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Phosphopeptide Enrichment Using Offline Titanium Dioxide Columns for Phosphoproteomics

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Summary

Identification of phosphoproteins or phosphopeptides as cancer biomarkers is an emerging field in phosphoproteomics. Owing to the low stoichiometric nature of protein phosphorylation, phosphoproteins or phosphopeptides must be enriched prior to downstream mass spectrometry analysis. Titanium dioxide (TiO₂) has been prevalently used to enrich phosphopeptides from complex proteome samples due to its high affinity for phosphopeptides and the method is straightforward. In this protocol, an offline phosphopeptide enrichment procedure using TiO₂ columns is described. Peptides from a proteome lysate are loaded onto a TiO₂ column in an acidic environment, followed by column washing with aqueous, organic, and ammonium glutamate (NH₄Glu) buffers at acidic conditions. Phosphopeptides are eluted using an ammonia solution at high pH. Use of NH₄Glu significantly reduces non-specific bindings while a high recovery rate (84%) of phosphopeptides is retained. The method is optimized for large-scale phosphoproteomic analysis and phosphoprotein biomarker discovery starting from sub-milligram or milligrams of proteome samples.

Keywords

Phosphoproteomics; Phosphopeptide enrichment; Titanium dioxide; Biomarker; Proteomics; Ammonium glutamate; Mass spectrometry; MS/MS; Liquid chromatography; Cancer

1. Introduction

Mass spectrometry (MS)-based phosphoproteomic technologies have been increasingly applied to the understanding of basic biological processes and mechanisms of disease development. An emerging field in phosphoproteomics has been the identification of phosphoproteins or phosphopeptides as cancer biomarkers. Specific phosphoprotein signatures would facilitate disease diagnosis and patient stratification, predict, and monitor therapeutic outcomes for personalized cancer therapy. Recent development in

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phosphoproteomic technologies (1) provide great opportunities in applying such tools to the discovery of phosphoprotein biomarkers. Several phosphoproteins/kinases have been approved by regulatory agencies as cancer biomarkers (2). Initial endeavors have been made to identify phosphoproteins from body fluids, and hundreds of phosphopeptides have been identified (3–5).

Owing to the low stoichiometric nature of protein phosphorylation, phosphoproteins or phosphopeptides must be enriched prior to downstream MS characterization and quantitative analysis. Several approaches have been developed and compared for the enrichment of phosphopeptides, including Immobilized Metal Affinity Chromatography (IMAC), Phosphoramidate Chemistry (PAC), and Titanium Dioxide (TiO₂) chromatography (2, 6). Each approach has its own advantages and disadvantages. Among them, TiO₂ has been prevalently used in phosphoproteomics because it is compatible with a wide range of reagents used in biological sample preparation, including salts, detergents, and other small molecules (7). In addition, the procedure for phosphopeptide enrichment by TiO₂ is relative simple and straightforward compared to IMAC. TiO₂ chromatography has been incorporated into sequential elution from IMAC (i.e., SIMAC) to purify mono-phosphorylated peptides (8). Both online and offline TiO₂ chromatographic approaches have been employed in phosphoproteomics (9–11).

While TiO₂ has high affinity for phosphopeptides (10, 12–14), significant non-specific bindings from non-phosphopeptides become an issue when digested proteome samples are applied to TiO₂ columns (11). Recent studies revealed that these non-specific bindings can be minimized by conditioning and/or washing the TiO₂ column before and/or after sample loading with a buffer containing 2,5-dihydroxybenzoic acid (DHB) (10), aliphatic hydroxy acids (15), or ammonium glutamate (NH₄Glu) (11), etc. While several of these reagents might result in the loss of phosphopeptides, TiO₂ column wash with NH₄Glu retain a high recovery rate (e.g., 84%) of phosphopeptides in terms of amounts (11).

In this chapter, an offline phosphopeptide enrichment procedure using a TiO₂ column is described. The method incorporates a column wash with an NH₄Glu buffer. This offline approach is flexible for both analytical and preparative scale isolation of phosphoproteomes by using different size of TiO₂ columns. A large scale enrichment of phosphopeptides from 1–2 mg of digested proteome sample is suitable for current multi-dimensional liquid chromatography-tandem mass spectrometry (LC-MS/MS) approaches. As a demonstration of this protocol, 0.5 mg of peptides from a HeLa cell lysate is loaded onto the TiO₂ column in an acidic environment. The column is then washed with aqueous, organic, and NH₄Glu buffers at acidic conditions. Phosphopeptides are eluted with a high recovery rate using an ammonia solution at high pH (~11.7).

2. Materials

2.1. Cell Culture of HeLa Cells

1. Human cervical epithelial cancer cell line HeLa (ATCC).

2. DMEM-10 medium: Dulbecco's modified Eagle's medium supplemented with 10% cosmic calf serum (CCS), 100 units/mL penicillin, 100 µg/mL streptomycin, and 2 mM L-glutamine.
3. Phosphate saline buffer (PBS).
4. T75 flasks: sterilized cell culture flasks (75 cm²).
5. Cell scrapers.
6. Falcon 15-mL conical centrifuge tubes.

2.2. Proteome Sample Preparation

1. Lysis buffer: 8 M urea in 50 mM Tris-HCl, pH 8.3, with 100-fold dilution of phosphatase inhibitor cocktail 1 (Sigma-Aldrich) and 100-fold dilution of phosphatase inhibitor cocktail 2 (Sigma-Aldrich) (see Note 1).
2. Digital Sonifier cell disruptor with 1/8" microtip (Branson Ultrasonics Corporation, Model 250).
3. Bicinchoninic acid (BCA) protein assay kit (Pierce).
4. 50 mM Tris-HCl, pH 8.3.
5. Sequencing grade modified trypsin (Promega).
6. Water purified from a Barnstead Nanopure system (ThermoFisher Scientific) (ultrapure water) (see Note 2).

2.3. Solid Phase Extraction (SPE)

1. Extract-Clean™ high capacity 200 mg C18 cartridges (Alltech Associates Inc.).
2. One vacuum manifold for SPE.
3. HPLC grade acetonitrile (CH₃CN).
4. SPE washing buffer: 0.1% trifluoroacetic acid (TFA).
5. SPE elution buffer: 80% acetonitrile, 0.1% TFA.

2.4. TiO₂ Chromatography

1. One TiO₂ (5 µm particle size) column and one cartridge holder: column size is 4 mm inner diameter (ID) and 10 mm long (GL Sciences Inc., Part No. 5020-08690).
2. One syringe pump: Harvard Pump 11 Plus single or dual syringe pump (Harvard Apparatus, Part No. 702208 or 702209).

¹Phosphatase inhibitors should be added in the lysis buffer to prevent phosphate groups from being removed from the proteins during cell lysis. If the phosphatase inhibitors cocktails are prepared in-house, the inhibitors should cover a broad range of phosphatases. Highest purity of chemicals should be used throughout the whole phosphopeptide enrichment experiment.

²All the solutions should be prepared using Type 1 ultrapure water with resistivity of ~18.2 MΩ-cm such as those obtained from Barnstead Nanopure™ or Milli-Q systems.

3. Syringes: 1-2 syringes of 5 mL (22 gauge needle) capacity for column washing, 2-3 syringes of 2.5 mL (22 gauge needle) capacity for sample loading, column washing, and phosphopeptide elution (Hamilton Company, Part No. 81516 and 81416).
4. PEEK (polyetheretherketone) polymer tubing: 1/16" OD (outside diameter) x 0.015" ID x 5' (IDEX Health & Science, Part No. 1565).
5. Two-piece fingertight fittings for 1/16" OD tubing (IDEX Health & Science, Part No. F300x).
6. One Rheodyne® needle port adapter: the needle port adapter has a 10-32 threaded nut, a PEEK ferrule, and a Teflon liner (IDEX Health & Science, Part No. 9013).
7. One polymer tubing cutter for 1/16" and 1/8 OD" tubing (IDEX Health & Science, Part No. A-327).
8. Sample loading buffer: 50% CH₃CN /0.1% TFA.
9. TiO₂ washing buffer 1: 0.1% TFA.
10. TiO₂ washing buffer 2: 200 mM L-glutamic acid ammonium salt (NH₄Glu), pH 2.0 (see Note 3).
11. TiO₂ washing buffer 3: 80% CH₃CN /0.1% TFA.
12. TiO₂ elution buffer: 1.5% ammonium hydroxide (NH₄OH), pH 11.7 (see Note 4).

3. Methods

The protocol described in this chapter is aimed to enrich phosphopeptides from complex proteome samples as illustrated in the use of the tryptic digest from a HeLa cell lysate. It is highly efficient in isolating phosphopeptides with a high recovery rate and a significant reduction of non-phosphopeptides. The method yields enough phosphopeptides for subsequent multi-dimensional liquid chromatographic separation and MS analysis, which is usually employed for phosphoprotein biomarker discovery and phosphoproteome analysis, as demonstrated by using sub-milligram or milligrams of protein. The experimental setup and principle of TiO₂ phosphopeptide enrichment are illustrated in Figure 1. Instead of isolating phosphopeptides from each fraction after liquid chromatographic fractionation, the current method is time-efficient since phosphopeptide purification is performed for a single un-fractionated sample from the total cell lysate. The protocol is simple and straightforward, and can be applied to any mixtures of peptide digests of complex proteome samples. Since the flow rate of sample loading and column wash is strictly controlled, the procedure for phosphopeptide enrichment and experimental results are highly reproducible.

³. Attention should be paid to avoid precipitation during the preparation of 200 mM L-glutamic acid ammonium salt (NH₄Glu), pH 2.0. To prepare 50 mL of this buffer, weigh 1.64 g NH₄Glu and transfer to a beaker. Add ultrapure water to a volume of ~25 mL. Adjust pH to 2.0 by adding ~12% HCl drop-wise. Make up to 50 mL with ultrapure water using a cylinder.

⁴. The TiO₂ elution buffer is prepared by diluting ~28% ammonium hydroxide (NH₄OH), which is commercially available. A Syringe may be used to measure the 28% NH₄OH solution.

3.1. HeLa Cell Culture and Lysis

1. Grow human cervical epithelial cancer cell line HeLa in T75 flasks in DMEM-10 media at 37 °C in a humidified 5% CO₂ atmosphere to reach 90% confluence.
2. Wash the cells with ice-cold PBS buffer three times. Add 3 mL of ice-cold lysis buffer per flask of cells to directly disrupt the cells in the flasks. Harvest the cell lysate using a cell scraper and transfer the lysate to a 15-mL Falcon tube (see Note 5).

3.2. Proteome Sample Preparation and Digestion

1. Sonicate the cell lysate at ~20% output five times 10 s on ice with one-minute intervals to further disrupt the cells using a digital sonifier cell disruptor (see Note 6 and Note 7).
2. Centrifuge the lysate at 18 000 *g* for 15 min at 4 °C, and collect the supernatant.
3. Determine the protein concentration using a BCA assay kit according to the manufacture's instruction (see Note 8).
4. Dilute the protein sample four folds with 50 mM Tris-HCl, pH 8.3 (see Note 9), and add sequencing grade modified trypsin at a ratio of 40:1 (w/w, protein/trypsin).
5. Incubate the sample for 8 h at 37 °C.

3.3. Reversed-phase Solid Phase Extraction (RP-SPE)

1. Condition the high capacity C18 columns with CH₃CN. Use 3 mL of CH₃CN for each column (see Note 10).
2. Wash each column with 5 mL of SPE washing buffer (0.1% TFA).
3. Dilute the digested protein sample with equal volume of ultrapure water and load the samples onto the columns slowly (~1 drop/s).
4. Repeat Step 2.
5. Elute the peptides with 2 mL of SPE elution buffer (80% CH₃CN/0.1% TFA) for each column, and collect the eluates.
6. Lyophilize the eluates in a speed vacuum concentrator to dryness.

⁵Procedures to detach and collect the cells first and then lyse the cells could affect protein phosphorylation status since phosphorylation changes were observed within a minute of treatment (16). Therefore, *in situ* cell lysis is preferred. After the cell lysate is collected, the sample may be stored at -80 °C if the procedure needs to be interrupted.

⁶The time interval between each sonication can be extended to avoid the sample getting warm or hot.

⁷DNA is broken down during sonication, so the sample is no longer glutinous after this step.

⁸Sample dilution may be needed so that the protein concentration is within the measurable standard range. Other protein assays such as the Bradford assay can be used as long as the lysis buffer is compatible with the assay.

⁹Alternatively, dilution can be performed using 25–50 mM ammonium bicarbonate (NH₄HCO₃). Four-fold dilution of the sample is to bring down the urea concentration to 2 M or less in which trypsin can work effectively without significant loss of activity.

¹⁰SPE should be performed using a vacuum manifold and is suggested to be in a chemical hood.

3.4. Assemble the TiO₂ Cartridge Column and Set up the Syringe Pump

1. Confirm the flow direction of the TiO₂ column. Insert the column into the cartridge holder and strongly tighten the holder using hands.
2. Confirm the flow direction of the assembled cartridge column. Cut 2 inches of PEEK tube (1/16" OD x 0.015" ID) using a polymer tubing cutter. Insert the tube into a 1/16" ferrule, and connect the tube to the outlet of the TiO₂ column using a 1/16" nut (see Note 11) by finger-tightening. Ensure the PEEK tube is bottomed in the outlet port of the cartridge.
3. Connect the Rheodyne[®] needle port adapter to the inlet of the TiO₂ column by finger-tightening (see Note 12). Ensure the Teflon tube of the adapter is bottomed in the inlet port of the cartridge.
4. Insert the needle of a 5-mL syringe into the needle port adapter of the assembled TiO₂ cartridge column, and place them onto the syringe pump.

3.5. TiO₂ Phosphopeptide Enrichment from Digested HeLa Cell Lysate

1. Configure the syringe pump flow rate unit as mL/min. Set the syringe inner diameter as 10.3 mm for the 5-mL syringe, and set the flow rate as 0.2 mL/min and volume as 3 mL (see Note 13). Condition the TiO₂ column with TiO₂ washing buffer 1 (0.1% TFA) using a 5-mL syringe (see Notes 14-16).
2. Change the syringe inner diameter to 7.28 mm for the 2.5-mL syringe, and set the flow rate as 0.05 mL/min and volume as 1 mL for the syringe pump (see Note 17). Dissolve 0.5 mg of peptides from the HeLa cell lysate in 1 mL of the sample loading buffer (50% CH₃CN /0.1% TFA) (see Note 18). Load the sample onto the column using a 2.5-mL syringe (see Note 19).
3. Change the syringe pump flow rate to 0.2 mL/min and volume to 0.75 mL. Wash the column with TiO₂ washing buffer 1 using a 2.5-mL syringe.
4. Set the syringe pump parameters to the same as in Step 1. Wash the column with 3 mL of TiO₂ washing buffer 2 (200 mM NH₄Glu, pH 2.0) at 0.2 mL/min using a 5-mL syringe.

¹¹The orientation of ferrule to nut on tubing should be correct. The blunt end of the ferrule must face nut threads.

¹²The tightness of the needle port should be adjusted using the syringe needle. Insert the syringe needle into the Teflon tube and tighten the fittings into the port until an increased resistance is felt during the insertion of the syringe needle. Do not over tighten the fittings.

¹³Each time when a syringe of different size is used, the parameter of syringe inner diameter should be changed for the syringe pump, and the flow rate and volume settings need to be adjusted to meet the desired values.

¹⁴The TiO₂ column is designed for use in the pH range of 2-12. Be cautious that all the solvents used do not exceed this pH range.

¹⁵Make sure the TiO₂ cartridge column is properly tightened and connected without leaking. This can be done by checking the connections at the column inlet and outlet, and by comparing the solvent volume delivered by the syringe to that collected at the outlet of the column.

¹⁶The TiO₂ column can be re-usable after re-conditioning. If this step is for re-conditioning, it is important to ensure the column is fully re-equilibrated by 0.1% TFA by checking the eluate pH.

¹⁷The flow rate for sample loading is much slower than that for column washing to ensure all the phosphopeptides are retained on the column.

¹⁸Fifty percent of CH₃CN facilitate dissolving of complex proteome samples. Up to 80% of CH₃CN can be used.

¹⁹The capacity of the TiO₂ column for phosphopeptides is very high. However, it is suggested to use multiple such columns for milligrams of peptide samples, and each is loaded, for example, with 0.5 mg of peptides. The phosphopeptides enriched from each column are then pooled and analyzed by multi-dimensional LC-MS/MS.

5. Change the syringe inner diameter to 7.28 mm for the 2.5-mL syringe, and set the flow rate as 0.2 mL/min and volume as 0.75 mL for the syringe pump. Wash the column with TiO₂ washing buffer 1 using a 2.5-mL syringe.
6. Change the syringe pump volume to 1.5 mL. Wash the column with TiO₂ washing buffer 3 (80% CH₃CN/0.1% TFA) at 0.2 mL/min using a 2.5-mL syringe.
7. Elute the phosphopeptides with 1.5 mL of TiO₂ elution buffer (1.5% NH₄OH) at 0.2 mL/min using a 2.5-mL syringe.
8. Lyophilize the sample in a speed vacuum concentrator.
9. Redissolve the dried phosphopeptides in 150 µL of 0.2% phosphoric acid (H₃PO₄). After a quick spin, the sample is ready for capillary reversed-phase LC-MS/MS analysis.

An example of nanoflow RPLC-MS²-MS³ analysis the phosphopeptides enriched from a HeLa cell lysate using this protocol is shown in Figure 2. The analysis is for 5 µL injection of phosphopeptides onto a 75 µm ID capillary column coupled online with a linear ion trap MS (LTQ, Thermo Electron, San Jose, CA). MS/MS (MS²) and phosphate neutral-loss dependent MS/MS/MS (MS³) data are acquired. The lower panel is the base peak chromatogram of the analysis. The upper panel shows the MS³ or MS² spectra of the phosphopeptides eluted from the chromatographic peaks at 13.8 min, 15.2 min, and 24.8 min, respectively. Phosphopeptides were effectively enriched as demonstrated from the relatively high abundance levels of the LC chromatographic peaks from which these phosphopeptides were eluted.

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Figure 1.

The principle of assembling the TiO_2 cartridge column, setup of the syringe pump, and phosphopeptide enrichment. (A) A TiO_2 column, a cartridge holder, 1/16" OD PEEK tubing, 1/16" OD fittings, a Rheodyne® needle port adapter, a polymer tubing cutter, and 2-3 syringes are needed to assemble the TiO_2 cartridge column device for phosphopeptide enrichment. (B) The TiO_2 column is assembled in a cartridge holder by finger-tightening. (C) A ~2 inches of 1/16" OD PEEK tube is cut using a polymer tubing cutter. (D) Insert the tube into a 1/16" ferrule, and connect the tube to the outlet of the TiO_2 column using a 1/16" nut by finger-tightening. (E) Connect the Rheodyne® needle port adapter to the inlet of the TiO_2 column by finger-tightening. (F) Insert the needle of a syringe into the needle port adapter of the assembled TiO_2 cartridge column and place them onto the syringe pump. The device is ready for phosphopeptide enrichment. (G) Schematic diagram showing the

principle of TiO₂ enrichment of phosphopeptides with a column wash of 200 mM NH₄Glu, pH 2.0.

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Figure 2.

Nanoflow reversed-phase liquid chromatography coupled online with a linear ion trap mass spectrometer for the analysis of TiO₂-enriched phosphopeptides from a HeLa cell lysate using collision-induced dissociation (CID) for tandem MS and phosphate neutral loss-dependent MS/MS/MS (RPLC-MS²-MS³). The analysis is for 5 μ L injection of phosphopeptides onto a 75 μ m ID capillary column. The lower panel is the base peak chromatogram of the analysis. The upper panel shows the MS³ or MS² spectra of the phosphopeptides ALVAT*PGKK, SGAQASSTPLS*PTR, and LLT*PTHSFLAR

identified, respectively, from nucleolin, lamin-A/C, and microtubule-associated protein 7. The relatively high abundance levels of the LC chromatographic peaks, from which these phosphopeptides were eluted, demonstrate that phosphopeptides were effectively enriched from the HeLa cell lysate. The asterisk marks the phosphorylated Ser or Thr residues.