

Separation of isobaric phosphopeptides by High field asymmetric waveform ion mobility spectrometry (FAIMS) confirmed by LTQ Orbitrap XL ETD mass spectrometry

Yue Xuan¹, Andrew Creese², Julie Horner³, Helen Cooper², Thomas Moehring¹

¹ Thermo Fisher Scientific, Bremen, Germany; ² University of Birmingham, School of Biosciences, Birmingham, UK; ³ Thermo Fisher Scientific, San Jose, US

Overview

Purpose: Separating isobaric phosphopeptides by using High Field asymmetric waveform ion mobility spectrometry (FAIMS).

Methods: FAIMS connected to LTQ Orbitrap XL ETD system.

Results: The results show that FAIMS is capable of at least partially separating isobaric phosphopeptides.

Introduction

Phosphorylation is a widespread and biologically significant protein post-translational modification involved in numerous cellular processes. Localization of sites of phosphorylation is key in determining their functional significance. The occurrence of isobaric phosphopeptides presents problems in liquid chromatography tandem mass spectrometry (LC-MS/MS) analyses as the species often co-elute. Separation of isobaric species is particularly pertinent to the characterisation of protein phosphorylation.

An intriguing feature of FAIMS is its ability to separate isobaric species. High field asymmetric waveform ion mobility spectrometry (FAIMS) has been applied to analysis of the phosphopeptides APLpSFRGSLPKSYVK (pSer1), APLSFRGpSLPKSYVK (pSer2), and APLSFRGSLPKpSYVK (pSer3). The results show that FAIMS is capable of at least partially separating isobaric phosphopeptides. Separation was confirmed by coupling FAIMS with LTQ Orbitrap XL ETD mass spectrometry.

Methods

Sample

Phosphopeptides APLpSFRGSLPKSYVK, APLSFRGpSLPKSYVK, and APLSFRGSLPKpSYVK were synthesized by AltaBiosciences (Birmingham, UK) and used without further purification.

ESI-FAIMS-ETD mass spectrometry

All FAIMS analyses were performed on a Thermo Scientific LTQ Orbitrap XL ETD hybrid mass spectrometer equipped with electrospray ionization source

FAIMS

DV: -5 kV
Outer bias voltage: 30 kV
Inner electrode temperature: 50 °C
Outer electrode temperature: 100 °C
Carrier gas
Flow rate: 4 L/min
Composition: 50% N₂ and 50% He

MS

Triply charged precursor: [M+3H]³⁺ (*m/z* 577.6)
Doubly charged: [M+2H]²⁺ (*m/z* 865.9)
Isolation in the linear ion trap
Fragmentation
ETD with supplemental activation
ETD reaction time: 80ms (3+ ions)
120 ms (2+ ions)
Mass spectra comprise a single microscan

Results

Individual phosphopeptides were electrosprayed into the FAIMS device and subjected to full-scan mass spectrum compensation voltage (CV) scanning from -50 V to -9 V in steps of 0.3 V (DV = -5 kV). The different charge states can be separated by FAIMS. The optimum CV for the 2+ charge state was -20.56 V and for the 3+ charge state was -37.73 V. See Figure 1.

Binary mixtures (1:1) of the peptides were introduced into system. The compensation voltage was scanned from -50 V to -23 V in steps of 0.3 V (DV = -5 kV). At each CV, an ETD mass spectrum was acquired in the Orbitrap™ mass analyzer. ETD results in cleavage of the backbone N-Cα bond to give N-terminal c (or c') fragments and C-terminal z⁺ (or z) fragments. The *m/z* ratios of the predicted ETD fragments are given in Table 1.

For mixture of pSer1 and pSer2, at CV = -42.64 V, the fragments observed in this mass spectrum originate from pSer2 only, but no pSer1. See Figure 2.

For mixture of pSer1 and pSer3, the ETD mass spectrum obtained at CV = -39.17 V contains peaks corresponding to fragments of pSer1 only, but no pSer3. See Figure 3.

FIGURE 1. Full scan mass spectrum compensation voltage scanning of PLpSFRGSLPKSYVK peptide ions (DV = -5 kV). Extracted ion chromatogram for [M+3H]³⁺ ions (top) and [M+2H]²⁺ ions (bottom). Inset: mass spectra obtained at CV = -37.73 V (top) and CV = -20.56 V (bottom). Data recorded in LTQ.

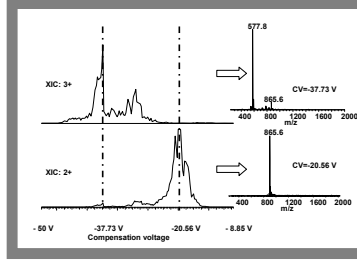


Table 1. Predicted fragments for the three phosphopeptides

APLpSFRGSLPKSYVK				APLSFRGpSLPKSYVK				APLSFRGSLPKpSYVK			
AA	C	Z	AA	C	Z	AA	C	Z	AA	C	Z
A	89.07093934	-	15.00	A	89.07093934	-	15.00	A	89.07093934	-	15.00
P	186.1237032	1642.85	14.00	P	186.1237032	1642.85	14.00	P	186.1237032	1642.85	14.00
L	299.2077671	1545.80	13.00	L	299.2077671	1545.80	13.00	L	299.2077671	1545.80	13.00
S*	466.2061255	1432.71	12.00	S	386.2397955	1432.71	12.00	S	386.2397955	1432.71	12.00
F	613.2745394	1265.71	11.00	F	533.3082094	1345.68	11.00	F	533.3082094	1345.68	11.00
R	769.37565	1118.64	10.00	R	689.4092205	1198.61	10.00	R	689.4092205	1198.61	10.00
G	826.3971142	962.54	9.00	G	746.4307842	1042.51	9.00	G	746.4307842	1042.51	9.00
S	913.4291426	905.52	8.00	S*	913.4291426	985.49	8.00	S	833.462813	985.49	8.00
L	1026.513207	818.49	7.00	L	1026.513207	818.49	7.00	L	946.5468765	898.46	7.00
P	1123.56597	705.41	6.00	P	1123.56597	705.41	6.00	P	1043.59964	785.37	6.00
K	1251.660913	608.35	5.00	K	1251.66093	608.35	5.00	K	1171.694603	688.32	5.00
S	1338.692962	480.26	4.00	S	1338.692962	480.26	4.00	S*	1338.692962	560.22	4.00
Y	1501.75629	393.23	3.00	Y	1501.75629	393.23	3.00	Y	1501.75629	393.23	3.00
V	1600.824704	230.16	2.00	V	1600.824704	230.16	2.00	V	1600.824704	230.16	2.00
K	-	131.09	1.00	K	-	131.09	1.00	K	-	131.09	1.00

FIGURE 2. ESI-FAIMS-ETD mass spectrometry of a 1:1 mixture of phosphopeptides APLpSFRGSLPKSYVK and APLSFRGpSLPKSYVK. Compensation voltage scanned from -50 V to -23 V (DV = -5 kV). (a) Extracted ion chromatograms for APLpSFRGSLPKSYVK fragments (c6, c7, z9* and z10*) (red) and APLSFRGpSLPKSYVK fragments (c4, c6, z10* and z11) (blue). ETD mass spectra obtained at (b) CV = -42.64 V; (c) CV = -33.34 V; and (d) CV = -23.63 V.

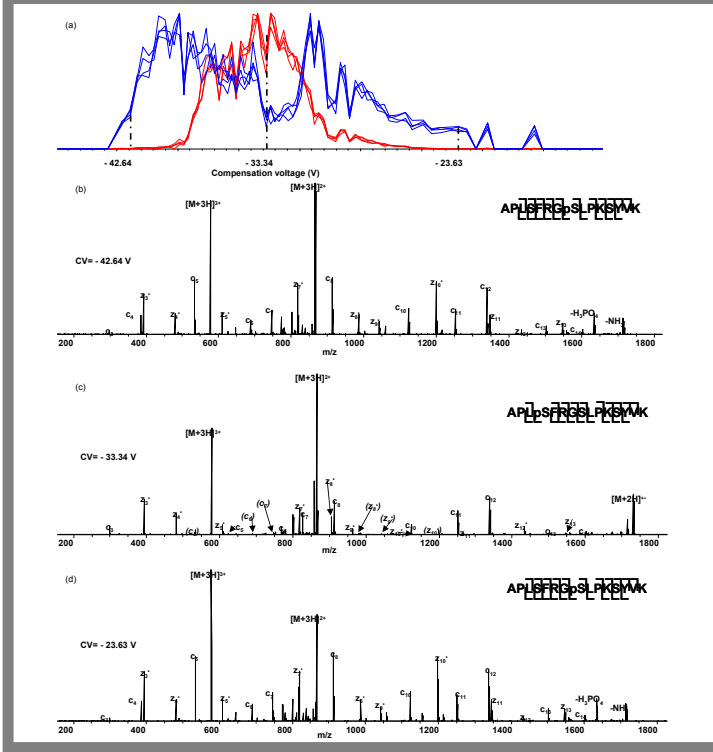


FIGURE 3. ESI-FAIMS-ETD mass spectrometry of a 1:1 mixture of phosphopeptides APLpSFRGSLPKSYVK and APLSFRGSLPKpSYVK. Compensation voltage scanned from -45 V to -19 V (DV = -5 kV). (a) Extracted ion chromatograms for APLpSFRGSLPKSYVK fragments (c6, c7, z8* and z9*) (red) and APLSFRGSLPKpSYVK fragments (c7, c8, z7* and z8) (green). ETD mass spectra obtained at (b) CV = -39.17 V; and (c) CV = -29.36 V.

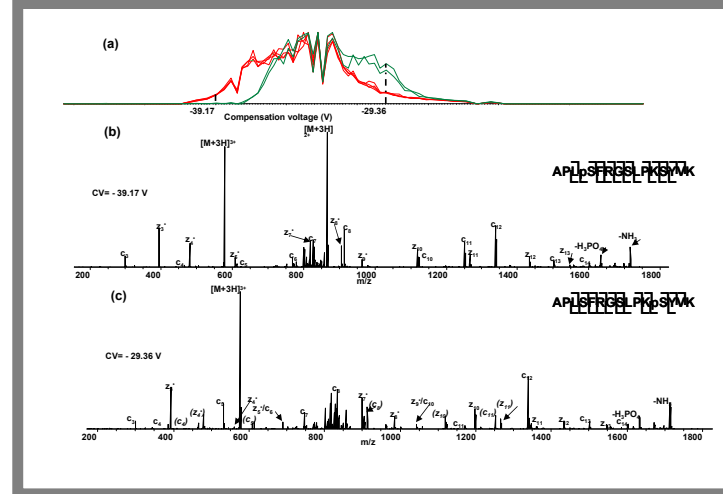
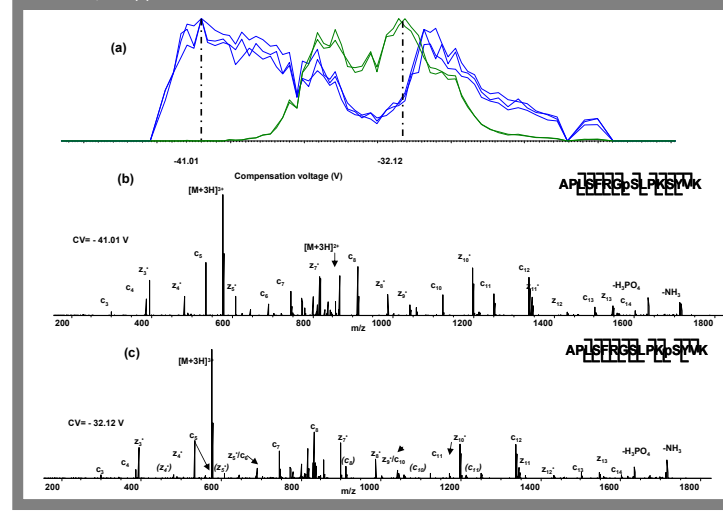


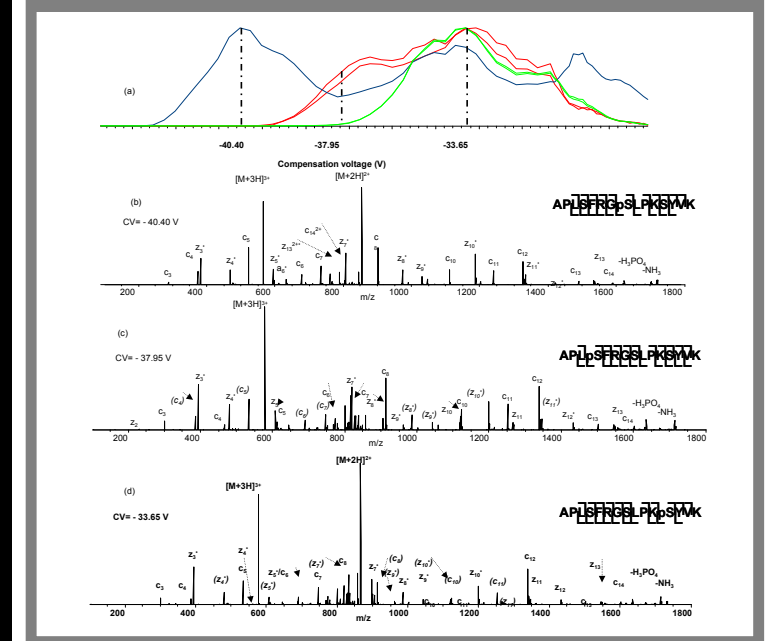
FIGURE 4. ESI-FAIMS-ETD mass spectrometry of a 1:1 mixture of phosphopeptides APLSFRGpSLPKSYVK and APLSFRGSLPKpSYVK. Compensation voltage scanned from -45 V to -21 V (DV = -5 kV). (a) Extracted ion chromatograms for APLSFRGpSLPKSYVK fragments (c10, c11, and z7*) (blue) and APLSFRGSLPKpSYVK fragments (c8 and z7*) (green). ETD mass spectra obtained at (b) CV = -41.01 V; and (c) CV = -32.12 V.



For mixture of pSer2 and pSer3, the ETD mass spectrum obtained at CV = -41.01 V suggests that at this CV value only 3+ ions of pSer2 are transmitted and the fragments observed in the mass spectrum derive from pSer2 only. See Figure 4.

These findings were corroborated by the FAIMS-ETD analysis of a mixture of all three phosphopeptides (1:1:1). The compensation voltage was scanned from -45 to -25 V in steps of 0.3 V and an ETD mass spectrum was recorded in the orbital trap at each step. See Figure 5.

FIGURE 5. ESI-FAIMS-ETD mass spectrometry of a 1:1:1 mixture of phosphopeptides APLpSFRGSLPKSYVK, APLSFRGpSLPKSYVK and APLSFRGSLPKpSYVK. Compensation voltage scanned from -45 V to -25 V (DV = -5 kV). (a) Extracted ion chromatograms for APLpSFRGSLPKSYVK fragments (c6 and z9*) (red), APLSFRGSLPKpSYVK fragments (c8 and z7*) (green), and APLSFRGpSLPKSYVK (or APLSFRGSLPKpSYVK) fragment (z10*) (blue). ETD mass spectra obtained at (b) CV = -40.40 V; (c) CV = -37.95 V; and (d) -33.65 V.



Conclusions

We have shown that it is possible to partially separate phosphopeptides with identical sequences but differing sites of modification by FAIMS and to confirm the separation by ETD mass spectrometry. The results obtained for binary mixtures of the peptides showed that (a) it is possible to separate pSer2 from pSer1 (but not vice versa, i.e. at certain CV values only pSer2 is transmitted; the same is not true for pSer1); (b) pSer1 can be separated from pSer3 (but not vice versa); and (c) pSer2 can be separated from pSer3 (but not vice versa). These findings were corroborated by the FAIMS-ETD analysis of a mixture of all three phosphopeptides. It was possible to completely separate peptide pSer2 from both pSer1 and pSer3. Peptide pSer1 could be separated from pSer3 but not pSer2. It was not possible to separate pSer3 from its isobars.

The results have implications for the incorporation of FAIMS in phosphoproteomic LC-MS/MS methods, and the investigation of the gas-phase structure of phosphopeptides.