutes post-dissection. Levels of each phospho-protein were determined in each sample by Western blotting (n=5).

Figure 5. Enzymatic activity in snap-frozen samples compared to heat-stabilized samples. Phosphatase activity was measured using colorimetric pNpp phosphatase kit (AnaSpec). Snap-frozen samples were solubilized in buffers with and without phosphatase inhibitors using microtip sonication.

Figure 6. Data extracted from "Thermal stabilization of tissues and the preservation of protein phosphorylation states for two-dimensional gel electrophoresis"[3].

The 18 most phosphorylated peptides from a 144 peptide array in the tyrosine kinase activity assay of frozen, unstabilized brain homogenate. Only three out of 144 peptides produced measurable phosphorylated in heat stabilized samples. Of the remaining 126 peptides not shown, 73 additional peptide substrates were phosphorylated in snap frozen homogenates.

Results

Phosphorylation states are highly sensitive to disturbances and can change rapidly due to kinase and phosphatase activity that may continue after sampling. This is demonstrated clearly by the rapid changes in phosphorylation states on a variety of different proteins (pAKT1, pCREB, pATM/ATR substrate and pGSK3b) detected only minutes after excision, using RPPA (Fig 1).

Heat stabilization of protein-phosphorylation states was shown on ERK1 and JNK1 using Western blot. Levels are conserved for 30 minutes in heat-stabilized tissue and reduced to 25% in snap-frozen tissue (Fig 2)[1]. Similar results were found after 2 hours in room temp on proteins CREB, GSK, and MAPK. However, levels of these phosphorylated proteins were much lower when left in room temp for only 10 min prior to heat stabilization (Fig 3)[2].

The post-sampling degradation effect on endogenous peptides in snap-frozen samples is evident using MALDI imaging analysis. The neuropeptide Pep-19 and two of its ex-vivo degradation products have been imaged in rat brain sections. In heat-stabilized samples, full length Pep-19 can be identified at higher levels than in sections from
comparative snap-frozen samples. In addition to the full length Pep-19 neuropeptide, two fragments of Pep-19 have been exclusively detected in snap-frozen sections (Fig 4)[4].

Figure 5 and 6 show the level of enzymatic activity still present in snap-frozen samples in comparison with enzymatic activity present in samples that have been rapidly heat stabilized. There is little or no enzymatic activity in the heat-stabilized samples. This together with the recent report of hyperphosphorylation of proteins in snap-frozen tissue samples and the elimination of 99.6% kinase activity in heat-stabilized tissue samples[3], we can conclude that it is highly important to quickly inactivate both phosphatases and kinases to ensure reliable measurement of phosphorylation states without interference from post-mortem events.

Conclusion

We conclude that post-mortem changes, not only in phosphorylation states, may distort our view of in vivo proteomic profiles. Adequate and sustained suppression of enzymatic activity e.g. proteases, phosphatases and kinases is important to ensure reliable measurement without interference from post-mortem events. Heat stabilized samples keep their integrity throughout the sample preparation and beyond and will thereby enable higher statistical power or reduced number of replicates. We believe that the use of rapid heat stabilization, as an alternative to conventional snap-freezing in combination with inhibitors, instantly and permanently stops enzymatic activity. In this way stabilization prevents post-mortem changes and enables the sample to reflect the in vivo status as closely as possible. This approach may help us refine our experiments and differentiate true biomarkers from those proteins found in any situation where cells are under stress.

References

[1] M Ahmed et al, Journal of Neuroscience Methods. 2011 Mar 15;196(1):99-106
[2] M. Svensson, et al., J Proteome Res, 8 (2), 974-981(2009)
[3] Smejkal et al., Electrophoresis 2011, Volume 32, pages 2206–2215
[4] Goodwin R.J.A., et al., Journal of Proteomics 2012, 4912-20

Figure 7. About Stabilizor system
- Eliminates enzyme activity from the moment of sampling
- Stops degradation permanently - without using additives
- Standardizes sample handling - to improve reproducibility of analytical workflows
- Links upstream processing to downstream results - traceable treatment parameters
- Facilitates accurate sample comparison and data interpretation