PHOSPHOPEPTIDE ENRICHMENT AND ANALYSIS USING A COMBINATION OF METAL OXIDE AFFINITY PURIFICATION AND LC-MALDI MS

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INTRODUCTION

- **Enrichment strategy for phosphopeptide using a novel metal oxide sorbent.**
- **Samples of differing complexity are tested to show the improvement of detection of phosphopeptides.**
- **After enrichment, phosphopeptide solutions were cleaned-up using microcolumns or fast nanoscale Ultra Performance Liquid Chromatography.**
- **All MS experiments are carried out using a MALDI Q-Tof mass spectrometer.**

Phosphorylation is an important regulator of cell function in eukaryotes. This post translation modification can alter protein localization, regulate protein function and stabilize and mediate their interactions. Due to their associated negative charge, phosphopeptides are often poorly ionized compared to their non-phosphorylated counterpart and their analysis is often complicated due to their low cellular abundance. Therefore, it is critical to selectively enrich the phosphopeptides prior to MS analysis. A widely used[1] technique, IMAC (Immobilized Metal-Ion Affinity Chromatography) purifies phosphorylated peptides by their affinity to metal ions like Fe^{3+} or Ga^{3+}. However, non-phosphorylated peptides containing multiple acidic residues tend to also bind to the metal ion and therefore are enriched as well in the process. Recently a new strategy using titanium dioxide (TiO_2)[2] followed by reversed-phased desalting step has been reported to overcome this issue and is more specific for phosphopeptides.

In this study we have evaluated a new way of enriching phosphopeptides by using a metal oxide based solid phase extraction (SPE) micro scale device. This sorbent has a high affinity for phosphopeptides and the problem of acidic adsorption is greatly minimized. Furthermore, recent developments in MALDI and LC-MALDI spotting devices, allow the coupling of the off-line chromatographic separation step to the subsequent MS analysis. This approach has been compared to a homemade microcolumn desalting approach on protein digest mixtures. We have also used a high pressure nanoUPLC sample preparation strategy (as described in Poster P115A-T) which allows us to use elevated flow rates combined with nanoscale columns and to reduce considerably the elution time without compromising the separation. The UPLC separated samples were analysed by MALDI using an orthogonal acceleration time-of-flight mass spectrometer equipped with a 200Hz laser.

METHODS

Samples

Experiments were conducted on:
- ß-casein (Sigma Aldrich, St Louis, MO, USA) digested using trypsin (Promega, Madison, MI, USA) was mixed 1:1 with a mixture of four protein digests (ADH, BSA, Enolase and Phosphorylase B) (Waters, Milford, MA, USA) at 500 fmol/µL.
- A 6 protein mixture containing tryptic digests of serum albumin, carbonic anhydrase, β-lactoglobulin, β-casein *(phosphorylated protein)*, α-casein *(phosphorylated protein)* and ovalbumin *(phosphorylated protein)* at 500 fmol/µL.
- A 12 protein mixture containing tryptic digests of serum albumin, carbonic anhydrase, ß-lactoglobulin, RnaseB, Alcohol dehydrogenase, Myoglobin, Transfferin, Lysozyme, α-amylase, ß-casein *(phosphorylated protein)*, α-casein *(phosphorylated protein)* and ovalbumin *(phosphorylated protein)* at 500 fmol/µL.

Affinity SPE µElution plate protocol

Vacuum Manifold Settings: 2 -10 in. Hg

Condition the well	Condition the well with 200 µl of Milli-Q water first, then 200 µl of MeOH.
Prepare the sample	Solubilize the sample in 0.5% TFA in 80% MeCN solution, the final volume is between 200 to 400 µl. - For the 6 and 12 proteins mixture, load the sample in 200 mg/mL of Enhancer 0.5% TFA in 80% MeCN solution
Sample loading	Load the sample into a well, and let gravity pull the sample through the well (no need to turn on the vacuum for this step), collect the breakthrough solution using a collection plate. It takes about 15 to 25 minutes.
Washing the well	Wash the well with: -200 µl Enhancer solution (if used when loading) - 200 µl 0.5% TFA in 80% MeCN (repeat this step if necessary) -200 µl Milli-Q water.
Elution	Elute with 3 x 50 µl of 100 mM diammonium phosphate (pH ~ 8)
Neutralization	Neutralize the eluent with 2 µl of TFA.

Acidified eluents were cleaned-up using either homemade reverse-phase microcolumns[3] or reverse-phase nanoscale fast UPLC, followed by spotting onto MALDI targets using a CTC-PAL MALDI spotter (CTC analytics, CH).

Nanoscale UPLC Conditions

Nanoscale UPLC separations were performed on a 1.7 µm BEH 75µm x 50mm BEH column using a nanoACQUITY™ UPLC™ system (Waters Corporation). The column was maintained at 60ºC and a flow of 1.4 µL/min produced a typical back pressure of 5900 psi. A gradient over 2 minutes from 10% to 60% B was used where the mobile phase was A: 0.1% formic acid and B: MeCN. The total cycle time, injection to injection, was 8 minutes.
Matrix: 2.5-Dihydroxybenzoic acid (DHB) (Fluka, Switzerland) 20mg/mL (1% phosphoric acid in pure Ethanol) added using auxiliary pump at 2.5 µL/min mixed with eluent prior to spotting in a Y-piece. The spotting time was 10 seconds per spot with a solvent delay of 1.5 minutes.

MS Conditions

The Q-Tof Premier mass spectrometer was operated with a MALDI source. External calibration was performed using a PEG standard and data were lock mass corrected post-acquisition using Glu-fibrinopeptide B, to ensure high mass accuracy.

RESULTS

1) *Single* Phosphoprotein in a five protein mixture

The first experiment was carried out on one phosphoprotein containing 5 phosphoserines, added to a mixture of four digested proteins. This solution was spotted at 125 fmol on target, mixed with the matrix, DHB. Potentially there are 361 peptides present in the solution (1 missed cleavage, between 800 and 3500 Da). As seen in figure 1(a), the spectrum contained a great number of peaks, specially below 2000 Da. 10 µL of this mixture were loaded onto the µ-elution plate. After elution with 150 µL of 100 mM diammonium phosphate solution, the entire eluent was loaded onto a homemade reversed-phase microcolumn using a GELoader tip. After a washing step using 10 µL of 0.1% TFA, the retained peptides were eluted using 1 µL of matrix solution directly onto the MALDI target. The spectrum obtained can be seen in figure 1 (b). Clearly there is a noticeable simplification of the spectrum

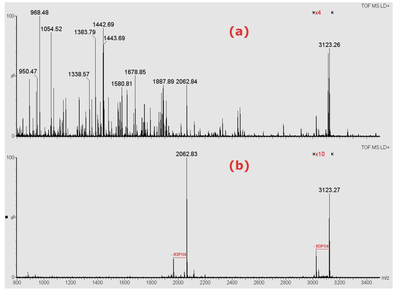

*Figure 1. (a) Spectrum of **Mix 1 + ß –Casein** digest mixture spotted directly onto MALDI target. (b) Spectrum of the same solution after µ-elution phosphopeptide enrichement.*

and the two major peaks are phosphopeptides from ß-Casein: the mono-phosphopeptide at m/z 2061.83 and the tetra-phosphopeptide at m/z 3122.27. Also observed in the spectrum is the loss of H_3PO_4.

2) *Three* Phosphoproteins in a six protein mixture

The second experiment was carried out on a six protein mixture which contains three phosphoproteins: α-casein (S1 and S2), β-casein and ovalbumin. These three phosphoproteins were mixed with unphosphorylated proteins (serum albumin, carbonic anhydrase, β-lactoglobulin) to give a mixture with a large number of unphosphorylated peptides.

Theoretically there are 30 phosphopeptides when digested with trypsin, allowing one missed clevage, between 800-3500 Da.

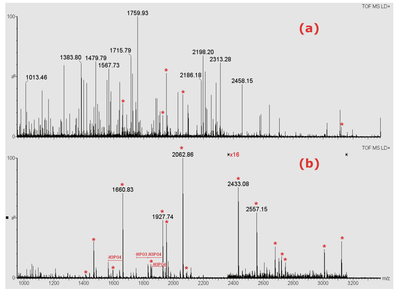

*Figure 2. (a) Spectrum of **six protein** digest mixture spotted directly onto MALDI target. (b) Spectrum of the same solution after µ-elution phosphopeptide enrichement.*

Nine more peptides have been identified by M.R.Larsen *et al*[2]. Figure 2 shows the spectra before (a) and after (b) phosphopeptide enrichment using the µ-elution plate. Before enrichment, five phosphopeptides can be identified in a densly populated mass spectrum. After enrichment, a simplied spectrum can be observed. 16 phosphopeptides were identified and these are the most intense peaks of the spectrum.
Some smaller peaks are observed, especially between 1000-1300 Da; these have not yet been identfied.

3) *Three* Phosphoproteins in a twelve protein mixture

The third experiment was carried out on a twelve protein mixture which contains the same three phosphoproteins as described previously. The difference here is that nine unphosphorylated protein digests were added to the mixture (serum albumin, carbonic anhydrase, β-lactoglobulin, ADH, Myoglobin,

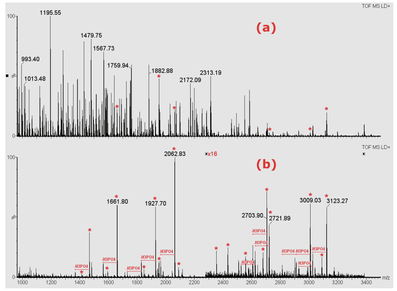

*Figure 3. (a) Spectrum of **twelve protein** digest mixture spotted directly onto MALDI target. (b) Spectrum of the same solution after µ-elution phosphopeptide enrichement.*

Transferin, Lysozyme and α–amylase). Figure 3 shows the spectra before (a) and after (b) phosphopeptide enrichment using the µ-elution plate. In theory, the spectrum obtained after clean-up of this sample shoud be similar to the spectrum in figure 2(b) as the same three phosphoproteins are present in both samples.
Figure 3 shows the spectra before (a) and after (b) phosphopeptide enrichment using the µ-elution plate.
Six phosphopeptides were observed before enrichment with the metal oxide solid phase sorbent. Like figure 2(a), this spectrum contained a great number of peaks, especially below 2300 Da.
Once more, a clear enrichement can be observed in figure 3 (b). After enrichment, 18 phosphopeptides were observed, two more than in the six protein experiment. 2432.05 Da is a β-casein phosphopeptide. 2703.9 Da has not been identified but has been analysed by MS/MS and a clear loss of 98 Da (H_3PO_4) was observed.

4) Fast LC-MALDI-MS analysis of the twelve protein digest mixture

The eluent from the phosphopeptide enrichment of the twelve protein mixture was dried down and reconstituted in 10 µL 0.1%TFA. 2 µL was injected onto the column. The

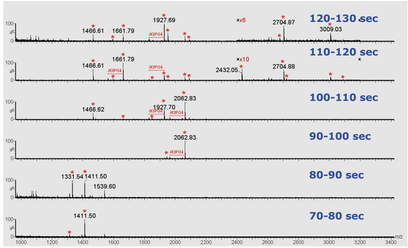

Figure 4. MS spectra of fast nanoUPLC separation of 12 protein mixture after phosphopeptide enrichment. Fractions shown between 70 and 130 seconds.

phosphopeptides eluted after 70 to 130 seconds as shown in figure 4.
Table 1 shows a summary of the phosphopeptides observed from the six protein mixture, cleaned-up by microcolumn and the twelve protein mixture, cleaned-up by microcolumn and by fast nanoUPLC.
Several differences could be observed depending on whether phosphopeptides are desalted using a microcolumn or are chromatically separated. Indeed the 1331.53 Da and 1539.6 Da peaks were only observed by nanoUPLC whereas 2352.85 Da, 2556.11 Da, 2678.01 Da and 3122.27 Da peaks are only observed after desalting by microcolumn.
The identification of new phosphopeptides after LC separation could be due to the reduction of the complexity of the mixture, reducing any ion suppression effects.

The absence of some of the phosphopeptides in the LC MALDI experiments could be due to their high affinity for the chromatographic column material or with metal surfaces within the UPLC system.

Protein	Mass	Position	Modifications	Peptide sequence	6 protein mixture	12 protein mixture	
					µ-column	µ-column	Fast NanoUPLC
α-casein 2	1331.53	141-151	PHOS:144 or PHOS: 146	EQLSTSEENSK			•
α-casein 2	1411.5	141-151	PHOS: 144, 146	EQLSTSEENSK	•	•	•
α-casein 2	1466.61	153-164	PHOS: 158	TVDMESTEVFTK	•	•	•
α-casein 2	1539.6	141-152	PHOS: 144, 146	EQLSTSEENSKK			•
α-casein 2	1594.71	152-164	PHOS: 158	KTVDMESTEVFTK	•	•	•
α-casein 1	1660.79	121-134	PHOS: 130	VPQLEIVPNSAEER	•	•	•
α-casein 1	1847.69	58-73	PHOS:61 or PHOS: 63	DIGSESTEDQTMEDIK	•	•	•
α-casein 1	1927.69	58-73	PHOS:61, 63	DIGSESTEDQTMEDIK	•	•	•
α-casein 1	1951.98	119-134	PHOS: 130	WKVPQLEIVPNSAEER	•	•	•
β-casein	2061.83	48-63	PHOS: 50	FQSEEQQQTEDELQDK	•	•	•
Ovalbumin	2088.91	341-360	PHOS: 345	EVVGSAEAGVDAASVSEEFR	•	•	•
β-casein	2352.85	7-25	PHOS: 15, 17, 18, 19	NVPGEIVESLSSSEESITR		•	
β-casein	2432.05	45-63	PHOS: 50	IEKFQSEEQQTEDELQDK	•	•	•
	2556.11		mono phosphopeptide		•	•	
α-casein 1	2678.01	52-73	PHOS: 56, 61,63		•	•	
	2703.9		tetra phosphopeptide			•	•
α-casein 1	2735.91	74-94	PHOS: 79, 81,82,83,90	GMEAESISSSEEIVPNSVEQ K	•	•	•
α-casein 2	2747	16-36	PHOS: 23, 24,25,31	KNTMEHVSSSEESIISQETY K	•		
α-casein 2	3008.03	61-85	PHOS: 71, 72,73,76	NANEEEYSIGSSSEESAEVA TEEVK	•	•	•
α-casein 2	3087.99	61-85	PHOS: 68, 71, 72,73,76	NANEEEYSIGSSSEESAEVA TEEVK		•	•
β-casein	3122.27	16-40	PHOS: 30, 32,33,34	RELEELNVPGEIVESLSSSE ESITR	•	•	

Table 1. Summary of phosphopeptides observed after six and twelve protein mixtures were desalted and after the twelve protein mixture was separated by fast nanoUPLC.

CONCLUSION

- **The use of an efficient new device for phosphopeptide enrichment from complex peptide mixtures is demonstrated.**
- **Enrichment of phosphopeptides prior to MALDI analysis is greatly improved.**
- **Enriched phosphopeptide fractions were chromatographically separated using rapid UPLC technique in under ten minutes.**
- **Differences were observed between homemade microcolumn and chromatographic sample clean-up methods.**

ACKNOWLEDGEMENTS

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