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USE OF THE MALDI-TOF WITH A CURVED FIELD REFLECTRON FOR THE IDENTIFICATION OF PHOSPHORYLATION SITES IN A PROTEIN

KOMPACT

APPLICATION NOTE

USE OF THE MALDI-TOF WITH A CURVED FIELD REFLECTRON FOR THE IDENTIFICATION OF PHOSPHORYLATION SITES IN A PROTEIN

INTRODUCTION

The study of phosphorylated proteins is important for understanding the transmitting of signals intracellularly to the nucleus. Knowing the sites of phosphorylation on the expressed proteins can help the understanding of this process. Protein kinases phosphorylate a wide range of mammalian cell proteins and identifying the specific hydroxyl groups on a large protein substrate for phosphorylation can be difficult. In the past, elucidation of the phosphopeptides from protein digests was carried out using radioactive ^{32}P and column purification to obtain the single peptides for sequencing. Another difficulty is the use of Edman chemistry with these peptides due to extra problems from the phosphate groups.

The use of Mass Spectrometry for this technique has several advantages. Information can be obtained a lot faster as there is no need to purify the protein digest. Using the Kompact MALDI 4[(i)], sequence information and the presence of phosphorylation sites can be determined very quickly with seamless data acquisition using the unique patented Curved Field Reflectron [(ii)]. (See Figure 1). This technique of single pass acquisition also results in maximum sensitivity when using small amounts of materials.

To demonstrate this technique a well-known protein β-casein was used which is rich in phosphoserines. This was trypsin digested and the peptide fragments subjected to seamless-Post-Source Decay (s-PSD)[(iii)] to obtain sequence information.

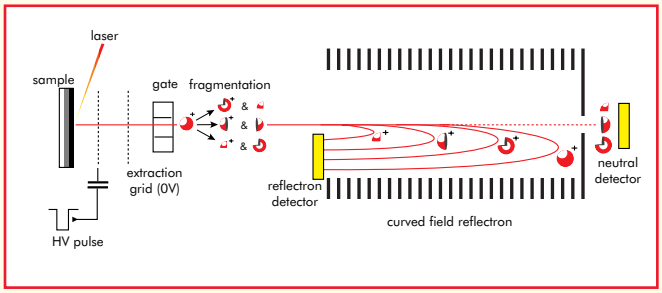

Figure 1: Schematic of the curved field reflectron

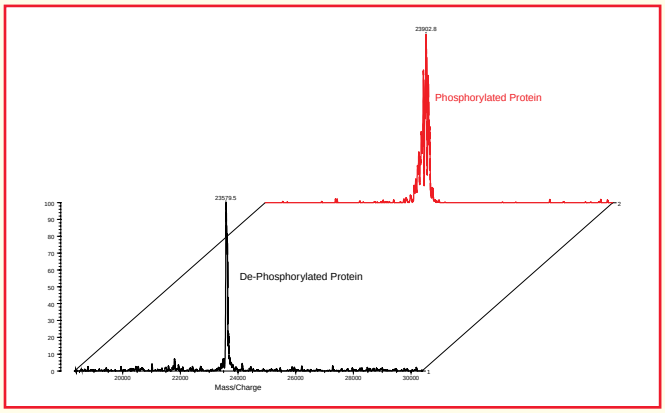

Figure 2: Comparison of phosphorylated and de-phosphorylated β-casein

EXPERIMENTAL

Solutions of β-casein (10mg/ml in 0.1M ammonium bicarbonate pH 8.3) were digested with L-1-tosylamide-2-phenylethyl-chloromethylketone (TPCK)- treated trypsin at 37°C for 2 hours. The ratio of enzyme to substrate was 1:100. 0.5 μl aliquots were taken directly from the digest mixture and added on to a 20-position sample slide with 0.5 μl α-cyano-4-hydroxycinnamic acid (10 mg/ml in 2:3 double distilled water:acetonitrile). Addition of the acidic matrix at pH 2 stopped the reaction. Preparations of dephosphorylated and phosphorylated forms of β-casein were analysed before digestion to look for the modification in mass. These were examined using the Kompact MALDI 4 in linear mode using Pulsed Extraction[(iv)] to get the best resolution and mass accuracy.

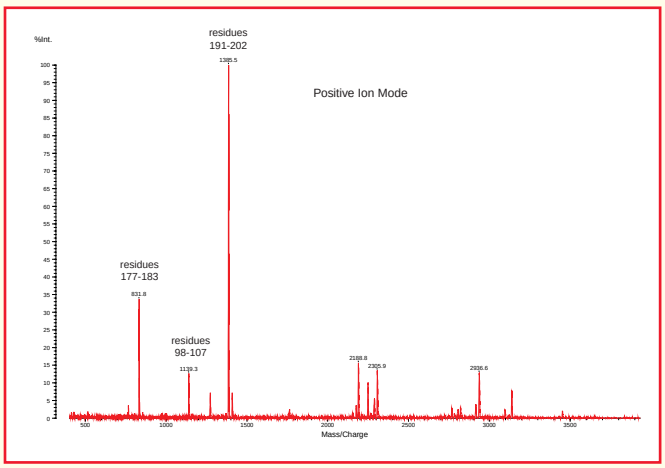

Figure 3: Tryptic peptide map for β-casein - positive ion mode

RESULTS

The results for the phosphorylated form of the protein show a mass increase of about 400 Da which would correspond to five phosphorylation sites which is as expected for the known protein. Some dephosphorylated forms were resolved from the phosphorylated form at 23980 Da. (See Figure 2).

The peptide map for β-casein gave the following masses in positive linear mode. (See Figure 3) The two most intense peptide peaks were gated and fragmented by increasing the laser power to obtain sequence information. The use of selected ion gating allows individual peaks from the peptide map to be selected for seamless PSD without the need to HPLC purify.

One of the advantages of trypsin digests is that all the fragments have a lysine or arginine at the C-terminus. The incidence of N- terminal (a or b) or C-terminal (y'') fragment ions is charge directed. This means that in protonated precursors the location of the charge carrier, a basic residue determines the type of fragment ions. In the absence of a basic residue then the charge carrier is located at the N-terminus hence a or b ions would be dominant. Therefore in this sequence an arginine at the C-terminus will induce easily interpretable y'' ion fragmentation.

The peak at 831.9 Da gave the following PSD spectrum (See Figure 4). PSD of this peptide gave the full sequence in a very short acquisition time (about 20 seconds). The peptide of 1383.8 Da also with a C-terminal arginine gave most of its sequence (See Figure 5). The sequences match to the expected peptide map for this protein using the Rockefeller University Protein Database[v].

As none of the phosphopeptides can be seen the sample was run in negative ion mode (See Figure 6) and this showed three phosphopeptides. The most intense signal was given by the peptide at 2058 Da, position 33-48 from the casein sequence. This mass was gated in the MALDI 4 and the s-PSD spectrum shown below (See figure 7). This is a negative ion s-PSD spectrum.

The peptide contains a phosphoserine residue that can be seen by the loss of 98 Da from the parent ion. This corresponds to $[M\text{-}H\text{-}H_3PO_4]^-$. This is not the dephosphorylated peptide but a dehydroanaline residue in place of the phosphoserine. This proves a serine phosphorylation rather than a tyrosine because this ion is more abundant than the $[M\text{-}H\text{-}HPO_3]^-$ which is a loss of 80 Da from the parent ion. In the case of phosphotyrosines the $[M\text{-}H\text{-}HPO_3]^-$ ion is more abundant than the $[M\text{-}H\text{-}H_3PO_4]^-$ ion. Therefore this is a quick method for determining the type of phosphorylation present.

Due to the instability of the phosphoserine residue location of the site is made difficult. Some of the sequence can be applied using the integral Kompact peptide software. a and b ions can be seen for the intact phosphorylated peptide. b3 which contains the phosphoserine loses the phosphate group to give $[b3\text{-}HPO_3]^-$. There is a further loss of H_2O to give $[b3\text{-}H_3PO_4]^-$. This confirms that the phosphate group is located on the first three residues from the N-terminus.

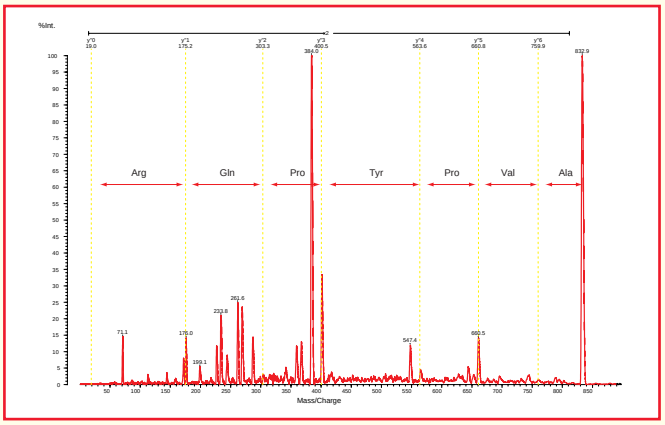

Figure 4: s-PSD spectrum of fragment 177-183

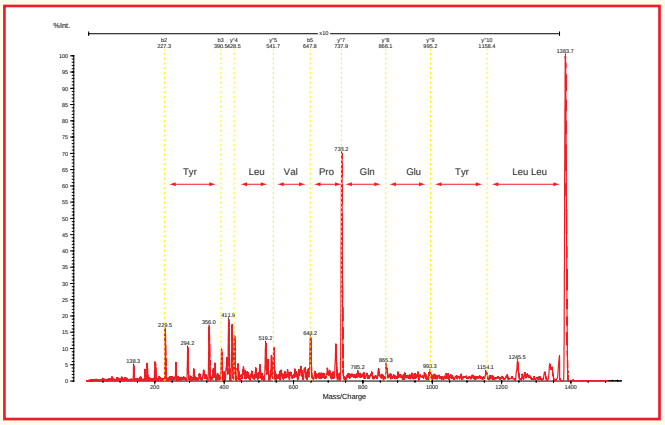

Figure 5: s-PSD spectrum of fragment 191-202

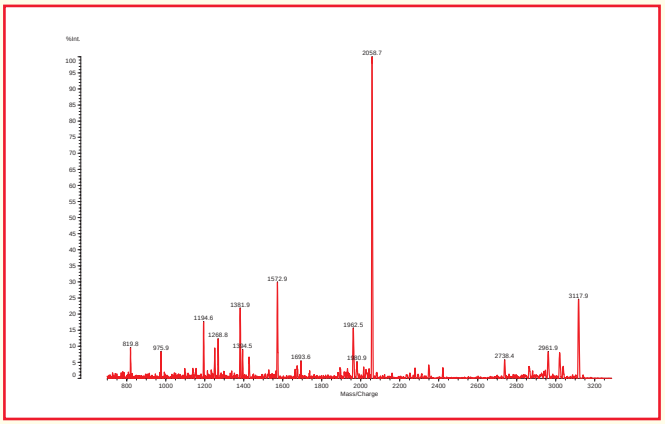

Figure 6: Tryptic peptide map for β-casein - negative ion mode

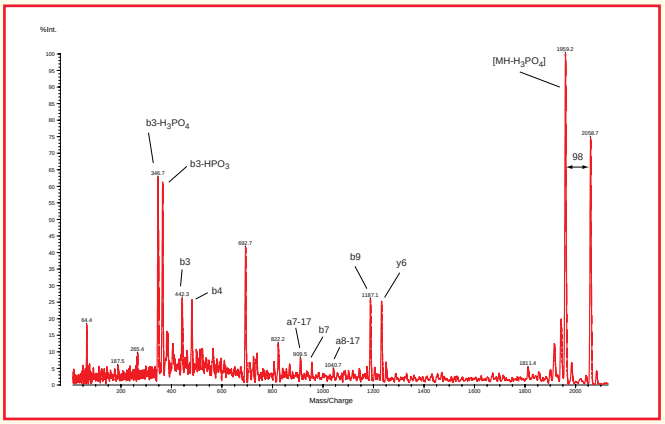

Figure 7: Negative ion s-PSD spectrum of fragment 33-43

CONCLUSIONS

The use of a dedicated s-PSD instrument such as the Kompact MALDI 4 shows definite advantages with this technique. The power of the Curved Field Reflectron is unique in the fact that fragmentation studies can be carried out without the time consuming need to stitch data of each focused fragment ion. A full fragment spectrum can be acquired in one run and the ability to analyse positive and negative ion spectra with equal facility is essential in dealing with phosphated species. Phosphopeptides can be analysed from digests to show the presence of a phosphate site, the type of phosphorylation and the likely position in the sequence.

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v http://chait-sgi.rockefeller.edu/

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