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PHOSPHOPEPTIDE ANALYSIS USING IMAC SAMPLE PREPARATION FOLLOWED BY MALDI-MS AND MALDI PSD MX

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Overview

- The analysis of phosphopeptides is one of the most challenging areas of biological mass spectrometry.
- Phosphopeptide purification and enrichment using IMAC is described.
- The quality of data obtained desalting via the MassPREP PROtarget is compared to that obtained using a ZipTip™ for phosphopeptides
- A new 'parallel' post source decay technique PSD MX™ is escribed and the analysis of phosphopeptides by PSD MX is presented

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).

In this study, we have employed IMAC purification in combination with analysis by MALDI MS We present here the analysis of phosphopeptides from ß-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, St. Louis, MO, USA), trypsin (Promega, Madison, MI, USA), ACTH (18-39 clip) (Sigma Aldrich, St. Louis, MO, USA), tryptically digested alcohol dehydrogenase (ADH) (Waters, Milford, MA, USA), tryptically digested phosphorylase B (Waters, Milford, MA, USA), 0.1 M acetic acid, acetonitrile, methanol, iron (III) chloride, EDTA, NaCl, trifluoroacetic acid (TFA), RapiGestTM (Waters, Milford, MA, USA), Geloader Eppendorf tips, µ-C18 ZipTip's (Millipore, Bedford, MA, USA), Water's Stainless Steel Target, Mass PREP PRO Target (Waters, Milford, MA, USA). A 1ml plastic syringe. Milli-Q system (Millipore, Bedford, MA, USA.) The chromatographic resin used was trilotriacetic acid (NTA)-silica (16-24 µm particle size) (Qiagen, Valencia, CA, USA).

Sample preparation

Preparation & purification of phosphopeptides 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[1].
- 10 – 20 µL of phosphopeptide sample were loaded slowly onto the IMAC column using slight backpressure from the syringe. Loading times were ~ 30 minutes or more to reduce non-specific binding
- The column was washed first with 10 µL of 0.1 M acetic acid, then with 10 µL of 0.1 M acetic acid and acetonitrile (3:1, v/v) and lastly with another 10 µL of 0.1 M acetic acid.
- Retained phosphopeptides were then eluted using two times 5 µL volumes of pH 10.5 solvent (Milli-Q water adjusted to pH 10.5 by addition of 25% ammonia corresponding to approximately 0.1% NH_3).
- The eluted phosphopeptides were then acidified immediately by the addition of TFA to aid ionization.
- Two different sample clean-up strategies were employed:
  - o C_{18} ZipTip, followed by spotting of the sample onto a stainless target plate. Samples were mixed 1:1 with 2,5-Dihydroxy benzoic acid (DHB) dissolved in 1:1 acetonitrile: 1% phosphoric acid (10 mg/mL).
  - o Waters MassPREP PROtarget [2]. DHB dissolved in 9:1 Acetonitrile: 10% phosphoric acid (2 mg/mL) was used as matrix.

Mass Spectrometry

All MS data were acquired using a MALDI micro™ MX (Waters, Manchester, UK). MALDI TOF MS and MALDI PSD MX spectra were recorded. Data were acquired in positive ion mode using automated software control. In MS mode, alcohol dehydrogenase (ADH) digest was used to generate a multi-point external calibration and subsequently an external lock mass correction using ACTH (18-39 clip) was applied. In PSD MX mode, data were calibrated using PSD fragments from ACTH (18-39 clip).

Results

Validation of the method

1.During this study, we initially evaluated the method using the standard mono-phosphopeptide (FQpSEEQQQTEDELQDK), which was added to a solution containing 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. Figure 1, spectrum a) shows that the mono-phosphopeptide peak is slightly more intense than the ACTH peak before IMAC (Ratio of 1:1.7). In Figure 1, spectrum b) shows the same sample after IMAC purification. The mono-phosphopeptide peak is more intense than the ACTH peak (ratio of 1:10). These results show that the IMAC method pre-concentrated the phosphorylated species.

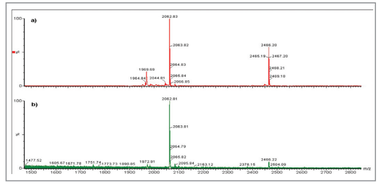

Figure 1: a) Mono-phosphopeptide ([M+H]+=2061.8291 Da) FQpSEEQQQTEDELQDK at 500 fmol on target with ACTH at 4 pmol on target. b) 20 µL of the same mixture after IMAC micro-column purification.

2. In a further experiment, the same mono-phosphopeptide was added to a more complex solution, ADH digest. Again the concentration 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.

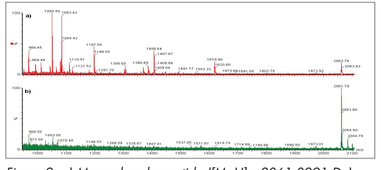

Figure 2: a) Mono-phosphopeptide ([M+H]+=2061.8291 Da) FQpSEEQQQTEDELQDK at 100 fmol on target with ADH digest at 100 fmol on target. b) 20 µL of the same mixture after IMAC enrichment.

3. The IMAC enrichment method was also performed using ß-casein. After digestion using RapiGest SF and trypsin, two phosphopeptides are known to be generated: A mono-phosphopeptide with a [M+H]+=2061.8291 Da and a tetra-phosphopeptide with a [M+H]+=3122.3 (RELEELNVPGEIVEpSL pSpSpSEESITR). A ß-casein solution of 100 fmol/µL was used. In figure 3, spectrum a) neither phosphopeptide peak is intense, especially in comparison to the peak at 2186 Da. In figure 3, spectrum b) the phosphopeptide peaks were the two most intense peaks in the mass spectrum and other digest peaks were significantly reduced.

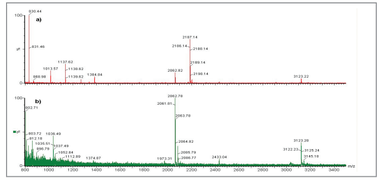

Figure 3: a) ß-casein digest at 100 fmol on target. b) 20 µL of the same solution after IMAC micro-column treatment.

4. In a final experiment a very complex mixture was produced, 15 standard proteins including ß-casein were digested, diluted to 500 fmol/µL and then spotted onto a stainless steel target (figure 4.a). The sample is initially so complex that, only a fraction of the total number of peptides present in the sample is 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 4.b). The other peptides were almost fully removed by the enrichment protocol.

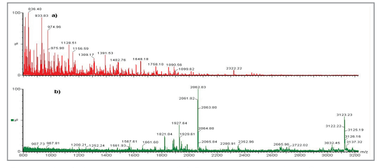

Figure 4: a) A tryptic digest of 15 proteins including ß-casein at 500 fmol on target. b) 20 µL of the same solution after IMAC micro-column enrichment.

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 which 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 (10 fmol/µL) was 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, whereas the second half was spotted onto the MassPREP PROtarget. The results obtained (figure 5) show that the MassPREP PROtarget improves sensitivity, as the two phosphorylated peptides were detected with minimal background noise. Also, 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.

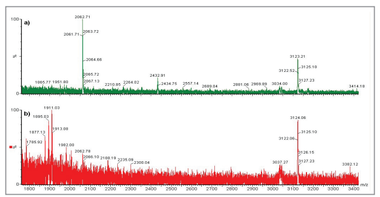

Figure 5: a) 100 fmol ß-casein digest loaded on IMAC and desalted using MassPREP PROtarget. b) The same digest desalted using ZipTip.

Phosphopeptide analyzed by Parallel PSD [3]

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. PSD MX 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 PSD MX technique - provides greater confidence in assigning PSD peaks over traditional PSD methods.

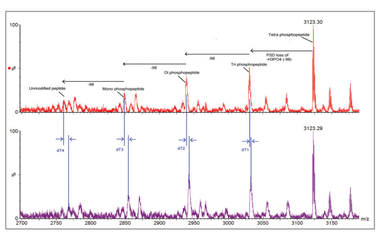

Figure 7: a) Major and b) Minor time-of-flight spectra of tetra-phosphopeptide analyzed by parallel MALDI PSD

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

The molecular ion MH^+ 2061.83 includes the modified Serine ($^+HPO_4$=+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.

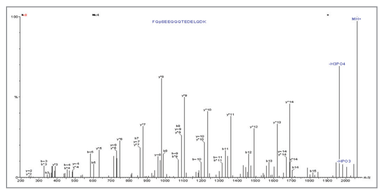

Figure 8: MALDI micro MX PSD MS/MS spectrum of mono-phosphopeptide

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 PSD MX 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.
- Acquisition of 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. Stensballe A, Andersen S, Jensen ON. Characterization of phosphoproteins from electrophoretic gels by nanoscale Fe(III) affinity chromatography with off-line mass spectrometry analysis. Proteomics. 2001 Feb; 1(2): 207-22.
2. MassPREP PROtarget Plates with ActiveWell Technology (Waters Part No. 720000747EN)
3. D. Kenny, J. Brown, M. Snel, Technical note: Multiplexed Post Source Decay (PSD MX) A novel technique explained (Waters Part No. 720000948EN)