A NOVEL MULTI-DIMENSIONAL METHOD FOR THE AFFINITY ENRICHMENT OF PHOSPHOPEPTIDES

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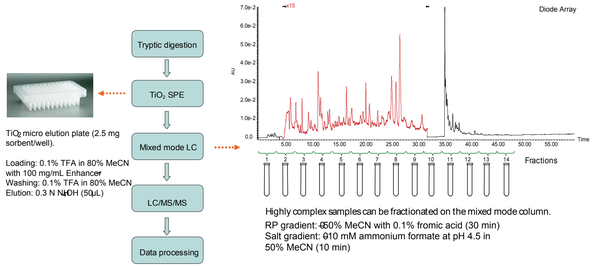
INTRODUCTION

IMAC and TiO_2 type of affinity chromatography is widely used to isolate phosphopeptides. However, some non-phosphorlated peptides (*e.g.*, acidic peptides) also bind to the affinity sorbent; the degree of non-specific binding increases with increased sample complexity. We present an improved phosphopeptide enrichment/separation method that combines TiO_2 and a prototype mixed mode (reversed-phase and strong cation exchange) liquid chromatography media prior to tandem mass spectrometry analysis.

- Phosphopeptides are weakly retained on the mixed mode LC column due to the negatively charged phosphate. A reversed-phase gradient elutes phosphopeptides according to the number of phosphate groups and their hydrophobicity.
- Non-phosphorylated peptides retain strongly on the mixed mode LC column due to ionic interaction. A rapid shallow salt gradient is used to elute these peptides.
- The isolation of phosphopeptides are further improved by processing complex samples using a TiO_2 SPE device prior to the mixed mode LC separation.

METHODS

Scheme 1. The work flow of enriching and separating phosphopeptides from a complex protein digest for LC/MS/MS analysis



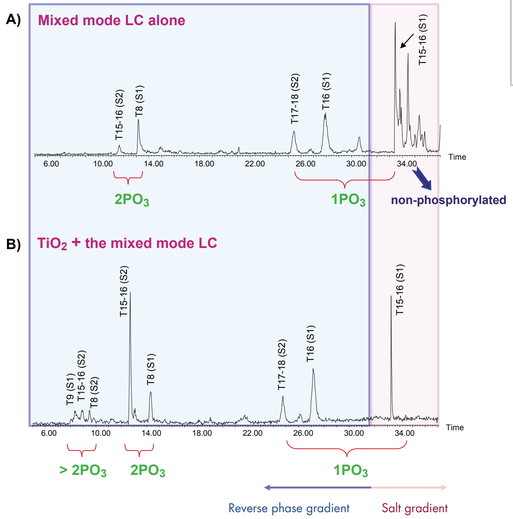
Instruments and software

Waters nanoACQUITY™ UPLC with 75 µm nanoACQUITY columns
Waters QTof API US Mass Spectrometry
Waters Alliance HPLC 2795
Waters ZQ Mass Spectrometry

MassLynx 4.0 software is used to control LC/MS experiments
Protein Global Server v. 2.2 for data processing and database search

RESULTS

Separation of phosphopeptides using the mixed mode LC



Alpha-casein S1

T16	VPQLEIVPNpSAEER
T15-16	YKVPQLEIVPNpSAEER
T8	DIGpSEpSTEDQAMEDIK
T9	QMEAEpSIpSpSpSEEIVPNpSVEQK

Alpha-casein S2

T15-16	EQLpSTpSEENSKK
T17-18	TVDMEpSTEVFTKK
T3	NTMEHVpSpSpSEESIIpSQETYK
T8	NANEEEYSIGpSpSpSEEpSAEVATEEVK

Figure 1. Alpha casein tryptic peptides were separated using A) the mixed mode LC column alone and B) the mixed mode LC column after TiO_2 SPE extraction.

- *Most phosphopeptides are separated and eluted under a reverse phase gradient (0—50% MeCN); while other peptides are retained unless a salt gradient is applied.*
- *The elution order of phosphopeptides is determined by their charge and hydrophobicity; the early eluting phosphopeptides contain more negatively charged phosphate groups than the later ones.*
- *TiO_2 SPE was used before the mixed mode LC separation to improve the detection of phosphopeptides, especially the singly phosphorylated peptides which retain more strongly on the mixed mode column.*

•Acidic peptides (pI < 4.5) are separated from phosphopeptides

Mixed mode column: acidic peptides are separated from phosphopeptides; these acidic peptides are problematic for IMAC type of affinity separation.

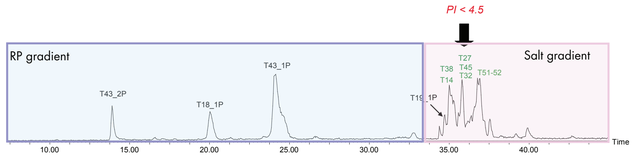


Figure 2. A yeast Enolase tryptic digest contains four synthetic phosphopeptides (see Table 1) are separated using the mixed mode LC. Three out four phosphopeptides are eluted during a reversed phase gradient while the non-phosphopeptides are eluted during a salt gradient. Acidic peptides that have pI less than 4.5 are labeled in green.

Table 1.

Phosphopeptide Description	Sequence	$[M+H]^+$	$[M+2H]^{2+}$
T18_1P	NVPLgIYK	813.39	407.20
T19_1p	HLADLpSK	863.40	432.21
T43_1p	VNQIGpTLSESK	1368.68	684.84
T43_2P	VNQIGTLpSEpSK	1448.64	724.83

•Complex Sample (Yeast YPD Cytosol Tryptic Digest)

Yeast sample preparation:
Yeast Type 1 was purchased from Sigma. The cytosolic fraction of the yeast was digested using trypsin, followed by enrichment using TiO_2 SPE and the mixed mode LC fractionation (14 fractions were collected). A total of 1.6 mg of yeast digest was used.

LC/MS/MS instrumentation:
LC: nanoLC (Waters nanoACQUITY) equipped with a 75 µm nanoACQUITY column
MS: QTOF (API US) MS, estimated injection is about 320 µg of yeast peptides.
Data Dependent Acquisition was performed on ions with (+2, +3, +4) charges.

Data Processing and search → Mascot Distiller (2.0) and Mascot database search using Yeast database (peptide score cut off at > 24)

Results
A total of 218 non-redundant phosphopeptides with 293 unique phosphorylation sites were identified using the yeast database. More than one third of the identified peptides are multiply phosphorylated (Table 2).

An example of MS/MS fragmentation of a multiply phosphorylated peptide

Mascot search results: PDR12_YEAST (Q02785); peptide mass: 943.35 (2+), score 47

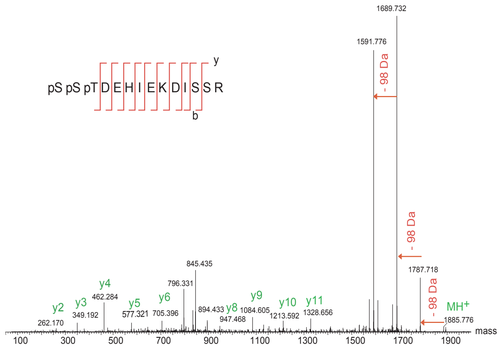


Figure 3. MS/MS fragmentation of a triply phosphorylated peptide from Fraction 5 was shown as an example. The masses were deconvoluted. All y series ions were labeled. Three consecutive loss of phosphate group (H_3PO_4, 98 Da) was observed, which clearly indicates that this peptide is triply phosphorylated. The phosphorylation sites are located at the first three amino acids.

Table 2. Number of phosphopeptides identified in each fractions were listed.

Mixed mode LC fractions	No. of phosphopeptides
F3	13
F4	29
F5	43
F6	37
F7	26
F8	17
F9	11
F10	8
F11	16
F12	63
F13	19
F14	5
Total	287
Overall unique	218
Multiply phosphorylated peptides (%)	31%

RP gradient

Salt gradient

CONCLUSION

- Affinity chromatography (IMAC or TiO_2) alone is not sufficient for isolating phosphopeptides from highly complex samples. A combination of TiO_2 SPE and the mixed mode LC separation/fractionation improves the overall phosphopeptide selectivity and reduces the sample complexity for LC/MS/MS analysis and data processing.
- The advantage of using the mixed LC column is to further separate phosphopeptides and non-phosphorylated peptides (mostly acidic peptides) *via* a combination of ionic and reversed-phase interactions. Also phosphopeptides with similar charge properties are further separated by their hydrophobicity.
- Yeast cytosolic fraction was digest and processed using TiO_2/mixed mode LC combination. A total of 218 non-redundant phosphopeptides with 459 unique phosphorylation sites were identified using Mascot.

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