

# Targeted Hypothesis-Driven Mass Spectrometry: A Synthesis of Methods for the Detection and Identification of Phosphopeptides

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## OVERVIEW

### Purpose:

Development of a simple and rapid method for detecting and sequencing phosphopeptides in proteolytic digests.

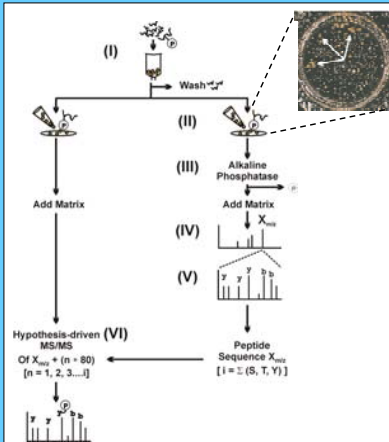
### Approach:

1. Perform on-target phosphatase reactions with proteolytic peptides affinity-bound to IMAC beads.
2. Identify the de-phosphorylated peptides by direct MALDI MS/MS on the phosphatase-treated IMAC beads.
3. Based on the identified sequences, generate a hypothetical mass list containing the potential phosphorylation states of each peptide.
4. Identify sites of phosphorylation by MALDI MS/MS on IMAC beads that HAVE NOT been treated with phosphatase.

### Results Summary:

THD-MS was used successfully to identify 4 novel *in-vivo* phosphorylation sites that could not be detected by numerous alternative approaches.

## METHODS



**Phosphoproteins:**  $\beta$ -casein (Sigma) was dissolved to 10 $\mu$ M in 25mM ammonium bicarbonate (ABC) and digested with 1 $\mu$ g sequencing-grade trypsin (Promega) at 37°C overnight. 1 $\mu$ L (10 pmoles) of the digest was used for IMAC analysis.  
*Drosophila melanogaster* stem loop-binding protein (dSLBP) was expressed in and purified from baculovirus, followed by C4 Zip-Tip purification (Millipore) and subsequent digestion with endopeptidase Lys-C (Wako) in 50mM ABC at 37°C overnight. Peptide digests were desalted by C18 Zip-Tip purification and eluted in 50% MeCN / 0.1% TFA. Approximately 3g of protein was used for IMAC analysis.

**IMAC:** Metal-free NTA beaded agarose (Sigma) was manually charged with Fe(III) in a compact reaction column (USB). Fe(III)-charged beads were incubated overnight with the specified sample (equilibrated to 50mM acetic acid) at RT and 600 rpm shaking. Reacted beads were washed with 30 $\mu$ L 0.1% acetic acid, 30 $\mu$ L 30% MeCN / 0.1% acetic acid, and 30 $\mu$ L 0.1% acetic acid. Between 1% to 10% (10 to 100 beads) of reacted IMAC beads were dispensed per spot for THD-MS analysis.

**On-Target De-Phosphorylation:** On-target de-phosphorylation was accomplished by spotting 0.25 $\mu$ L sample immediately followed by 0.25 $\mu$ L calf intestinal phosphatase (NEB) diluted in 100mM ammonium bicarbonate. The phosphatase reaction was allowed to progress for 5 minutes at RT, after which 0.5 $\mu$ L saturated CHCA in 50% MeCN / 0.1% TFA (or 0.5% H<sub>3</sub>PO<sub>4</sub>) was added and allowed to crystallize at RT.

**Mass Spectrometry:** All mass spectra were generated on an ABI-4700 MALDI-TOF/TOF mass spectrometer (Applied Biosystems).

## INTRODUCTION

Phosphopeptides can be difficult to detect and sequence by mass spectrometry (MS) due to low ionization efficiency and suppression effects in the MS mode, and insufficient fragmentation in the tandem MS (MS/MS) mode, respectively. To address this problem, we have developed a technique called Targeted Hypothesis-Driven MS (THD-MS), which combines on-target phosphatase reactions, direct MALDI-MS/MS on IMAC beads, and hypothesis-driven MS (HD-MS).

In this method, on-target de-phosphorylation experiments are conducted on IMAC-bound phosphopeptides, because dephosphorylated peptides have, in general, higher MS sensitivities than the corresponding phosphopeptides. The detected dephosphorylated peptides are sequenced by MS/MS, which identifies the protein and gives the total number of potential phosphorylation sites within each peptide. Based on this information, a mass list containing every possible phosphorylation state of each peptide (where 1 HPO<sub>3</sub> = 80 Da) is used to direct MALDI-MS/MS on the phosphorylated IMAC beads at each theoretical mass from the list. If the peptide is present, the resulting MS/MS spectrum reveals the exact site(s) of phosphorylation in the peptide.

We have demonstrated the applicability of THD-MS to the detection of *in vivo* phosphorylation sites on the *Drosophila* Stem Loop Binding Protein (dSLBP), and the complementarity of this new technique to conventional MS phosphorylation site mapping methods, since the phosphorylation sites in dSLBP could not be detected by other methods.

## RESULTS

### Proof-of-Concept

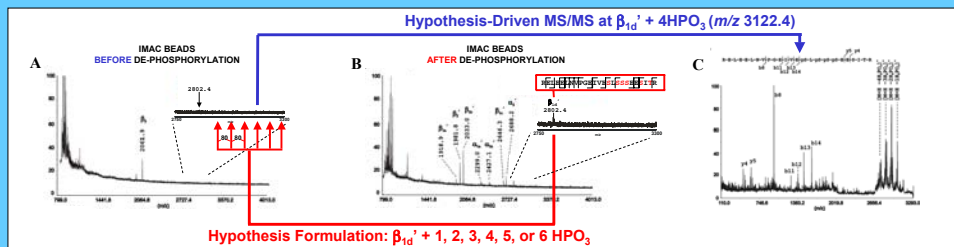


Figure 1. THD-MS analysis of  $\beta$ -casein.

(A) MALDI-MS spectrum of a fraction of IMAC beads after incubation with a tryptic digest of  $\beta$ -casein.

(B) MALDI-MS spectrum of a fraction of IMAC beads (as in A) AFTER on-target de-phosphorylation. All labeled peaks correspond to derivatives of phosphopeptides from  $\beta$  or  $\alpha$  casein.

(C) Hypothesis-driven MALDI-MS/MS spectrum of a fraction of IMAC beads (without on-target de-phosphorylation) at  $m/z$  3122.4 [ $= 2802.4 + (4 \text{ HPO}_3)$ ].

### Analysis of *In-vivo* Phosphorylated Protein

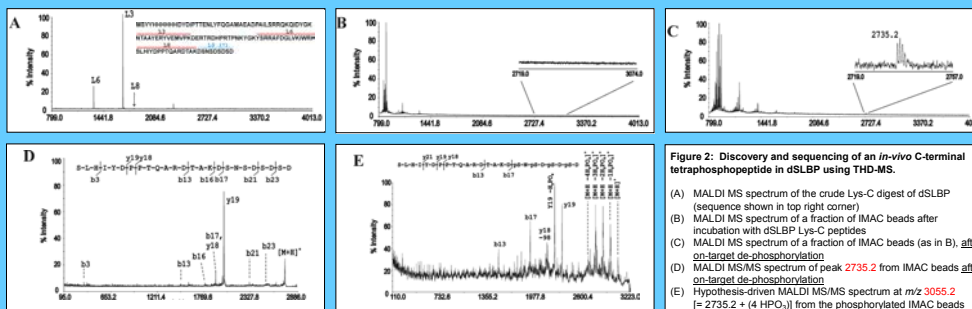


Figure 2: Discovery and sequencing of an *in-vivo* C-terminal tetraphosphopeptide in dSLBP using THD-MS.

(A) MALDI MS spectrum of the crude Lys-C digest of dSLBP (sequence shown in top right corner)  
(B) MALDI MS spectrum of a fraction of IMAC beads after incubation with dSLBP Lys-C peptides  
(C) MALDI MS spectrum of a fraction of IMAC beads (as in B), after on-target de-phosphorylation  
(D) MALDI MS/MS spectrum of peak 2735.2 from IMAC beads after on-target de-phosphorylation  
(E) Hypothesis-driven MALDI MS/MS spectrum at  $m/z$  3055.2 [ $= 2735.2 + (4 \text{ HPO}_3)$ ] from the phosphorylated IMAC beads

## CONCLUSIONS

Analysis of peptides that are directly bound to IMAC beads can improve detection of multiply-phosphorylated and highly negative peptides that cannot be eluted from IMAC beads using traditional approaches.

Using THD-MS, we sequenced 4 novel *in-vivo* phosphorylation sites in dSLBP from *Drosophila melanogaster* that could not be detected/sequenced by other complementary methods including: Top-Down FTICR-MS, LC-MS (Q-TOF), and bind/elute IMAC strategies.

THD-MS simplifies the assumptions necessary for hypothesis-driven MS by targeting the hypotheses toward only those peptides that appear after IMAC and phosphatase treatment, rather than assuming that the entire protein is phosphorylated.

Future work will involve testing THD-MS as a proteomic tool for discovering phosphopeptides in yeast cell extract proteolytic digests.

### Acknowledgements

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