

Absolute Quantification (AQUA™) of Phosphorylation Levels in Biological Samples using Immobilized Metal Affinity Chromatography Enrichment (IMAC)

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Overview

The analysis of phosphorylated peptides by mass spectrometry has often been hindered by the intrinsically low abundance and poor ionization of phosphopeptides. Immobilized Metal Affinity Chromatography (IMAC) has been shown to be one methodology to increase phosphopeptide signal levels, and thereby allow confident determination of phosphorylation. We have developed an (IMAC) based microspin column that has been optimized for selectivity and sensitivity through the matching of a chelate immobilized on silica with Ga³⁺ metal. Thus, when AQUA (absolute quantification) peptides were spiked into a cell lysate prior to tryptic digestion and subsequently enriched using the IMAC microspin column, the phosphopeptides were easily identified. While traditional methods of determining phosphorylation levels in protein samples have been cumbersome, the combination of IMAC and AQUA would be comparatively simple. Additionally, the IMAC technology permits identification of even low-abundance phosphoproteins within complex samples. As a result, the enrichment methodology is highly beneficial for the discovery of novel phosphorylated species, and when paired with AQUA, allows for their quantification under varying biological conditions.

Introduction

The addition and removal of phosphate groups by kinases and phosphatases, respectively, are dynamic signaling events which play key roles in a number of cellular processes, and offer insight into the inner workings of the cell. The traditional study of phosphosignalling is accomplished through an enrichment process which is specific for the phosphomonoester and greatly reduces the complexity of the sample prior to analysis. Thus far, the most popular tool which is utilized for phosphopeptide enrichment, IMAC, has been shown to be invaluable in the global analysis of phosphorylation levels. However, inherent biases within the IMAC technology, have demonstrated that the technique is not amenable to the quantitative determination of individual phosphopeptides.¹

AQUA is rapidly becoming a powerful tool in the molecular biologist's arsenal, allowing for the mass spectrometric quantification of peptides within complex biological samples and thereby facilitating the determination of protein expression levels in the context of different system perturbations.² To date, AQUA has been underutilized for the determination of levels of post-translational modifications (PTMs) such as phosphorylation.

We have chosen to principally demonstrate the combination of a novel IMAC technology paired with an AQUA workflow, which would allow determination of phosphorylation levels within a complex sample. The novel IMAC technology has been developed in an ideal format for workflows involving low levels of sample. In the initial studies presented here, AQUA peptides, derived from epidermal growth factor receptor (EGFR) were spiked into an *E. coli* tryptic digest at varying levels, to determine both the column's effectiveness and lower limits of enrichment.

Methods

E. coli cells were lysed and the protein fractions were extracted using CellLytic B as per the manufacturer's instructions. Quantitation of the resulting solution was accomplished via bicinchoninic acid (BCA) assay. The resultant solution was dried, reconstituted in 6M guanidine hydrochloride, and heated to 60 °C for 5 minutes. An equivalent volume of 100 mM ammonium bicarbonate was added and the resultant denatured proteins were reduced (tributyl phosphine) and alkylated (iodoacetamide) under standard protocols. The solution was subsequently diluted 1:1 by the addition of enzyme reaction buffer (100 mM ammonium bicarbonate). Proteolytic cleavage of the proteins was accomplished using proteomics grade trypsin at a 1:40 weight ratio at 37 °C overnight. *E. coli* lysate solutions were dispensed into vials as 3 µg aliquots and dried via speedvac.

Phosphorylated and non-phosphorylated AQUA peptides were custom ordered from Sigma Genosys and used as received.

IMAC enrichments were all performed in duplicate using the phosphopeptide enrichment kit from Sigma-Aldrich. The *E. coli* peptide fractions were initially dissolved into 50 µL of the supplied Bind/Wash solution, and the pH of the solutions were verified to be ≤ 3.0. AQUA peptides were then spiked into *E. coli* fractions at levels of 39 ng, 3.9 ng, and 390 pg, corresponding to loads of 1.3, 0.13, and 0.013 weight percent, respectively. The samples were loaded onto an equilibrated PhosphoProfile™ Gallium Spin Column by spinning gently (500 × g) in a microcentrifuge. The samples were incubated for 15 minutes at room temperature, after which time the columns were centrifuged and washed with 150 µL total of the Bind/Wash Solution to remove unbound peptides (flow-through fraction). A water wash of 50 µL was employed to remove any residual Bind/Wash Solution prior to elution. A total of 50 µL of the provided Elution Solution was used to elute the phosphopeptides.

All samples were separated by reverse phase on an Agilent capillary 1100 HPLC employing a peptide trap column for peptide preconcentration and desalting prior to introduction to the 150 mm × 0.5 mm C18 column. This separation was performed with a gradient of water and acetonitrile both of which were acidified with formic acid. Peptides were detected on a Thermo-Finnigan LTQ linear ion trap mass spectrometer set for full scan MS or selective reaction monitoring (SRM) experiments.

Materials

All materials were obtained from or prepared at Sigma-Aldrich, unless noted.

- Trypsin, Proteomics Grade (Cat. No. T6567)
- QuantiPro™ BCA Assay Kit (Cat. No. QPBCA)
- ProteoPrep™ Reduction and Alkylation kit (Cat. No. PROT-RA)
- PhosphoProfile™ Phosphopeptide Enrichment Kit (Cat. No. PP0410)
- CellLytic™ B (Cat. No. B7435)
- Non-phosphorylated AQUA peptide, GSTAENAEYL(¹⁵N,¹³C)R (Sigma-Genosys Custom Order)
- Phosphorylated AQUA peptide, GSTAENAEpYL(¹⁵N,¹³C)R (Sigma-Genosys Custom Order)

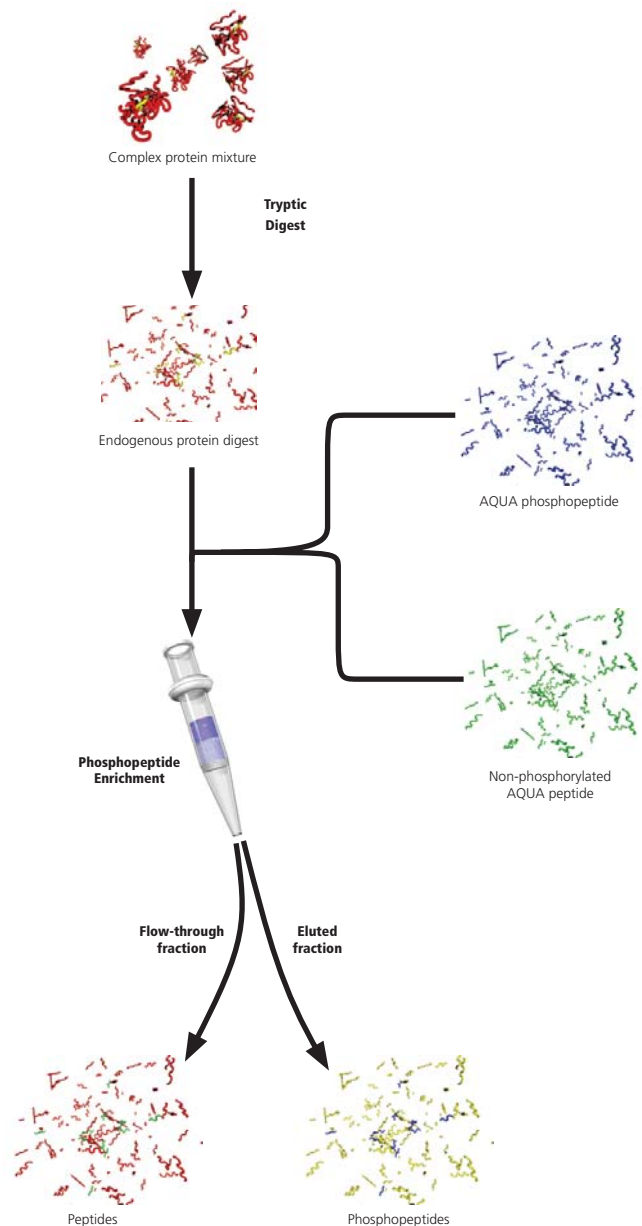


Figure 1: Workflow highlighting the use of phosphopeptide enrichment kit for selective enrichment of the phosphorylated species.

Results and Discussion

The combination of the AQUA technology with IMAC enrichment of phosphopeptides presents a unique and powerful method by which PTM (post-translational modification) levels can be quantitated. However, the technique is highly dependant upon a few key factors: most importantly, the rigor and effectiveness of the IMAC technique. As has been shown previously, traditional IMAC technologies are often plagued with problems of non-specific adsorption of acidic peptides, as well as bias in the enrichment of specific phosphopeptides, both of which ultimately raise doubt as to the reliability of the enrichment technique.³ However, the combination of Ga³⁺ metal and nitrilotriacetic acid (NTA) chelate, when incorporated into a silica matrix, was recently shown to minimize non-specific adsorption by non-phosphorylated species, in addition to showing little bias in the enrichment of specific phosphorylated amino acids.⁴

Herein, we examine the sensitivity levels of the novel IMAC technology, by employing a control mixture containing phosphorylated and non-phosphorylated AQUA peptides corresponding to amino acids 1165–1175 of the human epidermal growth factor receptor. The sequence has been shown to be endogenously phosphorylated on a single tyrosine residue.⁵ The peptides were spiked at three different levels (39 ng, 3.9 ng, and 390 pg) into an *E. coli* protein digest (3 µg total protein), and tested for enrichment in the phosphopeptide enrichment kit. Analysis of the resulting elution fractions using extracted ion currents indicates that the 39 and 3.9 ng level spikes are easily observed with little background, while the 390 pg level has an unacceptable signal-to-noise level. Accordingly, the signal intensity of the phosphopeptide peak in each chromatogram shows the appropriate trending signal levels (Figure 2).

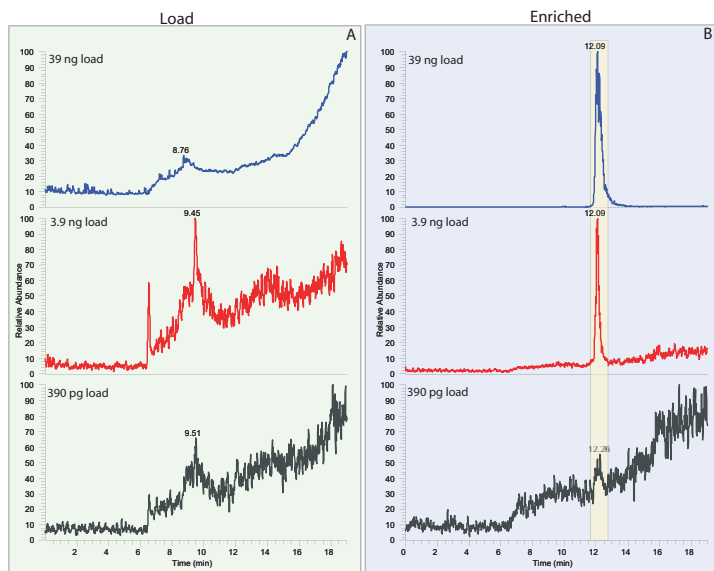


Figure 2: Extracted ion currents of AQUA phosphopeptides contained in the load and elution fractions from the phosphopeptide enrichment kit. The sample load prior to enrichment (A) demonstrates significant noise levels with little signal. Following IMAC enrichment of 39 ng, 3.9 ng, and 390 pg loads, the enriched (elution) fractions appropriately show decreasing signal levels of the phosphopeptide (B).

Further analysis of the 390 pg column load by SRM indicated that the phosphopeptide was observable with an acceptable signal-to-noise level and would allow for quantification using the AQUA methodology. SRM development and chromatogram of the phosphopeptide spiked at the 390 pg load level are shown in (Figure 3).

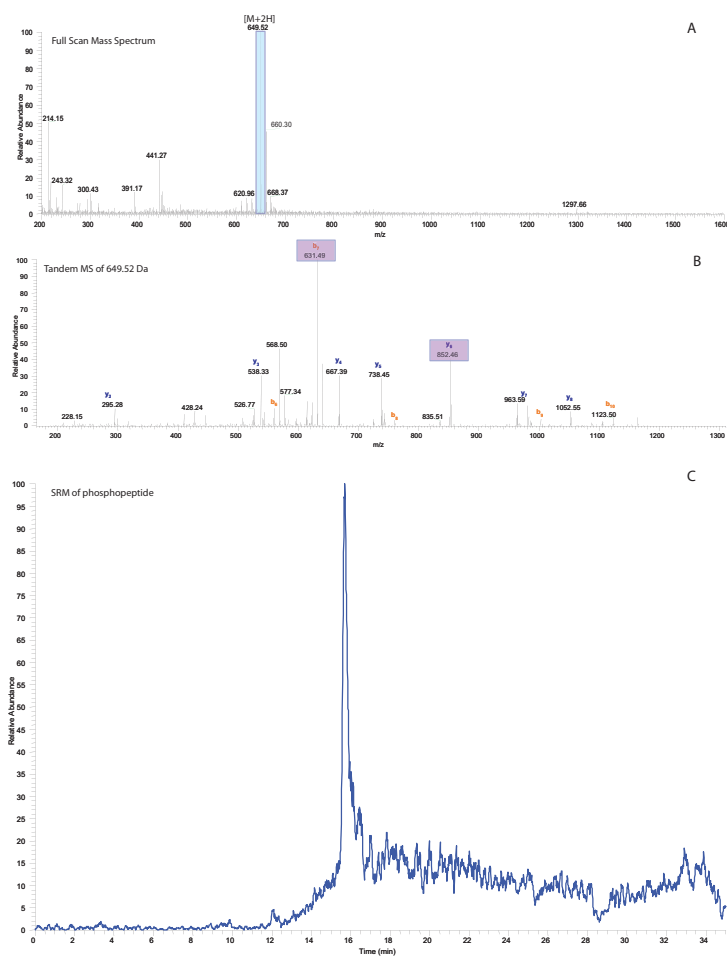


Figure 3: SRM development of AQUA phosphopeptides to examine low levels of phosphorylation within a biological sample.

The AQUA phosphopeptide was analyzed by tandem MS in order to determine appropriate daughter ions. b7 and y6 ion were chosen as shown (B). The sensitivity of SRM facilitates detection of even a 390 pg IMAC load (C).

Conclusions

- The novel IMAC resin presented herein is a powerful technique for the analysis of phosphorylation levels within a biological sample, demonstrating remarkable sensitivity and selectivity to enrichment of low levels of phosphopeptides.
- Residual loss upon application to the column is minimal and still allows for signal detection even at the 390 pg load level, when monitored by SRM.
- The signal levels observed by mass spectrometry were found to be appropriately load dependant.

Acknowledgments

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