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Note d'application

Phosphopeptide Analysis Using IMAC Sample Preparation Followed by MALDI-MS and MALDI PSD MX

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Abstract

The analysis of phosphopeptides is one of the most challenging areas of biological mass spectrometry. Phosphopeptide purification and enrichment using IMAC, prior to MALDI MS, is described. The quality of data obtained desalting phosphopeptides using the MassPREP PROtarget is compared to that obtained using a ZipTip. A new parallel post source decay technique (PSD MX) is used in the analysis of phosphopetides.

Benefits

IMAC method can be used to enrich phosphopeptides, whether they are contained in a simple peptide solution or a very complex peptide solution such as a 15 protein digest

Introduction

Phosphorylation is an important regulator of cell function in eukaryotes. It plays a well-established role in cellular signaling and can alter protein localization, regulate protein function, and stabilize and mediate their interaction. In recent years, the interest in studying protein phosphorylation has grown significantly. Unfortunately, phosphopeptides are often poorly ionized in comparison to their non-phosphorylated counterpart due to their associated negative charge. In addition, the analysis of phosphopeptides is often complicated, due to their low cellular abundance. In order to overcome these two issues, enrichment methods have been developed, especially Immobilized Metal-Ion Affinity Chromatography (IMAC). IMAC is a separation technique that uses covalently bound chelating compounds on solid chromatography supports to entrap metal ions that serve as affinity ligandsfor phosphoralyted species. Metal ions such as Cu(II), Ni(II), Fe(III), and Ga(III) have reversible affinities, especially towards the phosphate group carried on a phosphoprotein or phosphopetide. Iminodiaceticacid (IDA) and Nitrilotriaceticacid (NTA) coupled to a resin are the two main metal-chelating stationary phases used in IMAC enrichment methods.

UKIn this study, we have employed IMAC purification in combination with analysis by MALDI MS. We present the analysis of phosphopeptides from beta casein contained within peptide mixtures of differing levels of complexity. This method has been optimized for the purification and analysis of low level phosphopeptides. Finally, the analysis of phosphorylated peptides was performed using a new MALDI MS/MS technique, PSD MX, which provides structural information for peptides and phosphopeptides.

Experimental

Chemicals

Standard mono-phosphopeptide positive control set bovine casein and bovine ß-casein (Sigma Aldrich), trypsin (Promega), ACTH (18–39 clip) (Sigma), tryptically digested alcohol dehydrogenase (ADH) (Waters), tryptically digested phosphorylase B (Waters), 0.1 M acetic acid, acetonitrile, methanol, iron (III) chloride, EDTA, NaCl, trifluoroacetic acid (TFA), Waters RapiGestTM SF, Geloader eppendorf tips, µ-C_{18} ZipTips (Millipore), Waters' Stainless Steel MALDI Target, Waters MassPREP PROtarget. A 1 mL plastic syringe. Milli-Q system (Millipore, Bedford, MA, USA) The chromatographic resin used was trilotriaceticacid (NTA)-silica (16–24 µm particle size, Qiagen, Valencia, CA, USA).

Sample Preparation

PreparationPreparation & purification of phosphopetides using IMAC

For Fe(III)-IMAC, Qiagen silica resin based columns were used. The IMAC resin was prepared as previously described by Stensballe ***et al***[1]. IMAC columns were prepared and equilibrated.

- The phosphopeptide sample to be analyzed was diluted in 0.1 M acetic acid and 10–20 µL of the resultant solution was loaded slowly onto the IMAC column using slight backpressure from the syringe
- Loading times were 30 minutes or more to reduce non-specific binding. Once the sample was loaded, the column was washed, first with 10 µL of 0.1 M acetic acid, then with 10 µL of 0.1 M acetic acid: acetonitrile (3:1, v/v) and lastly with 10 µL of 0.1 M acetic acid
- Retained phosphopeptides were then efficiently eluted using two times 5 µL volumes of aqueous pH 10.5 solvent (Milli-Q water adjusted to pH 10.5 by addition of 25% ammonia corresponding to approximately 0.1% NH_3–tested with a pH paper)
- The eluted phosphopeptides were then acidified immediately by the addition of concentrated trifluoroacetic acid (TFA) to aid ionization
- Analysis of the eluted sample, showed the presence of high salt levels, (data not shown). Two different sample clean-up strategies were employed
- The commonly used strategy was one of desalting the sample with a C_{18} ZipTip, followed by spotting of the sample onto a stainless target plate. The sample was mixed 1:1 with 2,5-dihydroxy benzoic acid(DHB) dissolved in 50:50 acetonitrile: 1% phosphoric acid (10 mg/mL)

- An alternative sample clean-up technique was to desalt using a Waters MassPREP PROtarget plate
- Desalting using this method has been described in a previous application note[2]. In this case, DHB dissolved in 90: 10 Acetonitrile: 10% phosphoric acid (2 mg/mL) was used as the matrix. The MassPREP PROtarget was used when maximum sensitivity was required

Mass Spectrometry

The Waters MALDI micro MX incorporates a MALDI source and axial Tof mass analyzer with reflectron detector for recording of both MS and parallel PSD data. Data was acquired in positive ion mode using automated software control. In MS mode, alcohol dehydrogenase (ADH) tryptic digest was used to generate a multi-point external calibration; subsequently, an external lock mass correction using ACTH (18–39 clip) was applied. In parallel PSD mode, data was calibrated using PSD fragments from ACTH (18–39 clip).

Results and Discussion

Validation of the IMAC Method

During this study, we initially evaluated the IMAC method using standard mono-phosphopeptide (FQpSEEQQQTEDELQDK). This was added to two peptide solutions, to provide samples of different complexity. The first peptide solution contained only one other peptide, ACTH (18–39 clip). The ratio of the ACTH concentration to the phosphopeptide concentration was optimized to obtain similar signal intensities from both species. This optimization was required, as the ionization efficiency of the two peptides is significantly different.

1. In Figure 1, spectrum a shows that the mono-phosphopeptide peak is slightly more intense than the ACTH peak before IMAC (1.7:1). Spectrum b shows the same sample after IMAC purification. The mono-phosphopeptide peak is more intense than the ACTH peak (intensity ratio 10:1). These results show that the IMAC method pre-concentrated the phosphorylated species.

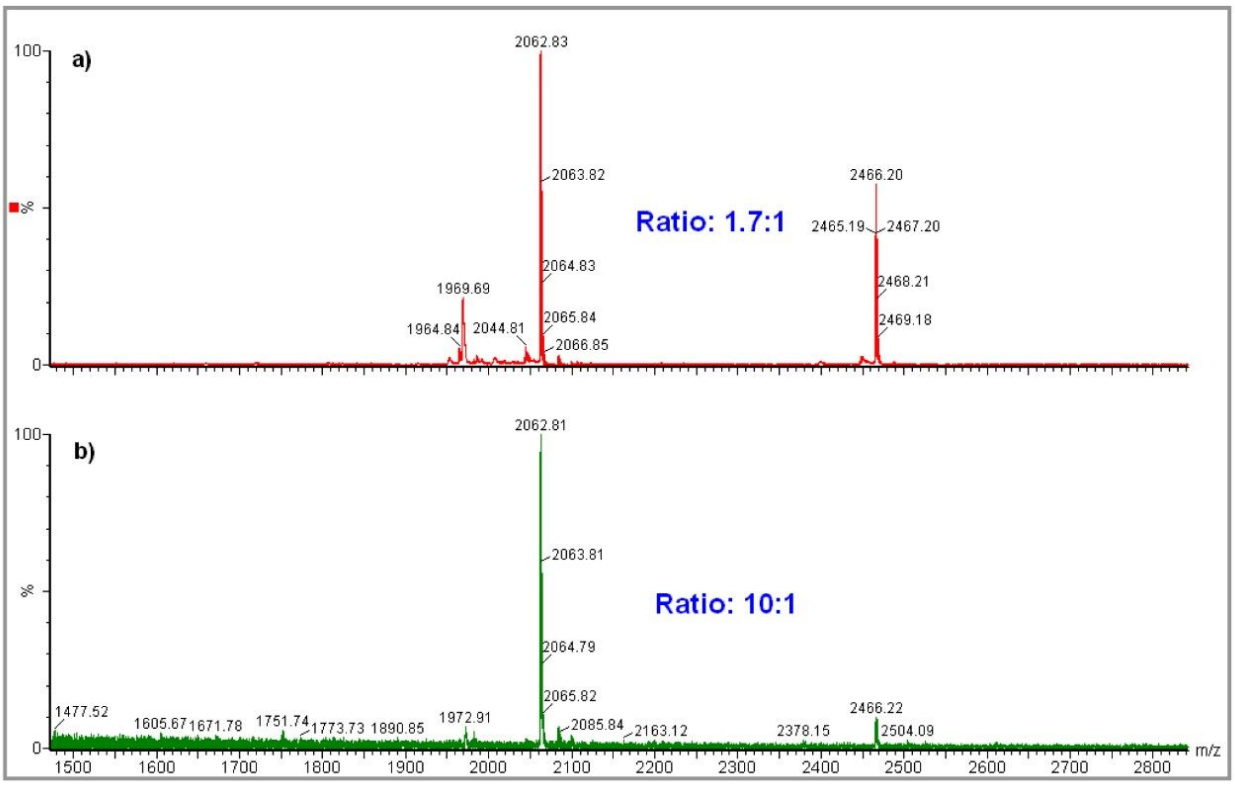

Figure 1. a) Mono-phosphopeptide ($[M+H]^+$=2061.8291 Da) FQpSEEQQQTEDELQDK at 500 fmol on target with ACTH at 4 pmol on target. b) 20 µL of the same mixture after IMAC micro-column purification.

2. In a further experiment, the same mono-phosphopeptide was added to a more complex ADH digest solution. Again, the ratio was optimized to obtain similar peak intensities. Figure 2 shows that after IMAC micro-column treatment, the major peak in the spectrum represents the mono-phosphopeptide. The ADH peptides have either been completely removed or are present at very low level, below the limit of detection.

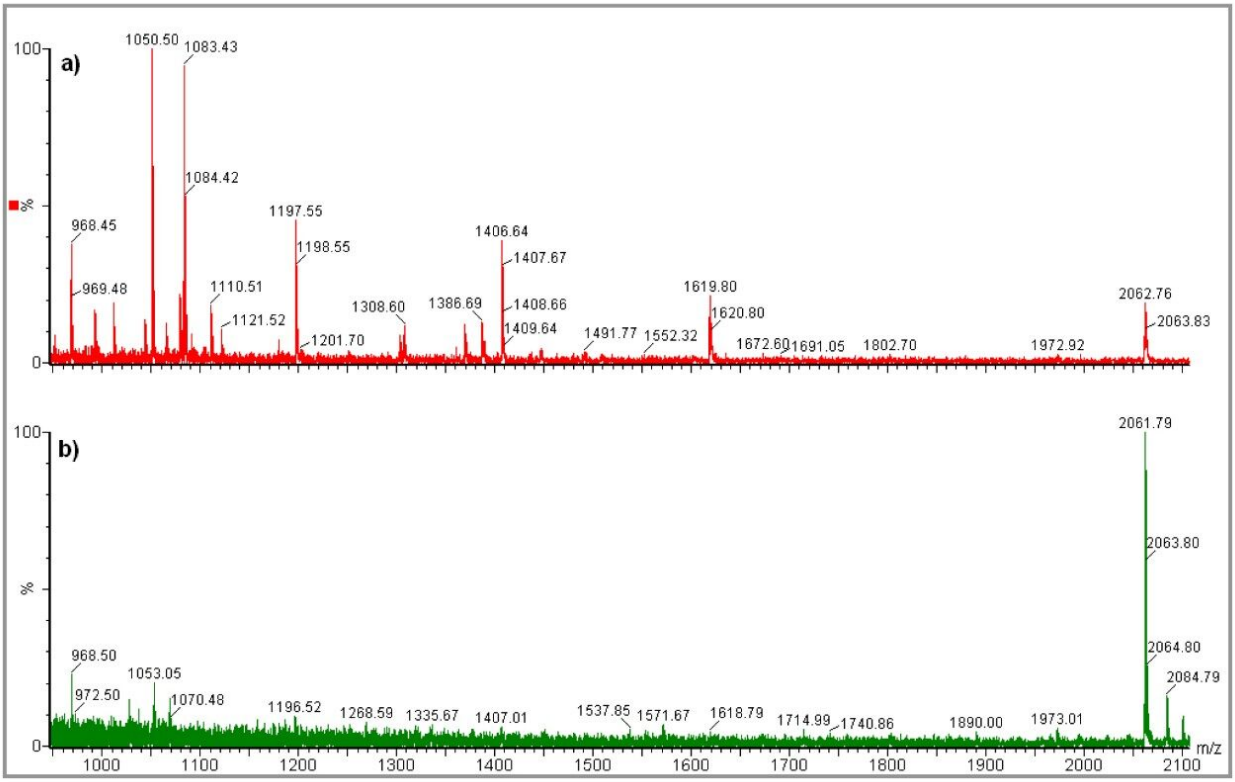

Figure 2. a) Mono-phosphopeptide ([M+H]$^+$=2061.8291 Da) FQpSEEQQQTEDELQDKat 100 fmol on target with ADH digest at 100 fmol on target. b) 20 µL of the same mixture after IMAC enrichment.

3. The IMAC enrichment method was also performed using ß-casein. After digestion using Rapigest and trypsin, two phosphopeptides are known to be generated: A mono-phosphopeptide with a [M+H]$^+$=2061.8 Da and a tetra-phosphopeptide with a [M+H]$^+$=3122.3 (RELEELNVPGEIVEpSLpSpSpSEESITR). Digest stock solution was diluted to 100 fmol/µL. Figure 3 shows the analysis of 1 µL of 100 fmol/µL spotted onto a stainless steel target. Neither phosphopeptide peak is intense, especially in comparison to the peak at 2186 Da. When 20 µL of the 100 fmol/µL ß-casein digest solution was bound onto the IMAC micro column and eluted, both of the phosphopeptide peaks were the most intense peaks in the mass spectrum while other digest peaks were significantly reduced (cf.Figure 3.b).

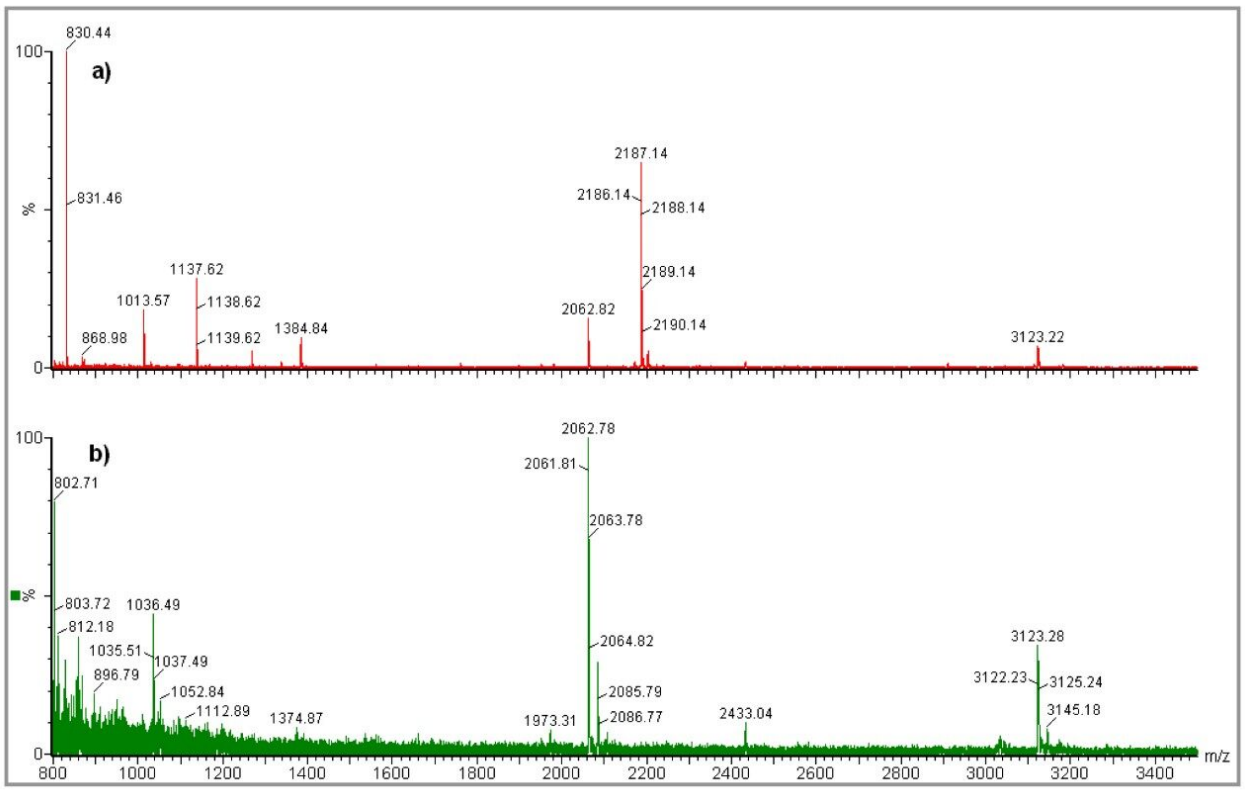

Figure 3. a) ß-casein digest at 100 fmol on target. b) 20 µL of the same solution after IMAC micro-column treatment.

4. A more complex sample was produced by combining the ß-casein digest solution with a phosphorylase B digest. Both digests were present in the mixture at 500 fmol/µL. Figure 4 shows that before IMAC enrichment, neither of the phosphopeptide peaks was apparent in the mass spectrum. After IMAC enrichment, the two most intense peaks represent the two phosphopeptides from ß-casein. The other, non-phosphorylated-peptides are again significantly reduced.

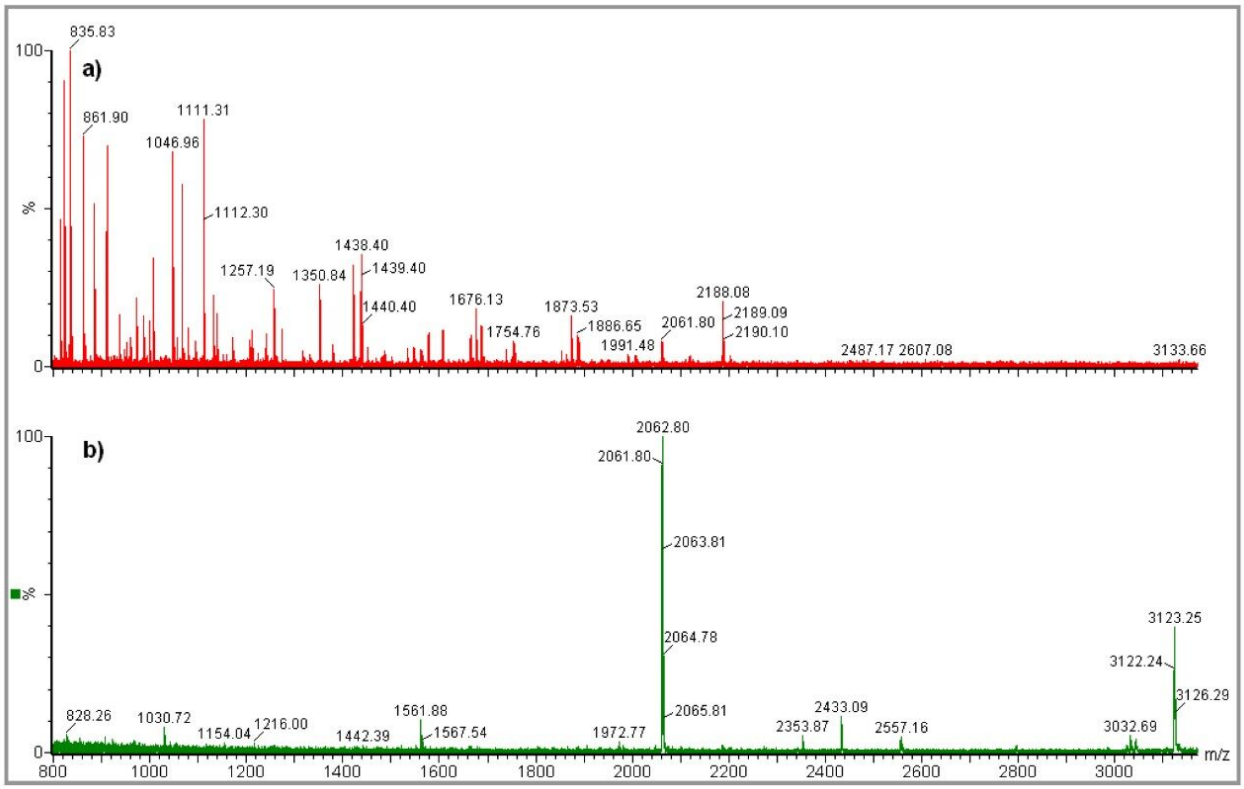

Figure 4. a) A mixture of ß-casein digest at 500 fmol on target with phosphorylase B digest at 500 fmol on target. b) 20 µL of the same solution after elution from an IMAC micro-column.

5. In the final experiment, a very complex mixture was produced, 15 standard proteins included ß-casein were digested, diluted to 500 fmol/µL and then spotted onto a stainless steel target (Figure 5.a). The sample was initially so complex that only a fraction of the total number of peptides present in the sample was observed in the mass spectrum. After the same solution was purified via IMAC, the two phosphopeptides from ß-casein gave the most intense peaks (Figure 5.b). The other peptides were almost fully removed by the enrichment protocol.

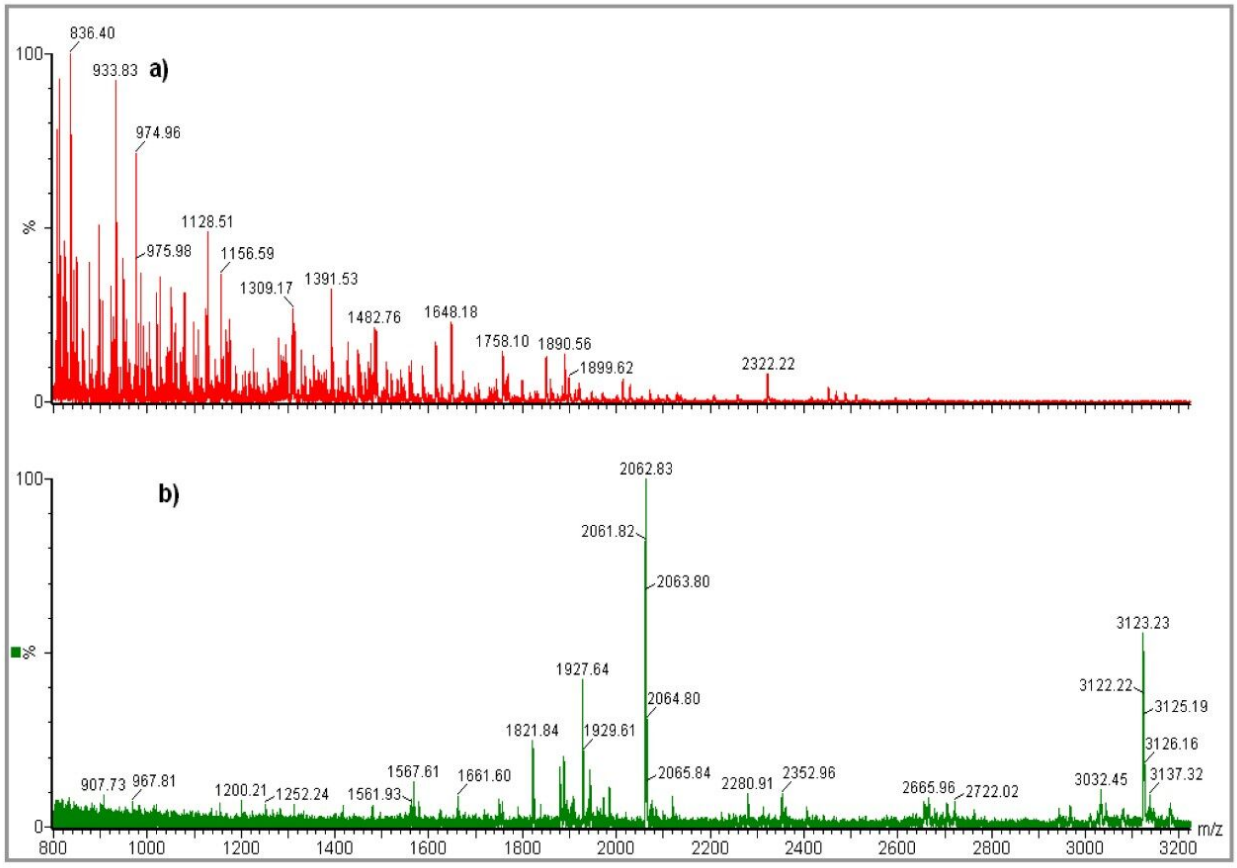

Figure 5. a) A tryptic digest of 15 proteins including ß-casein at 500 fmol on target. b) 20 µL of the same solution after IMAC micro-column enrichment.

Comparison of ZipTip and MassPREP PROtarget for Sample Purification

In the previous experiments described, the amount of material loaded onto the IMAC column was in the picomole to nanomole range. The lowest amount of sample that gave satisfactory results for the ß-casein digest was 200 fmol loaded onto the IMAC column, with a loading volume varying between 5 and 20 µL. Comparison of two desalting methods was made to improve the limits of detection. ZipTip sample clean-up followed by spotting onto stainless steel target was directly compared with spotting and clean-up using the MassPREP PROtarget.

10 µL of ß-casein digest (10 fmol/µL) were loaded onto the IMAC column, then eluted and acidified. The resultant solution was divided into two equal portions. One half was desalted using the ZipTip route; the second half was spotted onto the MassPREP PROtarget. The results obtained (Figure 6) show that the MassPrep PROtarget improves sensitivity, as the two phosphorylated peptides were detected with minimal

background noise. Additionally, it was possible to use lower laser energy when acquiring data from the MassPREP PROtarget than was required when acquiring from the stainless steel target. With the stainless steel plate, only the tetraphosphorylated peptide peak was detected.

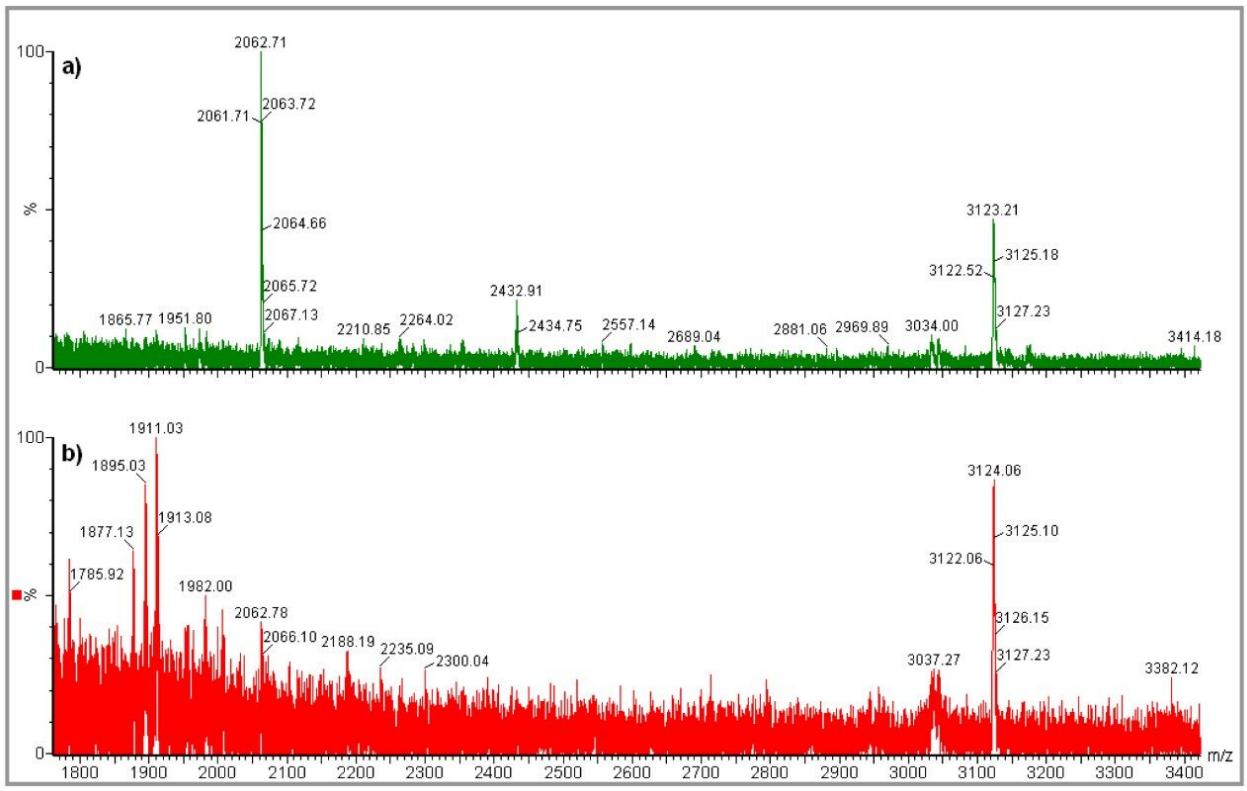

Figure 6. a) 100 fmol ß-casein digest loaded on IMAC and desalted using MassPREP PROtarget. b) The same digest desalted using ZipTip.

Phosphopeptide Analysed by Parallel PSD

Parallel PSD Technology

In a conventional PSD experiment, one precursor ion at a time is selected to pass through an ion-gate. Subsequent dissociation via PSD provides fragment ions. In contrast, in a parallel PSD experiment there is no ion gate and all precursor ions are transmitted and those precursors that fragment favourably by PSD are recorded.

- As fragment ions from different precursor ions are detected simultaneously, it is necessary to match the fragments to their associated precursor. This is achieved by acquiring two spectra, but at slightly different reflectron voltages. Fragment ions have a unique combination of mass and kinetic energy, which is

related to the mass of the precursor. By measuring the shift in time-of-flight between the same fragment ion in the two spectra it is possible to determine the precursor of each fragment

- Typically, one spectrum is acquired at the same reflectron voltage as for conventional PSD and is referred to as the Major spectrum. The second, or Minor spectrum is acquired at a reflectron voltage approximately 4% lower
- In both traditional and parallel PSD experiments, small low energy fragment ions do not penetrate as deeply into the reflectron as their respective precursors and consequently are not as well focussed. This limitation is overcome by acquiring several Major and Minor spectra (commonly known as segments) at reduced reflectron voltages. Fragment ion spectra are then formed by "stitching" together the focussed regions of each segment

The highest abundance of PSD fragment peaks were obtained from the tetra-phosphopeptide standard (MH^+3122.3), data were acquired in positive ion mode with CHCA as the matrix. Parallel PSD data were acquired for this sample, Figure 7 illustrates the change in time-of-flight (TOF) between the major and minor TOF spectra acquired at two slightly different reflectron voltages The differences in the flight times (dT1, dT2, dT3, dT4) observed between the same fragment ions in the major and minor TOF spectra provide secondary confirmation that the PSD peaks correspond to sequential losses of H_3PO_4 (-98 Daltons). This secondary confirmation -inherent to the parallel PSD technique -provides greater confidence in assigning PSD peaks over traditional PSD methods.

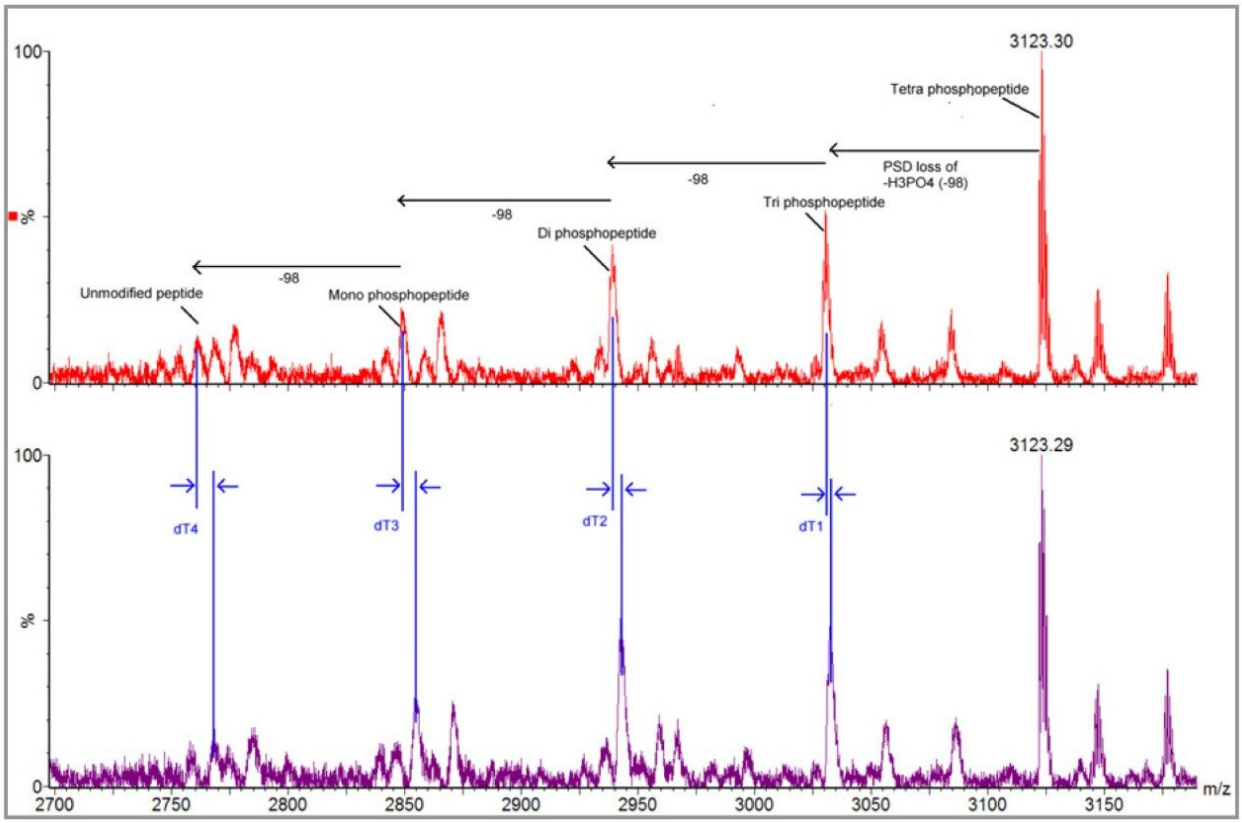

Figure 7. a) Major and b) Minor time-of-flight spectra of tetra-phosphopeptide analysed by parallel MALDI PSD.

The mono-phosphopeptide (FQpSEEQQQTEDELQDK) as identified from the tryptic digest of β-casein was analysed by parallel PSD. The isolated phosphopeptide was combined with CHCA and acquired in positive ion mode by parallel PSD on the MALDI micro MX (Figure 8).

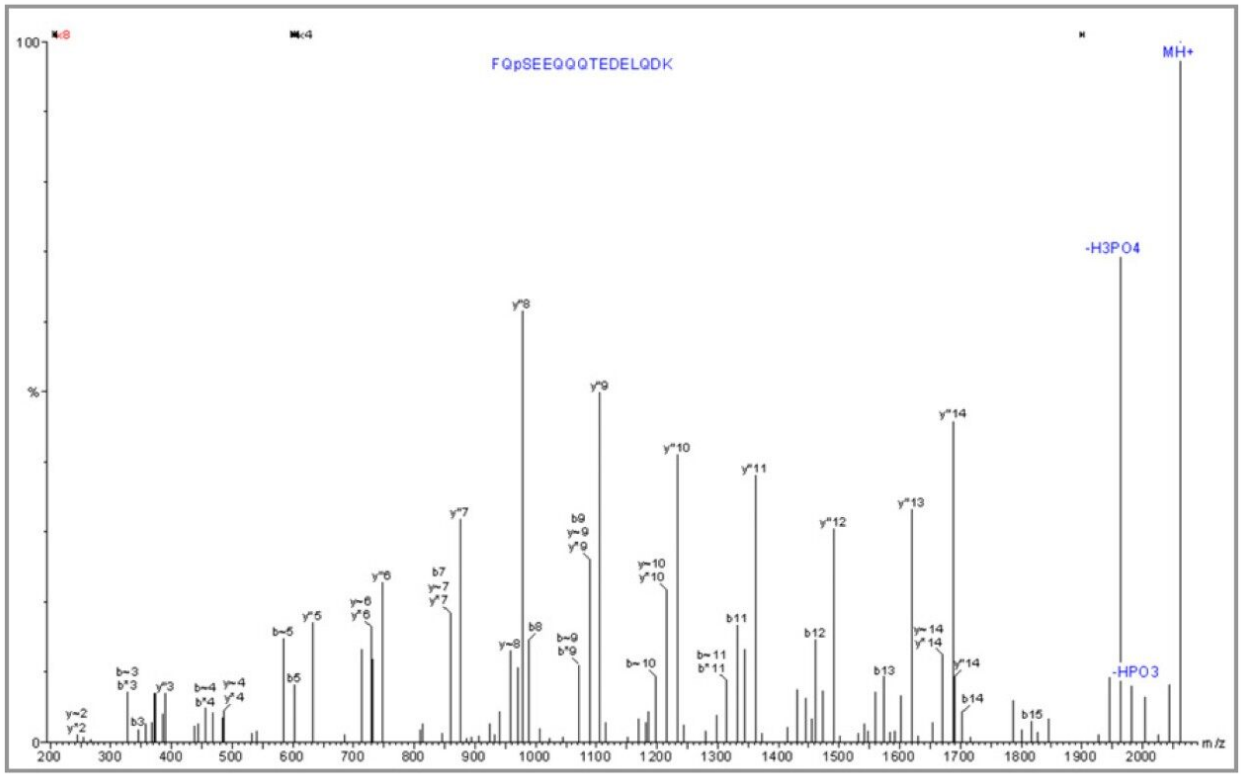

Figure 8. MALDI micro MX PSD MS/MS spectrum of mono-phosphopeptide.

The molecular ion MH^+2061.83 includes the modified Serine (+HPO_3=+80) however under fragmentation this ion incurred a total loss of H_3PO_4(-98) corresponding to a modified Serine mass of 69 provided in the sequence annotation.

Conclusion

- It has been shown that the IMAC method can be used to enrich phosphopeptides, whether they are contained in a simple peptide solution or a very complex peptide solution such as a 15 protein digest
- Desalting using the MassPREP PROtarget improved the absolute sensitivity and data quality obtained compared to ZipTip desalting
- The new parallel PSD approach provides fragment ion spectra from all precursor ions simultaneously, thus removing the serial nature of a MALDI MS/MS experiment. Valuable time and sample is not wasted

acquiring data from peptides that do not fragment by PSD

- Acquisitionof PSD data at two similar reflectron voltages provides unambiguous identification of phosphopeptide loss of 98 Daltons by correlating the TOF shift for the same fragment ions

References

1. StensballeA, Andersen S, Jensen ON. Characterization of Phosphoproteins from Electrophoretic Gels by Nanoscale Fe(III) Affinity Chromatography with Off-line Mass Spectrometry Analysis. *Proteomics*. (2001) 1(2) p 207–222.
2. MassPREP PROtarget Plates with ActiveWell Technology (Waters Literature 720000747EN < https://www.waters.com/webassets/cms/library/docs/720000747en.pdf>).

720001150, April 2005