A Novel Silica-Based Immobilized Metal Affinity Chromatography (IMAC) Technology for the Enrichment of Phosphopeptides

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Abstract

Protein phosphorylation is a dynamic post-translational modification that plays a critical role in the regulation of numerous cellular events including signal transduction, gene expression, and apoptosis. Phosphopeptides are often found in low natural abundance and ionize poorly, complicating their identification by mass spectrometry. Immobilized metal affinity chromatography (IMAC) is a tool that has been developed to aid in the isolation and subsequent identification of phosphorylated molecules.

A novel silica matrix for IMAC applications utilizing a proprietary nitrilotriacetic acid (NTA) analog has been developed and its use optimized after being complexed with gallium. This poster highlights the advantages of this matrix, including enhanced recovery and selectivity, as compared to similar phosphopeptide enrichment technologies, when each are tested against a standardized peptide mixture.

Introduction

Phosphorylation of proteins and peptides *in vivo* is a reversible process controlled by the action of kinases and phosphatases for the addition and removal, respectively, of phosphate moieties. The most common phosphorylation events in eukaryotes occur on three amino acids: serine, threonine, and tyrosine. However, as many as six other amino acids hold the potential for modification, adding complexity to the study of protein phosphorylation and further emphasizing the importance of the identification of phosphorylation sites for characterization of signaling events.

Three popular techniques exist which are designed to aid researchers in the enrichment and subsequent analysis of the phosphoproteome. The first of these, immunoprecipitation (IP) is a fairly commonly utilized method for the enrichment of phosphotyrosines. However, IP has been less reliable and more costly when used for the enrichment of either phosphoserines or phosphothreonines.[1] A second and more recent technology which relies on chemical replacement of the alkyl phosphate group with an enrichable tag[2] has shown promise, but requires harsh alkaline treatment of the protein which causes issues of non-specific functionalization. The third enrichment type, IMAC, is the most frequently utilized enrichment technique and works for all phosphorylated species, but has been problematic in areas of both specificity and recovery.

Traditional IMAC technologies consist of two components: an immobilized chelating group and a metal species. The chelating group binds to and presents one face of the metal, allowing interaction and separation of phosphorylated species. A variety of metals have been tested in conjunction with IMAC and, generally, Ga^{3+}, Fe^{3+}, Al^{3+} and Zr^{4+} have been shown to be most effective.[3] Through an iterative process, an optimized pairing of Ga^{3+} metal with a nitrilotriacetic acid (NTA) chelate analog placed onto a silica matrix has been developed and conveniently supplied in a spin column format.

Methods

Bovine serum albumin (BSA) was formulated at a concentration of 10 µg/µL in a choatropic reagent containing urea, thiourea, Tris, and the detergent C7BzO. Disulfide bonds were reduced and alkylated using tributylphosphine and iodacetamide, respectively, as per the manufacturer's instructions. The protein concentration was subsequently adjusted to 1 µg/µL by dilution with 100 mM ammonium bicarbonate and digested using trypsin immobilized in a spin column format. Column and sample preparation were completed as per the manufacturer's instructions. A 100 µL protein sample (100 µg) was applied to each trypsin spin column. Following a 15 minute incubation/digestion period, the tryptic peptides were eluted by applying 150 µL of 100 mM ammonium bicarbonate and centrifuging (800 × *g*) for 90 seconds.

Standard phosphopeptides were used as received. The lyophilized solids were first dissolved in water and an approximately equimolar mixture of the peptides was formulated. The phosphopeptides were chosen to contain phosphoserine, phosphothreonine, or phosphotyrosine, the three most common sites of phosphorylation. Each phosphopeptide was added to the BSA digest at a weight ratio of ~1.7% to produce a total phosphopeptide content of ~5% by weight.

All IMAC enrichments were performed in duplicate using each manufacturer's instructions. For the novel silica-based spin column (Sigma Phosphopeptide Enrichment Kit), samples were lyophilized following tryptic digestion and reconstituted to obtain a final volume of 50 µL using the supplied Bind/Wash Solution. The samples were loaded onto an equilibrated PHOS-Select™ Gallium Spin Column by spinning gently (500 x *g*) in a microcentrifuge. The samples were incubated for 15 minutes at room temperature, after which the columns were centrifuged and washed with 150 µL total of the Bind/Wash Solution to remove unbound peptides. A water wash of 50 µL was employed to remove any residual Bind/Wash Solution prior to elution. A total of 75 µL of the provided Elution Solution was used to elute the phosphopeptides.

High-pressure liquid chromatography analysis was performed on a Shimadzu LC-6 using mobile phases of water (A) and acetonitrile (B), each containing 0.1% trifluoroacetic acid (TFA). A C18 column (25 cm × 4.6 mm) containing 5 µm silica was used. A linear gradient of 2–80% solvent B over a 60 minute period and a flow rate of 1.0 mL/min was employed for all chromatograms. Ultraviolet detection was performed at a wavelength of 200 nm. UV detection of chromatographic peaks was performed at a low wavelength to minimize biases of peptides containing aromatic functionalities. A typical chromatogram of the BSA digest/phosphopeptide test mixture is shown in **Figure 1**.

Materials

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

- Phosphotyrosine peptide (Cat. No. P3860)
- Phosphothreonine peptide (Cat. No. T8696)
- Phosphoserine peptide (Cat. No. P0995)
- Bovine Serum Albumin (Cat. No. A7030)
- Protein Extraction Reagent Type 4 (Cat. No. C0356)
- ProteoPrep™ Reduction and Alkylation Kit (Cat. No. PROTRA)
- Trypsin Spin Columns, Proteomics Grade (Cat. No. TT0010)
- Discovery® BIO Wide Pore C18 HPLC Column, 25 cm × 4.6 mm (Supelco Cat. No. 568223U)
- Phosphoprofile™ Phosphopeptide Enrichment Kit (Cat. No. PP0410)

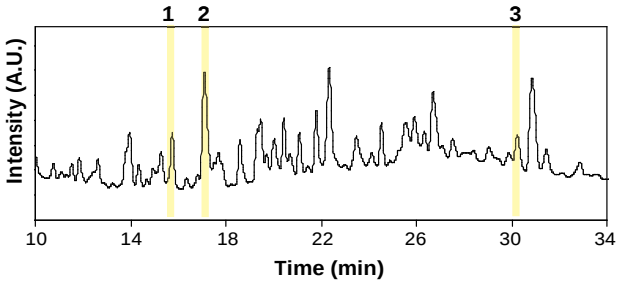

Figure 1: HPLC chromatogram of a tryptically digested BSA sample containing control phosphopeptides (highlighted in yellow). The phosphopeptides are representative of the three most common sites of phosphorylation: serine (1), threonine (2), and tyrosine (3).

Results and Discussion

IMAC enrichment technologies have long been plagued by two significant problems: specificity and recovery. Various commercial products have made attempts to minimize these issues by alterations in the binding metal, its chelate, and the substrate to which both are bound. In this study, a sampling of commercially available products were selected for comparison against a new Sigma product which is based upon a novel combination of gallium with a NTA chelate analog on a silica matrix. Competitor's products which were used in this study present Zirconium (competitor A), Gallium (with iminodiacetic acid [IDA] chelate, competitor B), or titanium dioxide[4] (competitor C) as the binding moiety. The enrichment protocol for each technology is similar (see workflow, **Figure 2**) and provides fractions corresponding to both phosphorylated (eluted) and non-phosphorylated (flow through) species.

HPLC analysis of the enriched and depleted fractions for each product allowed for the quantitation, and thus in-depth analysis, of the performance of each product. For this study, a standard mixture was utilized, which was comprised of a tryptic digest of BSA and three phosphopeptides. BSA is a non-phosphorylated protein (MW ~66 kDa) which is highly acidic in nature (pI ~5.7). A complete tryptic digestion of BSA produces > 60 distinct peptides, resulting in a poorly resolved and highly complex chromatogram **(Figure 1)**. Nearly 2/3 of the resulting peptides are acidic, a property which has been shown to contribute significantly to non-specific binding in previous IMAC technologies.[5]

In each of the IMAC technologies, the enrichment ability of the columns is dependent upon the proper binding and subsequent elution of the phosphopeptides. Inherent biases in each technology, however, means that what is recovered may not be fully representative of what was present in the original mixture. HPLC chromatograms of the relative phosphopeptide load and recovery for each of the products tested are shown in **Figure 3** and demonstrate the biases of each of the columns for phosphopeptide standards 1 and 2. These biases can arise from loss of the phosphopeptides due to irreversible binding within the matrix, or through non-retention within the column and passage into the flow through. Examination of the flow through fractions for each column by HPLC confirms that, for competitors B and C, a large percentage of the phosphopeptides showed no retention within the column **(Figure 4)**.

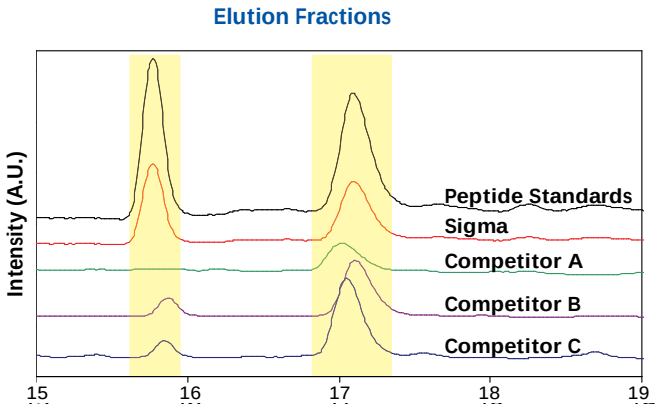

Figure 3: Selected portions of representative HPLC chromatograms demonstrating enrichment of phosphopeptides for each IMAC technology tested. Quantitation of these results are given in Table 1. Note that competitor A, B, and C technologies were biased in selecting Peptide 2, while Sigma's technology bound and eluted the peptides in approximately the same ratio as applied to the column.

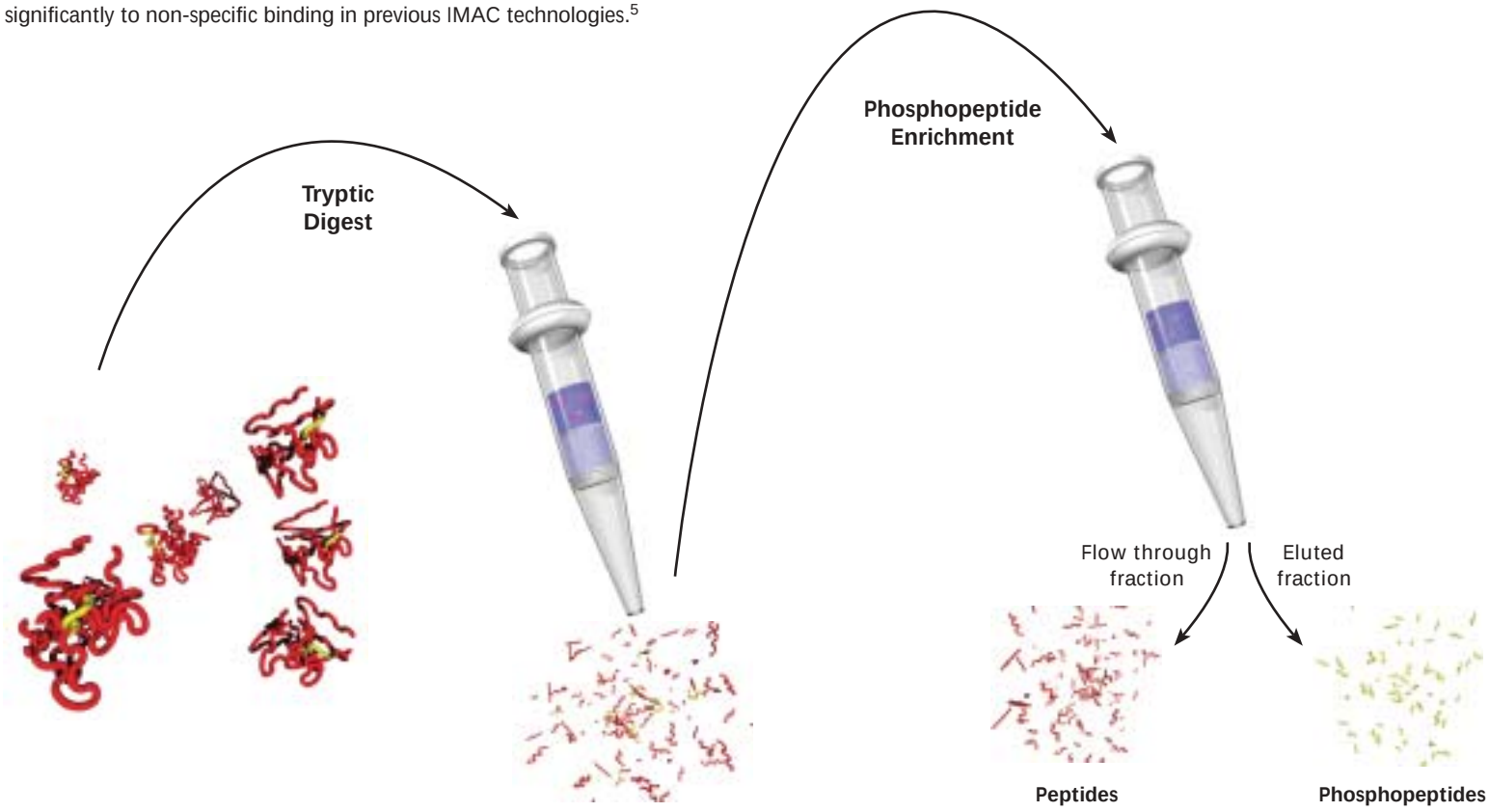

Figure 2: Workflow highlighting the use of the spin columns for digestion and selective enrichment of a protein sample.

The ability to bind, retain, and subsequently elute phosphopeptides is dependent in large part upon the column's specificity toward the phosphate functionality. Products which also have a strong tendency to bind carboxylate ions, for example, may display a high degree of non-specificity in addition to low phosphopeptide recovery. Quantitative analysis of both the individual and overall phosphopeptide recoveries, as well as the specificity for each product, is given in **Table 1**.

Overall, each of the competitor's products was found to give mixed results for the three phosphopeptides, with low-to-moderate overall recoveries. However, the Sigma Phosphopeptide Enrichment Kit was found to give the best phosphopeptide recovery of all products tested with little bias toward any one of the three phosphopeptide types. Additionally, the Sigma product was also found to have the smallest percentage of non-specific peptide retention.

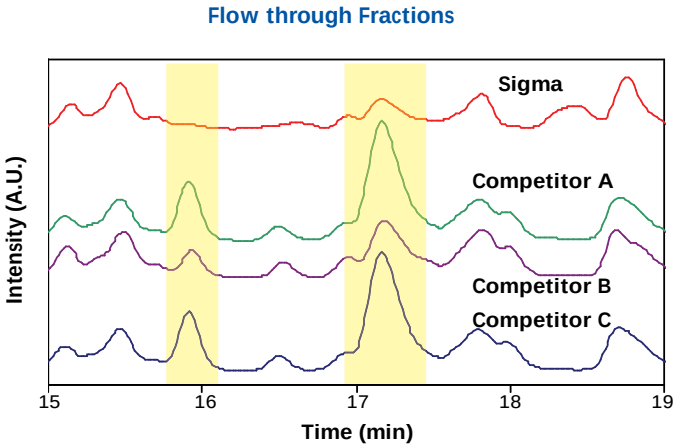

Figure 4: HPLC chromatograms of the flow through fractions of each of the IMAC products tested.
The highlighted (yellow) peaks represent phosphopeptides 1 and 2 and indicate non-binding within the columns. The Sigma product allowed the least loss of phosphopeptide to the flow through.

Kit	Recovery of phosphopeptide standards				Specificity*
	1	2	3	Total	
Sigma	59%	52%	74%	59%	50%
Competitor A	6%	19%	11%	13%	28%
Competitor B	39%	56%	17%	42%	28%
Competitor C	37%	65%	37%	46%	25%

Table 1: Performance summary of the IMAC technologies tested within this study.

*Specificity was measured as a percentage of the total HPLC peak area corresponding to phosphorylated peptides which appeared in the elution fractions.

Conclusions

- Four leading IMAC products were found to have variable performance in the enrichment of phosphopeptides from a standardized mixture.
- The Sigma Phosphopeptide Enrichment Kit demonstrated less bias in the enrichment of peptides containing three of the most commonly phosphorylated species: phosphotyrosine, phosphoserine, and phosphothreonine.
- Overall peptide recovery was variable for each product tested, ranging from 59% (Sigma) to 13% (Competitor A).
- The Sigma Phosphopeptide Enrichment Kit also demonstrated little retention of non-phosphorylated species, whereas the three competitors tested had nearly double the level of non-specific retention.

References

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