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Optimizing Phosphopeptide Sample Preparations for MALDI MS Analysis

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Overview

- An enolase digest spiked with four synthetic phosphopeptides was used to test sample preparation conditions recommended in the literature for MALDI TOF MS of phosphopeptides.
- MALDI sample preparation conditions were evaluated using the matrices CHCA, DHB, and THAP and solution additives or buffers ammonium citrate, phosphoric acid and hydrochloric acid.

Introduction

Phosphopeptides in peptide mixtures are often detected with low efficiency by MALDI mass spectrometry. A variety of sample clean-up and preparation methods can be used to improve these results. In this work the focus is on sample preparation, since the solution mixture applied is relatively clean. The sample mixture consisted of purified Enolase tryptic digest spiked with four synthetic phosphopeptides containing either one pY, pS, pY or two pS. In an effort to improve phosphopeptide response in MALDI MS, we systematically evaluated the effects of published sample preparation protocols [1-5] on MALDI MS and MALDI MS/MS results. These included different buffers, matrices, and additives. Results from this work were used to provide guidelines for method development, diagnostic tests and preliminary instrument optimization using a phosphopeptide standard. In this study, the effect of different sample preparation workflows on the detection of individual phosphopeptides and mixtures using MALDI MS are presented.

Experimental

Materials

Four synthetic Enolase phosphopeptides (**Table 1**) were obtained from GenScript Corp. (Piscataway, NJ) and were purified using a Waters Alliance® HPLC 2695 system. Waters MassPREP™ Protein Digestion Standard – Enolase (~ 1pmol/µL) was mixed with the purified phosphopeptides in various ratios to make custom mixtures. The optimized mixture (**Fig. 1**) for LC was different from the one used for MALDI. Since the LC mixture has a very strong, intact parent ion at *m/z* 1448 when analyzed by MALDI, the optimized MALDI mixture has a greatly reduced T43pp concentration. In **Figure 1**, the relative intensities of the

Synthetic Phosphopeptides

Phosphopeptide	Sequence	$[MH]^+$	$[M+2H]^{2+}$
T18p	NVPLpYK	813.3912	407.1995
T19p	HLADLpSK	863.4028	432.2053
T43p	VNQIGpTLSESIK	1368.6776	684.8428
T43pp	VNQIGTLpSEpSIK	1448.6439	724.8259

Table 1. Expected *m/z* values for enolase phosphopeptides detected by MALDI MS, $[MH]^+$, and LC MS, $[M+2H]^{++}$.

Waters MassPREP MALDI Matrices (CHCA, DHB, THAP) were used for MALDI sample preparation. Waters MassPREP Calibration Mix—MALDI Reflectron was used to calibrate the instrument in positive ion mode. Waters MassPREP Protein Digestion Standard—ADH was used to calibrate the instrument in negative ion mode.

Sample Preparation

For MALDI analysis, stock solutions (10 pmol/µL in 0.1% TFA) of the individual phosphopeptides were prepared. An enolase phosphopeptide mixture was prepared using the following sample concentrations: 1pmol/µL (T18p), 4 pmol/µL (T19p), 0.5 pmol/µL (T43p), 3 pmol/µL (T43pp) and 0.2 pmol/µL (enolase digest). 1 µL of this mixture was deposited on a stainless steel MALDI plate. The sample was mixed on-plate with a matrix solution (see Table 1). Some of the solutions contained additives ammonium citrate (AC), phosphoric acid (PA) or hydrochloric acid (HCl), where indicated.

For LC analysis, a four phosphopeptide mixture was prepared in water using the following concentrations: 4 pmol/µL (T18p), 4 pmol/µL (T19p), 2 pmol/µL (T43p), and 40 pmol/µL (T43pp). 1 mL of the mixture solution was transferred into a vial containing 1 nmol of enolase protein digestion standard, producing a solution with 1 pmol/µL of non-phosphorylated peptides. For LC chromatograms, 0.5 µL of the spiked solution was injected.

MALDI TOF MS

Instrument	Waters Micromass® MALDI micro MX™
Mode	Reflectron, positive or negative
Mass range	Between *m/z* 700 and 2000

MALDI QTOF MS/MS

Instrument	Waters Micromass® MALDI Q-Tof Ultima™
Mode	Positive
Mass range	Between *m/z* 150 and 2000

LC MS

Instrument	Waters nanoACQUITY UPLC™ system
Mobile phase A	0.1% formic acid in water
Mobile phase B	0.1% formic acid in acetonitrile
Gradient	0-56% B in 70 minutes
Column	75µm x 100mm nanoACQUITY Atlantis™ dC18, 3µm
LCT parameters	Micromass nanospray source, Source voltage = +2000V, Sample cone = 35 V, MCP = +2700V

Results

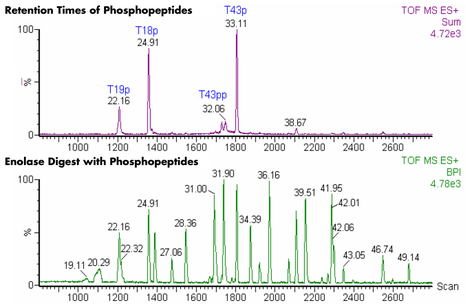

Figure 1. Retention times of phosphopeptides mixed with unmodified enolase peptides. LC chromatogram of phosphorylated enolase peptide mixture with 2 pmol T18p, 2 pmol T19p, 1 pmol T43p, and 20 pmol T43pp and 0.5 pmol enolase peptides.

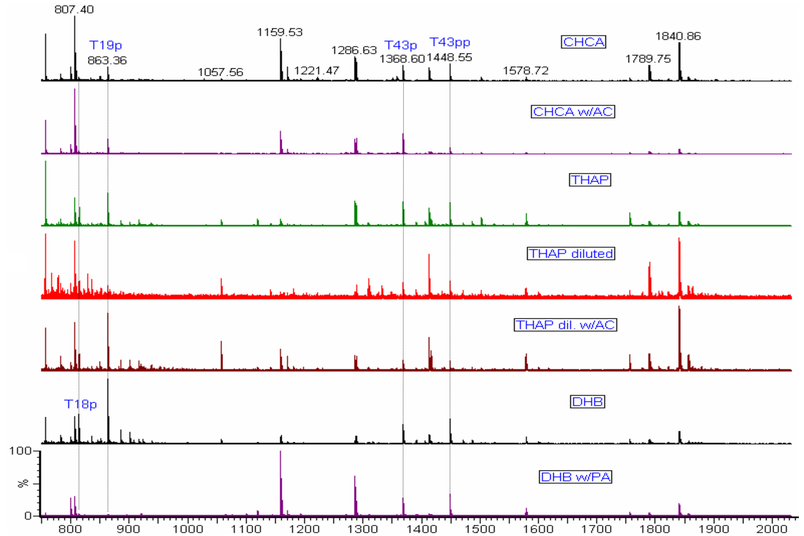

Figure 2. Comparison of phosphopeptide signal intensity as a function of matrices and additives (see **Table 2**). Positive mode MALDI TOF MS spectra of phosphorylated enolase peptide mixture with 1 pmol T18p, 4 pmol T19p, 0.5 pmol T43p, and 3 pmol T43pp and 0.2 pmol enolase peptides. Negative mode results exhibited lower sensitivity for most peptides.

Matrix	Concent.	Solution	Additive	T18p (pS)	T19p (pY)	T43p (pT)	T43pp (2 pS)	Comment
				(S/N of Parent Peak, +ve mode)				
CHCA	5 mg/mL	1:1 ACN:EtOH	n/a	2	12	25	40	Very strong T43p and T43pp detection; strong T19p; T43pp S/N better without AC in CHCA
CHCA	5 mg/mL	1:1 ACN:EtOH	5mM AC	2	18	50	20	Very strong T43p and T43pp detection; strong T19p; solution with AC improves intensity of T19p and T43p peaks in CHCA
THAP	10 mg/mL	1:1 ACN:H_2O	n/a	7	30	27	33	Very strong T19p, T43p, T43pp detection; higher concentration (10 vs. 1mg/mL) of THAP shows improved T43p, T43pp detection
THAP	1 mg/mL	1:1 ACN:H_2O	n/a	2	3	2	4	Not recommended
THAP	1 mg/mL	1:1 ACN:H_2O	20mM AC	7	30	6	6	Very strong T19p detection; greatly enhanced T19p detection w/AC in THAP; slight enhancement for other peptides
DHB	10 mg/mL	3:7 ACN:H_2O (0.1% TFA)	n/a	24	45	22	22	Very strong signal for all four phosphopeptides in mixture
DHB	10 mg/mL	3:7 ACN:H_2O (0.1% TFA)	1% PA	3	1	20	25	Very strong T43p, T43pp detection; compared with no additive, solution with PA suppresses T18p, T19p detection
CHCA	10 mg/mL	1:1 ACN:EtOH	1% PA					Poor signal in initial test mixtures
THAP	10 mg/mL	1:1 ACN:H_2O	1% PA					Poor signal in initial test mixtures
DHB	10 mg/mL	3:7 ACN:H_2O (0.1% TFA)	20mM AC					Signal in initial test mixtures suppressed compared to DHB alone and DHB with PA

Table 2. Comparison of signal to noise of $[MH]^+$ phosphopeptide peaks from **Figure 2**, comments on performance and additional information on sample preparations not shown due to relatively poor signal intensity. Matrix depositions using hydrochloric acid (1M) as an additive were also evaluated, but they did not enhance phosphopeptide peak intensity.

Matrix	Concent.	Solution	T18p (pS)	T19p (pY)	T43p (pT)	T43pp (2 pS)	Comment
			Primary Peaks Detected [m/z]				
CHCA	10 mg/mL	1:1 ACN:EtOH	600 813	765 863	1271 1369	289, 379, 335, 767, 865, 1271, 1449 (weak)	Relatively "hot" matrix that promotes more fragmentation than other two matrices; most pronounced with larger peptides T43p and T43pp, where parent ion is not primary peak detected. Adding AC to the CHCA sample does improve this slightly, with better parent ion detection, but fragments are still strong.
THAP	10 mg/mL	1:1 ACN:H_2O	600 813	765 863	1271 1369	386 865 1449	Similar to DHB results, but superior signal for T43pp parent ion.
DHB	10 mg/mL	3:7 ACN:H_2O (0.1% TFA)	600 813	765 863	1271 1369	273 865 1449 (weak)	Similar to THAP results, except for T43pp, where main peak in spectrum is 865.

Table 3. Phosphopeptide peaks detected on an Q-Tof before CID (MS only) using different matrices. Each phosphopeptide was prepared individually to isolate the effect of each matrix. Here, THAP is the best option for high intensity peaks for all four phosphopeptides. There were no significant differences in the spectra for T18p, T19p, and T43p with THAP or DHB with or without AC.

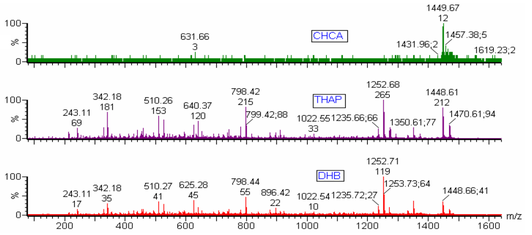

Figure 3. Fragmentation (MS/MS) of the phosphopeptide T43pp parent ion on a Q-Tof using different matrices. Peaks are labeled with m/z values and peak intensities. The precursor peak intensity using CHCA is too low.

Conclusions

- An enolase digest spiked with four synthetic enolase phosphopeptides was used to simulate a real sample.
- The best matrix in this evaluation for MALDI MS detection of the mixture is DHB with no additives (ammonium citrate, phosphoric acid, hydrochloric acid).
- Results show that other preparations, including other matrices, additives, and detection modes, can be used for preferential detection of the individual phosphopeptides.
- The best matrix for MALDI MS/MS fragmentation of the four phosphopeptides is THAP.

References

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