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Sequential phosphopeptide enrichment for phosphoproteome analysis of filamentous fungi: a test case using *Magnaporthe oryzae*

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Abstract

A number of challenges have to be overcome to identify a complete complement of phosphorylated proteins, the phosphoproteome, from cells and tissues. Phosphorylated proteins are typically of low abundance and moreover, the proportion of phosphorylated sites on a given protein is generally low. The challenge is further compounded when the tissue from which protein can be recovered is limited. Global phosphoproteomics primarily relies on efficient enrichment methods for phosphopeptides involving affinity binding coupled with analysis by fast high-resolution mass spectrometry (MS) and subsequent identification using various software packages. Here, we describe an effective protocol for phosphopeptide enrichment using an Iron-IMAC resin in combination with titanium dioxide (TiO₂) beads from trypsin digested protein samples of the filamentous fungus *Magnaporthe oryzae*. Representative protocols for LC-MS/MS analysis and phosphopeptide identification are also described.

Keywords

Mass spectrometry; phosphopeptide enrichment; *Magnaporthe oryzae*; Iron-IMAC; TiO₂; fungus

1 Introduction

Posttranslational modification (PTMs) of proteins such as phosphorylation adds a further layer of complexity to cellular regulation beyond transcription and translation. Although there are hundreds of possible PTMs of proteins, the addition of phosphate to serine, threonine or tyrosine is a primary and pervasive modification affecting protein activity, stability, location and interactions with other proteins [1]. Indeed, studies have suggested that half of an organism's proteome may be phosphorylated to some extent at any particular time [2, 3]. Given the importance and prevalence of protein phosphorylation in regulating proteins and cellular function, efforts in recent years have focused on protein phosphorylation dynamics at a global scale within cells and tissues [4–7]. Current techniques for exploration of the phosphoproteome are predominantly based on LC-MS/MS

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peptide analysis of trypsin digested protein samples following phosphopeptide enrichment [8, 9].

A number of challenges need to be overcome in order to achieve a holistic view of the phosphoproteome. Many proteins that are phosphorylated are regulatory proteins and of low abundance. Furthermore, the stoichiometry of phosphorylation of a given protein is low, thus modified peptides are buried in an excess of unmodified peptides [10]. Other issues are related to mass spectrometry. Loss of phosphate groups during phosphopeptide fragmentation can greatly affect the ability to identify phosphopeptides. Furthermore, assigning the exact location of the phosphorylation site in a peptide requires highly accurate MS/MS data. Many of these issues have been overcome through use of modern mass spectrometers such as the Q-Exactive HF Hybrid Quadrupole-Orbitrap mass spectrometer. Such instruments offer increased dynamic range and speed as well as the use of higher energy collision dissociation (HCD) which reduces neutral loss and enables the desired fragmentation for phosphopeptide identification [8, 11].

To overcome challenges associated with the relatively low representation of phosphoproteins in the proteome and obtain adequate coverage of modified sites, enrichment is needed. Protocols to enrich phosphoproteins are mainly based on use of antibodies and immunoprecipitation. Such strategies are unsuitable for large-scale studies and typically yield modest enrichment because only a small proportion of trypsin-derived peptides from a phosphorylated protein contain phosphate group(s) [12]. Strategies based on enrichment of phosphorylated peptides following trypsin digestion of proteins offer more flexibility and typically are superior, particularly when different phosphopeptide enrichment strategies are used in combination [13, 14].

Here, we describe a protocol developed for enrichment (and subsequent analysis) of phosphopeptides from the rice blast fungus *Magnaporthe oryzae*, the most destructive disease of rice worldwide. The protocol as shown in figure 1 employs sequential enrichment, first using immobilized metal affinity chromatography (IMAC) to capture phosphopeptides via interaction with chelated iron. Subsequently, the unbound fraction (or flow through) from the IMAC enrichment is subjected to metal oxide affinity chromatography (MOAC) using titanium dioxide (TiO₂) particles to capture additional phosphopeptides. The two phosphopeptide enriched samples are then pooled prior to LC-MS/MS.

The following references provide background on these enrichment approaches [9, 12]. IMAC has relatively poor selectivity for phosphopeptides; peptides with highly acidic residues such as aspartic and glutamic acids can bind non-specifically. However, binding of multi-phosphorylated peptides is enhanced under acidic conditions. Conversely, metal oxides such as TiO₂ interact with negatively charged phosphate groups particularly with singly phosphorylated peptides under acidic conditions. Although considered to provide better selectivity than IMAC, acidic side chains of other amino acids can form ionic interactions with TiO₂. Binding specificity can be enhanced by modifying the acidic conditions (see [9]). Careful attention to pH and composition of loading and elution buffers during the sequential binding to first IMAC followed by TiO₂ reduces non-specific binding and affords excellent enrichment of both singly and multi-phosphorylated peptides.

Using this approach to enrich phosphopeptides and using a nanoLC MS/MS high-resolution ion trap (Q-Exactive) mass spectrometer with data processed in MaxQuant, we identified and were able to examine changes in phosphorylation at 2,924 phosphosites on 1,914 phosphoproteins in conidia, mycelia and during conidial germination and appressorium formation of *M. oryzae* [15]. The protocol presented here is applicable to any fungal species or tissues and requires only 250 µg of protein input. This is of critical importance when protein samples from certain specialized tissues such as infection structures or sexual reproductive bodies are limited.

2 Materials

Various fresh or rapidly frozen tissues from *Magnaporthe oryzae* or other fungi can be used including but not limited to conidia, mycelium, developing appressoria, or sexual structures.

2.1 Protein extraction

1. Cell lysis buffer: 1 × phosphate-buffered saline (PBS), 0.1% SDS, 2 M urea and a PhosSTOP phosphatase inhibitor cocktail (Roche, Mannheim, Germany, 1 tablet per 10 mL buffer).
2. 0.5 mm zirconia/silica beads (BioSpec Products Inc., Bartlesville, OK).
3. 1.7 mL microcentrifuge tubes.
4. Bead mill homogenizer.
5. A refrigerated bench-top centrifuge (such as Eppendorf 5810R) equipped with a fixed angle rotor (such as F45–30-11, Eppendorf, Hauppauge, NY).
6. A bicinchoninic acid (BCA) assay kit.

2.2 Protein digestion and peptide purification by Filter Aided Sample Preparation (FASP).

1. 50 mM Dithiothreitol (DTT).
2. 8 M urea buffer: 8 M urea in 0.1 M Tris/HCl (pH 8.5).
3. 0.05 M iodoacetamide in 8 M urea buffer.
4. 0.05 M ammonium bicarbonate in H₂O (ABC).
5. Stock trypsin solution (0.25 µg/µL) in ABC.
6. 100% acetic acid.
7. 1.7 mL microcentrifuge tubes.
8. Vivacon 500 30 kDa MW cutoff filter units (Vivaconproducts, Littleton, MA).
9. 9. A refrigerated bench-top centrifuge. (See 2.1.5).
10. 10. Nanodrop 2000c (Thermo Scientific, Wilmington, DE).

2.3 Phosphopeptide enrichment by Immobilized Metal ion Affinity Chromatography (IMAC)

1. NTA Agarose (Qiagen, Valencia, CA).
2. 1% and 2% acetic acid.
3. 100 mM FeCl₃.
4. 10 micron filter paper.
5. 1.7 mL microcentrifuge tubes.
6. 10 mL Luer-Lock syringe.
7. 200 µL gel-loading tips.
8. A solution of 74% 100 mM NaCl, 25% acetonitrile and 1% acetic acid.
9. Molecular biology grade H₂O.
10. 5% NH₄OH.
11. Formic acid.
12. Benchtop tube rotator.

2.4 Phosphopeptide enrichment by Titanium Dioxide (TiO₂) Chromatography

1. TiO₂ beads (10 µm Titansphere, GL Sciences, Torrance, CA).
2. A solution of 2% acetic acid and 200 mg/mL lactic acid.
3. 200 µL gel-loading tips.
4. 1.7 mL microcentrifuge tubes.
5. Pipette tip boxes.
6. Microcentrifuge rack.
7. A refrigerated bench-top centrifuge. (See 2.1.5).
8. A solution of 74% 100 mM NaCl, 25% acetonitrile and 1% acetic acid.
9. Molecular biology grade H₂O.
10. 5% NH₄OH.
11. Formic acid.
12. Vacuum concentrator.
13. Mobile phase A (98% water, 2% acetonitrile and 0.2% formic acid).

2.5 Analysis by Mass Spectrometry

1. A high-resolution, high mass accuracy mass spectrometer capable of performing MS/MS such as an Orbitrap based instrument (LTQ Orbitrap XL or Q Exactive HF, Thermo Fisher Scientific) with an online nanoHPLC system (such as

Eksigent cHiPLC-nanoflex) using a 50–200 μm i.d. C18 column for peptide separation.

2. Mobile phase A: 98% water, 2% acetonitrile, 0.2% formic acid and mobile phase B: 98% acetonitrile, 2% water, and 0.2% formic acid.
3. Software for processing of MS/MS output data such as Mascot Distiller (Matrix Science), Proteome Discoverer (Thermo Scientific) and MaxQuant ([16], www.maxquant.org) for identifying proteins from peptide sequence databases with the ability to search for peptide modifications.
4. Genome sequence of *M. oryzae* (version 8) downloaded from NCBI BioProject PRJNA13840.

3 Methods

3.1 Protein extraction

1. Homogenize fungal tissue by bead beating for 2 min in 100 μL cell lysis buffer using ~ 150 mg of 0.5 mm zirconia/silica beads.
2. Heat the sample for 5 min at 95°C and centrifuge at $13,000 \times g$ at 4°C for 5 min.
3. Transfer the supernatant to a new tube.
4. Measure the protein concentration using a bicinchoninic (BCA) acid kit following manufacturer's protocol.
5. Store protein sample at -80°C before use.

3.2 Protein digestion and peptide purification by Filter Aided Sample Preparation (FASP).

1. Up to 250 μg of *M. oryzae* protein sample is dried down to 27 μL and 3 μL of 50 mM Dithiothreitol (DTT) is added to reach a final DTT concentration of 5 mM in a 1.7 mL microcentrifuge tube.
2. Incubate the sample for 30 min at 56°C to reduce the protein disulfide bonds.
3. Mix the sample with 100 μL of 8 M urea buffer and transfer to a Vivacon 500 30 kDa MW cutoff filter unit.
4. Centrifuge the filter unit at 21°C at $14,000 \times g$ for 15 min and discard the flow-through.
5. Add 200 μL of 8 M urea buffer and centrifuge the filter unit at 21°C at $14,000 \times g$ for 15 min. Discard the flow-through.
6. Add 100 μL of 0.05 M iodoacetamide to the filter unit and incubate for 30 min in the dark at room temperature for alkylation of the free thiols.
7. Centrifuge the filter unit at 21°C at $14,000 \times g$ for 15 min. Discard the flow-through.
8. Add 100 μL 8M urea buffer to the filter unit and centrifuge at $14,000 \times g$ for 10 min and discard the flow-through. Repeat this step two more times.

9. Add 100 μ L 0.05 M ammonium bicarbonate (ABC) to the filter unit and centrifuge at $14,000 \times g$ for 10 min and discard the flow-through. Repeat this step two more times.
10. Move the filter unit to new collection tube.
11. Add 30 μ L ABC and 10 μ L of stock trypsin solution (1 μ g of enzyme per 100 μ g of protein)) to the filter unit and vortex.
12. Incubate the filter unit for 16 h at 37°C.
13. Centrifuge the filter unit at 21°C and $14,000 \times g$ for 15 min. Do not discard the filtrate.
14. Add 40 μ L ABC and centrifuge the filter unit at 21°C and $14,000 \times g$ for 15 min.
15. Acidify the filtrate with 1.7 μ L of 100 % acetic acid (final acetic acid concentration ~ 2%).
16. Measure peptide concentration using Nanodrop 2000c (Thermo Scientific, Wilmington, DE).

3.3 Phosphopeptide enrichment by Immobilized Metal ion Affinity Chromatography (IMAC)

1. Add 400 μ L of NTA Agarose resin to 1.7 mL microcentrifuge tube.
2. Centrifuge at $500 \times g$ for 2 min and remove supernatant. (see Note 4)
3. Mix 1 mL of 1% acetic acid with the resin, centrifuge at $500 \times g$ for 2 min and remove supernatant.
4. To prepare the iron-IMAC resin, add 1 mL of 100 mM FeCl_3 in 1% acetic acid to the NTA Agarose resin and incubate for 4 h with rotation in the dark.
5. Store the iron-IMAC resin at 4°C before use.
6. Load 100 μ L of the iron-IMAC resin into a 200 μ L gel-loading tip fitted with a 10 micron filter paper plug. (see Note 5)
7. Wash the resin twice with 100 μ L of 2% acetic acid. (see Note 6)
8. Load total tryptic peptides onto the resin.
9. Collect the flow-through and reload it onto the column.
10. Set aside the flow-through for TiO_2 enrichment described below.
11. Wash the iron-IMAC resin twice with 100 μ L 2% acetic acid.
12. Wash the resin twice with 100 μ L of a solution containing 74% 100 mM NaCl, 25% acetonitrile and 1% acetic acid.
13. Wash the resin twice with 100 μ L of molecular biology grade H_2O .
14. Elute phosphopeptides twice with 100 μ L of 5% NH_4OH and combine eluents.
15. Acidify the eluate with 30 μ L of formic acid and store at -80°C .

3.4 Phosphopeptide enrichment by Titanium Dioxide (TiO₂) Chromatography

1. Suspend 1.5 mg of TiO₂ beads in a solution of 2% acetic acid and 200 mg/mL lactic acid in a 1.7mL microcentrifuge tube.
2. Load the TiO₂ beads into a 200 µL gel-loading tip fitted with a 10 micron filter paper plug. (see Note 5)
3. Wash the TiO₂ beads twice with 100 µL a solution of 2% acetic acid and 200 mg/mL lactic acid. (see Note 7)
4. Apply the flow-through from the iron-IMAC enrichments to the TiO₂ beads.
5. Collect the flow-through and reapply to the TiO₂ beads.
6. Wash the TiO₂ beads twice with 100 µL of a solution of 2% acetic acid and 200 mg/mL lactic acid.
7. Wash the beads twice with 100 µL of a solution of 74% 100 mM NaCl, 25% acetonitrile and 1% acetic acid.
8. Wash the beads twice with 100 µL of molecular biology grade H₂O.
9. Elute phosphopeptides twice with 100 µL of 5% NH₄OH and combine eluents.
10. Acidify the eluate with 30 µL of formic acid (pH should be < 4).
11. Subject the eluate to a second round of TiO₂ enrichment using the same protocol and fresh TiO₂ beads (Steps 1 – 10).
12. Pool the phosphopeptides from the iron-IMAC and TiO₂ enrichment steps and dry to near completion and store at –80°C.
13. 13. Resuspend the phosphopeptides in 25 µL mobile phase A prior to LC-MS/MS analysis.

3.5 Analysis by Mass Spectrometry

1. A representative setup involves a 15 cm x 75 µm nanoLC analytical column containing 3 µm C18 resin coupled to a high-resolution ion trap (Orbitrap Q-Exactive) mass spectrometer as described in our previous work [15]. Phosphopeptides are typically eluted from the nanoLC with a linear gradient of 5% to 40% acetonitrile (ACN) over 3 hours using mobile phase A and mobile phase B listed in materials. These conditions are optimal for use with a quadrupole Orbitrap mass spectrometer such as the Q-Exactive in data dependent mode using a top 12 method. Resolving power is set at 70,000FWHM at 200 m/z for MS acquisition and 17,500FWHM at 200 m/z for MS/MS acquisition. AGC settings for MS and MS/MS is 1E6 and 2E4, respectively (see [15]). The nanoLC setup may need modification for more complex samples and other mass spectrometers and may require longer columns with smaller chromatographic beads to improve phosphopeptide resolving power.
2. Phosphopeptide identification and analysis is performed using mass spectrometry software packages such as MaxQuant. MS/MS output data is first searched using

the Andromeda search engine within MaxQuant against the *M. oryzae* genome sequence with reverse database functioned enabled set to protein, peptide and site false discovery rates of 1%. The Phospho (STY) Site output file of MaxQuant is used to obtain phosphorylation site information. High confidence sites are defined as having a location probability greater than or equal to 0.75 and a score difference greater than or equal to five. The Perseus module of MaxQuant is used for quantification and to examine differential changes in protein phosphorylation as described in [15]. Further statistical tests can be performed using the JMP software package (SAS).

3 Notes

1. High quality chemicals should be used.
2. Urea and iodoacetamide solutions must be freshly made.
3. If necessary, preliminary or trouble shooting experiments can be carried out with radiolabelled model peptides such as ³²P Kemptide added to the peptide sample to evaluate efficiency of binding to IMAC and TiO₂. Eluates can be counted by liquid scintillation, compared to starting material and efficiencies calculated. Typically, we found our sequential enrichment method yielded >95% recovery.
4. The agarose beads are fragile and high speed centrifugation should be avoided.
5. To generate enrichment columns prepared using gel loading tips, small disks are punched from 10-micron filter paper and used to plug the gel loading tips. Any brand is suitable providing the tip is narrow. Remove the top of gel loading tips with a razor blade so that it provides a tight fit to 1 mL Luer-Lock syringe barrel. Check flow rate with 30 µL acetonitrile using gentle pressure on the syringe barrel. There should be no or little backpressure. See [17] for more details.
6. For IMAC enrichment, all washes are performed by forcing the solution through the resin using a 10 mL Luer-Lock syringe fitted to the top of the gel-loader tip.
7. For TiO₂ enrichment, all washes can be performed as indicated for IMAC, however, the flow rate will be slow. If necessary, washes can be performed by centrifugation using a bench-top centrifuge (such as a Sigma 4K15C) fitted with swinging bucket rotors (such as Sigma EXG-1020) and deep well microtiter plate holders (such as Sigma 09366). In this instance, a microcentrifuge rack is modified to fit inside a gel loading tip pipette box, which fits tightly in the microtiter plate holder. 1.7 mL microcentrifuge tubes are placed in the rack directly under each gel-loading tips. Samples are centrifuged at 1000 x g for a few minutes to collect eluent.
8. The method described here is suitable for processing between 1–12 samples simultaneously. For enrichment of more than 12 samples, collecting eluents from TiO₂ beads by centrifugation may be preferred, see Note 7.
9. Care should be taken in preparation and use of the tip loading columns to ensure no IMAC or TiO₂ resin leaks from the columns during phosphopeptide elution as

phosphopeptides may rebind to the resin. If necessary, the resin can be removed by centrifugation, prior to final acidification of the eluent.

10. Care must be exercised when performing quantitative phosphoproteome analyses involving different tissues or time course studies as protein levels may change. In such cases, global proteome studies should be conducted in conjunction with analyses of the phosphoproteome to enable accurate phospho-site normalization.

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Figure 1.
Work flow of the sequential phosphopeptide enrichment and analysis protocol.