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 phosphoproteomic workflows.

Methods: Lysates of K562 cells were digested with Lp-C and trypsin. Following the digestion, samples were enriched for phosphopeptides using Fe-NTA resin. Enriched phosphopeptides were fractionated in high-pH reversed-phase mode off-line with a Thermo Scientific™Pierce™Quantitative Colorimetric Reversed Phase Peptide Fractionation Kit using a bench top microcentrifuge. Absolute peptide quantities, including total post-enrichment yield and fractional distributions of phosphopeptides, were determined using Thermo Scientific™Pierce™Quantitative Colorimetric Reversed Phase Peptide Fractionation Kit analysis. 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™EASY-Spray™column heated at 50 °C. The objective of this study is to demonstrate the utility and benefits of high-pH reversed-phase fractionation of phosphopeptides in MS-based phosphoproteomic workflows.

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

Introduction

While phosphorylation of proteins is a common post-translational modification (PTM), 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 provide 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 elution (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 column for desalting and fractionation of enriched phosphopeptides, with the emphasis on maximizing 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 Lp-C and trypsin. Initial protein concentrations were determined using a BCA assay. Subsequent peptide quantification was performed using Pierce Colorimetric Peptide Quantitation Assay (Prod kit: 22270).

A digest sample corresponding to approximately 20 μg of peptide material was enriched for phosphopeptides using Pierce™Fe-NTA-Phosphopeptide Enrichment Kit (Prod kit: 89955). Briefly, the resin was washed and equilibrated using 1M water/acetate with 2% acetic acid solution. The sample was dissolved in 900 μl of 0.1% water/acetate with 2% acetic acid solution and split into three equal portions. To accommodate the large amount of sample peptide, three enrichment columns were custom made by pooling the resin material from three 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 twice with 300 μl of 1M water/acetate with 2% acetic acid solution to remove any unbound peptides. The columns were further washed twice with 300 μl of 1M water/acetate solution to remove any residual unbound acetic acid. Phosphopeptides were then eluted with 11 M water/acetate solution with 5% acetic acid.

Cooled 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 12.5 using sequential addition of small volumes of 10% trifluoroacetic acid solution, and 1/3 of the sample was 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 55 μl of 0.1% trifluoroacetic acid solution and fractional peptide content was determined using Pierce™Quantitative Colorimetric Kit. Absolute peptide concentrations in each fraction were converted to LC-MS analysis. All samples were analyzed on a Thermo Scientific™Orbitrap Fusion™Tribrid™ mass spectrometer. Liquid chromatography was performed using Thermo Scientific™EASY-Spray™column heated at 50 °C. A three-hour gradient was used in all experiments.

Data Analysis

All raw files were processed using SEQUEST™Proteomics 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 sample was achieved using the off-line high-pH reversed-phase fractionation approach shown in Figure 1. Figure 2A illustrates the fractionation of phosphopeptides identified in a very impressive manner in this study (Figure 2), which may be due to inactivating washing of the resin during enrichment, hydrolysis of phosphates during acidification, and/or sub-optimal MS method used, the antihype on most specifically the ability to effectively desalt phosphopeptide samples without sample loss and phosphopeptide fractionation of phosphopeptide samples, were properly evaluated here. Figure 2 and 3 show that reproducible fractionation in terms of unique peak 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 to ~25% (Figure 3). These salts can also be effectively eluted from the samples using our spin column (Figures 1 and 2), which leads to increased sensitivity when performing quantitative analysis. Figure 3 demonstrates that quantitative colorimetric phosphopeptide analysis can be used to determine fraction-specific enrichment factors for phosphopeptides following high-pH reversed-phase fractionation. The results from the colorimetric assay suggest the presence of interfering non-peptide material such as the phosphate used for phosphopeptide elution.

Discussion

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 and can be accurately estimated using quantitative colorimetric phosphopeptide assay.
- High-pH reversed-phase fractionation of phosphopeptides leads to significant improvement in phosphopeptide identification numbers in a highly reproducible manner suitable for comprehensive discovery and comparative analysis studies.

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