Data-Dependent™ Neutral-Loss Mass Spectrometry for the Identification of Protein Phosphorylation

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Introduction

Reversible protein phosphorylation plays a pivotal role in a multitude of regulatory mechanisms such as metabolism, cell division, growth and differentiation. Knowledge of the particular residues being phosphorylated in a protein can provide insight into the mechanisms of regulations. However, isolating and sequencing phosphopeptides from protein digests remains a labor-intensive and time-consuming challenge in conventional biochemical approaches. Traditionally, radio-labeled peptides from digested proteins were separated, with the "hot" peptide subjected to Edman protein sequencing to determine site of phosphorylation within the peptide. Non-radioactive techniques include: phosphopeptide enrichment, isotope affinity tagging of the phosphoprotein, and the mass spectrometery techniques of precursor ion scanning, mass mapping and neutral loss scanning.

Electrospray ionization (ESI) has been used with various types of mass analyzers to successfully identify phosphopeptides and phosphorylation sites. However, the identification of phosphopeptides in a complex mixture still remains a challenge. This is often due to the poor ionization efficiency and fast degradation of phosphopeptides. This report describes the use of a chip-based nanoelectrospray ionization technique, in combination with the recently developed Finnigan LTQ linear ion trap, for identification and characterization of phosphopeptides in complex mixtures.

Goal

The goal of this work was to design a robust technique for the identification of phosphopeptides in protein mixtures, using Data Dependent neutral loss MS[3] on the Finnigan LTQ.

Experimental Procedures

Preparation of proteolytic digests

Both α-casein and β-casein (Sigma, St. Louis, MO) were individually dissolved in 50 mM ammonium bicarbonate pH 7.8 at 1 mg/mL. A proteolytic enzyme in a 1 µg/µL stock solution was then added to the solution at an enzyme-to-substrate ratio of 1:60 (w/w). Digestions were performed at 37 °C for 16 hours and stopped by the addition of 0.1% (v/v) acetic acid, and were stored at –70 °C.

Enzymatic dephosphorylation of digested peptides

Calf intestinal phosphatase (CIP) (Biolabs, Boston, MA) was dialyzed against 20 mM ammonium bicarbonate pH 7.8 overnight at 4 °C using Slide-A-Lyzer® MINI Units. Four units of dialyzed CIP (8 units/µL) were added to 10 µL of the above digests and incubated at 37 °C for 2 hours. The CIP treated digests were stored at –70 °C until needed.

MS Conditions

The NanoMate® 100 was mounted to the Finnigan LTQ, and 5 µL samples were infused at a rate of approximately 100 nL/min.

Mass Spectrometer: Finnigan LTQ
Ionization Mode: Nano-electrospray
Ion Polarity: Positive and negative
Spray Voltage: 1.55 kV
Spray Pressure: 0.2 psi.
Capillary Temperature: 150 °C
Normalized Collision Energies: 20–25% for MS^n
Maximum Scan Time: 50 ms
Number of Micro Scans Summed for Each Scan: 2-3
Neutral loss MS[3] experiment activated for the loss of 98, 49 and 32.7 (singly, doubly and triply charged phosphopeptides).

Results and Discussion

The proteolytic digests of two standard phosphoproteins (bovine α-casein and β-casein) were analyzed with tandem mass spectrometry before and after CIP treatment. The amino acid sequence and the phosphorylation sites of α-casein and β-casein are shown in Figure 1. The CIP enzymatically removed all phosphate groups from the proteolytic peptides. Phosphorylated peptides could be identified by comparing the proteolytic mass fingerprints of the treated and untreated proteins (Figure 2). For α-casein, the additional peaks at m/z = 1161.7, 884.3, and 791.0 can be identified by tandem mass spectrometry as peptides QMEAESISSSEEIVPNSVEQK (T8), DIGSESTEDQAMEDIK (T7), and VPQLEIVPNSAEER (T14), respectively. The MS/MS spectrum of peptide (T8) is illustrated in Figure 3, showing an excellent correlation between observed and predicted peptide fragmentation pattern. For β-casein, the additional peaks at 1401.9, 991.7, and 935.0 can be identified by tandem mass spectrometry as peptides RELEELNVPGEIVESLSSSEESITR (T1, doubly charged), FQSEEQQQTEDELQDK (T4), and RELEELNVPGEIVESLSSSEESITR (T1, triply charged), respectively. Figure 4 shows the MS/MS fragmentation spectra for peptide T1. Again, the experimental data correlates extremely well with the predicted fragmentation pattern. The strategy of CIP treatment, followed by differential mass analysis, allows the detection of all potential phosphorylated peptides together with assigning the number of phosphorylation sites per peptide. However, the exact position of the phosphorylation cannot be determined solely with this approach.
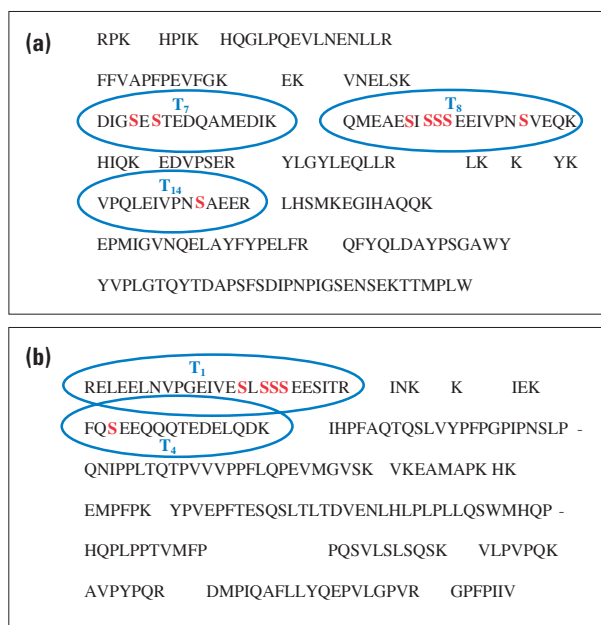
To determine the exact position of the phosphorylation site, the two bovine caseins were additionally analyzed with tandem mass spectrometry, employing automated neutral loss MS[3] mapping. This scan mode relies on the observed behavior of phosphopeptides subjected to MS/MS analysis in an ion trap. Rather than fragment along the peptide backbone to create y- and b-ion series that can be used to identify the peptide, they tend to lose the phosphate group, leaving the peptide intact. MS[3] analysis is then required to create ions that can be used for peptide identification.
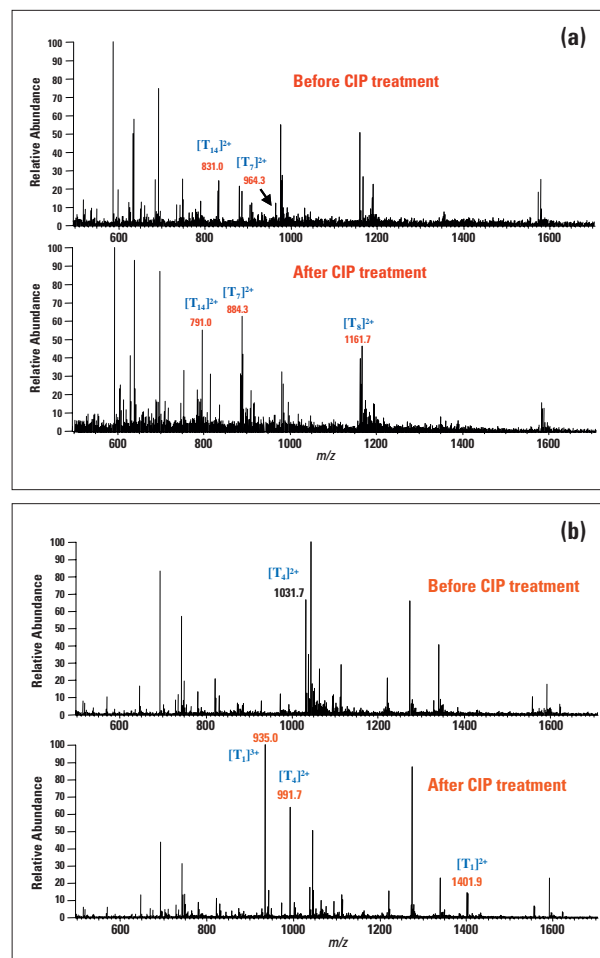

Figure 2: Full mass spectra of (a) 100 fmol/µL α-casein digest and (b) 1 pmol/µL β-casein digest before and after CID treatment. In the full mass spectra of the untreated samples, the T_8 peptide ion of α-casein and the T_1 peptide ion of β-casein were not observed in positive scan mode, due to the complete ion suppression caused by the presence of multiple negatively charged phosphate-groups. However, in the negative full scan mode, the T_8 peptide ion of α-casein and the T_1 peptide ion of β-casein were detected and matched with the predicted peptide mass of additional five and four phosphate-groups respectively (data not shown here).
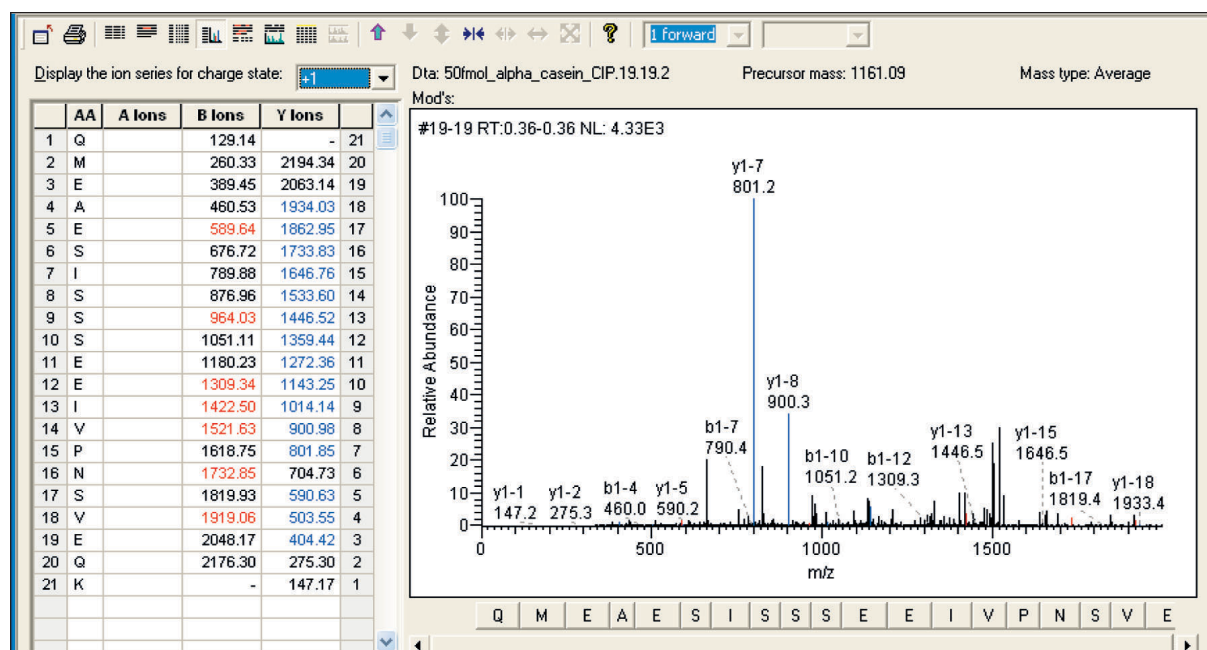

Figure 1: Amino acid sequence of (a) α-casein and (b) β-casein. The phosphorylation sites are marked in red and the proteolytic peptides are numbered from the N-terminus, assuming there is no cleavage before proline. Single amino acids are not counted as proteolytic peptides.

Figure 3: Tandem mass spectra derived by collision-induced dissociation of the (M + 2H)$^{2+}$ precursor ion of the α-casein de-phosphorylated peptide (T$_8$), m/z = 1161.1 and identification using BioWorks™ 3.1 software. The sample concentration was 50 fmol/µL and the Xcorr for peptide identification was excellent at 2.655
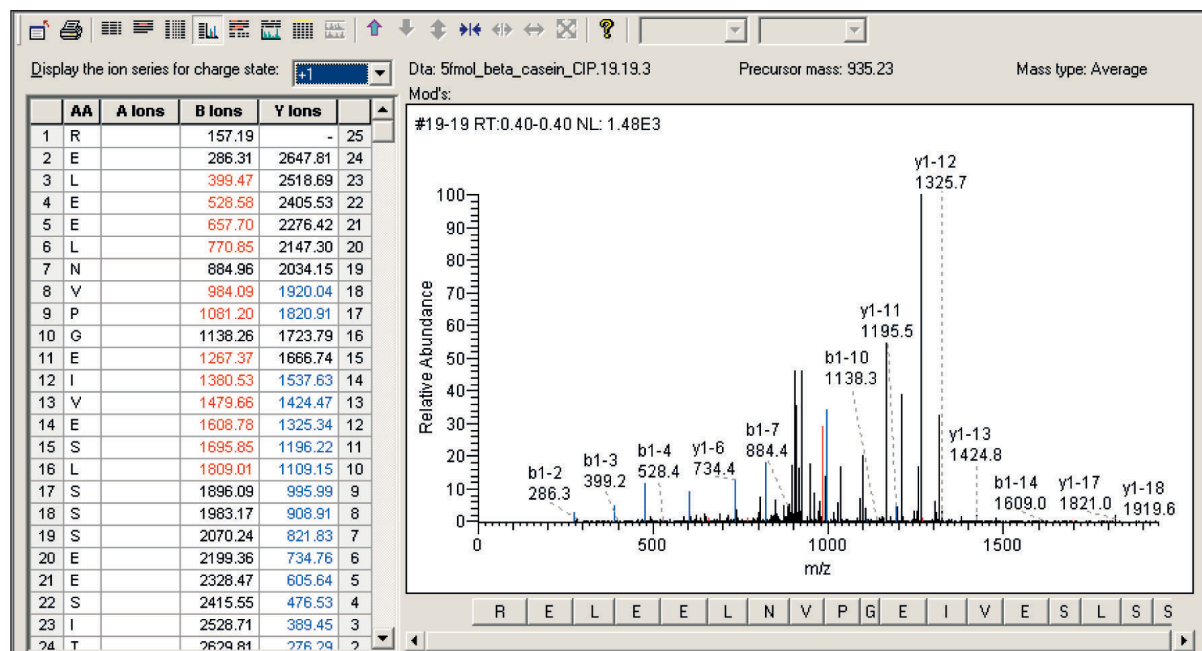

Figure 4: Tandem mass spectra derived by collision-induced dissociation of the (M + 2H)$^{3+}$ precursor ion of the β-casein de-phosphorylated peptide (T$_1$), m/z = 935.2 and identification using BioWorks 3.1 software. The sample concentration was 5 fmol/µL and the Xcorr value for peptide identification was 4.934.
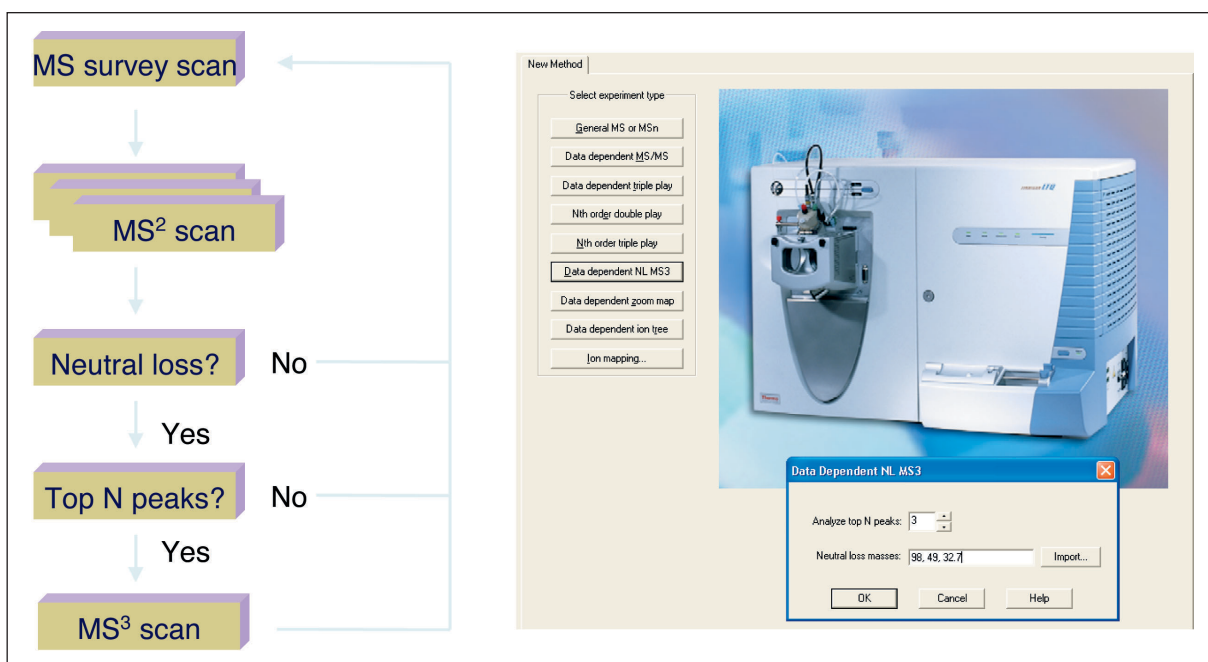
Figure 5: Schematic representation of the fully automated data-dependent neutral-loss scans. The detection of a peptide with a neutral loss of 98, 49, and 32.7 (corresponding to the phosphate loss of a singly, double, or triply charged phosphopeptides) triggered the MS^3 scan event. To prevent random MS^3 events, the intensity of the neutral loss fragment needed to be within the top three peaks.
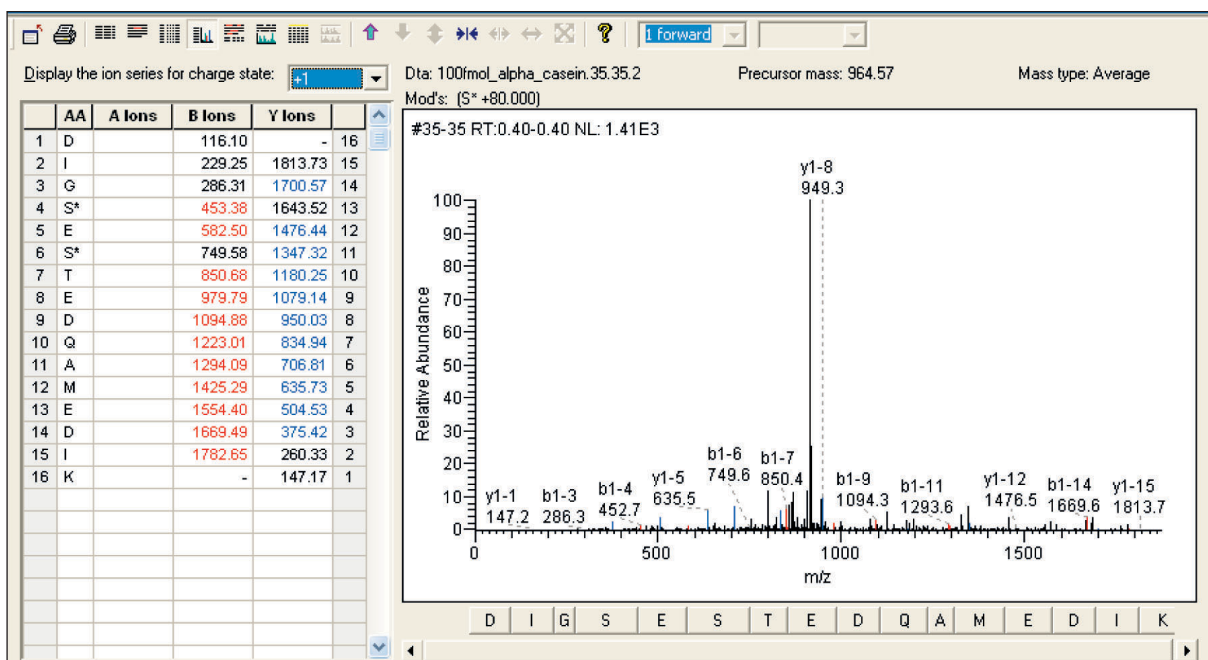

Figure 6a: Tandem mass spectra derived by collision-induced dissociation of the (M + 2H)$^{2+}$ precursor ion of the β-casein de-phosphorylated peptide (T$_7$), m/z = 964.6 and identification using BioWorks 3.1 software. The sample concentration was 100 fmol/μL and the Xcorr value for peptide identification was 3.578.

The detection of a neutral loss event of the masses of 98, 49, or 32.7 (corresponding to the phosphate loss from a singly, double, or triply charged phosphopeptide) triggered the MS³ scan, which was a result of an Xcalibur™ method (Figure 5). The threshold for triggering MS³ event required the neutral loss fragment to be within the top three peaks. The samples were analyzed at different concentrations to determine the detection limits for phosphorylated peptides in mixtures.
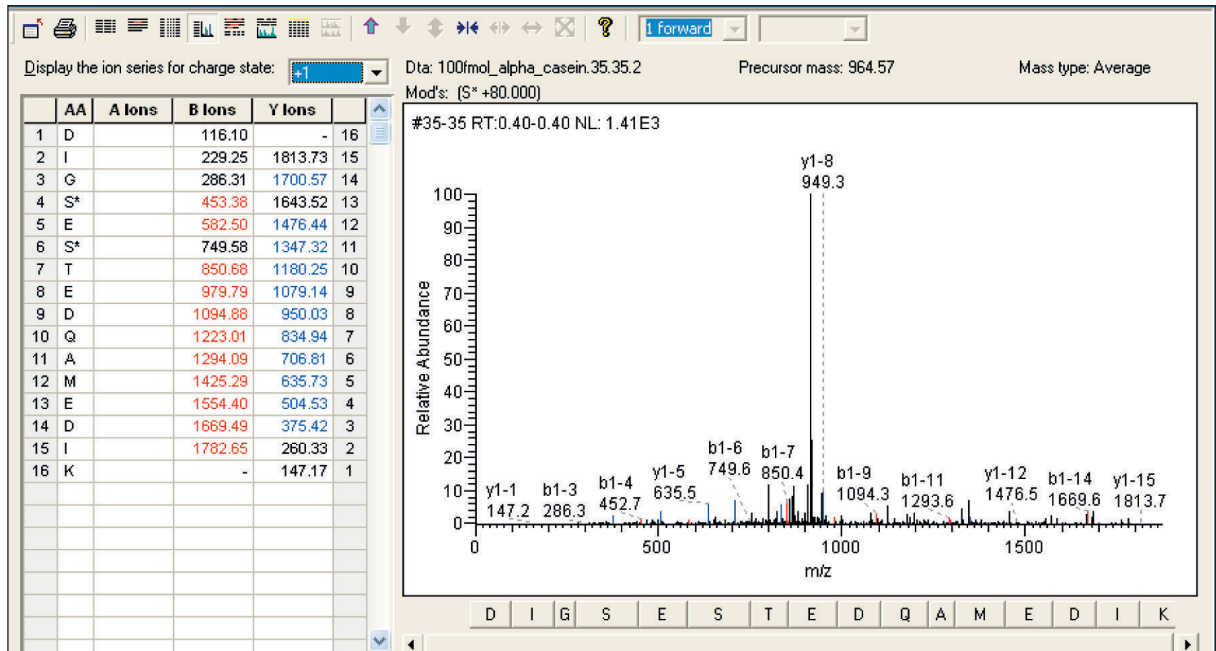

Figure 6b: MS³ spectra derived by collision-induced dissociation of the (M + 2H)²⁺ precursor ion of the β-casein phosphorylated peptide (T₇), m/z = 964.6 → 915.5 and identification using BioWorks 3.1 software. The sample concentration was 100 fmol/µL and the Xcorr value for peptide identification was 3.409.
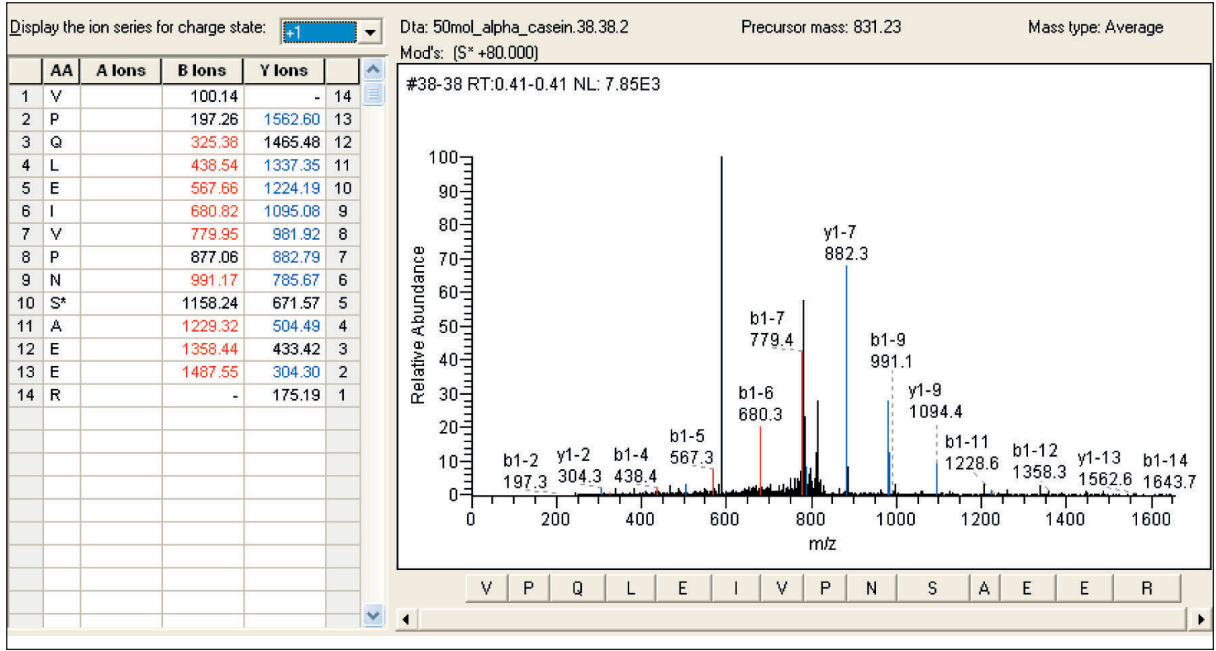

Figure 6c: Tandem mass spectra derived by collision-induced dissociation of the (M + 2H)²⁺ precursor ion of the β-casein de-phosphorylated peptide (T₁₄), m/z = 831.2 and identification using BioWorks 3.1 software. The sample concentration was 50 fmol/µL and the Xcorr value for peptide identification was 3.148.

The data was analyzed automatically using BioWorks 3.1 software with SEQUEST® search algorithm, taking into account dynamic phosphate modifications. For α-casein, the phosphorylated peptides could be identified in the MS[2] and MS[3] spectra at concentrations as low as 50 fmol/µL (Figures 6a-6d). Additionally, peptide DIGSESTEDQAMEDIK (T7) could be identified from a manually recorded MS[4] spectra (Figure 7).
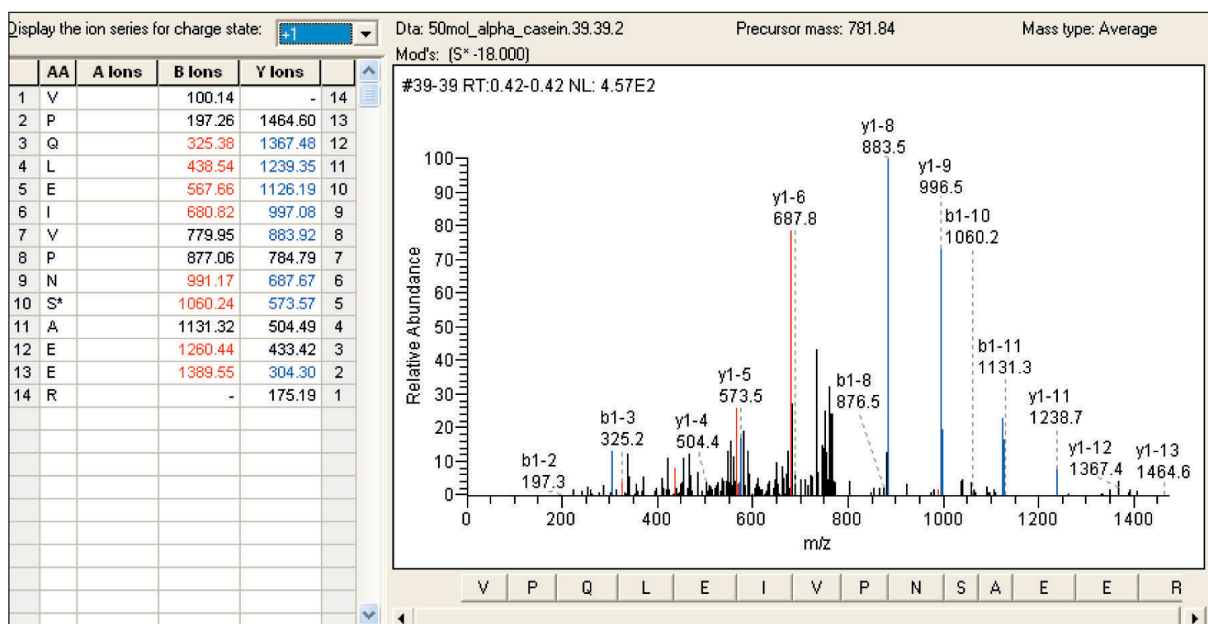

	AA	A Ions	B Ions	Y Ions	
1	V		100.14	-	14
2	P		197.26	1464.60	13
3	Q		325.38	1367.48	12
4	L		438.54	1239.35	11
5	E		567.66	1126.19	10
6	I		680.82	997.08	9
7	V		779.95	883.92	8
8	P		877.06	784.79	7
9	N		991.17	687.67	6
10	S*		1060.24	573.57	5
11	A		1131.32	504.49	4
12	E		1260.44	433.42	3
13	E		1389.55	304.30	2
14	R		-	175.19	1

Dta: 50mol_alpha_casein.39.39.2   Precursor mass: 781.84   Mass type: Average
Mod's: (S* -18.000)

Figure 6d: MS[3] spectra derived by collision-induced dissociation of the (M + 2H)[2+] precursor ion of the β-casein phosphorylated peptide (T$_{14}$), m/z = 831.2 → 781.8 and identification using BioWorks 3.1 software. The sample concentration was 50 fmol/µL and the Xcorr value for peptide identification was 3.126.
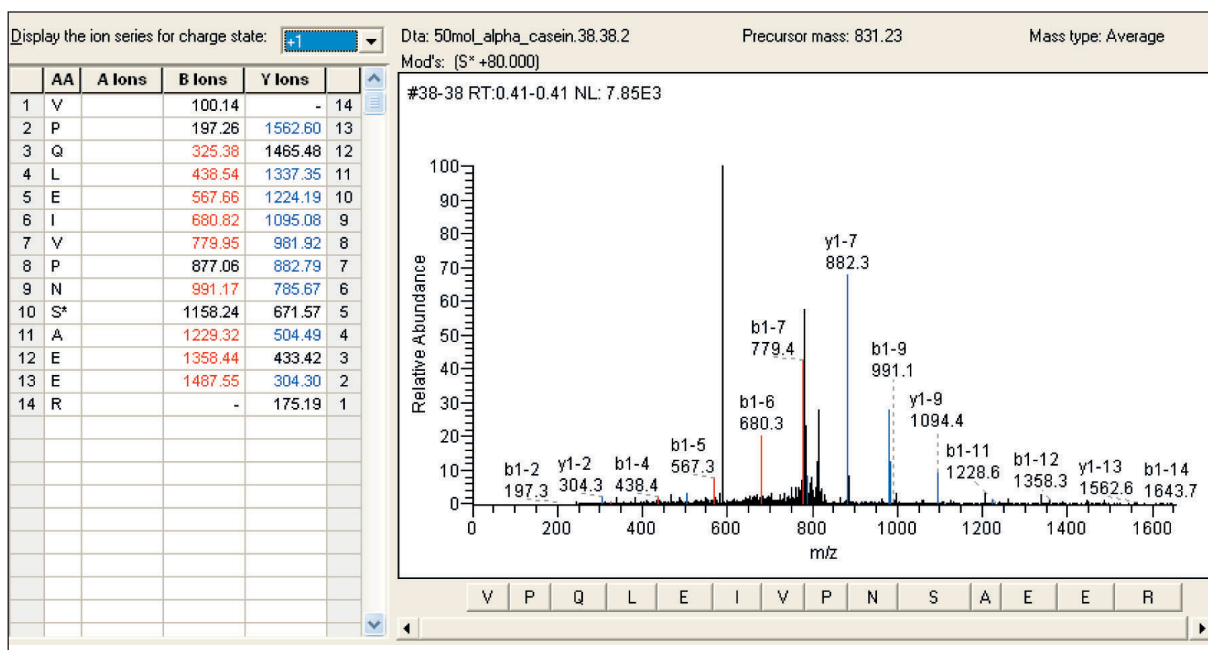

	AA	A Ions	B Ions	Y Ions	
1	V		100.14	-	14
2	P		197.26	1562.60	13
3	Q		325.38	1465.48	12
4	L		438.54	1337.35	11
5	E		567.66	1224.19	10
6	I		680.82	1095.08	9
7	V		779.95	981.92	8
8	P		877.06	882.79	7
9	N		991.17	785.67	6
10	S*		1158.24	671.57	5
11	A		1229.32	504.49	4
12	E		1358.44	433.42	3
13	E		1487.55	304.30	2
14	R		-	175.19	1

Dta: 50mol_alpha_casein.38.38.2   Precursor mass: 831.23   Mass type: Average
Mod's: (S* +80.000)

Figure 7: MS[4] spectra derived by collision-induced dissociation of the (M + 2H)[2+] precursor ion of the α-casein phosphorylated peptide (T$_7$), m/z = 964.6 → 915.5 → 866.0 and identification using BioWorks 3.1 software. The sample concentration was 100 fmol/µL and the Xcorr value for peptide identification was 3.352.
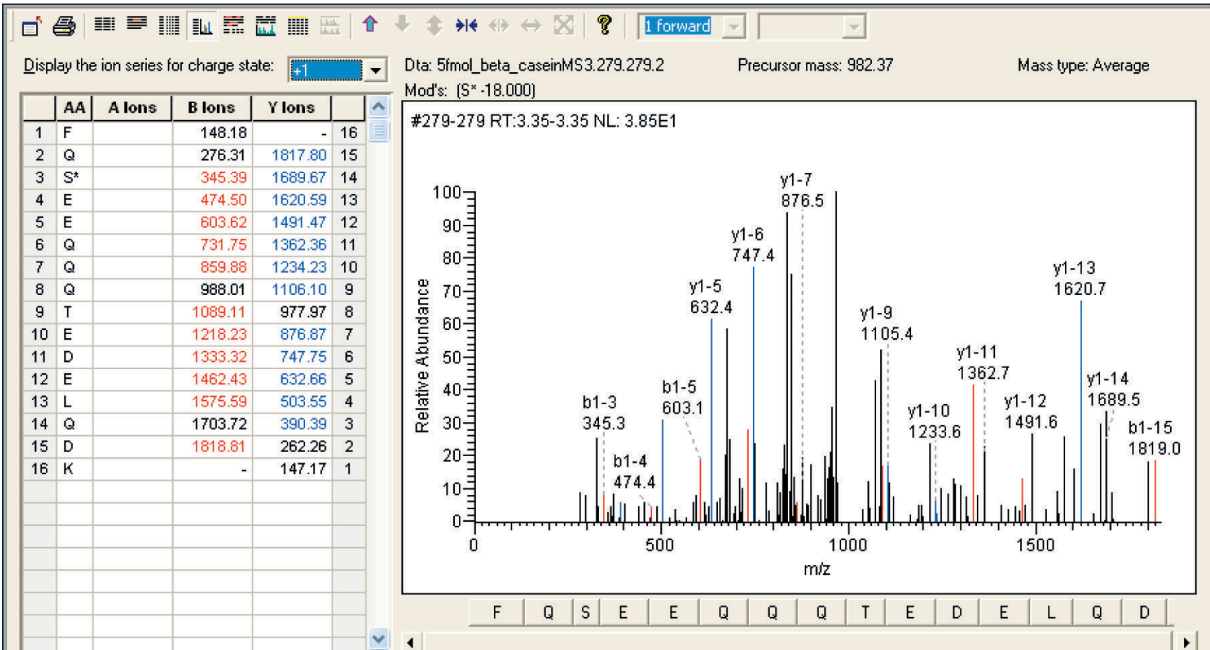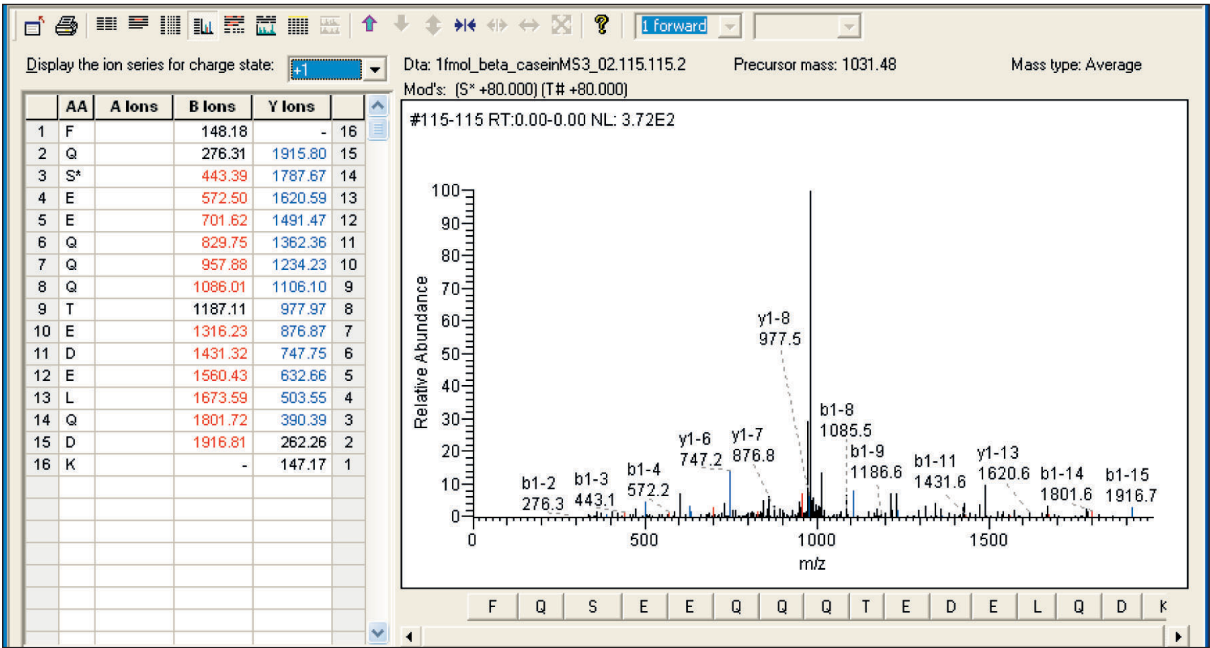
For β-casein, the phosphorylated peptides could be identified in MS² and MS³ spectra at concentrations as low as 1 fmol/μL. Based on an approximate flow rate of 100 nL/min, an estimated total sample consumption of only 500 attomoles for the fully automated identification of the phosphorylated peptide FQSEEQQQTEDELQDK was observed (Figures 8a and 8b).

Figure 8a: Tandem mass spectra derived by collision-induced dissociation of the (M + 2H)²⁺ precursor ion of the β-casein phosphorylated peptide(T$_4$), *m/z* = 1031.5 and identification using BioWorks 3.1 software. The sample concentration was 1 fmol/μL and the Xcorr value for peptide identification was 3.861.

Figure 8b: MS³ spectra derived by collision-induced dissociation of the (M + 2H)²⁺ precursor ion of the β-casein phosphorylated peptide (T$_4$), *m/z* = 1031.5 → 982.4 and identification using BioWorks 3.1 software. The sample concentration was 5 fmol/μL and the Xcorr value for peptide identification was 4.421.

Conclusion

The results demonstrate that the Finnigan LTQ linear ion trap is perfectly suited for sensitive analysis of phospho-peptides. The phosphorylation sites of peptides presented in mixtures at low fmol levels were mapped using Data Dependent neutral loss MS^3 scanning for targeted analyses of phosphopeptides. This technique coupled the powerful MS^n capabilities of the Finnigan LTQ with reproducible, stable, ESI chip nano-electrospray for extended analysis of very small sample volumes.

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