

AP-MALDI Ion Trap Mass Spectrometry Analysis of Selective Phosphopeptides Using Different Immobilized Metal Affinity Chromatography Materials

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INTRODUCTION

The identification of post-translational modification (PTM) in proteins remains a challenge in current proteomic research and analysis. Several methods have been developed to analyze the different phosphorylation sites on peptides and proteins [1-7]. There is, however, no universal method for the purification and analysis of modified peptides and proteins. Currently, different affinity chromatographic and other chromatographic materials such as immobilized metal affinity chromatographic (IMAC) - material, titanium dioxide (titanium), graphite carbon, anion exchanger, and antibody affinity purification are used for the purification of phosphopeptides [10]. 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 [8-11].

MATERIALS

Molecular Biology Grade Water from Biowatker (Walkersville, MD, USA) was used for the matrix and sample preparation solutions. Bovine β -Casein Ovalbumin and ProteoMass™ 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, α -cyano-4-hydroxycinnamic acid (4-HCCA) was obtained from Fluka (Buch, Switzerland). The synthetic phosphopeptides were obtained from Bachem Bioscience Inc. (King of Prussia, PA, USA). Trypsin beads (Poroszyme Bulk Immobilized Trypsin, PerSeptive Biosystems) was obtained from Applied Biosystems (Foster City, CA, USA). All materials were used as received, without any further purification or modification. ZipTip[®] pipette tips were obtained from Millipore (Bedford, MA, USA). Different affinity materials were obtained by different manufacturers and embedded in the microtipette tips by using Glygen (Columbia, MD, USA) Nutip Technology.

EXPERIMENTAL

Mass Spectrometry

Experiments were carried out on a Thermo Finnigan (San Jose, CA, USA) LCQ Deca XP ion trap MS integrated with an AP/MALDI ion source (MassTech Inc., Columbia, MD, USA). The AP/MALDI source is described in detail in ref. [11-12], but our specific arrangement is outlined here. A Thermo Laser Science Inc. (Franklin, MA, USA) Model 337 Si nitrogen (IV) laser was used. Its wavelength was 337 nm, laser pulse duration was about 4 ns, and the laser beam was focused to approximately 500 μ m size spot. The maximum laser energy on the target was measured to be about 140 μ J/pulse, using a Moletron Detector (Portland, OR, USA) Model EM-400 laser energy meter. During operation, the laser energy was attenuated to the level of about 60 μ 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. 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, the temperature of the LCQ input capillary was held at 280°C.

Method

Seven different IMAC-materials were charged with iron and gallium and used for selective enrichment of the phosphopeptides from tryptic digest of bovine β -Casein and chicken ovalbumin. Micro adsorptive pipette tips are used for the IMAC purification. Phosphopeptides (β -casein and chicken ovalbumin) were dissolved in a volatile buffer containing 100 mM ammonium bicarbonate. For adjusting the pH to 8.5, 3.0 ml of 1 M GAC2 was added. To enhance recovery of hydrophobic peptides, 5% acetonitrile was added. Aliquots of proteins 20 nmole were digested using 10 μ l trypsin beads with 1:100 v/v enzyme to substrate ratio. Digestion has been carried out at +37°C, overnight shaking. The Nutip were prepared with different IMAC materials and charged by gallium metal for enrichment of phosphopeptides obtained from tryptic digests. Phosphopeptides isolated from peptides mixture were eluted from immobilized gallium by 1.5 mM α -cyano-4-hydroxycinnamic acid (4-HCCA) matrix solution for analysis.

RESULTS

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 spectrometer.

Figure 1 shows positive ion AP-MALDI mass spectra of a tryptic digest β -Casein using the matrix 4-HCCA after the sample has been processed with the Ga (III) and different IMAC materials.

The data on β -Casein is presented below. The five phosphorylation sites are denoted with pS. The sequence purified from cow milk might also have a signal sequence. We conducted a detailed analysis on the β -Casein (Sigma, St. Louis, MO) product and found some contaminating phosphopeptides from alpha-casein, kappa-casein.

Figure 2 presents AP-MALDI spectra of tryptic digest Ovalbumin using the matrix 4-HCCA after the sample has been processed with different IMAC materials.

Ovalbumin was chosen for this study as a model phosphoprotein & glycoprotein. Chicken ovalbumin has 385 amino acids with a sequence mass of 42,748.2 Da, allowing for a single disulfide bond between Cys-73 and Cys-120. Ovalbumin contains two phosphorylated serines at positions 68 & 344. Tryptic cleavage of ovalbumin releases two phosphopeptides, LPFGFDySIEAQCCTGTVNVHSLR & EVVGGpSAEGVDAASVSEEEFR with expected masses of 2,354.11 and 2,088.91 Da, respectively.

Figure 1. AP-MALDI SPECTRA OF β -CASEIN

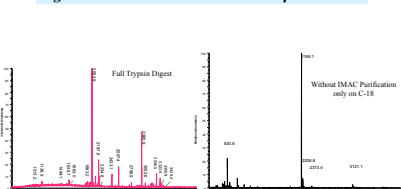
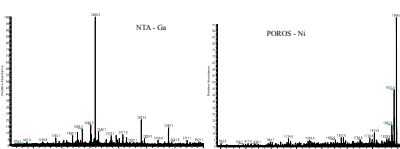
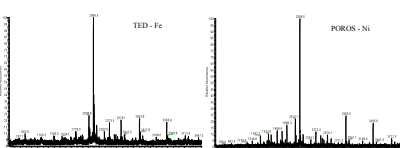
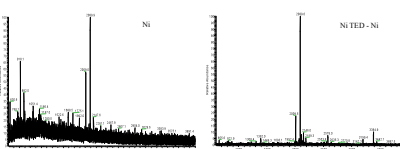
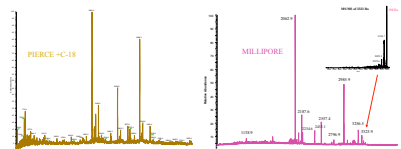
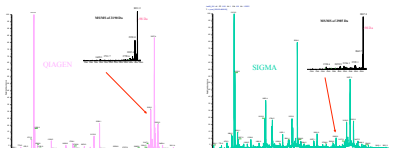
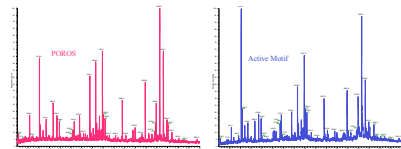
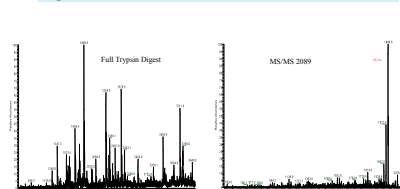


Figure 2. AP-MALDI SPECTRA OF OVALBUMIN



MassTech Search results, name and sequence of Ovalbumin are presented based on AP-MALDI data.

Start-End	Observed	Calculated	Sequence
127-142	232.09	232.09	DLNENWYDQADADAR
127-142	188.08	188.09	GLLGFNPYDADADAR
141-148	242.08	242.09	DLNENWYDQADADAR
141-148	186.50	186.49	AKKEDYDQADAR
141-148	182.50	182.49	YLVNINWYDAR
232-239	1774.60	1773.59	ISQVIAHIAKAEKGR
232-239	184.50	184.49	IRATVYDQADAR
248-259	2019.4	2018.12	EYVCSAAGCVDAASVE
191-201	2164.1	2163.48	SLVLPNPDQADADAVLR
61-64	232.14	232.35	DLNENWYDQADADAR
196-219	2282.9	2282.60	YLVNINWYDQADADAR

DISCUSSION

In this study, a simple test was performed by using different commercially available IMAC materials for the purification of phosphopeptides from β -casein and chicken ovalbumin that were analyzed with atmospheric pressure (AP) matrix-assisted laser desorption/ionization (MALDI) mass spectrometry (MS).

IMAC technology, which is used for the analysis of phosphopeptides, has its own variations. Different IMAC materials, which are commonly used for HIS-tagged protein purifications, are used for the purification of phosphopeptides. The chelating metal Nickel is used for the HIS-tagged proteins and for phosphopeptides. Iron and Gallium are commonly used as chelating metals for other protein purification methods. The different chelating groups such as IDA (iminodiacetic acid)-TRIDENATE, NTA (nitrilotriacetic acid)-TETRADENATE, and TED (tris(carboxymethyl) ethylenediamine) pentacetate are used for the purification of phosphopeptides.

This study shows that various materials provide different results and result in the identification of different phosphopeptides from bovine β -casein tryptic digest and ovalbumin tryptic digest. We did not find any correlation between tridentate, tridentate and pentadentate chelating agents. Furthermore, there is a difference in the results depending on whether the same chelating agent is immobilized on silica or polymer based chromatographic material.

The results (MSⁿ) showed that in positive ion mode or negative ionization mode without any labeling or modification AP-MALDI exhibited a neutral phosphate group loss of 98 Da (H3PO4) from peptides containing phosphoserine. Such phosphate loss was also observed on the MS/MS spectra of all other detected phosphopeptide peaks (data are not shown).

CONCLUSION

By using AP-MALDI-MS in combination with various micro affinity pipette tips (that contain a wide variety IMAC materials), a larger coverage of sequence of proteins as well as phosphorylation information about the post-translational modification has been obtained.

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