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ANALYSIS OF PROTEIN PHOSPHORYLATION USING A NOVEL "PARALLEL PSD" APPROACH ON A MALDI MASS SPECTROMETER

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OVERVIEW

- A new parallel post source decay (PSD) technique is presented.
- PSD fragment ion spectra from all precursor ions are recorded simultaneously, thus removing the serial nature of a conventional MALDI PSD experiment.
- This technology fitted to a new axial reflectron based MALDI-TOF system is compared to MALDI MS/MS on a Q-TOF mass spectrometer by analysis of Beta casein protein digest and phosphopeptide standards.

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 stability and mediate their interaction. In recent years, the interest in studying protein phosphorylation has grown significantly. The analysis of phosphopeptides can be difficult because often only a few copies per cell exist. In addition, phophopeptides are often poorly ionized in comparison to their non-phosphorylated counterpart. In this study, we introduce a new MALDI MS/MS technique which provides structural information for peptides and phosphopeptides. Traditional Post Source Decay (PSD) involves the selection of precursor ions with a timed electrostatic ion-gate. In this approach, the ion gate is not required as fragment ions from all of the precursor ions are acquired simultaneously. A deconvolution algorithm has been developed to match parent ions with fragment ions. This new technology provides a parallel approach to peptide sequencing and phosphopeptide identification.

MS data for Beta casein digest acquired using different MALDI matrices and ionization modes failed to produce a confident protein identification by peptide mass fingerprinting (PMF) however parallel PSD results from the digest provided unambiguous identification. PSD data for the mono-phosphopeptide were compared to that produced by collision induced disassociation (CID) on the MALDI-Q-TOF instrument. Additional data for the tetra-phosphopeptide standard were also acquired 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 disassociation 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.

EXPERIMENTAL

Sample preparation

Standard mono-phosphopeptide and tetra-phosphopeptide (Sigma, St Louis, MO) were each dissolved in 0.1% trifluoroacetic acid (TFA) to give final concentrations of 1 pmol/µL. The solution was mixed 1:1 with the matrix alpha-cyano-4-hydroxycinnamic acid (Waters, Milford, MA), dissolved in 50: 50 Acetonitrile: 0.1% TFA to a concentration of 5 mg/mL.

Beta casein protein (Sigma, St Louis, MO) was dissolved in ammonium bicarbonate, containing 0.2% (w/v) of RapiGest™ SF (Waters, Milford, MS). The protein was reduced with DTT (30 minutes at 60 °C), alkylated with Iodoacetamide (30 minutes in the dark at room temperature), prior to digestion with sequencing grade trypsin (Promega) for one hour at 37 °C. The stock concentration was 112 pmol/ µL. After a further dilution of 100 times, the solution was mixed 1:1 with the matrix alpha-cyano-4-hydroxycinnamic acid (CHCA) and also mixed separately 1:1 with matrix, 2,5-Dihydroxy benzoic acid (DHB) dissolved in 50: 50 Acetonitrile: water (1% phosphoric acid) to a concentration of 10 mg/mL.

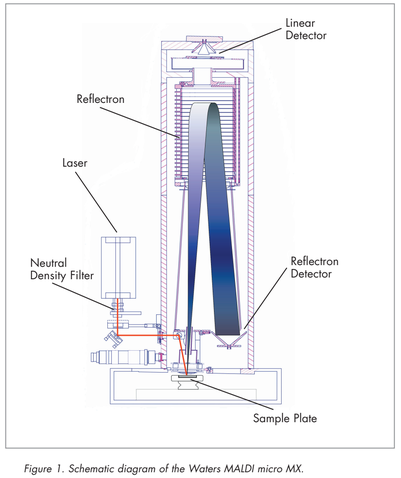

Figure 1. Schematic diagram of the Waters MALDI micro MX.

Mass Spectrometry

MALDI micro MX

- The MALDI micro MX (Waters) incorporates a MALDI source and axial TOF mass analyser with reflectron detector for recording of both MS and parallel PSD data. Data were acquired in positive and negative 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 parallel PSD mode, data were calibrated using PSD fragments from ACTH (18-39 clip). A schematic of the instrument is sh own in figure 1.

Q-Tof Ultima MALDI

- The Q-Tof Ultima MALDI (Waters) instrument combines a MALDI source, with a quadrupole as the first mass analyser, which provides for selection of the peptide precursor ions. They are transmitted to a hexapole gas cell where they undergo multiple low-energy collisions to induce collision decomposition (CID) using defined collision energy. The product ions produced in the hexapole collision cell are mass measured using the orthogonal acceleration time-of-flight mass spectrometer.

Data processing

Data acquired using Q-Tof Ultima MALDI were smoothed, background subtracted and deisoto ped using MaxEnt 3 (MaxEnt Solutions, UK) and ProteinLynx Global SERVER 2.1 (Waters). Peak lists (PKL files) were generated and database searched using Mascot (Matrix Science).

RESULTS

Figure 2 shows the MALDI micro MX precursor data from the Beta casein digest for a) +ve ion mode with CHCA matrix, b) -ve ion mode with CHCA matrix, c) +ve ion mode with DHB and d) -ve ion mode with DHB.The peaks labelled with * are the mono and tetra-phosphopeptides. The MS data in Figure 2a did not identify Beta casein by PMF.

Although the intensity of the phosphopeptides were significantly higher with DHB matrix in negative ion mode the degree of PSD was higher with the CHCA matrix in +ve ion mode.

The same sample well containing the Beta casein digest in CHCA was analysed further by parallel PSD. Six reflectron segments were acquired and processed. A peak list file (pkl format) was generated and the file was submitted to the Mascot MS/MS program with "semi-trypsin" as the digest enzyme. Figure 3 indicates a highly confident data base match for the MS/MS data produced by parallel PSD for three peptides at MH+ 1383.85, 2061.84 and 2186.19.

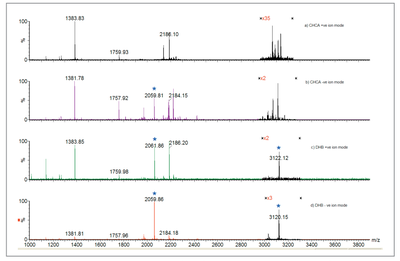

Figure 2. MALDI micro MX precursor data from the Beta casein digest for a) +ve ion mode with CHCA matrix; b) -ve ion mode with CHCA; c) +ve ion mode DHB and d) –ve ion mode DHB.

Three peptides were matched by MS/MS with a total score of 138. The results indicate that the 1383.8 Da peptide is a semi-tryptic peptide. The mono-phosphopeptide was also identified with a confident score. The high degree of modified and non specifically cleaved peptides within the Beta casein digest explains why the standard approach of PMF did not provide identification, however with the use of parallel PSD, confident identification was provided.

For the tetra-phosphopeptide *standard* at MH+3122.3 (another semi-tryptic miscleaved peptide), the positive ion spectra with CHCA provided the highest abundance of PSD fragment peaks. Parallel PSD data was acquired for this sample and Figures 4a and 4b illustrate the change in TOF between the major and minor spectra acquired at two slightly different reflectron voltages The differences in the times of flight (dT1, dT2, dT3, dT4) for the same fragment ions between the major and minor spectra provide secondary confirmation that the PSD peaks actually 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 previously reported methods.

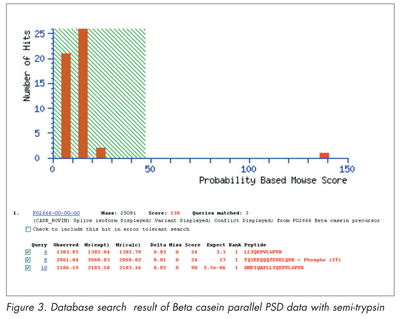

Figure 3. Database search result of Beta casein parallel PSD data with semi-trypsin enzyme parameter.

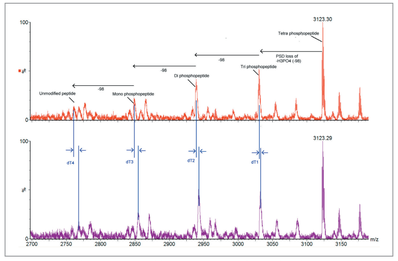

Figure 4a and 4b. (a) Major and b) Minor spectra of tetra-phosphopeptide (RELEELN VPGEIVEpSLpSpSpSEESITR) [M+H⁺]=3122.3 Da analysed by parallel MALDI PSD.

By comparing the times of flight for the same fragment ions between the major and minor spectra provide secondary confirmation that the PSD peaks actually 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 previously reported methods.

The same mono-phosphopeptide (MH+ 2061.84) as identified within the tryptic digest by parallel PSD was studied further. The standard was loaded separately with CHCA and acquired in positive ion mode by CID on the Q-Tof MALDI Ultima and by parallel PSD on the MALDI micro MX. Figure 5 and 6 show both MS/MS spectra respectively.

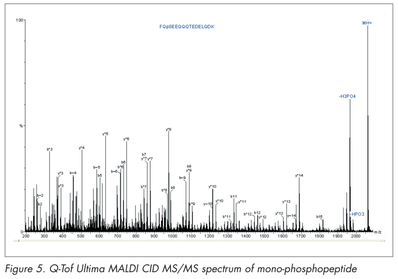

Figure 5. Q-Tof Ultima MALDI CID MS/MS spectrum of mono-phosphopeptide (FQpSEEQQQTEDELQDK) [MH⁺]=2061.8 .

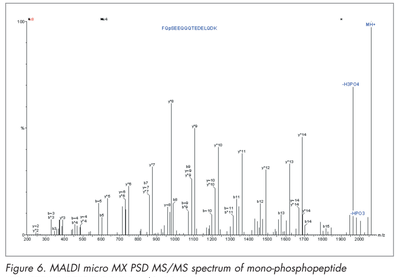

Figure 6. MALDI micro MX PSD MS/MS spectrum of mono-phosphopeptide (FQpSEEQQQTEDELQDK) [MH⁺]=2061.8.

By database searching both sets of MS/MS data, both the PSD and CID data were matched with very high MOWSE scores to the correct phospho peptide - FQpSEEQQQTEDELQDK (Serine is modified to phosphoserine). The molecular ion MH+ 2061.83 includes the modified Serine (+HPO3=+80) however under fragmentation this ion incurred a total loss of H3PO4 (-98) corresponding to a modified Serine mass of 69 provided in the sequence annotation.

SUMMARY

- 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.
- The PSD experiment is simplified because it is not necessary to program and select ions with an ion gate.
- Peptide mass fingerprint data for Beta casein did not provide positive identification because many of the peptide ions were non-specifically cleaved however parallel PSD data provided highly confident identification of the protein when searched against MS/MS fragment ion databases.
- 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.
- MS/MS results generated using this approach compared favourably to those obtained from a MALDI Q-Tof instrument.

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