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High-pH Reversed-Phase Sample Fractionation for Phosphoproteomic Workflows.

Sergei I. Snovida[1], Jae Choi[1], Ryan Bomgarden[1], Julian Saba[2], Rosa Viner[2], John C. Rogers[1], [1]Thermo Fisher Scientific, Rockford, IL, USA; [2]Thermo Fisher Scientific, San Jose, Ca, USA

ABSTRACT

Purpose: The objective of this study is to demonstrate the utility and benefits of high-pH reversed-phase fractionation of phosphopeptides in MS-based phosphoproteomics workflows.

Methods: Lysates of K562 cells were digested with Lys-C and trypsin. Following the digestion, samples were enriched for phosphopeptides using Fe-NTA resin. Enriched phosphopeptides were fractionated in high-pH reversed-phased mode off-line with a Thermo Scientific™ Pierce™ High pH Reversed Phase Peptide Fractionation Kit using a bench top microcentrifuge. Absolute peptide quantities, including total post-enrichment yields and fractional distributions of enriched peptides, were determined using Thermo Scientific™ Pierce™ Quantitative Colorimetric Kit. LC-MS analysis was performed using Thermo Scientific™ Orbitrap Fusion™ Tribrid™ mass spectrometer.

Results: The use of Thermo Scientific™ Pierce™ High pH Reversed Phase Peptide Fractionation Kits enables efficient, loss-free desalting of phosphopeptides following enrichment. High-pH reversed-phase fractionation of phosphopeptides leads to ~3-fold increase in phosphopeptide identifications relative to unfractionated samples.

INTRODUCTION

While phosphorylation of proteins is a common post-translational modifications (PTMs), only a very small subset of the total peptides found in a complex digest sample carry these modifications. This necessitates enrichment of phosphopeptides in a context of a comprehensive proteomic study. Off-line fractionation of complex peptide mixtures using a high-pH reversed-phase approach followed by low-pH LC-MS has been shown to improve protein identification numbers and provides better site-specific information with respect to modifications.

In our preliminary experiments, we observed that phosphopeptides in our enriched samples were not retained well on the hydrophobic resin used for high-pH reversed-phase fractionation with most of the peptides present in the flow-through, wash and first few acetonitrile elution fractions. While it is well known that phosphorylation modifications render peptides more hydrophilic, we were surprised by the poor retention of enriched peptides under low-pH ion-pairing (0.1% TFA) loading conditions compared to unenriched samples.

To investigate this phenomenon further, we set out to optimize sample loading conditions onto our high-pH reversed-phase spin columns for desalting and fractionation of enriched phosphopeptides, with the emphasis on minimizing sample loss and obtaining good fractional resolution and analytical reproducibility.

MATERIALS AND METHODS

Sample Preparation

Protein extracts from K562 cell lysates were digested sequentially with Lys-C and trypsin. Initial protein concentrations were determined using a BCA assay. Subsequent peptide quantitation was performed using a Pierce Colorimetric Peptide Quantitation Assay (Product# 23275).

A digest sample corresponding to approximately 20 mg of peptide material was enriched for phosphopeptides using Pierce™ Fe-NTA Phosphopeptide Enrichment Kit (Product# 88300). Briefly, the resin was washed and equilibrated using 1:1 water/acetonitrile with 2% acetic acid solution. The sample was dissolved in 900 µL of 1:1 water/acetonitrile with 2% acetic acid solution and split into three equal portions. To accommodate the large amount of sample peptides, three enrichment columns were custom made by pooling the resin material from five columns into one. Samples were loaded and the columns were vigorously shaken for 30 minutes to maximize phosphopeptide binding. Flow-through was then collected and the columns were washed three times with 500 µL of 1:1 water/acetonitrile with 2% acetic acid solution to remove any unbound peptides. The columns were further washed twice with of 1:1 water/acetonitrile solution to remove any residual unbound acetic acid. Phosphopeptides were then eluted with 1:1 water/acetonitrile solution with 5% piperidine.

Collected phosphopeptide fractions were pooled and dried in a vacuum centrifuge. Prior to high-pH reversed-phase fractionation, the samples were dissolved in 500 µL of 0.1% trifluoroacetic acid solution and pH was adjusted to ~2.5 using sequential addition of small volumes of 10% trifluoroacetic acid solution, and 1/3 of the sample were each subjected to fractionation on a separate high-pH reversed-phase fractionation column.

Test Method

Post-enrichment peptide yields were estimated using Thermo Scientific™ Pierce™ Quantitative Colorimetric Kit according to the recommended protocol. Upon fractionation, contents of each fraction were dissolved in 50 µL of 0.1% trifluoroacetic acid solution and fractional peptide content was measured using Thermo Scientific™ Pierce™ Quantitative Colorimetric Kit . The remainder of sample in each fraction was committed to LC-MS analysis. All samples were analyzed on a Thermo Scientific™ Orbitrap Fusion™ Tribrid™ mass spectrometer. Liquid chromatography was performed using Thermo Scientific™ Dionex™ Ultimate™ 3000 Nano LC system, utilizing a 50 cm C_{18} Thermo Scientific™ EASY-Spray™ column heated at 60˚ C. A three-hour gradient was used in all experiments.

Data Analysis

All .raw files were processed using Thermo Scientific™ Proteome Discoverer™ 1.4 software. Data was searched against a custom human/yeast database using SEQUEST® HT search engine using Percolator with 1% FDR.

RESULTS

Excellent fractionation of the phosphopeptide-enriched samples was achieved using the off-line high-pH reversed-phase fractionation approach shown in Figure 1. While the overall numbers of phosphopeptides identified was not very impressive in this particular study (Figure 2), which may be due to inadequate washing of the resin during enrichment, hydrolysis of phosphates during acidification, and/or sub-optimal MS method used, the attributes on trial, specifically the ability to effectively desalt phosphopeptide samples without sample loss and efficient/reproducible fractionation of phosphopeptide samples, were properly evaluated here. Figure 2 and 3 show that reproducible fractionation in terms of unique peptide/phosphopeptide identification numbers, as well as fraction-specific peptide content, can be achieved using our off-line high-pH reversed-phase fractionation approach.

Presence of residual salts in the sample resulted in overestimation of total post-enrichment peptide yield by ~20-25% (Figure 3). These salts can also be effectively eliminated from the samples using our spin-columns (Figures 1 and 3), which leads to improved accuracy when performing quantitative colorimetric peptide assay to assess the peptide yields. No peptides were detected/identified in the flow-through (FT) and wash (Wash1 and Wash2) fractions from MS analysis and the results from the colorimetric peptide assay suggest presence of assay-interfering non-peptide material, such as the piperidine used for phosphopeptide elution.

Figure 1. Off-line spin-column high pH reversed-phase fractionation workflow.

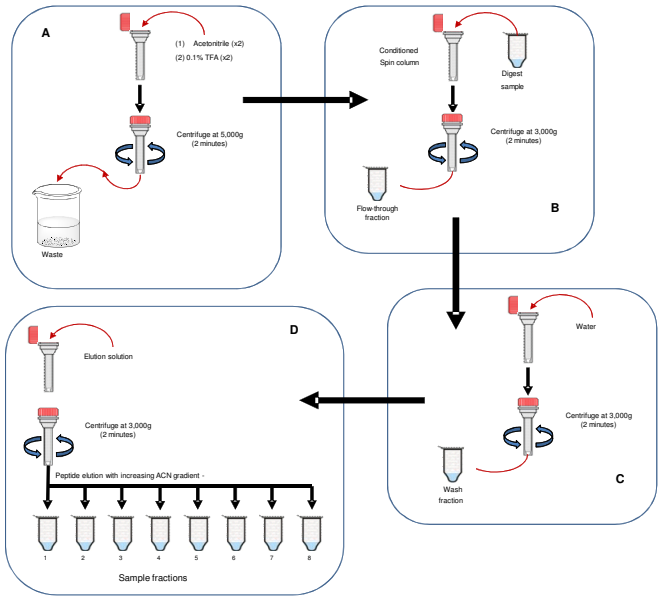


Figure 2. Fractional profiles of phosphopeptide-enriched samples. Unique peptides are the total unique peptides identified. Wash1 and Wash2 correspond to fractions obtained by washing the column with 0.1% trifluoroacetic acid solution and water, respectively.

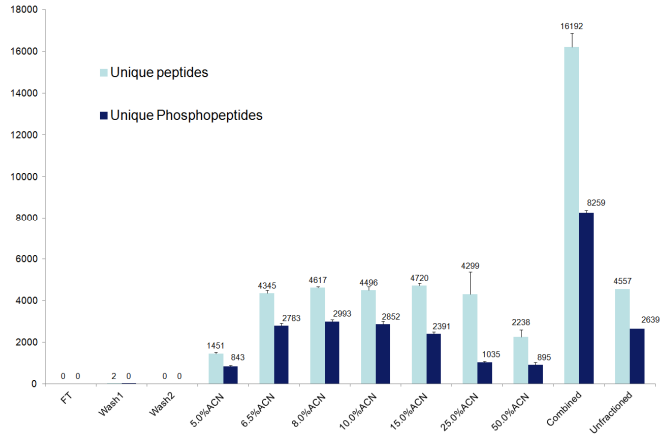


Figure 3. Fractional resolution and fractionation reproducibility. Overlaps in unique peptide IDs in three consecutive fractions within a set (A) and replicate fractions across three different sets (B).

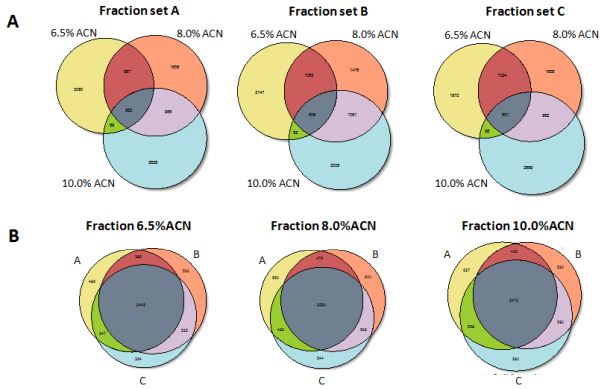
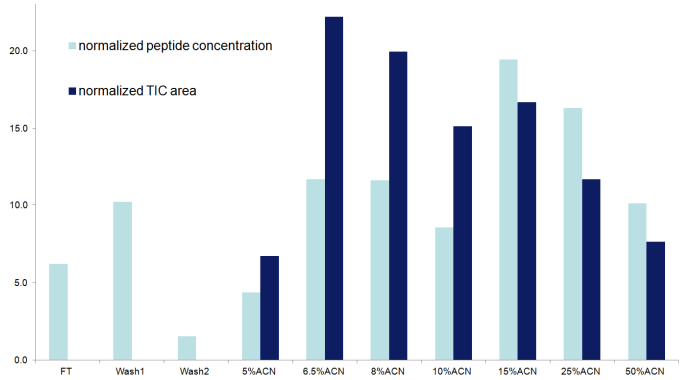


Figure 4. Comparison of normalized peptide fractional concentration as measured by the quantitative colorimetric peptide assay and fractional TIC areas from the LC-MS analysis. "Peptide" signal in the FT and Wash samples is due to interfering substances that are removed from the samples with the high pH fractionation columns.



CONCLUSIONS

- Careful control of pH and proper ion-pairing conditions enable good retention of phosphopeptides on hydrophobic resins.
- Post-enrichment desalting of phosphopeptide samples is required for accurate estimation of peptide quantities using quantitative colorimetric peptide assay.
- High-pH reversed-phase fractionation of phosphopeptides leads to a significant improvement in phosphopeptide identification numbers in a highly reproducible manner suitable for comprehensive discovery and comparative analysis studies.

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TRADEMARKS/LICENSING

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