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EVALUATION OF DIFFERENT METHODS TO IDENTIFY PHOSPHOPEPTIDES USING A NEW MALDI Q-TOF MASS SPECTROMETER

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OVERVIEW

- This poster shows an evaluation of the use of novel MS technology for the identification of phosphopeptides
- Enhanced duty cycle (EDC) was used to increase the detection efficiency of low mass diagnostic fragment ions on a Q-Tof mass spectrometer.
- Detection of PO_3^- was evaluated as an indication of peptide phosphorylation.

INTRODUCTION

The analysis of phosphorylated peptides presents a unique challenge. It is well accepted now that phosphopeptides, often present at only low copy numbers per cell, ionize less efficiently than their non-phosphorylated counterparts, due to negative charge interference. This may result in poor detection of phosphopeptides relative to the 'background' of un-phosphorylated peptides. An effective method for improving the detection of phosphopeptides is the use of an affinity enrichment protocol, producing enriched samples *e.g.* using IMAC, TiO_2 *etc.* An example of data obtained from a sample purified using a TiO_2 protocol is shown in **Figure 1.** Following enrichment almost all un-phosphorylated species have been removed from the mixture.
The focus of the work presented here is the implementation of a classic "parent ion scanning" experiment using a MALDI quadrupole Tof geometry instrument. This approach has the potential to identify phosphopeptides from MALDI spots without enrichment, *e.g.* from LC separated digest samples. The technique employed makes use of enhanced duty cycle (EDC) technology to increase the detection efficiency of low mass diagnostic fragment ions. This approach has the potential to be expanded to cover other post translational modifications.

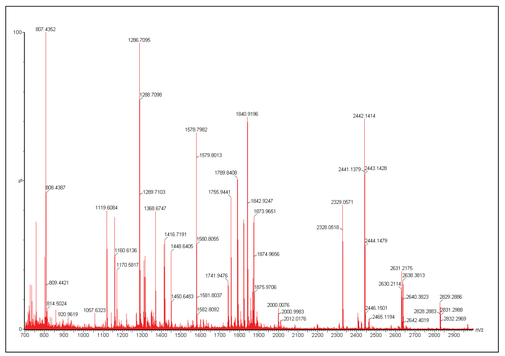

Figure 1. *Enriched phosphopeptide sample using TiO_2 protocol.*

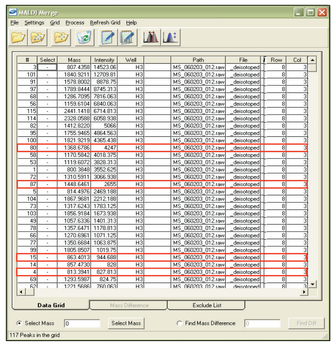

Figure 2. *List of peptides in intensity order displayed in MALDI merge software.*

METHODS

Samples: Enolase (MassPREP™ tryptic digest standard - Waters, Milford, MA), synthetic phosphopeptide standards, NVPLpYK, HLADLpSK, VNQIGpTLSESIK, VNQIGTLpSEpSIK (Waters, Milford, MA) were used for evaluation. The samples were dissolved in HPLC grade water. The enolase digest and phosphopeptide standards were mixed and diluted to a concentration of 200 fmol/µL.
TiO_2 purified phosphopeptide samples were kindly donated by the University of Southern Denmark, Odense.

Matrix: high purity DHB matrix (Waters, Milford, MA) was made up to 100 mg/mL in 7:1 0.1 % orthophosphoric acid:MeCN.

Sample preparation: matrix and peptide solution were mixed 1:1 and 1µL portions were spotted onto a stainless steel MALDI target plate (Waters, Manchester, UK).

MS Conditions

All MS and MS/MS experiments were performed on a MALDI source equipped Q-Tof™ Premier mass spectrometer (Waters, Manchester, UK).

Initially MS spectra of the phosphopeptide containing digest sample were acquired in positive ion mode. The data were lockmass corrected using an external standard (Glu-fibrinopeptide B) to ensure exact mass accuracy (RMS error sub 3 ppm).

A de-isotoped (m/z of ^{12}C isotope only) list of all peaks detected was generated using MALDI merge software (Waters, Manchester, UK). The list was arranged in intensity order (see **Figure 2**)and an include list (for a negative ion mode MS/MS experiment) was generated by subtracting the mass of H_2 (2.0141 Da) from all peptide masses.
Short negative ion MS/MS scans at a fixed collision energy of 100 eV (10 sec.) were performed on the 60 most intense peaks.

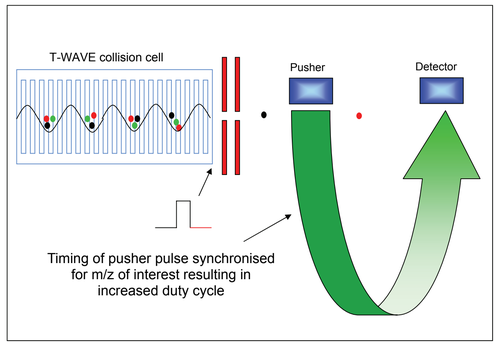

Figure 3: *Schematic illustration of Enhanced Duty Cycle (EDC) operation.*

Enhanced Duty Cycle (EDC)

For the MS/MS experiments, data were collected in EDC mode. EDC is a novel way of operating an orthogonal TOF mass spectrometer to increase the duty cycle of the instrument. This results in increased sensitivity for a selected mass range. Duty cycle enhancement is achieved by timing the application of the pusher voltage pulse to coincide with particular masses exiting the collision cell of the mass spectrometer (see **Figure 3**). For the purpose of enhanced detection of PO_3^- EDC was set for m/z 79. The effectiveness of analysis with and without EDC is shown for VNQIGpTLSESIK, $(m\text{-}H)^-$ 1366.7 Da, in **Figure 4**.

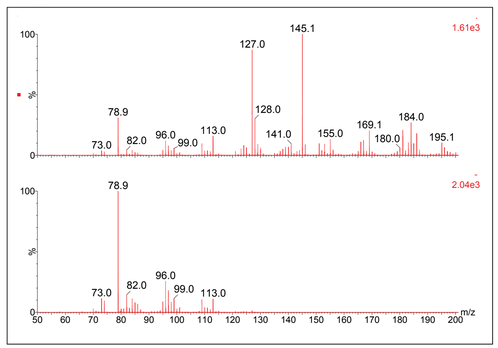

Figure 4. *Effect of EDC on the MS/MS spectrum of VNQIGpTLSESIK, $(m\text{-}H)^-$1366.7 Da. Top spectrum no EDC, bottom spectrum EDC set to m/z 79.*

RESULTS AND DISCUSSION

A typical MS spectrum of the phosphopeptide spiked enolase digest is shown in **Figure 5**. The phosphopeptides are marked with arrows. Although present at the same concentration (100 fmol/spot) as the enolase digest, as expected the phosphopeptides are of lower intensity than the enolase peptides.

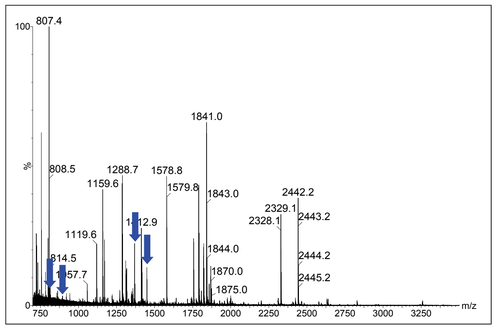

Figure 5. *100 fmol of enolase digest spiked with 100 fmol each of NVPLpYK (813.4 Da), HLADLpSK (863.4 Da), VNQIGpTLSESIK (1368.7 Da) and VNQIGTLpSEpSIK (1448.6 Da) (marked with arrows).*

Peptides containing a phosphorylation site produce an intense fragment ion at m/z 78.9 (see **Figure 6**). To facilitate identification of potentially phosphorylated peptides pseudo parent ion mass spectrum was produced. Here the mass displayed is that of the precursor ion but the intensity of each peak is the intensity of the associated 78.9 Da fragment (**Figure 7**). The four phosphopeptides spiked into the mixture are clearly identified (marked with arrows). In this representation the three most intense peaks are phosphopeptides. One of the phosphopeptides is of lower intensity (m/z 861.4 Da). This lower intensity was attributable to inhomogeneity of the sample surface leading to a lower intensity spectrum for this peptide. Although some other peptides also produced fragments with an m/z of 78.9 Da the number of possible phosphopeptide candidates is greatly reduced using this method. Early results indicate that a subsequent fast MS/MS analysis in positive ion mode of the possible phosphopeptides specifically looking for the neutral loss of H_3PO_4 can be used to rapidly confirm phosphopeptides.

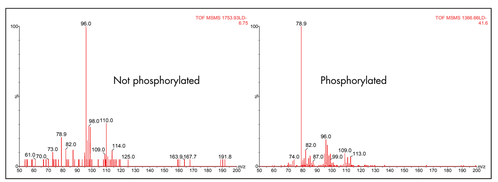

Figure 6: Examples of EDC MS/MS spectra from phosphorylated and unmodified peptides.

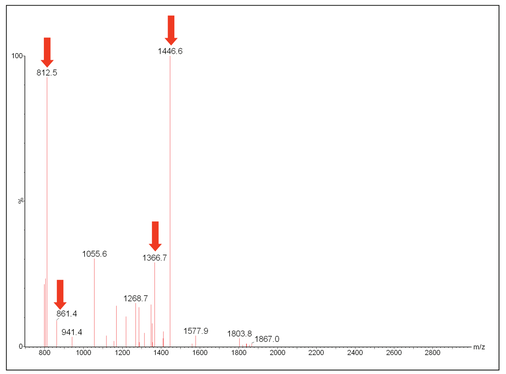

Figure 7. *Pseudo Parent ion mass spectrum where m/z of precursor is displayed with the intensity measured for the fragment ion at 78.9 Da. Known phosphopeptides are marked with arrows.*

CONCLUSIONS

- We have shown a novel method for identifying phosphopeptides from a mixture of peptides.
- Proof of principle was provided using a well characterized standard mixture choosing the diagnostic PO_3^- ion for targeting phosphopeptides.
- Phosphoserine, phosphotyrosine and phosphothreonine peptides were detected.

FUTURE WORK

- The scope of the experiments will be widened to consider other diagnostic fragment ions for phosphopeptides as well as other classes of compounds.
- Initial work on neutral loss experiments will be explored further.
- Alternative sample preparation protocols will be investigated.

ACKNOWLEDGMENTS

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