

Targeting the Phosphotyrosine Proteome Using an Immobilized Metal Affinity Chromatography Spin Column and MALDI Matrix Optimization for Mass Spectrometric Enhancement

Jessica M. Moeller, John G. Dapron, Angela S. Crawford, Ned Watson, Justin Wildsmith, Holly A. Chapman, Richard J. Mehigh, Graham B.I. Scott, and William K. Kappel

Overview

Purpose

A novel gallium IMAC (immobilized metal affinity chromatography) spin column has been developed with demonstrated utility in the capture of tyrosine-phosphorylated peptides, in addition to serine and threonine-phosphorylated peptides, from crude cell extracts. An optimization of the capture, release and analysis methodologies was performed.

Methods

The ultra-micro (50 microliter resin volume) spin column is comprised of a porous silica resin substrate conjugated with a gallium NTA (nitrilotriacetic acid) chelate analog via a neutral spacer. Gallium was selected based on its superior capacity and selectivity performance when compared to iron and aluminum based supports (data not presented).

Results

Ammonium hydroxide was compared to phosphoric acid as spin column eluants. Both exhibited comparable efficiency in terms of phosphopeptide recovery; however, the phosphoric acid provided enhanced MALDI (matrix assisted laser desorption/ionization) phosphopeptide signal when matched with DHB (dihydroxybenzoic acid) matrix.

Model tyrosine-phosphopeptides, including a phospho-tyrosine containing peptide from EGFR (epidermal growth factor receptor) in addition to the serine linked mono and tetra-phosphopeptides from β casein were utilized to evaluate the various parameters.

Significant enrichment specificity was demonstrated using a phospho-tyrosine-peptide spiked into a tryptic digest of an *E. coli* extract.

Introduction

Tyrosine phosphorylation plays a key role in cell signaling for higher eukaryotes. It is estimated that only 0.05% of total protein phosphorylation occurs at tyrosine, so enrichment for tyrosine phosphorylation components is a critical aspect of sample preparation prior to analysis by mass spectrometry. IMAC has been successfully utilized to provide significant enrichment of phosphopeptides. It has not been commonly recognized however that the metal, chelate, and substrate must be matched to provide optimal results. We have developed a gallium chelate silica substrate in a spin column format that has demonstrated potential to provide significant enrichment of tryptic phosphopeptides from various mixtures. In particular, we have shown the utility of this affinity substrate to select phospho-tyrosine-peptides. Finally, since phosphorylated peptides will poorly ionize under standard MALDI conditions, we have confirmed the enhancement effect of using phosphoric acid in combination with DHB for sample spotting.

Materials

- PhosphoProfile™ Phosphopeptide Enrichment Kit (Sigma-Aldrich Product Code PP0410)
- Model peptides:
 - BSA peptides (derived from tryptic digests of Sigma-Aldrich Product Code A0281)
 - β Casein peptides (derived from tryptic digests of Sigma-Aldrich Product Code C6905)
 - [pTyr¹¹⁶³]-Insulin Receptor Fragment (Sigma-Aldrich Product Code I2031)
 - Human EGFR protein AQUA peptide: GSTAENAEY[LC13N15]R (Sigma-Genosys)
 - Human EGFR-phosphorylated protein AQUA peptide: GSTAENAE[pTyr][LC13N15]R (Sigma-Genosys)
- Trypsin, TPCK treated (Sigma-Aldrich Product Code T1426)
- ProteoPrep™ Reduction and Alkylation Kit (Sigma-Aldrich Product Code PROT-RA)
- Discovery C-18 Reversed Phase HPLC column 15 cm \times 1 mm, 5 μ (Supelco 41201-01)
- All other reagents used were obtained from or prepared at Sigma-Aldrich

Methods

Phosphopeptide Enrichment

- Peptide mixtures were enhanced specifically for phosphopeptides following the instructions provided with the PhosphoProfile Phosphopeptide Enrichment Kit as follows. Samples were dried following tryptic digestion then reconstituted to obtain a final volume of 50 μ L using the supplied Bind/Wash Solution (250 mM acetic acid in 30% acetonitrile). The samples were loaded onto an equilibrated PHOS-Select™ Gallium Spin Column by briefly spinning (500 – 1000 rpm) in a microcentrifuge. The samples were incubated for 15 minutes at room temperature. Following incubation, the columns were spun and washed with 150 μ L total of the Bind/Wash Solution to remove unbound peptides. Prior to elution, a water wash of 50 μ L was employed to remove any residual Bind/Wash Solution prior to elution. After placing the columns into new microcentrifuge tubes, a total of 50 μ L of the provided Elution Solution (10% phosphoric acid) was used to elute the phosphopeptides.
- Variations from the instructions are detailed for an individual experiment in the appropriate figure legends.

MALDI-TOF MS

- Samples were combined with an equal volume of either DHB or ACCA (α -cyano-4-hydroxycinnamic acid) and spotted onto a MALDI target.
- MALDI-TOF-MS data was acquired using a Kratos Axima-CFR™ Plus mass spectrometer in positive ion reflection mode.

LC-MS

- Samples were separated on a 15 cm \times 1 mm C-18 reverse phase column using a 33 minute gradient of water (Buffer A) and acetonitrile (Buffer B) both containing formic acid at the concentration of 0.1% and 0.08%, respectively.
- Positive ESI, neutral loss scanning and tandem MS was performed on a Thermo Electron LTQ instrument.

Results

Comparison of Elution Buffers for Increased Recovery Efficiency

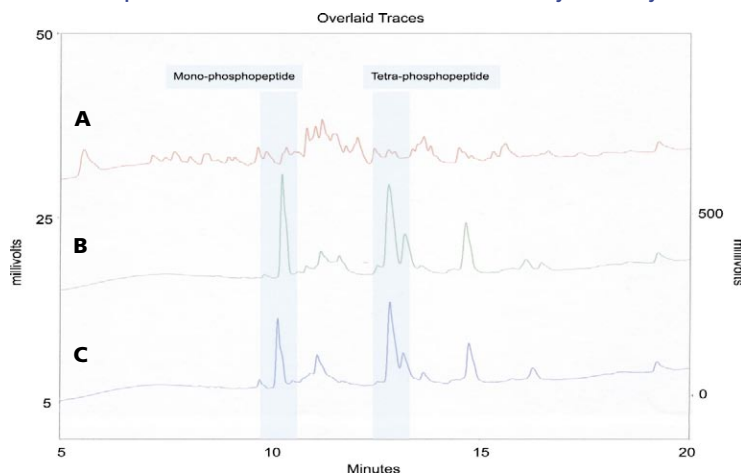


Figure 1. 10% Phosphoric acid elutes phosphopeptides comparably to 0.4 M ammonium hydroxide, as demonstrated by HPLC. Prior to tryptic digest, BSA and β casein samples were denatured in 0.05 M Bicine, 8 M Guanidine HCl, pH 8.5, and reduced and alkylated as per the PROT-RA kit. To prepare samples for enzymatic digestion, each reaction mixture was dialyzed against running de-ionized water, followed by an addition of ammonium bicarbonate to obtain a final concentration of 0.01 M and a pH of 8.5. TPCK-treated trypsin was added at a concentration of 1 mg/ml in 0.001 M HCl. Samples were digested for 5 hours at 37 °C. Peptides from BSA and β casein were combined at a ratio of 4:1 respectively. A sample of mixed BSA and β casein peptides (A) of approximately 500 μ g of total weight was reconstituted, applied to the spin column, which was washed to remove non-retained material, and eluted by either 10% phosphoric acid (B) or 0.4 M ammonium hydroxide (C) to enrich for phosphopeptides as per the kit instructions.

- By eluting with either 10% phosphoric acid or 0.4 M ammonium hydroxide, one can successfully elute phosphopeptides from the IMAC based enrichment media.

Enhanced Phosphopeptide Signals with Phosphoric Acid Elution in Combination with DHB for MALDI

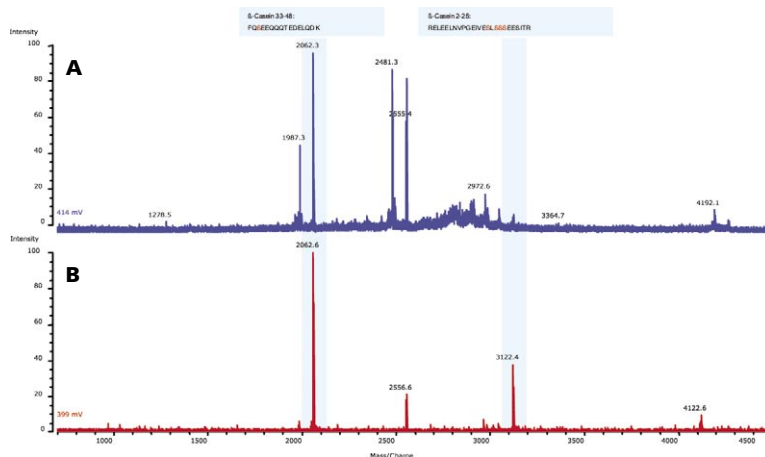


Figure 2. Using 10% phosphoric acid to elute from the spin column, with the addition of DHB as the MALDI matrix, accomplished enhanced phosphopeptide signal. The ACCA matrix (A) was compared to the DHB matrix (B) after elution of β casein peptides with 10% phosphoric acid during MALDI-TOF MS analysis.

- The mono-phosphopeptide from β casein was evident in both samples; however, the tetra-phosphopeptide signal was masked by suppression effects. When DHB was used as the matrix, an intact multi-phosphorylated species was readily observed.
- Additionally, we have demonstrated that DHB can be included in the elution buffer to reduce sample handling before analysis and not adversely affect signal enhancement (data not shown).

Separation of an EGFR Phosphopeptide From Its Non-phosphorylated Counterpart

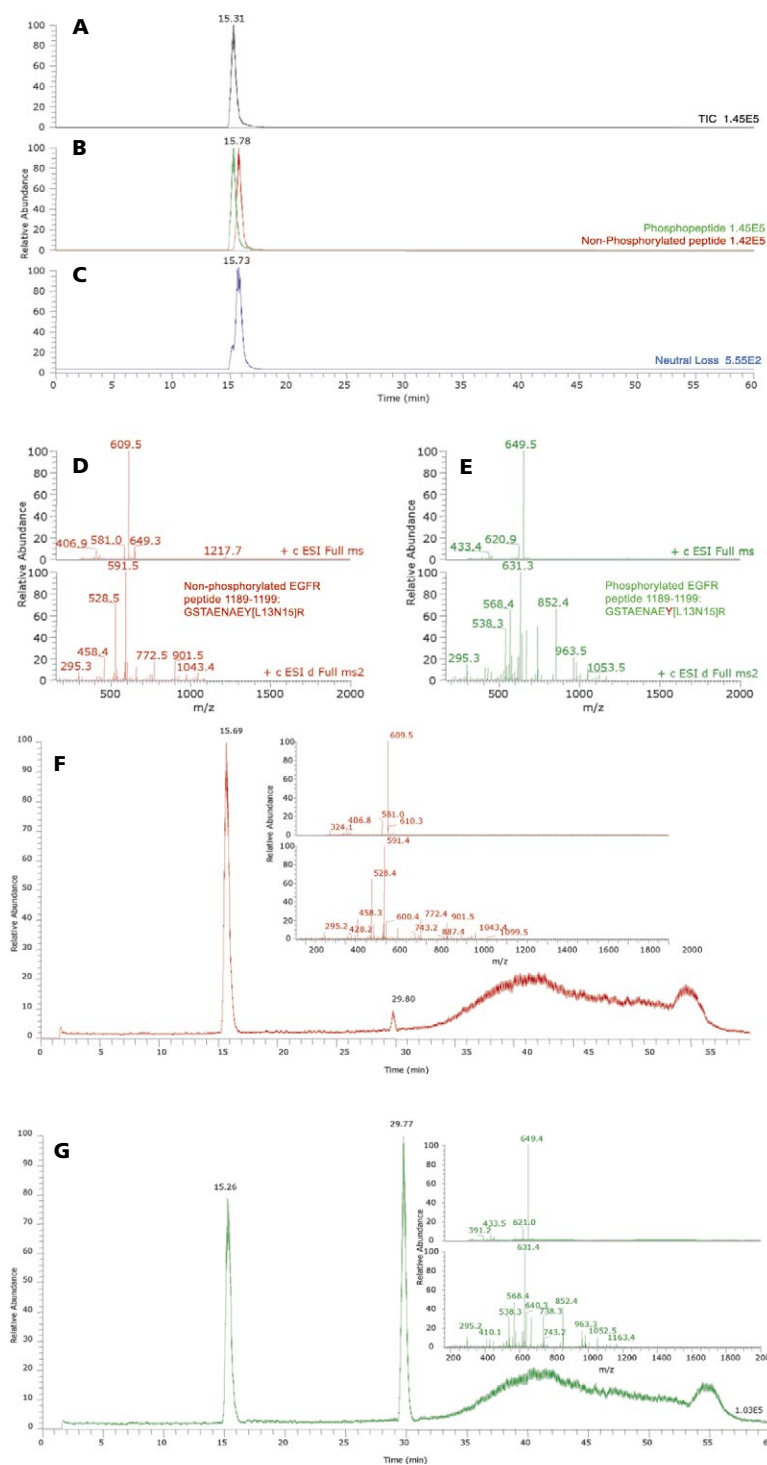


Figure 3. Peptides differing only by a phosphorylated tyrosine are successfully separated following IMAC based enrichment. An equal mixture of two synthetic labeled EGFR peptides (A–E) identical in amino acid sequence one having a phosphorylated tyrosine and the other non-phosphorylated, was applied to the PHOS-Select™ Gallium Spin Column. The total and restricted ion currents (A–B) represent the combined signal for the two peptides that were used in the application. The neutral loss spectrum (C) indicates the phosphorylated peptide by detecting the loss of the phosphate moiety. The MS/MS spectra (D–E) yields the sequence information of the two peptides. During the wash steps, the non-phosphorylated peptide (F) was successfully recovered. The elution step recovered the phosphorylated EGFR peptide (G). Identification was confirmed by MS/MS (F–G inlays).

- This experiment illustrates that both phosphorylated and non-phosphorylated peptides can be separated for analysis using IMAC based enrichment.

Phosphopeptide Enrichment From a Complex System

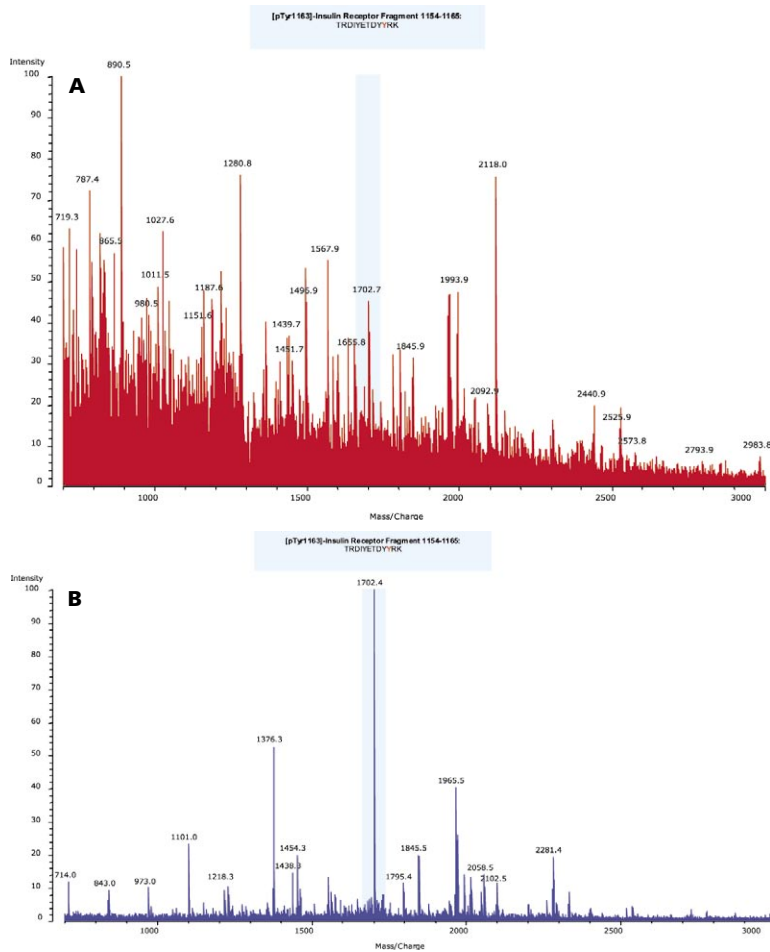


Figure 4. To demonstrate the utility in a complex system, a tyrosine-phosphorylated control at a level of 0.5 μg was spiked into a trypsin digest of an *E. coli* cell extract that had been reduced, alkylated, and typically digested. The sample was reconstituted in Bind/Wash Solution, applied to the spin column, which was washed to remove non-retained material, and the phosphopeptide was eluted as per the instructions from the kit. A total of 130 μg of material was applied to the column.

- As highlighted, the [pTyr¹⁶³]-Insulin Receptor Fragment is not observed when in the complex extract (A), but becomes highly enriched and readily apparent after use of the PHOS-Select Gallium Spin Column (B).

Conclusions

- Gallium matched with a NTA analog chelate bonded to a silica substrate was optimal in the spin column format for enrichment of phosphopeptides.
- Ammonium hydroxide and phosphoric acid are comparably efficient eluents.
- Phosphoric acid, in combination with DHB matrix, enhances signal intensity of phosphorylated peptides with the greatest enhancement shown for multi-phosphorylated peptides.
- The IMAC based enrichment works well with phospho-tyrosine peptides, which are typically low abundance, relative to serine and threonine phosphorylated peptides.

References

1. Brill, L., et al., Robust Phosphoproteomic Profiling of Tyrosine Phosphorylation Sites from Human T Cells Using Immobilized Metal Affinity Chromatography and Tandem Mass Spectrometry. *Anal Chem.*, **76**, 2763-2772, (2004).
2. Mann, M., et al., Analysis of protein phosphorylation using mass spectrometry: deciphering the phosphoproteome. *Trends in Biotechnol.*, **20**, 261-268, (2002).
3. Kjellstrom, S., and Jensen, O., Phosphoric acid as a matrix additive for MALDI MS analysis of phosphopeptides and phosphoproteins. *Anal. Chem.*, **76**, 5109-5117, (2004).

