Comparison of Fractionation Strategies Post-Enrichment by Tithonia used in the Study of the Phosphoproteome of a Green Alga

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Phosphorylation is a ubiquitous protein post-translational modification of pivotal importance in numerous cellular processes. It occurs at low levels and is easily destroyed or lost during analytical processing. In addition, the liability of the phosphate modification in the mass spectrometer means phosphoproteins often fragment poorly. Analysis is further hindered by the presence of more abundant non-phosphorylated peptides and this has led to the development of phospho-enrichment affinity strategies using immobilized metal or metal oxides. Subfractionation of phosphopeptide pools also helps improve identification and this is most often achieved through one of two orthogonal approaches:

• Hydrophilic Interaction Chromatography (HILIC) where peptides which are dissolved in a high concentration of organic phase are bound to a hydrophilic stationary phase and eluted according to their polarities by decreasing the organic content.

• Strong Cation Exchange chromatography (SCX): Peptides are desorbed in a low salt buffer (pH 2.7) with up to 30% organic. Application of a salt gradient releases peptides from the stationary phase in order of charge.

Here, during the phosphoprotein analysis of the green alga, Chlamydomonas reinhardtii, we compare these two chromatographies by using identical aliquots of phosphoproteins and identical acquisition parameters incorporating extended 2 h gradients. Past experience with 1 h gradients has shown the overlap to be minimal (~40%), so the expectation is that this should increase.

Results: Overall Statistics

Phosphorylated peptides fractionated by HILIC or SCX were identified using Mascot and grouped into protein family using Scifit with a protein false discovery rate of 1%. The phosphosite localization confidence level was set at 95% and using Scifit-TPD employing the Ascorn algorithm.

Figure 4. Enrichments statistics (line) for identical sample separated by both HILIC and SCX chromatographic separations. The samples were from a single enrichment and therefore identical, but the overall statistics show:

• an 85% phosphopoenriment figure for SCX compared to 75% for HILIC

The difference in phosphosite selectivity between the two different fractionation methods was examined further by outputting each localized site as a 13 amino acid sequence with the phosphorylated residues centrally positioned. Duplicate sites were then removed.

Figure 5. (A) HILIC and (B) SCX chromatographic separations showing the number of unique phosphosites per fraction and the cumulative scores across the separation.

• The distribution of phosphopeptides through the HILIC fractionation is more homogeneous (Fig 5A) than the SCX fractionation (Fig 5B) where they elute in groups.

• Some SCX fractions (see Fig 2B fractions 7, 8 & 9) clearly saturated the mass spectrometer. Nevertheless more phosphosites are still found by SCX even though there is an extra deassembling step post-fractionation which might lead to losses.

• Unique phosphosites are found across all fractions for both techniques in high numbers.

Figure 6. Overlay of unique phosphosites between HILIC and SCX

• The total number of unique phosphosites identified is 16,752 with an overlap of just 37% between HILIC and SCX (Fig. 6).

• Combining the SCX phosphosites to those from HILIC increases the number of phosphosites identified by 54%.

The full phosphoprotein sequences from the non-overlapping regions in Fig 6, were further used to see if there were any obvious features in the data that could give rise to such a big difference.

Calculation of the isoelectric point (pI) distribution of these phosphopeptides by fraction identified a clear pattern (Fig. 7).

• In HILIC, peptides are clustered around a pI of ~4.5

• The average pI for those of SCX cover a larger range (pI 2.5 to 6.5)

Figure 7. Average isoelectric point (pI) of peptides unique to either HILIC or SCX

Conclusions:

• Indications are that HILIC gives a more hydrophobic-rich peptide dataset.

• Regardless of mass spectrometer acquisition time the overlap between SCX and HILIC phosphopeptides remains low (~40%) in spite of expectations.

• Combining the SCX dataset to the HILIC added 54% extra phosphopeptides.

• SCX finds more multiply phosphorylated phosphopeptides.

More work is required to explain the differences seen at the isoelectric point level.

References

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Introduction:

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Methods:

Cells of the ch-15 cell wall-deficient mutant of C. reinhardtii were cultured in standard triacetate-phosphate (TAP) medium up to an OD750 of 0.60, then harvested and proteins extracted using a phenol method. Protein pellets were then processed as in Figure 1.