

Identification of Phosphoproteins and sites of Phosphorylation using a Rapid IMAC Procedure combined with Automated LC/MSⁿ

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Overview

Purpose: A method for enrichment and analysis of phosphoproteins is described.

Methods: Ga³⁺-IMAC magnetic beads were used to capture phosphopeptides from a model system based on β -casein, and then on an enzymatic digestion of a human A431 cell lysate. The peptides were released from the beads and analyzed by reversed phase C18 LC/MS/MS with an LCQTM Deca XP Plus ion trap mass spectrometer.

Results: We present a fast, simple protocol for enriching phosphopeptides in a complex mixture using magnetic bead-based IMAC prior to LC/MSⁿ analysis in an ion trap mass spectrometer. Beginning with 100 μ g of cell lysate digest, this procedure was used to successfully enrich and identify more than a dozen phosphoproteins from A431 cells.

Introduction

Reversible phosphorylation is an important post-translational modification associated with many proteins that have a regulatory function in cell cycle control, receptor-mediated signal transduction, cell differentiation, and nutrient metabolism. Although approximately 20% of all cellular proteins are estimated to be phosphorylated, their degrees of phosphorylation vary widely, from less than 1% to greater than 90%. Phosphoproteins are generally found in low quantities within cells and the phosphopeptides derived from these cellular proteins are relatively polar due to the presence of one or more phosphate groups, and in some instances, multiple acidic amino acids in the peptides. This low abundance, coupled with the higher acidity of phosphopeptides, may compromise the efficiency of their ionization in a mass spectrometer operated under positive ESI mode. Phosphoprotein analysis in a complex mixture is, therefore, technically challenging.

An efficient method of enriching phosphopeptides can greatly facilitate LC/MSⁿ characterization of phosphoproteins. Immobilized metal ion affinity chromatography (IMAC) is a technique that has been used to purify phosphoproteins and phosphopeptides [1-3]. In this procedure, the phosphate moieties of phosphoproteins/ phosphopeptides coordinate with Fe³⁺ or Ga³⁺ ions chelated on a matrix of chromatographic beads containing iminodiacetic or nitrilotriacetic acid ligands.

Methods

Chemicals: The following chemicals and reagents were purchased from the respective companies. **Sigma:** β -casein, DTT, guanidium hydrochloride, and gallium chloride; **Promega:** enzyme; **Mallinckrodt Chemicals:** H₂O and acetonitrile; **EM Biochemicals:** formic acid; **Pierce Chemicals:** iodoacetic acid; **Qiagen:** Ni-NTA magnetic beads.

Cell culture and preparation of total protein digests: Human A431 cells (an epidermoid carcinoma line) were cultured in a DMEM medium containing 10% FBS at 37°C under 5% CO₂. Cells were treated with 1 μ g/ml EGF for 10 minutes prior to harvest. Total protein extracts were prepared according to the method of Dignam *et al.* (4) and dialyzed in HEPES buffer (20 mM, pH 7.9) containing 100 mM KCl, 1mM MgCl₂, 0.5 mM PMSF, and 0.2 mM sodium orthovanadate. Whole proteins of A431 cells were reduced with DTT and methylated with IAA by standard procedures. The proteins were digested with enzyme in NH₄HCO₃ buffer and the mixture was acidified with formic acid (FA). β -casein was digested similarly, but without methylation or reduction.

IMAC Isolation of phosphopeptides: Gallium nitrilotriacetate (NTA) was prepared by replacing the Ni²⁺ ion on Ni-NTA magnetic beads with Ga³⁺ through buffer exchange. Figures 1 and 2 show the structure of this unique interaction, and the procedure for IMAC capture. Peptides were incubated with the beads for 30 minutes at room temperature and the beads were thoroughly washed with methanol-H₂O (50/50). Bound peptides were released by incubation at room temperature with 50mM sodium phosphate or ammonium phosphate, at pH 8.0.

LC/MS/MS analysis and database search: Following formic acid (FA) acidification, the released peptides were analyzed in an LC/MSⁿ system using an LCQ Deca XP Plus ion trap mass spectrometer. The peptides were eluted using a linear gradient (2% to 60% of solvent B into solvent A) in 45 minutes, followed by a 5-minute ramp to 60% of solvent B maintained by the Surveyor[®] HPLC system. Solvent A consisted of water containing 0.1% FA; solvent B consisted of acetonitrile containing 0.1% FA. A protein database search was carried out using TurboSEQUENT[™], within the BioWorks[™] 3.0 protein identification software suite.

Results

Using an enzymatic digest of β -casein as a model, IMAC conditions for capturing phosphopeptides were evaluated. Several factors affected the capturing efficiency, including the number of beads used, the concentration of protein digest, and the conditions used for washing and elution of the beads. We established a simple, rapid IMAC procedure, which takes less than two hours for sample preparation and does not require sophisticated equipment.

Figure 1 shows the complex structure of phosphopeptides, Ga³⁺ ion and IMAC stationary phase. The Ga³⁺ ion first binds to the nitrogen ions on the IMAC phase and then forms a complex with carboxylic acids on phosphopeptides. The phosphopeptides were retained by the IMAC phase while non-phospho peptides were not. After washing with methanol/water, the phosphopeptides were eluted by either base or phosphate buffer. The phosphopeptides were separated on a C18 column with a reversed phase gradient and analyzed by ion trap mass spectrometry. The data was analyzed by BioWorks 3.0 software and the peptides/proteins were identified by database searching. The analysis protocol is described in Figure 2.

FIGURE 1. The structure of IMAC support, Ga³⁺ ion and phosphopeptide complex.

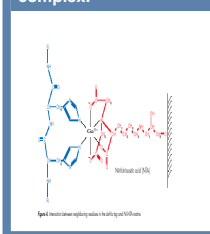
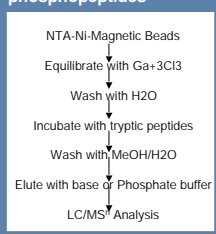
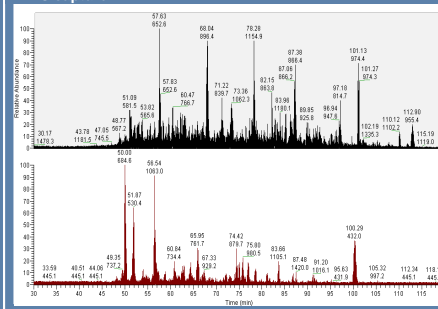


FIGURE 2. Procedure used for IMAC capture and LC/MSⁿ analysis of phosphopeptides



This technique was then applied to a complex biological sample, a human A431 cell line. Cells were lysed, and the released proteins were digested with enzyme. Figure 3 shows the chromatograms of A431 cell peptides with and without IMAC pretreatment. Without IMAC capture, all peptides were loaded onto the mass spectrometer and a complicated spectrum was obtained. A neutral loss search to identify phosphopeptides was unproductive because of co-elution of many species with similar m/z. With IMAC capture, the non-phospho peptides were washed away and a much cleaner chromatogram was obtained, allowing straightforward identification of phosphopeptides with a neutral loss search.

FIGURE 3. Base peak chromatograms of A431 cell digest peptides before (top panel) and after (bottom panel) Ga³⁺ IMAC capture.



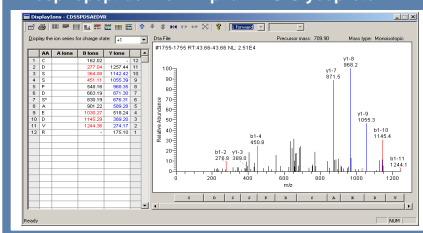
The MS/MS spectra of phosphopeptides were analyzed by BioWorks 3.0 software. The peptide sequence was determined by TurboSEQUENT, matching MS/MS spectra with those predicted by in silico digestion of a protein database, taking into account dynamic modification of serine and threonine. Figure 4 is an MS/MS spectrum for a peptide of alpha-2-HS-glycoprotein, showing excellent agreement with predicted values. The phosphorylation site determined for CDSSPDS⁺AEDVR of alpha-2-HS-glycoprotein is consistent with a recently published study (1).

Using 100 μ g of starting digest mixture, more than a dozen other phosphoproteins were identified, along with their sites of phosphorylation, and are listed in Table 1. The results are consistent with previous literature reports.

Table 1. Phosphoproteins from A431 Cells: Peptides and Sites of Phosphorylation as Determined by LC/MS/MS Analysis of Ga³⁺ IMAC Pretreated of Whole Cell Enzymatic Digest

Sequence	Protein Name
LTSE ⁺ SSDLSLVLPEPK	Ubiquitin-specific processing protease [Homo sapiens]
CDSSP ⁺ DAEDVR	Alpha-2HS-glycoprotein [Homo sapiens]
KETE ⁺ SEAEINDLDLEK	Ser/Arg-related nuclear matrix protein (plenty of proteins 101-like) [Homo sapiens]
IYHLPAE ⁺ SEDEDFKEQTR	SEPTIN 2 (NEDDS PROTEIN HOMOLOG)
LPS ⁺ SPVVEDAAAFK	Aplaxin [Homo sapiens]
DWDED ⁺ SDEMSNDFR	Inactive progesterone receptor, 23 KD [Homo sapiens]
SASPDDOGLSSNWEADLQNEER	small acidic protein [Homo sapiens]
MLP ⁺ HAPGVGMQAIPEADIPES ⁺ E	Histone deacetylase 1 [Homo sapiens]
GEDEEEDQPKR	
MESPAS ⁺ SPQSPMSPQSKGK	MAP kinase kinase 3b [Homo sapiens]
PLLH ⁺ MLLN ⁺ TLNK	Ac-like transposable element [Homo sapiens]
IWK ⁺ SATALDQGLKGL	Transcription elongation factor B (SII), polypeptide 3 (110kD, elongin A) [Homo sapiens]
IWRPLCAAAGGAF ⁺ SPAS ⁺ T ⁺ TYR	DKFZP586F1524 protein [Homo sapiens]
WLDE ⁺ SDAEMLR	Butyrate-induced transcript 1 [Homo sapiens]

Figure 4. Example MS/MS Spectrum of a Phosphopeptide From Alpha-2-HS-Glycoprotein



Conclusions

1. A rapid and efficient, magnetic bead-based method for the enrichment of phosphopeptides, based on Ga³⁺ IMAC, has been demonstrated.
2. This method was successfully demonstrated with β -casein and then applied to a complex biological system, a human cell line.
3. Phosphopeptides from an enzymatic digest of a human A431 cell line lysate were significantly enriched, improving MS/MS acquisition and allowing determination of sites of phosphorylation of many peptides.
4. Using this enrichment strategy, more than a dozen phosphoproteins were identified in excellent agreement with recent literature citations.
5. The described method is robust, rapid and compatible with high-throughput assay formats.

References

1. Anderson L and Porath J: Isolation of phosphoproteins by Immobilized Metal (Fe³⁺) Affinity Chromatography. *Anal. Chem.* 154, 250-254 (1986).
2. Posewitz MC and Tempst P: Immobilized Gallium (III) Affinity Chromatography of Phosphopeptides. *Anal. Chem.* 71, 2883-2892 (1999)
3. Ficarro SB, McClelland ML, Stukenberg PT, Burke DJ, Ross MM, Shabanowitz J, Hunt DF, and White FM: Phosphoproteome analysis by mass spectrometry and its application to *Saccharomyces cerevisiae*. *Nature Biotech.* 20, 301-305 (2002).
4. Dignam, JD, Lebovitz, RM and Roeder RG: Accurate transcription initiation by RNA polymerase II in a soluble extract from isolated mammalian nuclei. *Nucleic Acid Res.*, 11, 1475-1489 (1983).
5. Haglund AC, EK B, and EK P: phosphorylation of human plasma α 2-Heremans-Schmid glycoprotein (human fetuin) *in vivo*. *Biochem J.* 367, 437-45 (2001).
6. Kim J, Vladimir N, Lu X, Bergmann A, Ren XM, Warth T, Richardson p, Kourina N, and Stubbs L: Discovery of a Novel, paternally Expressed Ubiquitin-specific Processing protease Gene through Comparative analysis of an Imprinted Region of Mouse Chromosome 7 and Human Chromosome 19Q13.4. *Genome Res.* 10, 138-1147 (2000).

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