Highly sensitive phosphopeptide analysis using Ettan MDLC and a linear ion trap mass spectrometer

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Ettan™ MDLC coupled to a linear ion trap mass spectrometer was used to characterize phosphopeptides in the low femtomole range. Phosphopeptides in a complex biological sample were sequenced, and the phosphorylation sites were identified.

Introduction

Because of the importance of phosphorylation and dephosphorylation to the function of many metabolically active proteins (1, 2), there is a great interest in improving the methods for reliable analysis of phosphopeptides. Unfortunately, phosphoproteins often exist in low abundance in cells, and the corresponding phosphopeptides resulting from digestion exhibit low ionization efficiency in positive electrospray ionization (ESI). Furthermore, the frequently low stoichiometry of phosphorylation in proteins (3) makes it important to increase the sensitivity of the analysis.

One way to increase the sensitivity is to ensure that no peptides are lost or degraded during the analysis. Because Ettan MDLC is a biocompatible system, there are no metal ions in the flowpath that can complex with phosphate groups (4). In addition, the biocompatible materials that make up the pumpheads and flowpath promote the longevity of the system, especially when used with salt-containing buffers.

We developed a method for femtomole-scale phosphopeptide analysis using Ettan MDLC and a Finnigan™ LTQ™ linear ion trap mass spectrometer. The phosphopeptide analysis procedure consisted of the following steps:

- Desalting the peptide sample on an RPC trap column using Ettan MDLC
- Separation of the peptide sample on a nanoscale analytical RPC column using Ettan MDLC
- Data-dependent neutral loss scan in the Finnigan LTQ mass spectrometer
- Phosphopeptide identification by database searching using TurboSEQUEST™ protein identification software

The method was developed using β-casein trypsin digest, either alone or mixed with a six-protein digest. To test the method, phosphorylation sites in a complex biological sample—mouse brain tissue—were identified.

Materials

Products used

Ettan MDLC	18-1176-44
NAP™ 10 Columns	17-0854-01
PlusOne™ DTT	17-1318-01
PlusOne Iodoacetamide	RPN6302
PlusOne Tris	17-1321-01
Trypsin, sequencing grade	17-6002-75

Other products required

Finnigan LTQ mass spectrometer (Thermo Electron)

TurboSEQUEST protein identification software (Thermo Electron)

PepMap™ C18 RPC trap column, 300 µm i.d. x 5 mm, 5 µm, 100 Å (LC Packings)

PepMap C18 RPC analytical column, 75 µm i.d. x 150 mm, 5 µm, 100 Å (LC Packings)

Zorbax™ 300-SB C18 trap column, 300 µm i.d. x 5 mm, 3 µm (Agilent)

Zorbax 300-SB C18 analytical column, 75 µm i.d. x 150 mm, 3 µm (Agilent)

β-Casein, bovine (Sigma-Aldrich)

Protein Mixture Digest (LC Packings)

Formic acid, ultrapure (Merck Suprapur™)

Water, HPLC grade

Acetonitrile, HPLC grade

Ammonium bicarbonate (Merck)

Methods

Sample preparation

Approximately 0.5 mg of mouse brain tissue was prepared according to the following procedure. The proteins were dissolved by adding 1 ml of 9 M urea with 50 mM DDT to the tissue and allowing it to incubate for 60 min at 20 °C. A 1-ml aliquot consisting of 8 M urea, 250 mM TrisHCl, and 125 mM iodoacetamide, pH 8.8, was added. The mixture was allowed to incubate for 60 min at 20 °C. A 1-ml aliquot of this mixture was buffer exchanged with 20 mM ammonium bicarbonate, pH 7.8, on a NAP-10 desalting column. The protein sample was digested with trypsin (concentration ratio of 50:1) for 4 h. The trypsin was then inactivated by adding formic acid to the sample.

β-Casein was dissolved in 50 mM ammonium bicarbonate, and then digested with trypsin.

A 1-fmol/µl solution of a six-protein digest (cytochrome C, lysozyme, alcohol dehydrogenase, bovine serum albumin, apotransferrin, and β-galactosidase; LC Packings) was mixed with a 1-fmol/µl solution of β-casein digest (equimolar ratio).

High-throughput nanoscale liquid chromatography

Ettan MDLC was used in the high-throughput configuration using two RPC trap columns 300 µm i.d. x 5 mm (PepMap, C18, 5 µm, 100 Å or Zorbax 300-SB C18) for online desalting and sample cleanup, followed by two nanoscale RPC analytical columns 75 µm i.d. x 150 mm (PepMap, C18, 5 µm, 100 Å or Zorbax 300-SB C18) for high-resolution separation. One set of RPC trap/analytical columns was equilibrated while the second set separated the sample. The mobile phases were A: HPLC-grade water with 0.1% formic acid, and B: 84% HPLC-grade acetonitrile with 0.1% formic acid. A gradient of 0–56% B was run at a flow rate of 250 nl/min for 48 min.

Mass spectrometry

A Finnigan LTQ linear ion trap mass spectrometer equipped with a nanospray interface was used as the detector for the peptides that eluted from the RPC column. The MS method consisted of a cycle combining one full MS scan with three MS/MS events (25% collision energy) followed by an MS^3 event (35% collision energy) that was triggered upon detection of -98, -49, or -32.7 Da from the precursor (neutral loss of phosphoric acid). Dynamic exclusion duration was set to 30 s.

Peptide identification

The MS/MS and MS^3 spectra from all the runs were searched using TurboSEQUEST protein identification software (Thermo Electron). Modifications were set to allow for the detection of oxidized Met (+16), carboxyamidomethylated Cys (+57), phosphorylated Ser, Thr, and Tyr (+80), and dehydrated Ser and Thr (-18). The peptide matches were filtered based on cross-correlation scores (Xcorr) of 1.5, 2, and 2.5 for charge states 1^+, 2^+, and 3^+, respectively. All spectra in the result lists were manually confirmed. The spectra were considered good when 50% of the b and y ions were assigned, including amino acids with phosphate groups.

Results and discussion

A 200-fmol trypsin digest of β-casein was analyzed by LC-MS/MS for instrument tuning and method development. The averaged spectra at a retention time of 28 min are shown in Figure 1 (top). The signal observed at *m/z* 982.5 is due to the loss of the neutral fragment from the precursor ion at *m/z* 1032.4 (Fig 1, middle). The neutral loss in MS/MS automatically triggered an MS^3 experiment, which gives a very rich and clear peptide fragmentation (Fig 1, bottom). Interpretation of the data identified with very high confidence a β-casein phosphopeptide (FQpSEEQQQTEDELQD, *m/z* 1031).

Based on the findings from method development, we decided to lower the collision energy in MS/MS to 15–25% to enhance neutral loss and avoid complete fragmentation of the peptides. To obtain amino acid sequence information, the neutral loss ion was further fragmented in MS^3 mode. The collision energy for MS^3 was set to 35%, which led to good fragmentation of the selected phosphopeptides (5).

To determine whether phosphopeptides could be detected at low concentrations in mixtures, a mixture of β-casein trypsin digest and six-protein digest was analyzed by the described method. The sample was injected in different

concentrations corresponding to 20, 2, and 1 fmol of β-casein. The resulting spectra were subjected to database searches using TurboSEQUEST software. The phosphopeptide was easily identified (Xcorr > 3.2) even at 1 fmol, with as many as 15 out of 16 b ions and 15 out of 16 y ions assigned (Fig 2). The S/N of 1 fmol of phosphopeptide in the extracted ion chromatogram was from 5 to 10, which is above the detection limit. The phosphopeptide ion at *m/z* 1031 was easily detected in the averaged spectrum (Fig 3).

Table 1 illustrates the linearity of the method in the low femtomole range, indicating that there were no losses of the phosphopeptide in the system. This demonstrates the biocompatibility of Ettan MDLC: there are no metal ions in the system that can complex with phosphate groups. There was a slight deviation at 1 fmol, which was not surprising considering that the phosphopeptide concentration was close to the detection limit.

To test the method further, it was applied to a complex biological sample—mouse brain tissue—to search for phosphoproteins. We had earlier identified 2786 nonphosphorylated proteins in this sample by 2-D LC-MS/MS and TurboSEQUEST database searches. The base peak ion chromatogram and all MS^3 events are shown in Figure 4. The number of MS^3 events was typically around 50 per run. Notably, some of the neutral losses from precursors that eluted late in the LC run did not originate from a phosphopeptide. These false positives were clearly found during the manual spectra investigation but not always in the automatic database searches. In total, seven putative phosphorylation sites were assigned from the mouse database search of the MS^3 spectra (Table 2). At least two scans and four consecutive runs confirmed the sequences for the first two phosphopeptides shown in the list.

Table 1. LC retention times and area of the β-casein phosphopeptide for various amounts of β-casein/six-protein digest injected.

Run	Amount of protein injected (fmol)	Average peak area	Area %
1-4	1	39553	8.12
5-8	2	46627	9.57
9-10	20	487020	100.00

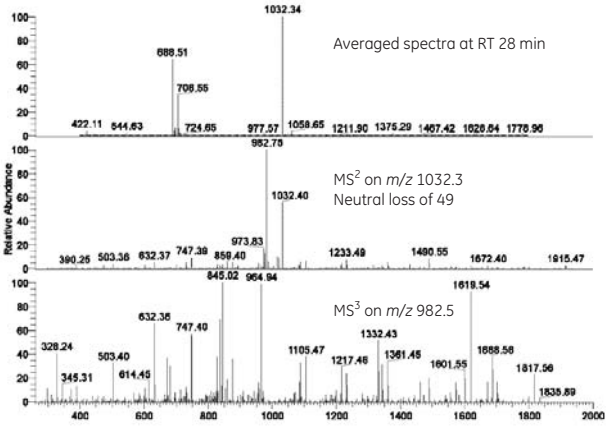

Fig 1. MS spectra from 200 fmol of β-casein digest. Averaged MS spectra from the peak at 28 min retention time (top). MS/MS neutral loss spectrum of the doubly charged phosphopeptide at *m/z* 1032.4 (middle). MS^3 spectrum of the MS/MS peak at *m/z* 982.5 (bottom).

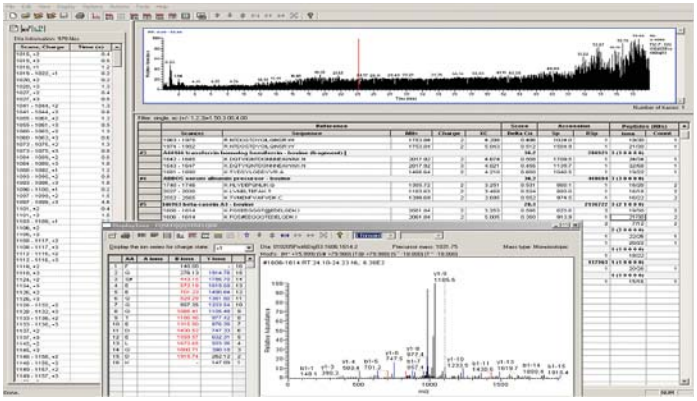

Fig 2. TurboSEQUEST search results from 1 fmol of β-casein/six-protein digest. The phosphopeptide was found and sequenced, and the phosphorylation site, serine 3, is denoted with #.

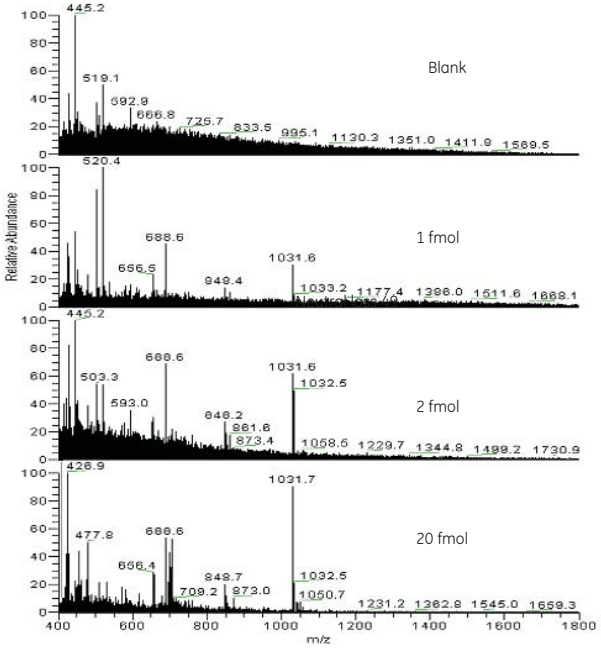

Fig 3. Averaged spectra at 24 min retention time of β-casein phosphopeptide from a blank, 1-fmol, 2-fmol, and 20-fmol injections of β-casein tryptic digest.

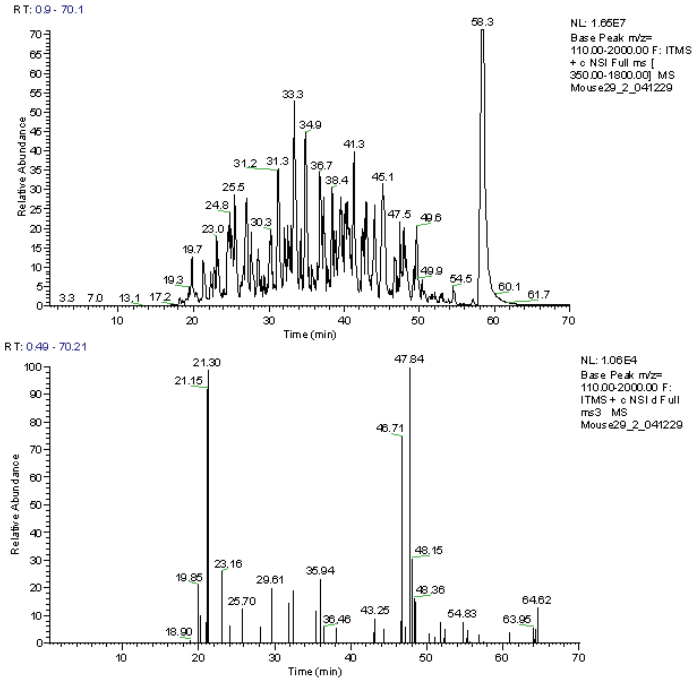

Fig 4. Total ion chromatogram (top) and MS^3 ion trace (bottom) of mouse brain tissue.

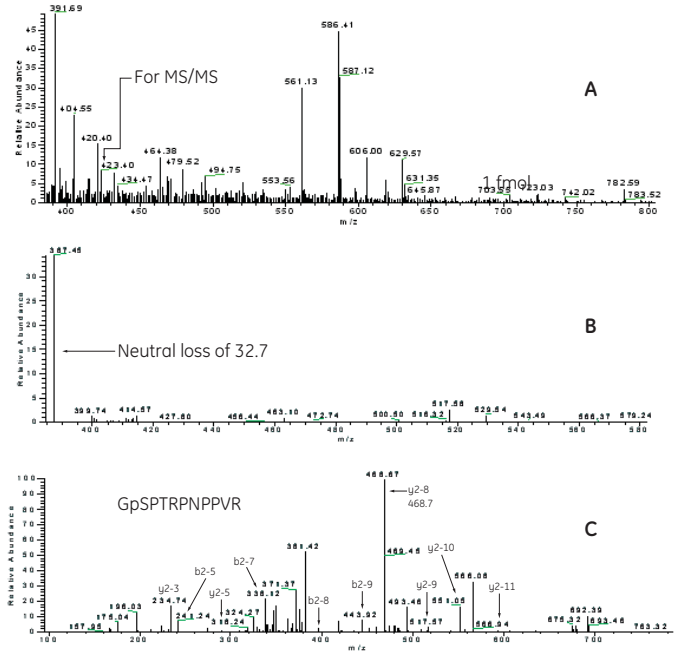

Fig 5. A) Full MS spectrum at 28 min retention time from a mouse brain sample. B) Resulting loss of phosphoric acid from the triply charged ion, *m/z* 420. C) MS^3 spectrum of the *m/z* 367 ion.

Where a phosphopeptide was identified in several runs, an average of the Xcorr values is shown in Table 2. Dihydropyrimidinase-like protein 3 (collapsing response mediator), myristoylated alanine-rich protein kinase C, and MARCKS-like protein (6) are all known phosphoproteins involved in cell signaling in the brain. Drebrin A is a neuron-specific protein that is downregulated in the brains of patients with Alzheimer's disease or Down's syndrome (7). Examples of the MS, MS/MS, and MS^3 spectra from the dihydropyriminidase-like protein 3 phosphopeptide are shown in Figure 5. In addition to the precursor ion, a b or y ion can also show a loss of phosphoric acid. This might account for some of the unknown intense ions in the spectra.

A TurboSEQUEST search was also performed on all MS/MS spectra. The phosphopeptides in Table 2 were confirmed in the MS/MS spectra. Many nonphosphorylated peptides from the phosphoproteins were also found. MS/MS spectra from phosphopeptides are often dominated by the neutral loss ion, as mentioned earlier, which results in less sequence information than from the nonphosphorylated peptides. Even so, more than 40 possible sequences with one or more phosphate groups—either indicated by dehydrated serine or threonine, or by the loss of phosphate—were identified apart from those in Table 2. Additional spectrum interpretation is needed to confirm this data.

Table 2. TurboSEQUEST result list of the putative phosphoproteins and phosphorylation sites from the MS^3 spectra of mouse brain tissue.

Protein	Sequence	Mass of MH^+ (Da)	Average Xcorr value
dihydropyrimidinase-like 3; unc-33-like phosphoprotein	GpSPTRPNPPVR	1159.6	2.63
T-cell receptor alpha V region t2c6	DSALYYCAL(p)SGD(p)SNNR	1787.7	2.83
myristoylated alanine rich protein kinase C subst	LSGFpSFKK	895.5	2.67
glutamyl-prolyl-^{t}RNA	SQGSGLpSSGGAGEGQGPK	1542.7	2.52
MARCKS-like protein	LSGLpSFKR	889.5	2.45
Similar to microtubule-associated protein 2	VAIIRpTPPKSPATPK	1557.9	3.06
Drebrin A	LSpSPVLHR	890.5	2.10

Conclusions

Ettan MDLC in combination with the Finnigan LTQ mass spectrometer is an ideal platform to study phosphopeptides because of the biocompatibility of Ettan MDLC and the speed and MS^n capabilities of the Finnigan LTQ system. Phosphopeptides could be sequenced and identified with a high level of confidence at low femtomole concentrations. Brain phosphoproteins were found in complex mixtures with a high level of confidence.

To further increase the sensitivity of phosphopeptide analysis, an enrichment strategy can be implemented, such as immobilized metal affinity chromatography (IMAC). The resolution of the analysis might also be increased by adding a cation-exchange column to the first-dimension separation (8).

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