Standardized Approach for Identification of Focal Adhesion Proteins' Phosphorylation Sites

Antonio F. M. Pinto, David M. Smalley, Li Ma, Pablo R. Grigera, Nicholas E. Sherman, J. Thomas Parsons and Jay W. Fox. University of Virginia, Charlottesville, VA.

Abstract

The Protein Discovery Initiative goal is to develop a census of the migration proteome by identifying interactions and post-translational modifications, i.e. phosphorylation, of novel molecules involved in cell migration. Over the past few years, identification of these phosphorylation sites have been done by a variety of methodologies, such as mutation of phosphorylation sites, phospho-antibodies, electrophoretic mobility shifts, immobilized metal ion affinity chromatography (IMAC), titanium dioxide (TiO_2) affinity chromatography and LC-MS/MS. Over the past year, we have developed a standard approach for phosphopeptide enrichment using direct LC-MS/MS and TiO_2 affinity chromatography coupled with LC-MS/MS.

Reverse phase chromatography in C18 column was used to analyze a tryptic digest of Wiskott-Aldrich Syndrome Protein (WASP). The phosphopeptide enrichment method modified from Canti et al. (Anal. Chem., 2007, 79:4666-4673) with or without the addition of 2,5-dihydroxybenzoic acid (DHB) was used to isolate and identify phosphorylation sites. Protein coverage and spectra counting of these three runs were used to compare the efficiency of the different methodologies.

Finally, we compared phosphorylation sites identified in the Protein Discovery Initiative with results found in databases (PhosphoSITE, http://www.phosphosite.org, and Phospho.ELM, http://phospho.elm.eu.org). Thus far, using our established methodologies, we have found a number of phosphorylation sites in the proteins studied which were not reported in the other two databases.

TiO_2 Methods

TiO_2 Columns

Capillary column (360x150) of about 15cm with Kasil frit in one extremity were used. Column was filled with 2cm of Titansphere (GL Sciences Inc.) packing material in 100% acetonitrile. Columns were rinsed with water prior to use or storage.

All column washing and loading was done at 500 psi with a flow rate of approximately 1-2 µL/mL.

TiO_2 enrichment method (modified from Canti et al., Anal. Chem., 2007, 79:4666-4673)

Reconstitute dried desalted sample in 20% acetonitrile/2% formic acid;
Wash TiO_2 column with 20 µL 0.5% NH_4OH/40% acetonitrile, pH 10.5
Equilibrate TiO_2 column with 20 µL 20% acetonitrile/2% formic acid
Load sample
Wash TiO_2 column with 20 µL 80% acetonitrile/2% formic acid
Wash TiO_2 column with 5 µL 2% formic acid
Elute TiO_2 column with 100 µL 200mM NH_4HCO_3, pH 9.0 into a C18 column
Wash C18 column with 2 mL 0.1M acetic acid (0.2 mL/min, 10 min)
Start 2 hour gradient then begin data acquisition

TiO_2 + DHB enrichment method

Reconstitute dried desalted sample in 50 mg/mL DHB in 20% acetonitrile/2% formic acid;
Wash TiO_2 column with 20 µL 0.5% NH_4OH/40% acetonitrile, pH 10.5
Equilibrate TiO_2 column with 20 µL 50 mg/mL DHB in 20% acetonitrile/2% formic acid
Load sample
Wash TiO_2 column with 20 µL 50 mg/mL DHB in 20% acetonitrile/2% formic acid
Wash TiO_2 column with 20 µL 80% acetonitrile/2% formic acid
Wash TiO_2 column with 5 µL 2% formic acid
Elute TiO_2 column with 100 µL 200mM NH_4HCO_3, pH 9.0 into a C18 column
Wash C18 column with 2 mL 0.1M acetic acid (0.2 mL/min, 10 min)
Start 2 hour gradient then begin data acquisition

Results

Table I. Results of different methods for phosphopetide identification using LC-MS/MS and SEQUEST analysis.

Method	Coverage	Phosphopetides	Total Peptides
C18	66%	15	380
TiO_2 enrichment	46%	49	102
TiO_2 + DHB enrichment	33%	46	71

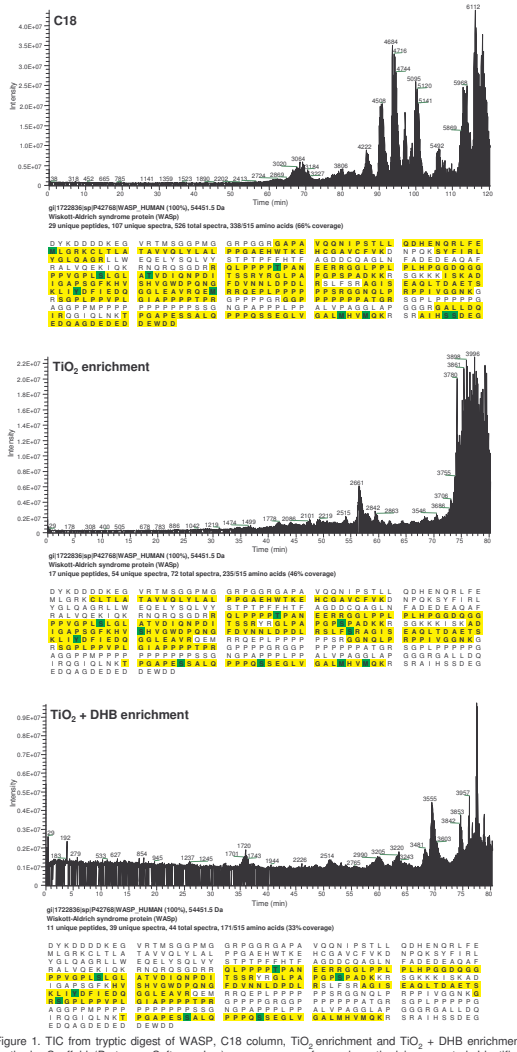


Figure 1. TIC from tryptic digest of WASP, C18 column, TiO_2 enrichment and TiO_2 + DHB enrichment methods. Scaffold (Proteome Software Inc.) coverage map for each method is presented. Identified residues are displayed in yellow. Oxidized methionine and phosphorylated serine, threonine and tyrosine are displayed in green.

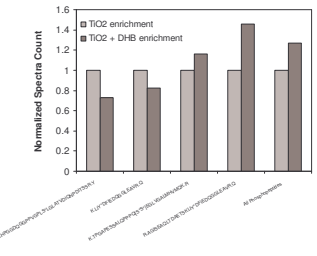


Figure 2. Comparison of the two TiO_2 enrichment methods. Approximately 1 µg of a tryptic digest of Wiskott-Aldrich Syndrome Protein (WASP) was loaded onto a TiO_2 column. TiO_2 enrichment was performed according to the different methods tested. Bound peptides were eluted into a C18 column and subjected to LC-MS/MS analysis. Peptide identification was performed using the SEQUEST algorithm. Spectra count results for the four most abundant phosphopeptides and the sum of all spectra counts of all phosphopeptides are presented. Spectra count results are normalized to those observed using the TiO_2 enrichment method.

Table II. Phosphorylation site coverage of seven proteins analyzed over the past year by the Protein Discovery Initiative. Cell Migration Consortium data are compared with two phosphorylation databases, PhosphoSITE (http://www.phosphosite.org) and Phospho.ELM (http://phospho.elm.eu.org). Total, unique and shared number of phosphorylation sites are indicated.

Protein	Protein Discovery Initiative total(unique/shared)	PhosphoSITE total(unique/shared)	Phospho.ELM total(unique/shared)
β–PIX	16(13/3)	6(3/3)	2(1/1)
CORTACTIN	18(12/6)	25(19/6)	8(7/1)
FAK	25(19/6)	14(8/6)	11(7/4)
GIT1	32(25/7)	10(3/7)	4(1/3)
PAK	3(0/3)	11(8/3)	1(1/0)
PAXILLIN	45(13/32)	51(19/32)	7(0/7)
TALIN	31(26/5)	8(4/4)	1(0/1)

Table III. Phosphorylation Sites in Paxillin. Phosphorylation sites identified by the Protein Discovery Initiative are compared to data from PhosphoSITE and Phospho.ELM databases. Phosphorylation sites are indicated in red; sites present in more than one database are highlighted in yellow.

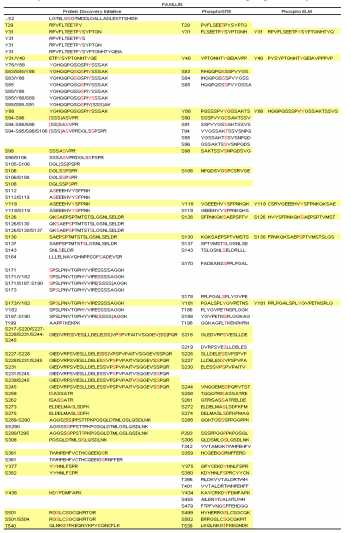
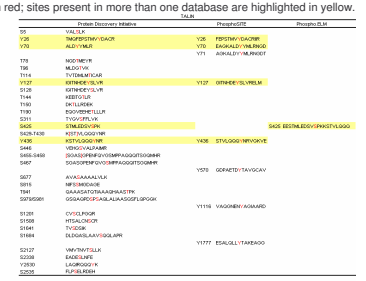


Table IV. Phosphorylation Sites in Talin. Phosphorylation sites identified by the Protein Discovery Initiative are compared to data from PhosphoSITE and Phospho.ELM databases. Phosphorylation sites are indicated in red; sites present in more than one database are highlighted in yellow.



Summary

- **Direct C18 analysis and TiO_2 enrichment method combined typically provide more than 85% sequence coverage. If not, additional proteinases can be used to reach acceptable coverage (85%).**
- **Addition of 2,5-dihydroxibenzoic acid (DHB) in the TiO_2 enrichment method decreases the non-phosphorylated peptide background present in the sample. This method may be particularly useful in the analysis of complex mixtures of proteins but is not of great utility in the identification of phosphorylation site in isolated proteins.**
- **The methodologies established in the Protein Discovery Initiative allow for an expedious approach to confidently identify phosphorylation sites in isolated proteins associated with cell migration.**
- **Future work will include stochiometric assessment of site specific phosphorylation using labeled and non-label methodologies.**