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OPTIMIZED MALDI MS SAMPLE PREPARATION AS A STRATEGY TO ENHANCE DETECTION OF PHOSPHOPEPTIDES

Grace M. Credo, Weibin Chen, John C. Gebler
Waters Corporation, Milford, MA 01757 USA

OVERVIEW

- An enolase digest solution spiked with phosphopeptides (from bovine β-casein or synthetic peptides based on tryptic yeast enolase) was used to test sample preparation conditions.
- Evaluated parameters included matrices, additives, sample deposition, ionization mode, contaminants, and sample enrichment.

INTRODUCTION

Difficulty detecting modified peptides inhibits efforts to accurately characterize protein structure and function. Phosphorylated peptides are often present at relatively low abundance and are also difficult to detect by mass spectrometry (MS) due to low ionization efficiency. In previously reported work from our group [1], published methods recommending procedures for optimized MALDI MS detection of β-casein phosphopeptides were evaluated on a custom mixture consisting of enolase digest spiked with relatively high concentrations of enolase phosphopeptides. For a relatively clean sample, DHB matrix was preferred for MALDI MS and THAP was preferred for MALDI MS/MS. In this work, we evaluated an expanded set of sample preparation conditions while reducing the relative concentrations of phosphopeptides in the samples.

METHODS

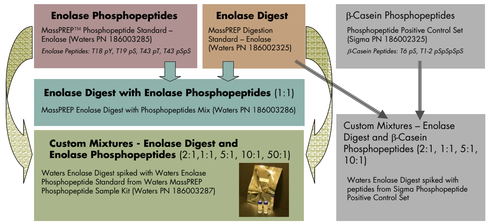
MALDI-TOF MS

Instrument	Waters Micromass™ MALDI micro MX™
Mode	Reflectron, positive or negative
Mass range	Between m/z 700 and 4000

LC-MS and LC-UV

Instrument	Waters Alliance® 2795 HPLC system
Mobile phase A	0.02% trifluoroacetic acid in water
Mobile phase B	0.016% trifluoroacetic acid in ACN
Gradient	0-56% B in 60 minutes
Column	BioSuite™ C_{18} 3μm, PA-A, 2.1mm x 150mm
Detectors	Waters ZQ™ 4000 with sample cone = 30 V; Waters 996 Photodiode Array with micro UV cell

SAMPLES USED

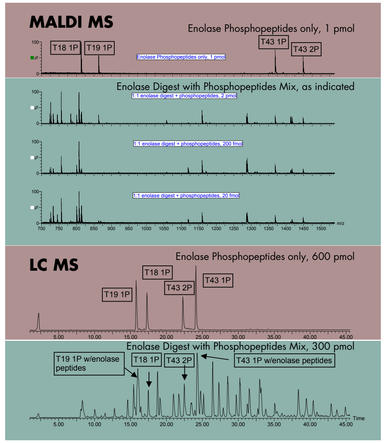


DEPOSITION AND ANALYSIS

- Used purified/recrystallized matrices; such as Waters matrices
- Deposition of aqueous sample (typical), in the following order:
  1) 0.7 μL sample
  2) 0.5 μL matrix solution (10-20 mg/mL, ~80% organic)
  3) 0.4 μL aqueous solution with additive
- Deposition of organic sample (e.g., ACN-based wash solutions):
  1) 0.7 μL sample
  2) 0.4 μL aqueous solution with additive
  3) 0.5 μL matrix solution (10-20 mg/mL, ~80% organic)
- Data Acquisition
  - Confirmed that deposition covered well area or redeposited
  - Automatic data acquisition with low and high thresholds
  - 200 shots/well (20 spectra per well, 10 shots each)

RESULTS

SAMPLE CHARACTERIZATION

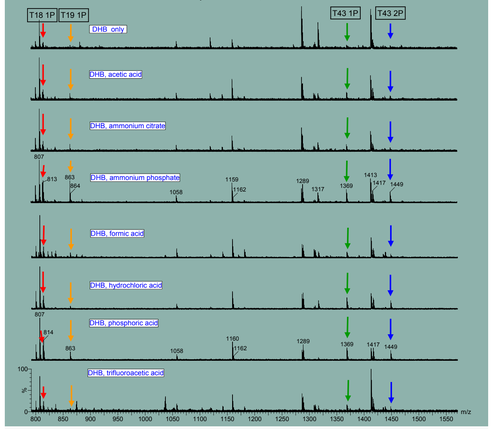


PHOSPHOPEPTIDES DETECTED

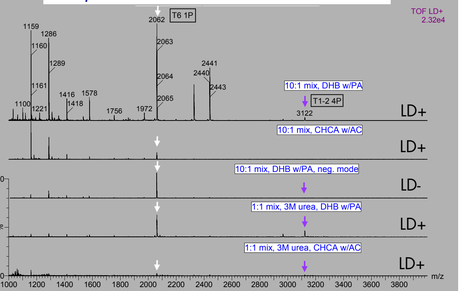
Phosphopeptide Description	Sequence	Amino Acid Residue	$[MH]^+$	pI	B&B index
Enolase T18 1P	NVPL[pY]K	126-131	813.3912	9.35	-1220
Enolase T19 1P	HLADL[pS]K	132-138	863.4028	7.35	-930
Enolase T43 1P	VNQIG[pT]LSESIK	346-357	1368.6776	6.41	-820
Enolase T43 2P	VNQIGT[pS]E[pS]pIK	346-357	1448.6439	6.41	-1370
β-casein T6 1P	FQ[pS]EEQQQTEDELQDK	33-48	2061.8212	3.71	6200
β-casein T1-2	RELEELNVPGEIVE[pS][pS][pS][pS][pS]EESITR	1-25	3122.2584	4.13	-120

MATRIX ADDITIVES

- Sample was 0.7 pmol of enolase digest and phosphopeptide mix (1:1)
- For DHB, ammonium phosphate and phosphoric acid (PA) were the best additives; usually used DHB 20 mg/mL in aqueous ACN with 0.4% PA
- Even with additives, THAP and CHCA were not as consistent as DHB in facilitating detection of all four enolase phosphopeptides; T19 1P was especially difficult and there is an enolase digest peptide at *m/z* 814 which is close to the T18 1P peak at *m/z* 813.



β-CASEIN PHOSPHOPEPTIDES

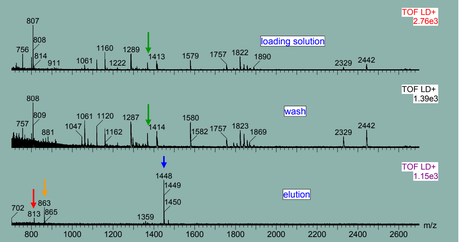


- Samples were 10:1 or 1:1 enolase digest to phosphopeptides
- T6 1P peptide very easy to detect; T1-2 4P peptide more similar to enolase phosphopeptides in response to different matrices, additives, contaminants
- Negative mode can be used to preferentially detect phosphopeptides but peak intensity is lower for all peptides (same intensity scale for all spectra)

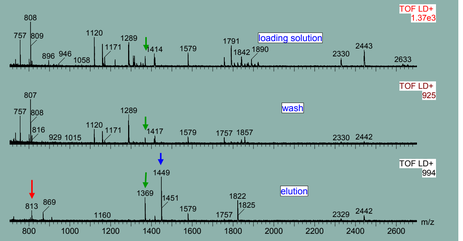
AFFINITY ENRICHMENT: Fe, Zr or TiO_2

- Loaded between 2 and 20 pmol of sample (1:1 mix) on bulk resin or particles; modified procedures [2,4] - did not use microcolumns
- TiO_2 retained all four phosphopeptides and was most selective, exhibiting the least retention of unmodified peptides; however, observed reduced T18 1P and T19 1P after reverse-phase sample clean-up recommended in [4]

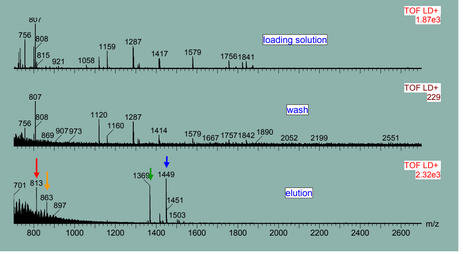
Method 1—Fe-containing resin [2]



Method 2—Zr-containing magnetic particles [3]

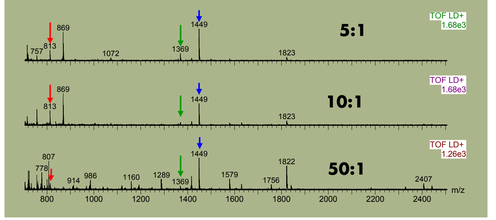


Method 3—Titanium dioxide particles, DHB loading [4]



ENRICHMENT from 2-20% MIXTURES

- Sample load on Zr-containing particles [3] was 4 pmol of phosphopeptide for each sample; enolase digest load adjusted accordingly
- 3 of 4 phosphopeptides detected in each sample (same as 1:1 mix)



ENOLASE PHOSPHOPEPTIDE STANDARDS

- 4 synthetic phosphopeptides based on modified tryptic enolase peptides
- 3 peptides phosphorylated at either serine, threonine or tyrosine; a fourth phosphopeptide is doubly phosphorylated at serine
- More diverse sample than two serine phosphopeptides from commonly used β-casein digest
- Reduced sample preparation times compared to bulk peptide purification and digestion of phosphorylated yeast enolase
- Lyophilized and conveniently packaged for long-term stability and storage

CONCLUSIONS

- New phosphopeptide standards facilitated method development for phosphopeptide analysis
- Synthetic enolase phosphopeptides in conjunction with more well-known β-casein phosphopeptides were used to verify and optimize recommended sample preparation methods in literature
- DHB with ammonium phosphate or phosphoric acid were the best MALDI matrix/additive combinations for detection of six different phosphopeptides with and without urea contamination
- Commercially-available enrichment products were not ideal for detection of four enolase phosphopeptides, while TiO_2–based enrichment was effective
- Sample enrichment is a useful strategy for detection in mixtures with less than 50% phosphopeptides (mol %)

REFERENCES

1. Credo, Chen, Gebler, ABRF 2005 Meeting (Savannah, GA), Poster. Available from Waters website - http://www2.waters.com/watprod.nsf/Newdocs/720001116EN
2. Used modified procedure from Morrice, PHOS-Select™ Iron Affinity Gel applications note, http://www.sigma-origins.co.uk/pdfs/articles/11072721S1.pdf
3. Used procedure from User Manual for EMD Biosciences ProteoExtract™ Phosphopeptide Capture Kit.
4. Used modified procedure from Larsen, Thinghølm, Jensen, Roepstorff, Jorgensen, *Molec. Cell. Proteom.* 2005, in press manuscript T500007-MCP200; used 20 μm particles from Sachtleben Chemie GmbH (Germany).

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