Sensitive phosphopeptide analysis: selective extraction and injection of phosphopeptides using capillary electrophoresis (CE) coupled ESI-MS

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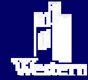


Overview

•Protein phosphorylation is an extremely important cellular event and its understanding is critical to gain further knowledge of signal transduction, gene expression, and numerous other cellular processes

•Phosphorylation is difficult to detect and its MS signal is often suppressed because phosphoylation generally occurs in very low levels relative to the rest of the sample

•To avoid sample adsorption issues that plague enrichment procedures based on solid sorptive phases we utilize the fact that phosphopeptides have characteristically low isoelectric points (pI) and draw them out of solution by the application of voltage

•The sample can then be collected and analyzed via ESI-MS using a metal coated nano tip emitter. Alternatively, the capillary can be directly interfaced with the ESI source using a 3-way splitter or a sheath flow interface.

•The entire tryptic digest of either β- or α-casein was analyzed by MS without any pre-separations and the recorded mass spectra were dominated by peptides with basic and neutral pIs; no phosphopeptides were detected

•When the selective injection technique was applied all phosphopeptides were successfully isolated and therefore detected: two phosphopeptides from β-casein (pIs: 3.29 and 1.37) and four phosphopeptides from α-casein (pIs: 3.62, 2.16, 4.20, and 0.72)

Introduction

•We describe a method to enhance phosphopeptide detection by ESI-MS coupled with CE. In this method we selectively inject low pI peptides, particularly phosphopeptides, into a capillary and then identify them with ESI-MS.

•Anionic phosphopeptides in a buffer, pH 3-5, are subjected to a positive electric field which is applied across the capillary. This field causes the anionic peptides to enter the capillary, while the cationic peptides remain in original sample vial.

•The peptides are then expelled from the capillary and analyzed with ESI-MS in positive ion mode

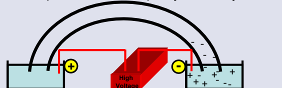


Figure 1: Schematic of the selective injection process. The capillary is filled with buffer and then the sample, in the same buffer, is placed at one end. Ions are drawn out of the solution toward the opposite electrode.

Methods

Instrumentation
•Agilent 3DCE, Beckman Coulter P/ACE MDQ Capillary Electrophoresis System, and Micromass Q-ToF Micro

Materials
•fused silica capillaries with OD 360µm and ID 50µm were used for all CE experiments

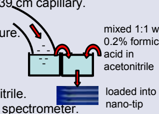
•capillaries were treated with a surfactant coating of 0.1mM DLPC (1,2-Dilauroyl-*sn*-Glycero-3-Phosphocholine) in 20mM Tris-HCl pH 7.4 to eliminate electroosmotic flow

•β- and α-casein, Adrenocorticotropic Hormone (ACTH)

•Proxeon borosilicate emitter tips, Agilent CE ESI-MS Sprayer, 3-way ESI sprayer

Experimentation
•A 10 pmol/µl tryptic digest of β-casein in 10mM ammonium-acetate buffer pH 4.0 was subjected to selective injection by applying 20 kV for 15 minutes across a 39 cm capillary.

•The selectively injected plug was then ejected into a micro vial with pressure. The injection was repeated 3-5 times to increase the sample volume to approximately 1µl for ease of handling.



•The collected sample was then mixed 1:1 with 0.2% formic acid in acetonitrile. Re-acidification was necessary to produce effective ionization in the mass spectrometer.

• The sample was subsequently loaded into a metal-coated nano tip for ESI-MS. A similar procedure was also carried out on a 10pmol/µl tryptic digest of α-casein at pH 5.2.

•ESI-MS data of the original digests without selective injection were also collected for comparison.

•In an attempt to integrate this procedure into an online setup selective injection of ACTH was performed using an anionic electrode in a 20mM ammonium-formate solution at pH 3.25. The capillary was extended from the CE and then connected to a 3-way junction to allow for a makeup solution containing 0.1% formic acid in acetonitrile to mix before ionization.

Results

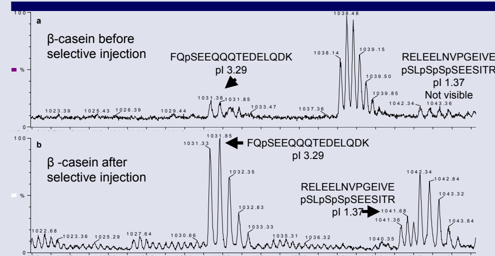


Figure 2: Spectra from collected fractions loaded into metal-coated nano-tips before (a) and after (b) the selective injection technique was applied.

•The offline nano tip method worked successfully however an online method was desired for handling purposes
•The pH range required for selective sampling, 3-5, was too high for effective ionization so the sheath flow method was explored as a way to re-acidify the sample

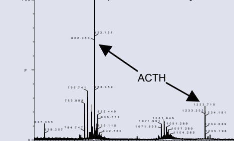


Figure 3: 1pmol/µl ACTH in a 20mM ammonium-formate buffer at pH 3.25 infused at 0.3µl/min with a sheath flow containing 0.1% formic acid in acetonitrile at a flow rate of 0.3µl/min. The Agilent CE-ESI-MS Sprayer was used for this experiment.

Figure 4: Agilent CE-ESI-MS Sprayer interfaced with a Micromass Q-ToF Micro. The Agilent sprayer is attached to the Micromass stage by a custom aluminum plate

•The Agilent sprayer was designed for a relatively high flow rate (a few uL/min). Flow rates as low as 0.6 uL/min were attempted (Fig 3) with continuous infusion of pure peptide solution, but the signal was poorly reproducible.

•To achieve lower flow rates a 3-way junction interface compatible with nano flow was used

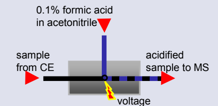


Figure 5: Schematic of the 3-way junction used to introduce the 0.1% formic acid in acetonitrile solution to the sample coming from the CE. Sample is selectively injected into the capillary as shown in figure 1.

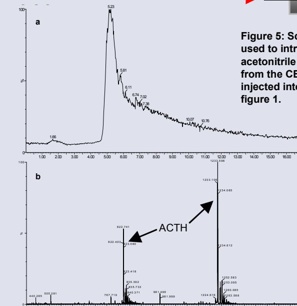


Figure 6: The chromatogram (a) and mass spectrum (b) of a 40pmol/µl solution of ACTH that has been selectively injected into a capillary (+20kV applied to the pH 3.25 sample for 30 minutes). After injection the capillary was connected to the 3-way junction and infused at 0.3µl/min along with the 0.1% formic acid in acetonitrile (make up flow) at 0.3µl/min.

•The 3-way junction produced a reliably stable signal at 0.3µl/min. Good signal was also obtained at the lowest possible flow rate delivered by the existing pumps. Further experiment on split flow is currently underway.

Conclusions

These results show that this selective sampling method is effective for low pI peptides and can be used to greatly enhance the ESI-MS signal of a sample. By making use of the low pI of phosphopeptides this method provides a practical and simple alternative to current methods for dealing with the inherent difficulties of phosphopeptide detection.

References & Acknowledgements

1. Cunliffe, J.M., Baryla, N.E., Lucy, C.A. Anal. Chem. **2002**, 74, 776-783.
2. Zhang, H., Yeung, K.K.-C. Anal. Chem. **2004**, 76, 6814-6818.

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