

Application Titanium Dioxide IMAC for Enrichment Phosphopeptides Prior to Tandem Mass Spectrometry

Jinghua Zhu and Qishan Lin

UAlbany Proteomics Facility, Center for Functional Genomics, Rensselaer, NY 12144

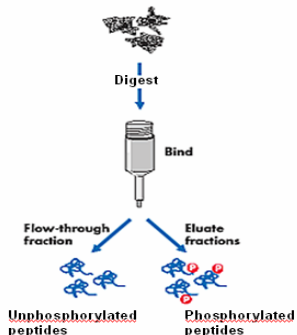
OVERVIEW

Phosphorylation of proteins on serine, threonine, and tyrosine residues is among the most important of the posttranslational modifications, playing a critical role in regulating many cellular processes. Despite progress made in instrumentation and techniques, locating site of phosphorylation in non-radioactive samples is still a formidable challenge. According to a recent survey from ABRF (Association of Biomolecular and Resource Facilities), only 3/67 (4%) facilities all over the world could locate the correct sites of phosphopeptide at the level of 1 pmol. This application describes how we used TiO₂ based immobilized metal ion affinity chromatography (IMAC) to enrich phosphopeptides prior to LC-MS/MS characterization of a phosphorylation site.

Matched peptides shown in **Bold Red**

1 MPFLPPTNN RPHIHETPEL SSQPSLDSNR QPRELCKKN **OLIVEGHAYE**
 51 LGDDRRMIDH FHCSCQNTSL GCSNDELVLG NGMLCSMCS YNCKQCGRIK
 101 DDLAILTGQQ AYCSNCFKCR SCNKIIEELR YARTSEGLFC MDCHQKLMK
 151 KKKTDAREKH LALLQEKRVQ LEREKQRELE QLEDDHGRSV NSSHNSLIQS
 201 YDNNQHQSG ELLYQQRNR SVTSVTSFKN KSLPLPPTST SPARPTPYSA
 251 NSYQSKESIE GRKQDTSLG SLSTSGTQSE HTNKGSTTDM SSTGLNPPPI
 301 FQSDNDEIE EVQNSSDSDS NDEKKSNT SLRKSMSNR SRVSPVNOY
 351 STPLPSDHL NDEISADTG ERTVLDVDT TPPEQADPL AGNQLKPALD
 401 VSPGRITEQK SEKELLSFN QFDNEFHNT RSPICDPTG IISNSQNT
 451 KAVNLVSDER PRSACPFPA KVMRQARVE TDEISDTGV DEGLAIGTPR
 501 QEHSRAGTLK SVSSPPKVP LPSTPSRGE ISKQSFVET PKGLGLEGV
 551 YDNDHQRSY IKSQQDQRH DISEKTSPTS DISTPRGVQ NEFPAVTHLE
 601 PKNSLSDMD QHKSQKQMN VSRASLKN LKHKSTSGN QNKLALETT
 651 GSKEDHRHGH TRQTSQGLF SSINRATYS PTINGALGR NHKSTSDST
 701 VLLSDHMY SVRVKQDIE QLNSSKVLLE EDVKLREDR HKLMEQLRIT
 751 QSKLSSSESR YDMLVREISE LQIQTKLTN ENKDLIEQK NRQASGATGK
 801 LSTSSSDSD PPSLTKGSV NTHLSPKSS SDTSVVSRE NEFSDHLSF
 851 DRHGGFQQH QYVQQYQQO PPOQLIDE QAKKATRLKF WRRPKVGTTO
 901 VISSNEYNRN GGHGNGARI PHSFTQALR IPARHSSGSF NRRGARRNQ
 951 QNRFSKSSSS NVLDSLLSGD SSVPLFMST LQORAEYKS QVPIITRLK
 1001 REVERKGLDF EGYIRSGGN SAIVSIEAF SLSNDPDK QLMRVDETL
 1051 VDHYGVTSL KRYLRLPDP VEPFLDQDF IKVSTKASHS KEVRIAELOK
 1101 IVMKPLPAMR ETLRLVNBH HLNVSLKIDN KMGYRNLVV FAPTLARDES
 1151 GVKENTDMGF RNDTELLT ESHRIF

PhosphoPeptide Purification Procedure



INTRODUCTION

Protein phosphorylation plays a significant role in regulating cellular processes such as signal transduction, cell division, cell motility, apoptosis, metabolism, differentiation, gene regulation and carcinogenesis. Typically, there are 10-20% of proteins which are phosphorylated. Due to the low level of phosphoproteins in the presence of overwhelming amounts of non phosphorylated proteins and proteins have a wide dynamic variation over time; identification (ID) phosphopeptides is still a formidable task. In addition, phosphopeptides often have poor ionization efficiency in MS analysis. Thus, highly sensitive detection method plus phosphopeptide enrichment is extremely important for a successful phosphopeptide ID. Currently, immobilized metal affinity chromatography (IMAC) is the method of choice for enriching phosphopeptides from complex biological samples. Typically, Nickel, iron and gallium based IMAC shows significant binding of non-phosphorylated peptides which have multiple acidic residues. Forest White, et al. used a kind of chemistry to put methyl esters onto those acidic groups (D and E) to solve the problem of non-specific binding to the IMAC beads. However, this approach brings in a lot of side reaction to that chemistry and issues of how complete the modifications are. Recently, several papers and posters have been published demonstrating the unique ability of titanium dioxide and zirconium dioxide to selectively retain phosphopeptides contained in complex biological mixtures (1, 2). In this application, TiO₂ based IMAC method was successfully developed to enrich phosphopeptides and adapted to a complex biological sample, Saccharomyces. Trapping phosphopeptides are demonstrated via the analysis protein CaO19_4593 (gi68466366), a GTPase-activator protein for Rho-like GTPases which contains lots of kinase binding domains

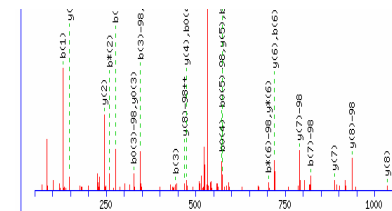
EXPERIMENTAL

The protein gel pieces were washed, reduced, alkylated and followed by in-gel tryptic digestion overnight. The peptide mixture was extracted, speed-vac followed by dissolving in a 20 µl of 5% formic acid +10% acetonitrile. The phosphopeptides were enriched by TiO₂ TopTip (Glygen, Inc). The bound peptides were eluted with 0.5% NH₃OH. Both flow-through and eluant were analyzed by Micromass Q-ToF2 LC-MS/MS. MASCOT 2.1 from Matrix Science (London, UK) was used to search all of the tandem mass spectra against the target protein with a MS and MS/MS mass tolerance of 1.0 Da and 0.5 Da respectively. PKL files were created using the software Masslynx 3.5 from Waters, which has a processing macro that smoothes, centroids, and assesses the quality of data. The parameters used for the searches were as follows: trypsin-specificity restriction with 2 missing cleavage site and variable modifications including oxidation (M), deamidation (NQ), alkylation (C), and phosphorylation (STY) with neutral losses of phosphoric acid.

Identified phosphopeptides after TiO₂ enrichment

NS*LIQSYMNNQNS*GES*LLYQQNRR (Total 2 sites S6 also possible)
 SLPLPPTSTS*PARPTPYASANYQSK (T10 also possible)
 VS*PFVNQYST*PPLSSDHLNDEISADTGER (S9 also possible)
 T*VYLDVIDTTPPEEQADPLAGNQLKPALDVS*PGR (T9 also possible)
 SFLLS*PNQFHDNEFHNR
 SPMIDS*PGT*IISNSNQTK
 S*ACPS*PFAK
 VVET*NDEISTDGVDEDLAIGT*PR (1 site)
 AGT*LKSVS*S*PPP
 SVSS*PPKVPLPST*PSR
 VPLPST*PSR (S5 also possible)
 KFS*FVET*PK
 GLGLEGVDDYDNDHQRS*YIK
 QHDIS*EKTPSSIMSTPK
 TS*PSSIMSTPK
 RVVGNFS*PAVTNLEPK
 QT*S*DG*S*LFSSIANAYIS*PPITNGALGGR (3 sites)
 LSTSS*S*NDS*DPDASTLGR
 SVNTHLS*PYK
 ENFEYSDHLS*PDR
 IPNS*FSTQALR

* Phosphorylation site



Monoisotopic mass of neutral peptide hr(cal): 1161.55
 Variable modifications: S9 : Phospho (STY), with neutral losses 97.98(shown in table), 0.00
 Ions Score: S1 Expect: 2.74e-007
 Matches (Bold Red): 29/136 fragment ions using 37 most intense peaks

#	b	b ⁺	b ⁺	b ⁺⁺	y	y ⁺	y ⁺	y ⁺⁺	y ⁺⁺	#				
1	129.10	65.05	112.08	56.54				K		9				
2	274.17	138.59	259.14	130.08		F	936.48	468.74	919.46	460.23	918.47	459.74	0	
3	345.19	173.10	328.17	164.59	327.18	164.09	S	709.41	395.21	772.39	386.70	771.40	386.21	7
4	492.26	246.63	475.23	238.12	474.25	237.63	F	720.39	360.70	703.37	352.19	702.38	351.69	6
5	591.23	296.17	574.20	287.65	573.32	287.16	V	573.32	287.17	556.30	278.65	555.31	278.16	5
6	720.37	360.69	703.34	352.18	702.36	351.68	E	474.26	237.63	457.23	229.12	456.25	228.63	4
7	821.42	411.21	804.39	402.70	803.41	402.21	T	345.21	173.11	328.19	164.60	327.20	164.10	3
8	918.47	459.74	901.45	451.23	900.46	450.73	P	244.17	122.39	227.14	114.07			2
9							K	147.11	74.06	130.09	65.55			1

Fig 2. Peptide view for the CID fragmentation of KFSQPFVETPK . Beautiful spectrum; long run of y ion series; Loss of H₃PO₄ from y₃ fragment after β-elimination was evidently observed.

CONCLUSIONS

- Phosphorylated peptides were selectively adsorbed to the TiO₂ beads
- Enrichment of the phosphopeptides from proteolytic digests in the presence of high abundant non-phosphorylated peptides was achieved
- Significant increase in phosphopeptide identification was achieved using TiO₂ beads for selective concentration and separation.

References

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