ANALYSING NATURE WITHOUT COSTING THE EARTH

KRATOS ANALYTICAL
A SHIMADZU GROUP COMPANY

EXPERIMENTAL STRATEGIES FOR ENHANCING PEPTIDE SEQUENCING

KOMPACT

APPLICATION NOTE

EXPERIMENTAL STRATEGIES FOR ENHANCING PEPTIDE SEQUENCING

INTRODUCTION

Since its inception in 1988[1], matrix-assisted laser desorption/ionization time of flight mass spectrometry (MALDI) has progressively increased its usefulness in the area of protein and peptide structural chemistry. With the advent of proteomics, the characterization of proteins using peptide mass fingerprinting by MALDI, followed by database searching is now a well established technique. However, with the continuously increasing size of these databases (each now typically contains over 300,000 protein entries), the likelihood of achieving a single protein match is rapidly decreasing. To address this problem, it has been necessary to include amino acid sequence or fragmentation data in addition to the peptide mass fingerprint in order to obtain an acceptable match.

The original purpose of the reflectron time of flight (TOF) analyzer design was the enhancement of resolution of the precursor ions when compared with linear TOF acquisition. A fortuitous side-effect of the reflectron is the formation, in the linear stage, of metastable ions. This spontaneous decay can be used advantageously in the structural characterization of biomolecules such as peptides and oligosaccharides.

Nevertheless in the conventional reflectron design[2], metastable fragments were not all focused at the detector simultaneously. This resulted in broad unresolved peaks, from which it was not possible to deduce a sequence. Two solutions were found to enable the acquisition of a fully focused, well resolved fragment spectrum (post source decay - PSD spectrum). The first involves the stepping down of the voltages applied to the reflectron device to create a number of well focused sections which can be stitched together to create an entire PSD spectrum. This process can be costly both in terms of time and sample consumed. The second solution involves the use of a curved-field reflectron[3] which offers the unique capability of simultaneously focusing all fragment ions at the detector without the need to step voltages (seamless PSD, sPSD). The net effect of using a curved-field reflectron is that sPSD spectra can be obtained as easily as a linear TOF spectrum.

A number of experimental strategies have been developed to explore the full potential of the spontaneous metastable decay in both linear and reflectron MALDI instruments as well as the ease of use of the curved-field reflectron for peptide sequencing.

METHODS AND MATERIALS

In-Source Decay (ISD) MALDI

Peptides were prepared for MALDI analysis by co–crystallization with α-cyano-4-hydroxycinnamic acid matrix (saturated in 1:1 acetonitrile/H_2O). The sample was analyzed in positive linear mode with pulsed extraction using a Kratos KOMPACT MALDI. In-Source Decay (ISD) was induced by increasing the time delay of the pulsed extraction by a factor of three. ISD fragmentation spectra of the peptides were acquired using the linear TOF detector.

Carboxypeptidase Y Digestion

Carboxypeptidase Y (Roche Molecular Biochemicals) was reconstituted in H_2O to a concentration of 1 μg/μl (sodium citrate buffer is already present with the enzyme). Peptides were diluted in H_2O. A 1 μl aliquot of carboxypeptidase Y was added to 10 μl of the peptide solution and incubated at room temperature for 30 min. Time points were taken at 0, 45 sec, 2, 10, 20 and 30 min by removing 0.5 μl sample, placing it on the KOMPACT MALDI sample plate and co-crystallizing it with α-cyano-4-hydroxycinnamic acid matrix.

Aminopeptidase Digestion

Aminopeptidase M enzyme (Roche Molecular Biochemicals) was reconstituted in H_2O to a concentration of 1 μg/μl (phosphate buffer is already present with the enzyme). This solution was further diluted ten fold to create a working solution at 0.1 μg/μl. Peptides were diluted to a concentration of 20–50 pmol/μl in H_2O. A 1 μl aliquot of aminopeptidase was added to 10 μl of peptide solution and incubated at 37°C for 90 min. Time points were taken at 0, 10, 30, 60 and 90 min by removing 0.5 μl sample, placing it on the KOMPACT MALDI sample plate and co-crystallizing it with α-cyano-4-hydroxycinnamic acid matrix.

Acid Hydrolysis

Peptide solutions were made to a 1 mg/ml concentration in 0.1% TFA (v/v). A 1 μl aliquot was then diluted into 10 μl of HCl solution of varied concentration (1N, 3N and 6N). Each sample was incubated at 96°C for 1 hour then evaporated to dryness to remove the HCl. Each hydrolysate was resuspended in 10 μl of 1:1 acetonitrile/H_2O. An aliquot of 0.5 μl of each was loaded onto a KOMPACT MALDI sample plate and co–crystallized with α-cyano-4-hydroxycinnamic acid matrix.

RESULTS AND DISCUSSION

In-Source Decay MALDI Analysis

Sequencing of intact, untreated peptides is not an application typically associated with linear MALDI systems. However, since the development of pulsed extraction - introduction of a time delay between ion formation and acceleration - linear MALDI instruments can be used under controlled conditions to produce fragment ion spectra for peptides. If metastable decay occurs in the drift tube, the fragments will not be observed as they arrive at the linear detector at the same time as their precursor and are hence assigned the same mass. However, if the fragmentation occurs prior to ion acceleration in the source region then metastable ions will be detected as separate ion species by the linear detector, thus allowing sequence interpretation.

Direct comparison between linear mode analysis and in-source decay analysis of oxidized insulin B chain is illustrated in Figure 1. The three fold increase in the pulsed extraction delay applied following ionization is sufficient to allow significant fragmentation to occur in the source. In total 15 consecutive amino acids were observed.

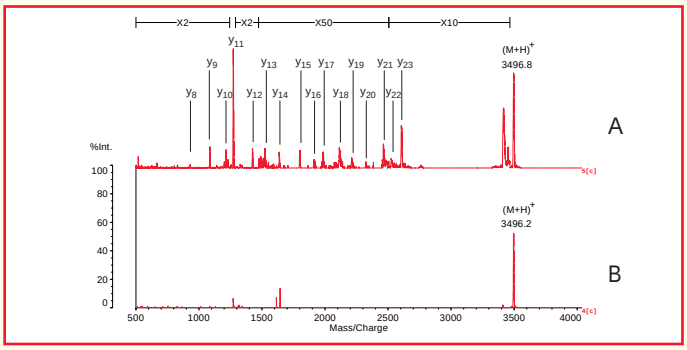

Figure 1. A) ISD MALDI spectrum of insulin B chain
B) Standard MALDI spectrum of insulin B chain

Partial Aminopeptidase and Carboxypeptidase Digestion

The use of rapid proteolytic cleavages can significantly enhance the amount of sequence information obtained from a peptide and the ease with which this can be deduced. Limited aminopeptidase or carboxypeptidase digestions create a nested set of 2 to 3 peptides of the same amino acid backbone with sequential truncations of either the C- or N-terminus. Linear MALDI analysis of these peptide mixtures enables the determination of the C- or N-terminal amino acid. sPSD sequencing of the consecutive truncations of a peptide provides several complementary fragmentation spectra from which the sequence can be deduced. To further aid in the characterization of unknown peptides, software has been developed to subtract the sPSD spectra from two sequential truncations (Nested-PSD™)[4][5]. The net effect of this is the separation into two spectra of the potential N-terminal ions (a, b, c ions series) and C-terminal ions (x, y, z ion series), thus significantly enhancing the degree of confidence in the deduced sequence.

The results described here show that the nested sets of peptides can be easily produced in less than 15 min for aminopeptidase (Figure 2) and as little as 45 sec for carboxypeptidase (Figure 3). This in conjunction with the speed of sPSD analysis using the curved-field reflectron MALDI instrument provides a rapid and reliable tool for the sequencing of peptides.

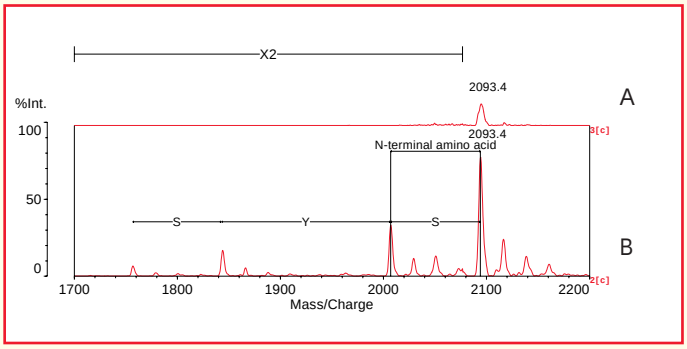

Figure 2. Rapid aminopeptidase M digestion
A) ACTH Clip 1-17 standard
B) ACTH Clip 1-17 aminopeptidase treated

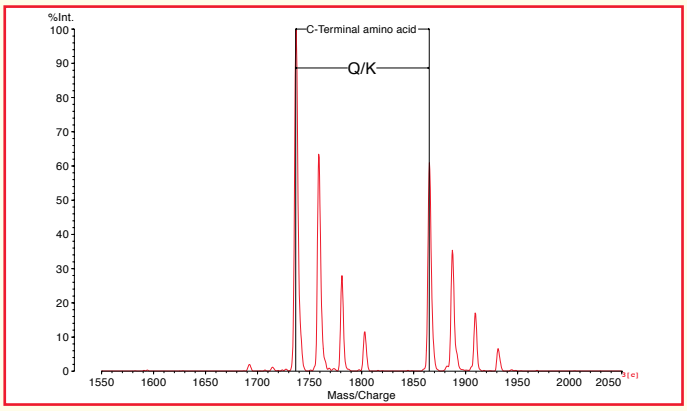

Figure 3. Rapid carboxypeptidase Y digestion of a synthetic peptide

Acid Hydrolysis and MALDI Analysis

Acid hydrolysis was originally used as a means of breaking down peptides to single amino acid residues to calculate the relative abundance of each component. In the experiments shown here the conditions for acid hydrolysis have been optimized to avoid complete degradation of the peptide, providing instead a nested set of peptides of similar backbone structure. Acid hydrolysis is particularly advantageous in the instance of peptides blocked at one or both termini, as proteolytic agents such as carboxypeptidase and aminopeptidase are unable to cleave protected termini. Acid hydrolysis treatment of luteinizing hormone releasing hormone analogue (LHRH) - containing pyroglutamate at the N-terminus and NH_2 at the C-terminus - resulted in a set of eight peptides with progressive truncations of the N-terminus (Figure 4).

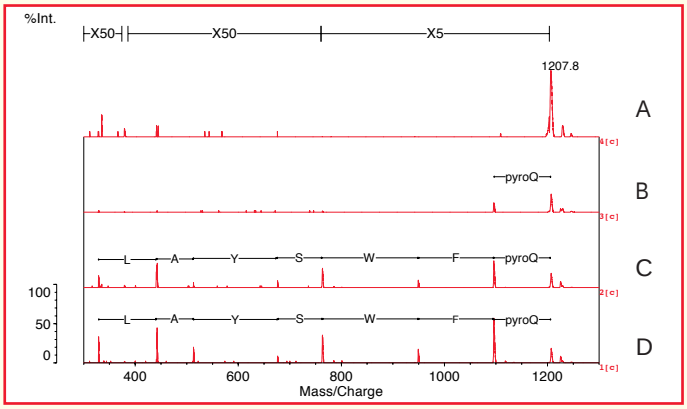

Figure 4. Acid hydrolysis of LHRH peptide analogue
A) No HCl treatment B) 1N HCl
C) 3N HCl D) 6N HCl

FURTHER RESULTS

The methods described above have been applied to many examples of peptides in order to establish optimal experimental conditions as well as to assess the usefulness of combining rapid, limited cleavage and Nested-PSD™ analysis using the curved-field reflectron MALDI. On average, with the conditions used, carboxypeptidase and aminopeptidase cleave the first two amino acid residues from the C- or N-terminus providing a set of three nested peptides for sPSD analysis. For the example shown (Figures 5 and 6) the full sequence of the peptide could be deduced by combining the results from the Nested-PSD™ experiment after removal of a single amino acid using carboxypeptidase Y.

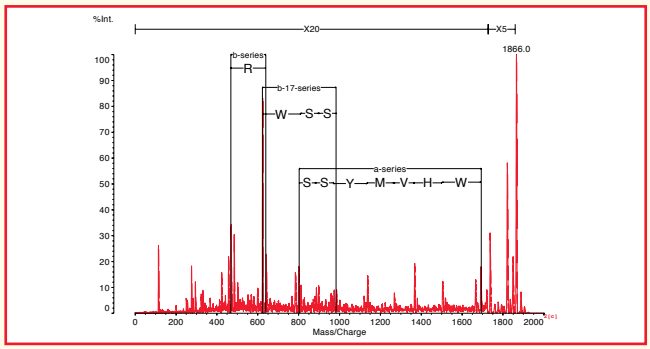

Figure 5. Nested-PSD™ sequencing of a synthetic peptide

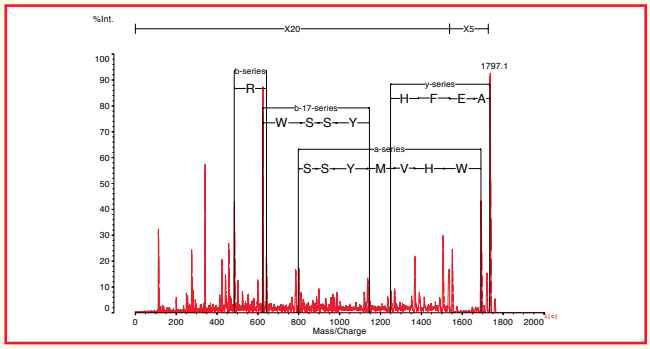

Figure 6. Nested-PSD™ sequencing of a synthetic peptide less a single amino acid

CONCLUSIONS

There are now a number of strategies available to the protein biochemist to enhance sequencing of peptides. Each of these methods has both advantages and disadvantages in isolation. However, we are now moving towards the combination of several of these methods with Nested-PSD™ to provide a rapid and easy to use tool for the effective sequencing of peptides and proteins using MALDI mass spectrometry.

BENEFITS

- In-source decay MALDI can be used successfully for the sequencing of pure peptides using only a linear MALDI instrument
- Limited proteolytic and chemical cleavage of peptides aids sequencing by creating a nested set of peptides of the same backbone structure
- These techniques combined with Nested-PSD™ software[5] provide an effective strategy for peptide sequence analysis by MALDI.

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