

Phosphopeptide Enrichment Using IMAC HyperCel™ Resin

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INTRODUCTION

Reversible phosphorylation of proteins is an essential part of cellular function as it plays a key role in the regulation of multiple cellular processes including proliferation, signal transduction, differentiation, apoptosis, and metabolic processes. Understanding these biological processes at the molecular level requires structural characterization and determination of the degree of peptide and protein phosphorylation. Phosphorylation generally occurs at very low levels, and enrichment of phosphorylated peptides or proteins is often a necessary prerequisite for their study. Immobilized metal affinity chromatography (IMAC) is typically used for enrichment of phosphorylated species, but these are frequently contaminated with acidic peptides. The choice of metal, IMAC resin, and bind/wash/elution conditions strongly influence the degree of enrichment of phosphorylated species.

This poster presents a highly selective enrichment method for phosphorylated peptides using IMAC HyperCel resin charged with iron (Fe³⁺) or Gallium (Ga³⁺). The method offers several advantages including: 1) flexibility of choosing either iron or gallium for enrichment of phosphopeptides; and 2) a reduction of binding of non-phosphorylated peptides to IMAC-Fe³⁺ and IMAC-Ga³⁺ while retaining a high binding affinity for phosphorylated peptides. The phosphopeptides enriched using the method are compatible with downstream mass spectrometry analysis.

MATERIALS AND METHODS

- IMAC HyperCel resin (PN 20093-010)
- FeCl₃, Ga₂(SO₄)₃, and Beta casein (Sigma)
- Nanosep® spin device with 0.45 µm GHP membrane (PN ODGHP34)

Table 1
Buffers Used for Phosphopeptide Enrichment

Buffers	Composition	Volume
Binding/Equilibration Buffer	0.1% acetic acid -pH 3.0	80 µL
Wash Buffer	0.1% acetic acid with 20% ACN	80 µL
Elution Buffer	100 mM ammonium bicarbonate pH 8.5	20 µL

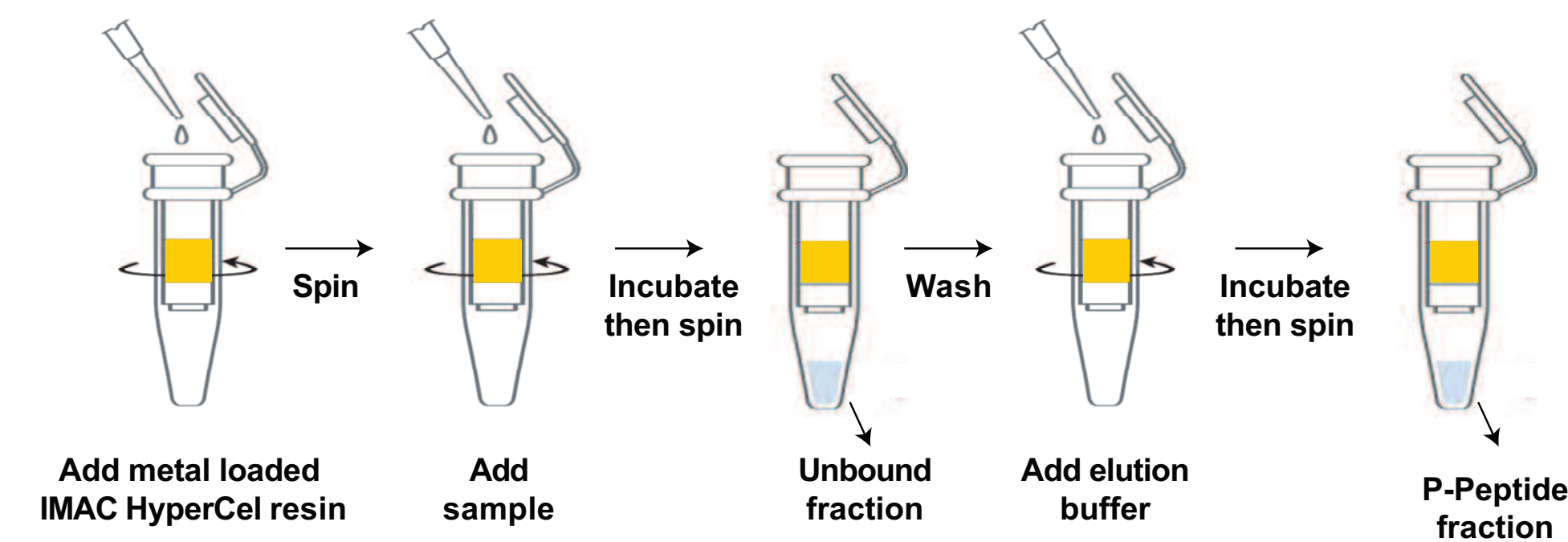
Charging of IMAC HyperCel Resin with Iron and Gallium

Incubate 1 mL 75% slurry of IMAC HyperCel resin (~0.75 mL resin) with 4 mL dH₂O (deionized water) for 10 minutes on a rotary shaker, then spin at 800 x g for 1 min. Remove water. Repeat this step three times to remove the storage buffer. Charge IMAC HyperCel with 1 mL of 0.15 M Iron (III) Chloride or Gallium (III) Sulfate, incubate for 20 minutes with shaking. Spin at 800 x g for 1 minute, then remove fluid. Wash the charged resin three times for 5 minutes each with 4 mL dH₂O. Spin at 800 x g, then remove fluid. Equilibrate the IMAC resin once with 4 mL of equilibration buffer, 5 minutes with shaking. Remove excess fluid, then add equal volume of fresh buffer to make a 50% slurry.

Enrichment of Phosphopeptides Using IMAC

The phosphopeptide enrichment is performed as shown in Figure 1. 40 µL of a 50% slurry of metal charged IMAC resin is placed into a Nanosep spin device and excess fluid removed by centrifugation. 3 µg of β-casein digest (acidified with 50 µL of 0.1% acetic acid) is added to the resin and incubated for 30 min. Unbound fraction is collected, then the resin is washed with 2x 40 µL wash buffer, 5 minutes per wash. The bound peptides are eluted with 3x 20 µL elution buffer, incubated for 5 minutes. Fractions are used directly for MALDI analysis.

Figure 1
Method Overview for Phosphopeptide Enrichment Using IMAC HyperCel Resin in a Nanosep Spin Device



MALDI-TOF (ABI Voyager DE Pro) Analysis of Phosphopeptides

A saturated solution of Alpha-cyano 4-hydroxy Cinnamic acid (HCCA) is prepared in matrix solution (33% acetonitrile in 0.1% TFA). The samples are diluted 1:5 in matrix solution. Equal volumes (1 µL) of diluted sample and saturated matrix solution are mixed and spotted using dried droplet method. All spectra are obtained by averaging 1000 laser shots fired at 10 different positions (100 shots each) in positive linear mode.

MATERIALS AND METHODS (continued)

Calculation of Phosphopeptide Enrichment

The relative peak intensities of phosphopeptide vs. other peptides can be used to assess the effectiveness of this enrichment method. A comparison of peak intensities of the most intense peptide in the starting material (m/z 1385) and the monophosphopeptide (m/z 2062) from the β-casein digest is performed using the following calculation.

$$f = \frac{(I_{\text{non-phosphopeptide}} / I_{\text{phosphopeptide}})_{\text{Starting Material}}}{(I_{\text{non-phosphopeptide}} / I_{\text{phosphopeptide}})_{\text{Enriched Eluate}}}$$

Enrichment factor (f) is the percent ratio of signal intensities for each peptide in both the Starting Material (I_s) and Enriched Eluate (I_e). This essentially normalizes the phosphopeptide peak to the non-phosphopeptide peak for each data collection. This analysis does not provide actual concentration of detected peptides, but the degree of improvement in MALDI detection does reflect real changes in concentration.

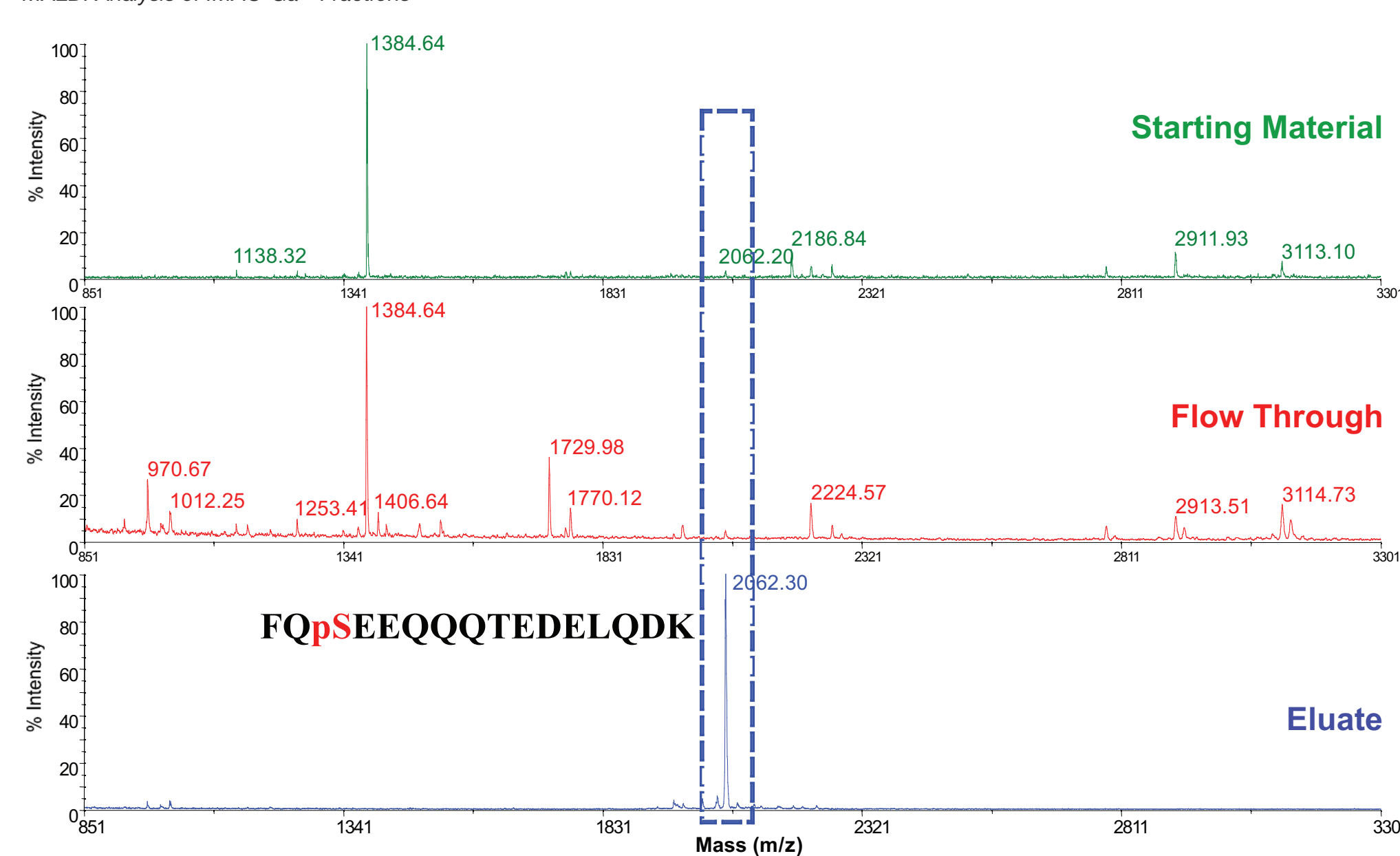
RESULTS

A tryptic digest of β-casein is used to optimize the protocol for phosphopeptide enrichment using IMAC HyperCel resin charged with iron or gallium. Several factors influenced the overall performance including the volume of resin, the amount of protein digest, and the wash and elution conditions. The final method is simple and fast (< 60 minutes) and is specific for the enrichment of the β-casein monophosphopeptide.

MALDI Analysis of IMAC-Ga³⁺ Fractions Demonstrate Highly Selective Capture of Phosphopeptides

MALDI-TOF analysis of IMAC-Ga³⁺ fractions from β-casein digest (Figure 2) clearly shows the selective retention (absence in flow through fraction) and elution (dominance in eluate fraction) of the phosphopeptide at m/z 2062.34. This phosphopeptide corresponds to the peptide sequence FQpSEEQQTEDELQDK (amino acid 48-63 in β-casein, phosphorylated at Ser 50). Many peptides are observed in the flow through fraction, the most pronounced with m/z of 970, 1385, 1730, 1770, 2225, and 2914. These peptides are not phosphorylated, thus demonstrating method selectivity. The purity of the phosphopeptide in the eluate fraction appears to be quite high as there are no other significant peaks detected in this fraction.

Figure 2
MALDI Analysis of IMAC-Ga³⁺ Fractions



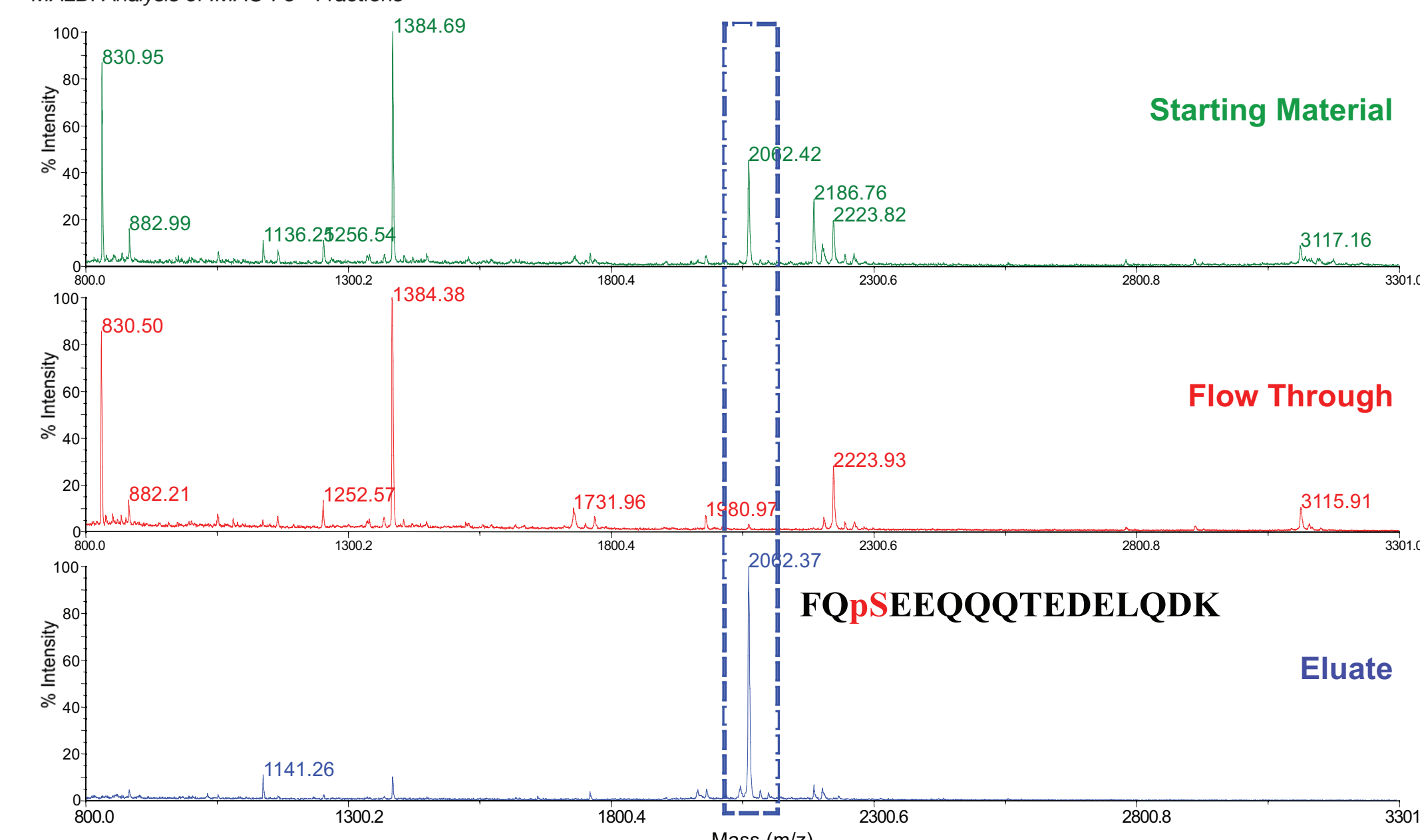
Total β-casein digest (Starting Material), the unbound fraction (Flow Through), and Eluate are indicated. Monophosphopeptide is indicated with a blue box. Amino acid sequence of the phosphorylated peptide is shown, with phosphorylated Ser in red.

MALDI Analysis of IMAC-Fe³⁺ Fractions Demonstrate Selective Enrichment of Monophosphopeptide

The same protocol was used to assess IMAC-Fe³⁺ for phosphopeptide enrichment. MALDI-TOF analysis of IMAC-Fe³⁺ fractions from β-casein digest (Figure 3) clearly show that the monophosphorylated peptide with m/z of 2062.42 is enriched (eluate fraction) while non-phosphorylated peptides are recovered in the flow through fraction. A peak with m/z of 1980.97 seen in the flow through fraction is the same peptide without the phosphate group. Note that in contrast to the IMAC-Ga³⁺ results, the phosphopeptide is readily detected in the starting material. However, the purity of this peptide is dramatically improved in the after enrichment using IMAC-Fe³⁺.

RESULTS (continued)

Figure 3
MALDI Analysis of IMAC-Fe³⁺ Fractions



Total β-casein digest (Starting Material), the unbound fraction (Flow Through), and Eluate are indicated. Monophosphopeptide is indicated with a blue box. Amino acid sequence of phosphorylated peptide is shown, with phospho-Ser in red.

Enrichment Factor Calculations Demonstrate that IMAC HyperCel Resin Charged with Iron or Gallium Effectively Enrich Phosphopeptides from a β-Casein Tryptic Digest

An estimate of relative enrichment of the phosphopeptide as compared to non-phosphorylated peptides in the β-casein tryptic digest is shown in Table 2. Results from both IMAC-Fe³⁺ and IMAC-Ga³⁺ are calculated. In both cases, the change in relative peak intensity of the phosphopeptide as compared to the non-phosphopeptide with the greatest peak intensity (m/z 1384.69) after enrichment is significant. The average enrichment factor for IMAC-Ga³⁺ is 101.6 and for IMAC-Fe³⁺ is 27.0. Thus, in this example with a β-casein tryptic digest the Ga³⁺ has a higher degree of enrichment. However, this is partly due to better detection of the phosphopeptide peak in the starting material (Figure 3). Different tryptic digestions were used and MALDI data collection was done separately for these two experiments. Differences in results could be partly due to experimental differences. However, it is evident that the traces of phosphopeptide observed in the flow through indicates effective capture of phosphopeptides.

Table 2
Relative Enrichment of Phosphopeptide vs. Non-Phosphorylated Peptide Using IMAC-Fe³⁺ and IMAC-Ga³⁺

IMAC-Fe ³⁺	Average Peak Intensity		
Fraction	Phosphopeptide (m/z 2062)	Non-Phosphopeptide (m/z 1385)	P-Peptide 2062/Peptide 1385
Starting Material	5370	17096	0.31
Flow Through	435	26753	0.01
Eluate	8686	1059	8.2
Enrichment Factor			f = 27

IMAC-Ga ³⁺	Average Peak Intensity		
Fraction	Phosphopeptide (m/z 2062)	Non-Phosphopeptide (m/z 1385)	P-Peptide 2062/Peptide 1385
Starting Material	778	2326	0.3
Flow Through	72	4805	0.0
Eluate	3514	160	22.0
Enrichment Factor			f = 74

The enrichment factor is calculated based on the average peak intensities.

CONCLUSIONS

The use of charged IMAC HyperCel resin provides a highly selective enrichment method for phosphorylated proteins. Benefits of this method include:

- A simple, rapid (< 1 hour) enrichment from a protein digest.
- The equilibration, wash, and elution buffers are specifically designed for highly selective capture and elution of phosphopeptides using IMAC HyperCel resin charged with Fe³⁺ or Ga³⁺.
- The phosphopeptide eluate fraction has a pH ~8.5, compatible with most downstream methods.
- The method can be readily adapted for high throughput formats such as AcroPrep™ 96-well filter plates.