

# Phospho- and Glyco-peptides Analysis Using Negative and Positive AP-MALDI Ion Trap Mass Spectrometry

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## INTRODUCTION

The identification of post-translational modifications to proteins remains a challenge in current proteomics research and analysis. Several methods have been developed to analyze the different phosphorylation and glycosylation sites on peptides and proteins. As of yet, no universal method for the purification and analysis of modified peptides and proteins exists. One of the main challenges in this area is purification and concentration of minuscule amounts of modified proteins of specific interest from thousands of other modified and unmodified peptides and proteins. Currently, different affinity chromatography methods, such as immobilized metal affinity chromatography for the purification of phosphopeptides, or lectin-based affinity chromatography for glycosites or glycopeptides, can be used to selectively isolate and concentrate modified proteins. After partial purification with micro pipette tips, peptides are analyzed by atmospheric pressure matrix-assisted laser desorption/ionization ion trap mass spectrometry. The results presented here show that by using negative and positive modes of ionization and by performing MS-MS of selected mass peaks, one can determine the structure of different phosphopeptides and glycopeptides.

## MATERIALS

Molecular Biology Grade Water from Biovintaker (Walkersville, MD, USA) was used for the matrix and sample preparation solutions. Bovine  $\beta$ -Casein and ProteoMax<sup>TM</sup> Peptide MALDI-MS Calibration Kit to calibrate AP-MALDI spectra up to m/z 4000 Da were obtained from Sigma (St. Louis, MO, USA). The matrix material,  $\alpha$ -cyano-4-hydroxycinnamic acid (4-HCCA) was obtained from Fluka (Buch, Switzerland). The synthetic phosphopeptides were obtained from Hakoten Bioscience Inc. (King of Prussia, PA, USA). Tryptin heads (Pierce) and Immobilized Tryptin, PerSeptive Biosystems were obtained from Applied Biosystems (Foster City, CA, USA). All materials were used as received, without any further purification or modification. ZipTip<sup>®</sup> pipette tips were obtained from Millipore (Bedford, MA, USA). Different affinity materials were obtained by different manufacturers and embedded in the micro pipette tips by using Glygen (Columbia, MD, USA) NanTip Technology.

## OPERATION AND SAMPLE PREPARATION

### Operation

Experiments were carried out in a repetitive laser shot mode (frequency 10 Hz). The laser trigger times were not synchronized with the LCQ operation. Operating conditions for all full MS and MS-MS experiments were as follows: automatic gain control (AGC) was off, ion injection time was 220 ns, the temperature of the LCQ input capillary was held at 200 °C. Typical high voltage applied to the target plate was 2000 V. Spectra were averaged over a 30 s x 3 s collection time. The LCQ was operated in high mass range mode (up to 4000 Da), and was calibrated using the AP-MALDI source and a standard protein.

### Sample Preparation

$\beta$ -Casein was dissolved in a volatile buffer containing 100 mM ammonium bicarbonate. For adjusting the pH to 8.5, 3.0  $\mu$ l of 1 M CaCl<sub>2</sub> was added. To enhance recovery of hydrophobic peptides, 5% acetonitrile was added. Since  $\beta$ -Casein is free of disulfide bridges, reduction and alkylation steps were omitted. Aliquots of  $\beta$ -Casein 20 nmole were digested using 10  $\mu$ l tryptin heads with 1:100 v/v enzyme to substrate ratio. Digestion has been carried out at +37 °C, overnight shaking.  $\beta$ -Casein samples were spun at 15,000 rpm for 30 min, supernatant was removed.

Immobilized metal affinity chromatography IMAC has been used for selective enrichment of phosphopeptides obtained from tryptic digests. In our experiments Ga (III) ions were selected for charging different affinity NuTips. Phosphopeptides isolated from bovine  $\beta$ -casein were eluted from immobilized gallium by alkaline solution (0.3 M ammonium hydroxide), then placed onto the target plate with 1.5 mM 4-HCCA matrix solution for analysis.

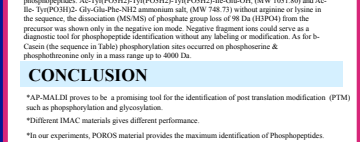
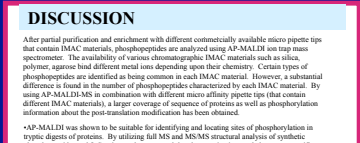
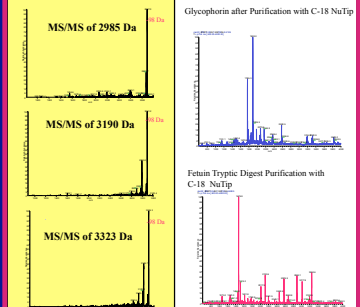
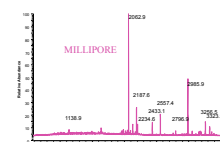
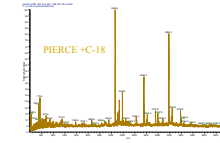
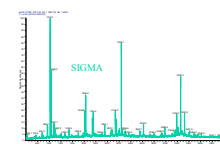
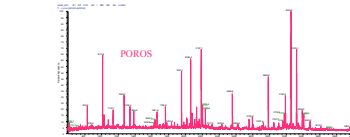
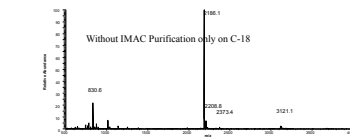
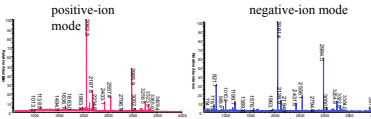
## RESULTS

In this poster, we have compared different commercially available IMAC materials for the purification of phosphopeptides. This study shows that various materials give different results as well as identification of different phosphopeptides from bovine  $\beta$ -casein tryptic digest.

In Figure 1, different AP-MALDI mass spectra are shown. In case of Pierce-IMAC material, further purification was necessary and was done using C-18 Tip before being analyzed by AP-MALDI. In all other IMAC materials, the phosphopeptides were directly applied to the MALDI target plate as described in methods without any further purification.

In Figure 2, we demonstrate the purification of tryptic digest peptides and glycopeptides from glycoprotein and from by using different NuTips.

## AP-MALDI SPECTRA OF $\beta$ -CASEIN



## DISCUSSION

After partial purification and enrichment with different commercially available micro pipette tips that contain IMAC materials, phosphopeptides are analyzed using AP-MALDI ion trap mass spectrometry. The availability of various chromatographic IMAC materials such as silica, polystyrene, agarose, and different metal ions depending upon their chemistry. Certain types of phosphopeptides are identified as being common in each IMAC material. However, a substantial difference is found in the number of phosphopeptides characterized by each IMAC material. By using AP-MALDI-MS in combination with different micro affinity pipette tips (that contain different IMAC materials), a larger coverage of sequence of proteins as well as phosphorylation information about the post-translational modification has been obtained.

\*AP-MALDI was shown to be suitable for identifying and locating sites of phosphorylation in tryptic digests of proteins. By utilizing full MS and MS-MS structural analysis of synthetic phosphopeptides and  $\beta$ -Casein, we demonstrated that the negative ion mode has some specific advantages over the positive ion mode. Moreover, for the investigated fragmentation of phosphopeptides: Ac-Tyr(PH)2-Cys(PH)2-Tyr(PH)2-Cys-Glu-OH (MW 1013.8) and Ac-Ile-Tyr(PH)2-Cys-Glu-Phe-NH2 ammonium salt (MW 748.73) without arginine or lysine in the sequence, the dissociation (MS-MS) of phosphate group ions of 90 Da (HPO4) from the precursor was shown only in the negative ion mode. Negative fragment ions could serve as a diagnostic tool for phosphopeptide identification without any labeling or modification. As for  $\beta$ -Casein (the sequence in Table) phosphorylation sites occurred on phosphoserine & phosphothreonine only in a mass range up to 4000 Da.

## CONCLUSION

\*AP-MALDI proves to be a promising tool for the identification of post translation modification (PTM) such as phosphorylation and glycosylation.

\*Different IMAC materials gives different performance.

\*In our experiments, POROS material provides the maximum identification of Phosphopeptides.

\*NuTip Technology can be very helpful for evaluating different chromatographic materials.

\*Leaching of metal ions from IMAC matrix does not interfere with the phosphopeptides analysis.

\*Atmospheric pressure matrix-assisted laser desorption/ionization (AP-MALDI) mass spectrometry is particularly beneficial for phosphopeptides analysis because of minimal fragmentation of analyte ions, large tolerance to the laser energy and the ability to produce primarily singly-charged ions.

## EXPERIMENTAL

### Mass spectrometry

Experiments were carried out on a Thermo Famosan (San Jose, CA, USA) LCX Duo XP ion trap mass spectrometer integrated with an AP-MALDI ion source (MassTech Inc., Columbia, MD, USA). The AP-MALDI source is described in detail in ref. [8-10], but no specific arrangement is outlined here. A Thermo Laser Science Inc. (Franklin, MA, USA) Model 337 Si nitrogen UV laser was used. Its wavelength was 337 nm, laser pulse duration was about 4 ns, and the laser beam was focused to approximately 200  $\mu$ m size spot. The maximum laser energy on the target was measured to be about 140  $\mu$ J/pulse, using a Mollectron Detector (Portland, OR, USA) Model EM-400 laser energy meter. During operation, the laser energy was measured to the level of about 60  $\mu$ J/pulse. Sample and laser spots were observed on a TV monitor at a viewing angle of 45° with a total magnification of about 100x using a CCD camera.

## TABLE MEASURED PHOSPHOPEPTIDES MASSES FROM BOVINE $\beta$ -CASEIN

Observed m/z	Start Peptide	Modification	Sequence
1138.13	166-173	2P(S)	(K)KTRKLTTEER (N)
1268.25	166-173	2P(S)	(K)KTRKLTTEER (N)
1338.38	166-173	2P(S)	(K)KTRKLTTEER (N)
2062.38	48-63	1P(S)	(K)FQSEHQQVTEDELQEK (N)
2432	43-63	1P(S)	(K)KDFGQVREKCCITTEHLEQ (N)
2456.88	17-36	3P(S)	(K)NTMREIVSSESSSISDRTYK (O)
2795.88	17-40	2P(S)	(R)RELELNVPQVETVLSSESSSETR (K)
2986.63	17-40	3P(S)	(R)RELELNVPQVETVLSSESSSETR (K)
3126	17-40	4P(S)	(R)RELELNVPQVETVLSSESSSETR (K)
3139	17-43	2P(S)	(R)RELELNVPQVETVLSSESSSETR (K)
3255.14	17-43	3P(S)	(R)RELELNVPQVETVLSSESSSETR (K)
3323.13	17-43	4P(S)	(R)RELELNVPQVETVLSSESSSETR (K)
3409	141-164	8P(S)	(R)RQLSTSEELNKKVIVIMSTESVTFK (K)