

# Comparison of Two IMAC Formats for the Enrichment of Phosphopeptides for Analysis by Mass Spectrometry

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## Overview

### Purpose

- Isolation and enrichment of phosphorylated peptides from a tryptic digest using two affinity capture formats

### Methods

- Apply crude digest containing phosphopeptides
- Capture peptides
- Wash to remove unbound contaminants
- Elute and analyze bound material by HPLC-MS or MALDI-MS

### Results

- Determined capacity
- Demonstrated enrichment for phosphopeptides
- Observed preferential binding of tetra over mono-phosphopeptide in a crude  $\beta$ -casein digest
- Observed differences in elution selectivity

## Introduction

Protein phosphorylation represents an important post-translational modification, playing a critical role in a multitude of cellular regulatory events. The identification and characterization of phosphorylation sites is an important aspect in gaining an understanding of signaling processes. Mass spectrometry (MS) of phosphopeptides obtained from tryptic protein digests is becoming a powerful tool for characterization.<sup>1</sup> However, there is generally a need to significantly enrich the phosphopeptide content to compensate for low abundance, poor ionization, and suppression effects.<sup>2</sup>

Immobilized metal affinity chromatography (IMAC) is commonly used for purification of phosphorylated compounds. The highest affinity and selectivity are demonstrated using Fe (III) and Ga (III) chelates.<sup>2</sup> In addition, the use of Fe (III) nitrilotriacetic acid (NTA) type chelates demonstrates greater specificity for phosphorylated peptides than iminodiacetic acid (IDA) chelates.<sup>3</sup>

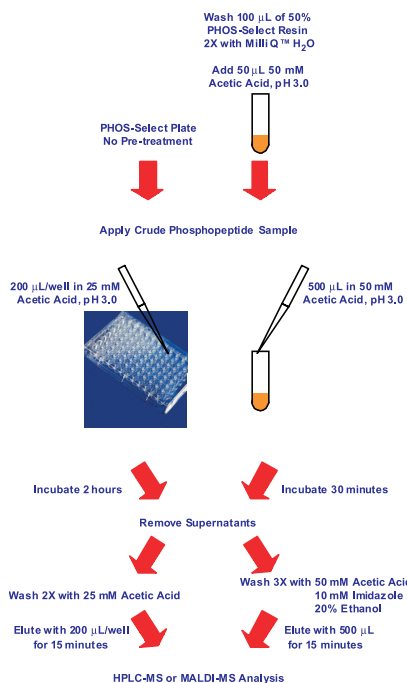
We have prepared Fe (III) NTA analog chelate ligands attached to both agarose gel and novel high capacity multiwell polystyrene plates. The two formats were compared for capacity and enrichment capabilities. Selectivity of binding for the mono-phosphopeptide (MPP) and tetraphosphopeptide (TPP) from a crude  $\beta$ -casein digest was evaluated under different loading concentrations. In addition, various eluents compatible with MS analysis were evaluated.

- Stensballe, A., et al. Electron Capture Dissociation of Singly and Multiply Phosphorylated Peptides., *Rapid Commun. Mass Spectrom.*, 14, 1793-1800 (2000).
- Zhou, W., et al. Detection and Sequencing of Phosphopeptides Affinity Bound to Immobilized Metal Ion Beads by Matrix-Assisted Desorption/Ionization Mass Spectrometry., *J. Am. Soc. Mass Spectrom.*, 11, 273-282 (2000).
- Newble, D., et al. Evidence for Phosphorylation of Serine 753 in CFTR Using a Novel Metal-Ion Affinity Resin and Matrix-Assisted Laser Desorption Mass Spectrometry., *Protein Sci.*, 6, 2346-2445 (1997).

## Materials

- All reagents used were purchased or prepared at Sigma-Aldrich
- HPLC-MS analysis was done using an HP 1100 (Agilent Technologies, CA) coupled to a Finnigan LCQ Classic Mass Spectrometer (ThermoFinnigan, CA)
- MALDI-MS analysis was performed using an Axima-CFR mass spectrometer (Shimadzu Biotech, UK)
- The MALDI matrix was 10 mg/mL  $\alpha$ -cyano-4-hydroxycinnamic acid, 25 mM ammonium citrate in 70% acetonitrile (ACN), 0.03% trifluoroacetic acid (TFA)

## Methods



## Results

### Binding Capacity Study

Species	Molecular Weight (Da)	Format	
		Plate (nmol/well)	Resin ( $\mu$ mol/mL)
4-methylumbelliferyl phosphate (MUP)	256,14	0,9	4,5
Monophosphopeptide ( $\beta$ -casein)	2061,96	0,5	8,0
Tetraphosphopeptide ( $\beta$ -casein)	3122,92	1,0	5,5

Figure 1. Binding capacities for the two formats were determined using purified phosphorylated molecules. Binding of MUP was determined via a fluorescence assay. Binding of the mono and tetraphosphopeptides was determined using HPLC-MS analysis.

## Plate Enrichment - HPLC

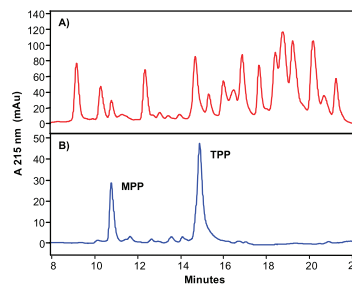


Figure 2. A) HPLC analysis of crude  $\beta$ -casein digest (4.5 pmol/ $\mu$ L, 50  $\mu$ L injection) prior to loading on the PHOS-Select Plate. B) HPLC analysis of purified phosphopeptides after elution from the PHOS-Select Plate using 200  $\mu$ L of 0.15 M  $NH_4OH$ .

## Plate Elution Selectivity - MALDI-MS

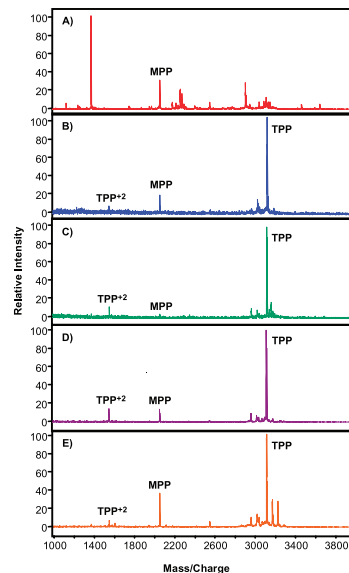


Figure 3. MALDI mass spectra of A) crude  $\beta$ -casein digest (4.5 pmol/ $\mu$ L) prior to loading on the PHOS-Select Plate and B-E) purified phosphopeptides after elution from the PHOS-Select Plate under various elution conditions. Eluents were as follows: B) 0.15 M  $NH_4OH$ , C) 0.4 M  $NH_4OH$ , neutralized with formic acid prior to analysis, D) 0.15 M  $NH_4OH/25\%$  ACN, and E) 5% formic acid/25% ACN.

## Resin Enrichment - HPLC

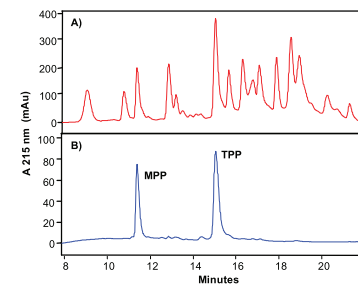


Figure 4. A) HPLC analysis of crude  $\beta$ -casein digest (113 pmol/ $\mu$ L, 5  $\mu$ L injection) prior to loading on the PHOS-Select Resin. B) HPLC analysis of purified phosphopeptides after elution from the PHOS-Select Resin using 500  $\mu$ L of 0.15 M  $NH_4OH/25\%$  ACN.

## Resin Elution Selectivity - MALDI-MS

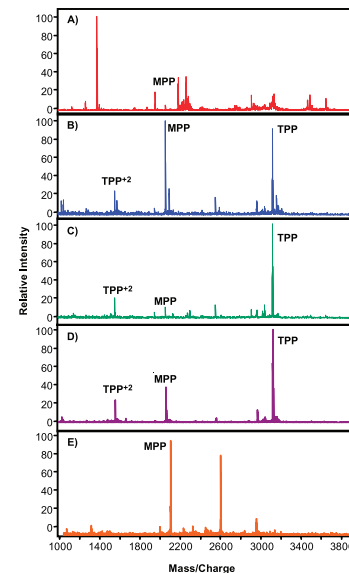


Figure 5. MALDI mass spectra of A) crude  $\beta$ -casein digest (113 pmol/ $\mu$ L) prior to loading on the PHOS-Select Resin and B-E) purified phosphopeptides after elution from the PHOS-Select Resin under various elution conditions. Eluents were as described in Figure 3.

## Plate Binding Selectivity

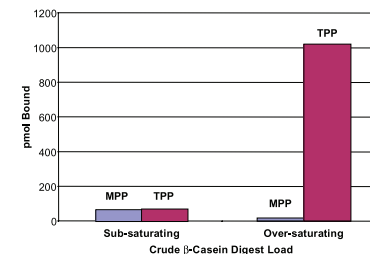


Figure 6. Binding of the mono and tetraphosphopeptides on the PHOS-Select Plate using a crude  $\beta$ -casein digest from over-saturating (3544 pmol) and sub-saturating (442 pmol) loading conditions. Binding was determined using HPLC-MS.

## Resin Binding Selectivity

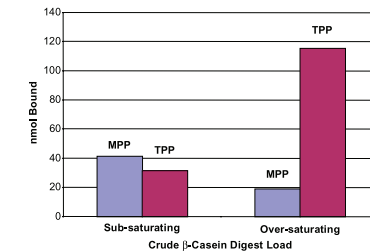


Figure 7. Binding of the mono and tetraphosphopeptides on the PHOS-Select Resin using a crude  $\beta$ -casein digest from over-saturating (770 nmol) and sub-saturating (56.3 nmol) loading conditions. Binding was determined using HPLC-MS.

## Conclusions

- Binding capacities of both formats are sufficient for capture and elution of phosphopeptides at concentrations detectable by MS analysis
- Both formats demonstrated significant enrichment of phosphopeptides from a crude  $\beta$ -casein digest allowing easy detection by MALDI-MS
- Differences in elution selectivity were observed between the two formats under the various elution conditions
- In sub-saturation loading conditions, both formats demonstrate co-capture of the tetra and monophosphopeptides from a crude  $\beta$ -casein digest
- In over-saturation loading conditions, both formats demonstrate preferential capture of the tetra over the monophosphopeptide from a crude  $\beta$ -casein digest