Identification of Phosphorylation Sites Using Automated Chip-Based Nanoelectrospray Coupled to a Linear Ion Trap Mass Spectrometer

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OVERVIEW

Purpose:

- To demonstrate and evaluate the NanoMate® system combined with the Finnigan LTQ™ mass spectrometer as a tool for identifying protein phosphorylation sites using data-dependent acquisition (DDA) and automated neutral-loss scanning.

Methods:

- Two standard phosphoproteins, bovine α-casein and β-casein, were used to demonstrate the method.
- Calf intestinal phosphatase (CIP) was used to treat the α-casein digest.
- The NanoMate and ESI Chip™ were used to infuse the samples into the LTQ for DDA analysis followed by automated neutral-loss scanning to identify phosphorylation sites.

Results:

- Definitive identification of phosphorylation sites of both α-casein and β-casein digests using chip-based nanoESI/MS^n with automated neutral-loss scanning was demonstrated.
- Phosphatase treatment prior to MS analysis is required for phosphorylation mapping for multiple phosphate groups in a single peptide.
- Chip-based infusion provides a stable spray with extended time for completing MS^n with automated neutral-loss scanning in a single analysis.
- The detection limit for phosphopeptides was demonstrated to be 5 fmol/μL for automated acquisition and 1 fmol/μL for manual acquisition.

INTRODUCTION

Reversible protein phosphorylation is a biological regulatory mechanism that is known to play an important role in various cell activities including growth, differentiation, division, and regulatory metabolism. Pinpointing phosphorylation sites in a protein is necessary to determine the mechanism of the post-translation modification. However, identifying the phosphorylation sites can be very difficult, requiring labor-intensive and time-consuming biochemical approaches. More recently, mass spectrometry methods have been used successfully to identify phosphopeptides and phosphorylation sites, yet identification of sites still remains a challenge because phosphopeptides are often present in very complex mixtures and at low stoichiometry. They are also subject to poor ionization efficiency and fast degradation. In this work, we describe a novel approach to identifying and characterizing phosphopeptides and phosphorylation sites in complex mixtures using automated chip-based nanoelectrospray coupled to a linear ion trap mass spectrometer.

METHODS

Preparation of enzymatic digest: α-casein and β-casein were directly dissolved in 50 mM ammonium bicarbonate, pH 7.8 at 1 mg/mL. Trypsin solution (1 μg/μL) was then added at an enzyme-to-substrate ratio of 1:60 (w/w). Digestions were performed at 37 °C for 16 hours and stopped with 0.1% acetic acid. The digest was stored at -70 °C until used.

CIP treatment of enzymatic digest: Four units of dialyzed CIP (8 units/μL) were added to 10 μL each of the α-casein digest and incubated at 37 °C for 2 hours. The CIP-treated digest was stored at -70 °C until used.

NanoMate mounted on a Finnigan LTQ

Experimental Conditions:

Samples:	CIP-treated and untreated α-casein and β-casein enzymatic digests (at various concentrations)
Solvent:	50:50 methanol/water with 0.1% formic acid

NanoMate (Advion BioSciences, Inc.):

Aspiration Volume:	5 μL
Flow Rate:	100 nL/min
Spray Voltage:	1.55 kV
Spray Pressure:	0.2 psi

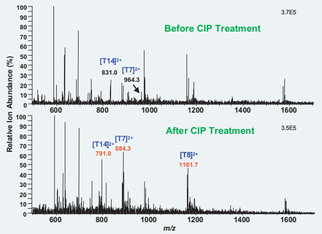
Finnigan LTQ (Thermo Electron Corp.):

Ionization Mode:	Positive ion
Acquisition Time:	5 minutes
Capillary Temperature:	150 °C
Collision Energy:	20 to 25% used for MS/MS and neutral-loss scanning
Ion Collection Time:	50 ms maximum
Microscans per Scan:	2 to 3 microscans
Search Database:	BioWorks 3.1/bovine.fasta

RESULTS

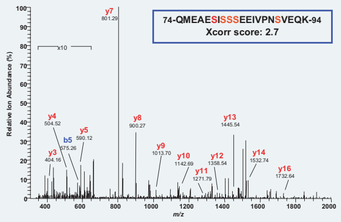
α-Casein

MS Spectra of 100 fmol/μL α-Casein Digest



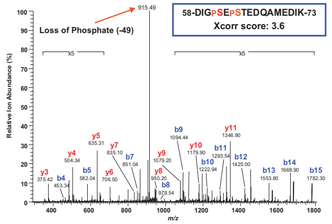
Top panel shows T14 and T7 phosphopeptides matched with predicted masses. Bottom panel shows that after CIP dephosphorylation all T14, T7, and T8 peptides were detected in positive ion full scan MS.

MS/MS Spectrum of *m/z* 1161.7 (T8) for 50 fmol/μL CIP-treated α-Casein Digest



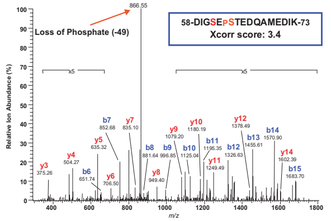
Data-dependent scan shows the MS/MS sequence of dephosphorylated T8 ion (m/z 1161.7) for CIP-treated digest containing five possible phosphorylated residues. It can be concluded that all five possible phosphorylated residues (S79, S81, S82, S83, and S90) are phosphorylated based on the mass difference between CIP-treated and untreated T8 ions in negative ion mode (spectra not shown).

MS/MS Spectrum of a Parent Ion at *m/z* 964.3 (T7) for 100 fmol/μL α-Casein Digest



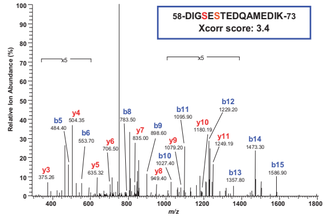
Data-dependent scan shows that T7 ion (m/z 964.3) is phosphorylated because it automatically triggered MS^3 neutral-loss ion mapping on m/z 915.5. The MS/MS sequence of T7 ion confirmed that both S61 and S63 are phosphorylated.

MS^3 Spectrum of a Neutral-Loss Ion at *m/z* 915.5 for 100 fmol/μL α-Casein Digest



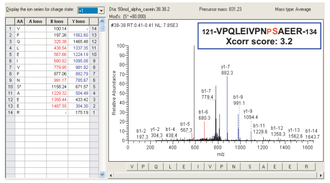
Data-dependent scan shows the automated neutral-loss mapping (MS^3) on m/z 915.5 which is from the original m/z 964.3 ion. The MS^3 sequence of m/z 915.5 confirmed that the S61 residue was neutrally lost while S63 is still phosphorylated.

MS^4 Spectrum of a Second Neutral-Loss Ion at *m/z* 866.6 for 100 fmol/μL α-Casein Digest



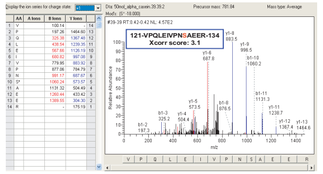
Product ion scan shows the second round of neutral-loss mapping (MS^4) on m/z 866.6 ion which is the neutrally lost ion m/z 915.5 that is from the original m/z 964.3 ion. The MS^4 sequence of m/z 866.6 confirmed that both S61 and S63 residues are neutrally lost.

MS/MS Spectrum of *m/z* 831.0 Ion (T14) for 50 fmol/μL α-Casein Digest



Database search for MS/MS spectrum on m/z 831.0 from DDA analysis using BioWorks 3.1 search engine with the bovine.fasta database.

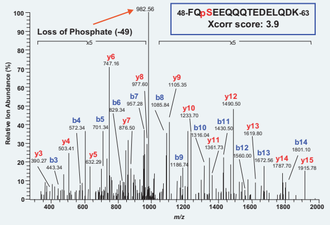
MS^3 Spectrum of a Neutral-Loss Ion at *m/z* 781.9 for 50 fmol/μL α-Casein Digest



Database search for MS^3 spectrum on m/z 781.9 ion from DDA-triggered neutral-loss scanning using BioWorks 3.1 search engine with the bovine.fasta database.

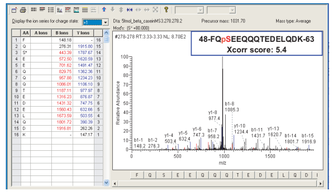
β-Casein

MS/MS Spectrum of Parent Ion *m/z* 1031.7 (T4) for 1 fmol/μL β-Casein Digest



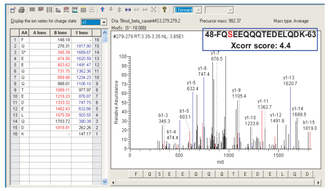
MS/MS sequence of a singly phosphorylated T4 ion (m/z 1031.7) that was generated by manually selecting the parent ion m/z 1031.7 in tune mode and summing the spectrum from 5-minute acquisitions. The MS/MS sequence result shows that a phosphorylation site can be determined in concentrations as low as 1 fmol/μL.

MS/MS Spectrum of Parent Ion *m/z* 1031.7 (T4) for 5 fmol/μL β-Casein Digest



Data-dependent scan shows that the T4 ion (m/z 1031.7) is phosphorylated because it automatically triggered MS^3 neutral-loss ion mapping on m/z 982.7. The MS/MS sequence of the T4 ion confirmed that S50 is phosphorylated.

MS^3 Spectrum of a Neutral-Loss Ion at *m/z* 982.7 for 5 fmol/μL β-Casein Digest



Database search for MS^3 spectrum on m/z 982.7 ion from DDA-triggered neutral-loss scanning using BioWorks 3.1 search engine with the bovine.fasta database.

CONCLUSIONS

- Unambiguous identification of phosphorylation sites in proteins by chip-based nanoESI/MS^n with automated neutral-loss scanning was demonstrated.
- The automated NanoMate system coupled with the Finnigan LTQ offers a powerful tool and effective approach for rapid and accurate identification of phosphorylation sites, including peptides containing multiple phosphates. The NanoMate allows an extended infusion time while maintaining stable spray and minimizing sample consumption, thus providing ample analysis time to perform MS^n in a single analysis.
  - For α-casein, all phosphorylation sites were identified in MS/MS and MS^3 spectra at concentrations as low as 50 fmol/μL.
  - For β-casein, a single phosphorylation site was identified in one analysis at concentrations of either 1 fmol/μL (manual acquisition) or 5 fmol/μL (automated acquisition).
- The combination of the NanoMate system with the Finnigan LTQ provides excellent sensitivity detection for phosphorylation identification through high-quality spectra even at the MS^4 level of a 100-fmol infusion of α-casein digest.
- The β-casein phosphorylated peptide, 48-FQpSEEQQQTEDELQDK-63, was fully identified in 500 attomoles of sample.
- For complex, multiple phosphate groups at adjacent residues in a single peptide, phosphatase treatment prior to MS analysis is required for phosphorylation mapping.

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