

Negative and Positive AP-MALDI Analysis of Synthetic Phosphopeptides and Bovine β -Casein Using Immobilized Metal Affinity Chromatography Ga(III) IMAC

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Introduction

The identification of phosphorylation sites is crucial for the understanding of various signaling events. The main challenges of analyzing phosphorylation by conventional MALDI are low sensitivity, poor stability of phosphoesters and the complex stoichiometry of the phosphorylation.

Comparing Atmospheric pressure (AP) MALDI to traditional MALDI shows that AP-MALDI is better suited for the analysis of "fragile" post-translational modifications, such as phosphorylation, oxydation, and glycosylation. Structural analysis of phosphopeptides by tandem mass spectrometry (MS/MS) is complicated by massive neutral loss of the phosphate $[M-98]^+$ and $[M-80]^+$ ions observed in positive mode, resulting in a low yield of the other structurally important fragment ions.

Methods

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 ms, and the temperature of the LCQ input capillary was held at 280 °C. Typical high voltage applied to the capillary was 3000 V. Spectra were averaged over a 30 s to 3 min collection time. The LCQ in a High Mass Range (up to 4000 Da) was calibrated using the AP/MALDI source and a standard protein mixture of four peptides. Four-point calibration with average peptide masses were: Angiotensin II (1047.20), Amyloid β protein fragment 1-16 (1955.06), ACTH fragment 18-39 (2466.72) and Andrenomedullin fragment 22-52 (3578.04).

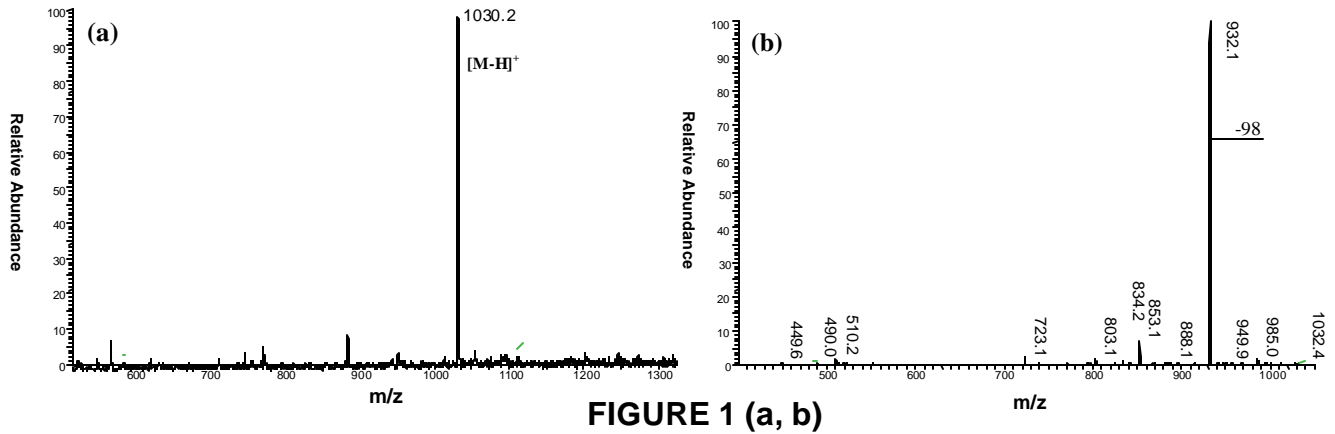
The samples of synthetic phosphopeptides for mass spectrometric analysis were prepared on the gold-plated target plate by mixing aliquots of 1.0 μ L of the peptide mixture with 1.0 μ L of a matrix solution and dried at room temperature. Matrix composition was 1.25 mg/mL of 4HCCA dissolved into a solution of 70% ACN and 0.1% TFA. Immobilized metal affinity chromatography Ga (III) IMAC was used for selective enrichment of the phosphopeptides obtained from tryptic digests. Optimal performance of the micro adsorptive pipette tips ZipTip_{MC} (10 μ L) is dependent on the peptide digest, media pH, and the metal ion used. Maintaining a low pH (2.5 to 5.5) is critical for enhancing phosphopeptide binding. The choice of metal (copper, nickel, gallium or iron) may also vary depending on the peptide digest and application. In our experiments Ga (III) ions were selected for charging of ZipTip_{MC} pipette tips. The manufacturer's protocol was modified to maintain pH as required for the preparation MALDI samples. Phosphopeptides isolated from bovine β -Casein were eluted from immobilized gallium by alkaline conditions such as 0.3 M ammonium hydroxide, and then the pH was adjusted to pH 4.5 by performing titration with 0.1% of formic acid. The derivative peptides were thoroughly washed and eluted onto the target plate with 1.5 mM 4 HCCA for analysis.

We used the Protein Prospector software (<http://prospector.ucsf>) to prove the feasibility of protein identification based on a peak list generated in AP-MALDI MS/MS experiments.

Results

The multiphosphorylated peptide (II)- Ac-Tyr(PO₃H₂)-Tyr(PO₃H₂) -Tyr(PO₃H₂)-Ile-Glu-OH (MW 1031.80) is the most active compound screened in a competitive ELISA to detect inhibitors of the SH3-SH2 domain binding to the epidermal growth factor receptor. Figure 1a represents AP-MALDI mass spectra of phosphopeptides II, obtained using 4-HCCA as a matrix in negative ionization mode. Figure 1b shows the MS/MS spectra of the parent ion at m/z 1031.8 of phosphopeptide II. 200 fmol was loaded. The spectrum

was obtained in negative ionization mode. A negative fragment ion loss of phosphoric acid (H_3PO_4) was evident for all three phosphorylated sites of phosphopeptide II. The MS^3 of the m/z 932 Da peak results in a second phosphate loss which corresponds to a fragment ion at m/z 834.1 Da. The MS^4 of the m/z 834.1 peak results in phosphate loss corresponding to the third fragment ion at m/z 736.3 Da.



Immobilized metal affinity chromatography Ga(III) IMAC has been used for selective enrichment and structural analysis of phosphopeptides obtained from the tryptic digests mixture of bovine β -Casein. Figure 2a shows AP-MALDI mass spectra of β -Casein after the sample has been processed with the Ga (III) IMAC ZipTips using 4-HCCA as a matrix in negative ion mode.

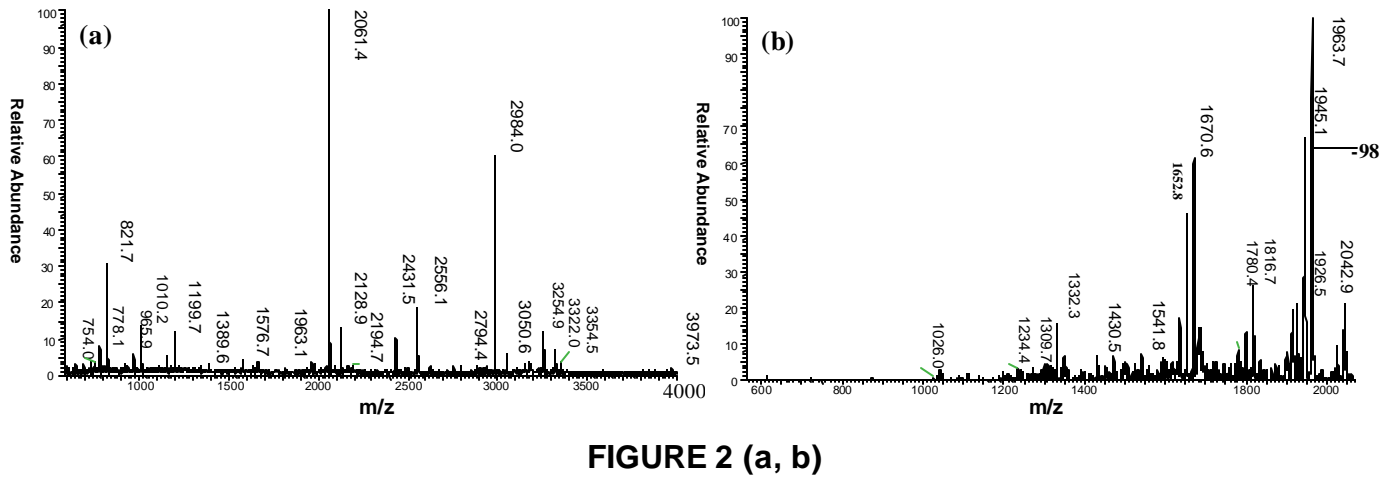


Figure 2b shows MS/MS spectra of monophosphorylated peptide FQpSEEQQTEDELQDK (m/z 2061.4) in negative ion mode.

Conclusion

AP-MALDI was shown to be suitable for identifying and locating sites of phosphorylation in the digest mixture of proteins. The results showed that in negative ion mode, AP/MALDI was able to demonstrate phosphate group loss resulting in a neutral loss of 98 Da (H_3PO_4) from peptides containing phosphotyrosine and phosphoserine without arginine or lysine in the peptide sequence, and without any labeling or modification.

Our work also proved that immobilized metal affinity chromatography Ga (III) IMAC can be used successfully for selective enrichment of phosphopeptides obtained from bovine β -Casein tryptic digest.