Comparison of phosphoprotein enrichment methods using DIGE

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Introduction

The amount of information obtained from a single 2-D Differential Gel Electrophoresis (DIGE) experiment can be greatly enhanced by combining pre-staining of proteins using CyDyes with post-staining of gels with fluorescent dyes[1]. We used this approach to validate and compare different methods for enrichment of phospho-proteins from rat brain synaptoneurosomes. Relative enrichment was determined for individual proteins from the phospho-protein enriched (Cy2 labeled) as well as the un-enriched (Cy5 labeled) sample. This information was complemented by post-staining of DIGE gels with a phospho-specific stain (Pro-Q Diamond, Molecular Probes) as well as a stain for total protein (Sypro Ruby, Molecular Probes), prior to identification of proteins and phosphorylation sites by MALDI-TOF/TOF mass spectrometry (Fig. 1).

Using this approach, we have compared the selectivity and yield of four methods for phospho-protein enrichment:

1) Immobilized Metal-Affinity Chromatography (IMAC) using Chelating Sepharose (GE Healthcare) charged with Ga^{3+} ions.

2) Pro-Q Phosphoprotein Enrichment Kit (Molecular Probes).

3) Metal-Oxide Affinity Chromatography (MOAC) using $Al(OH)_3$.[2)]

4) MOAC using $FeTiO_3$.

Metal oxides offer several advantages to traditional IMAC resins. Firstly, published results[2), 3)] suggest that metal oxides such as TiO_2 and $Al(OH)_3$ may compete with IMAC when it comes to purity and yield of the phospho-enriched fraction. Furthermore, the enrichment protocol is simplified by the fact that there is no need to charge the MOAC materials with metal ions, or to remove unspecifically or loosely bound ions prior to use. A quick rinsing step where the resin is equilibrated with the binding buffer is typically sufficient prior to incubation with the sample.

Materials and Methods

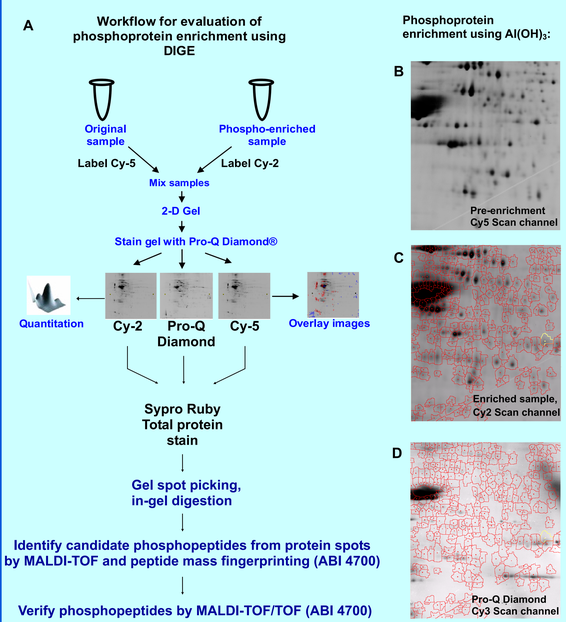
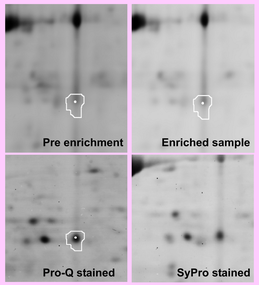


Figure 1. Evaluation of phosphoprotein enrichment from synaptoneuro-somes by DIGE. A. Workflow. An aliquot of the original sample is labeled with Cy-5, whereas the same amount of protein from an aliquot of the sample that has been enriched in phosphoproteins is labeled with Cy-2. Following mixing of the two samples and protein separation by 2-D gel electrophoresis, phospho-proteins are stained with Pro-Q Diamond (Molecular Probes), a phosphospecific stain. During scanning of the gel, the Cy-5, Cy-2, and Pro-Q Diamond signals are obtained simultaneously, by employing the Cy-3 channel for the Pro-Q Diamond signal. Simultaneous scanning of the three channels minimizes errors introduced during picking of gel spots for identification by MS and MS/MS. B. Sample of proteins from rat synaptoneurosomes stimulated with potassium chloride (Cy-5 scan channel). C. Sample enriched in phospho-proteins using Al $(OH)_3$ (Cy-2 scan channel). D. Pro-Q Diamond signal (Cy-3 scan channel). Figures B — D are from the same gel and the same scan, lowering the introduction of errors due to changes in gel size and shape during post-staining.

Results



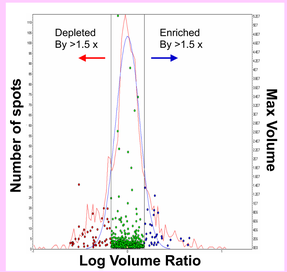


Figure 2. Phosphoprotein enrichment using the Pro-Q Diamond Phosphoprotein Enrichment kit (Molecular Probes). Although the yield of total protein is high with this method (~10%), changes in the overall pattern appear relatively small.

Of the four methods for phosphoprotein enrichment that were compared, highest yields of eluted proteins were obtained with the Pro-Q Phosphoprotein Enrichment Kit (Molecular Probes). IMAC using Chelating Sepharose charged with Ga^{3+} came second, followed by MOAC using $Al(OH)_3$ and MOAC using $FeTiO_3$. The selectivities of these methods for phosphoproteins followed the reverse sequence:

Yield

Pro-Q Enrichment Kit - Ga^{3+} IMAC - Al^{3+} MOAC - $FeTiO_3$ MOAC

Selectivity

References

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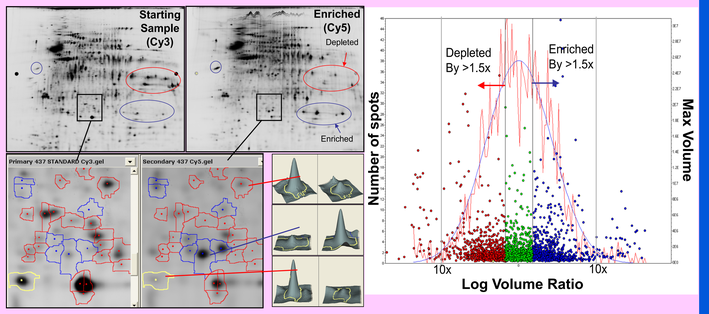

Figure 3. MOAC using $FeTiO_3$ was the method that generated the largest changes in protein concentration patterns. Blue outlines show the enriched spots or areas and the red spots show the depleted protein spots or areas of the gel. Both spots indicated with green arrows were identified as Creatine Kinase-B.

Conclusions

These results show that even with the most recent methodological developments, researchers attempting to enrich phosphoproteins from complex samples need to recognize the trade-off between high yield and high selectivity.

We urge researchers attempting to enrich phosphoproteins from complex samples to validate and compare different methods using a relevant sample matrix rather than choosing a method based on what works best with mixtures of standard proteins.

The experimental flow outlined in Figure 1 allows such validation and comparison based on quantitative data from a large number of proteins, the degree of phosphorylation of individual proteins, and mass spectrometric identification of individual proteins and their respective phosphorylation sites.

Acknowledgements

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