



Proteomics/Mass Spectrometry Services

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

Qishan Lin, Jinghua Zhu, UAlbany Center for Functional Genomics, Rensselaer, NY 12144

Background

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 (gi|68466366), a GTPase-activator protein for Rho-like GTPases which contains lots of kinase binding domains.

Methods & Materials

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 μ l 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 1.2 Da and 0.6 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.

Results

Fig 1 shows the Mascot analysis results for both flow through and eluant after the TiO₂ IMAC capture. After TiO₂ IMAC capture, most of the non-phosphorylated peptides were presented in the flow through and phosphopeptides were shown in the 0.5% NH₃OH eluant. In this project, almost 38 phosphopeptides were successfully identified. Fig 2 shows an example of MS/MS spectrum for phosphopeptide **KFS_pFVETPK**

<input checked="" type="checkbox"/>	89	573.28	1144.55	1145.57	-1.02	0	(33)	0.00019	1	K.SVNTHLSPYK.S + Deamidation (NQ)
<input checked="" type="checkbox"/>	91	576.26	1150.51	1150.55	-0.05	0	24	0.0012	1	K.TSPSSIMSTPK.R + Oxidation (M)
<input checked="" type="checkbox"/>	93	581.76	1161.51	1161.55	-0.04	1	(41)	4e-006	1	K.KFSFVETPK.G + Phospho (STY)
<input checked="" type="checkbox"/>	104	613.26	1224.50	1224.55	-0.05	0	48	3.7e-006	1	K.SVNTHLSPYK.S + Phospho (STY)
<input checked="" type="checkbox"/>	107	617.30	1232.59	1232.65	-0.06	0	49	2.6e-006	1	R.IPNSESTQALR.I
<input checked="" type="checkbox"/>	115	628.35	1254.68	1255.65	-0.97	0	35	0.00011	1	K.RVNLSEVDRPR.S + Deamidation (NQ)
<input checked="" type="checkbox"/>	116	630.34	1258.67	1258.73	-0.06	0	49	2.6e-006	1	K.NLSVVFAPTLAK.D
<input checked="" type="checkbox"/>	117	630.88	1259.74	1259.71	0.03	0	(18)	0.0025	1	K.NLSVVFAPTLAK.D + Deamidation (NQ)
<input checked="" type="checkbox"/>	125	640.82	1279.62	1280.67	-1.05	1	18	0.0038	1	R.ELQILEDHKKR.S + Deamidation (NQ)
<input checked="" type="checkbox"/>	129	648.25	1294.48	1294.53	-0.05	0	48	5e-006	1	K.GLFCMDCHQK.L + 2 Carbamidomethyl (C)
<input checked="" type="checkbox"/>	130	648.75	1295.48	1294.53	0.95	0	(16)	0.0079	1	K.GLFCMDCHQK.L + 2 Carbamidomethyl (C)
<input checked="" type="checkbox"/>	136	656.24	1310.47	1310.52	-0.05	0	(48)	3.5e-006	1	K.GLFCMDCHQK.L + 2 Carbamidomethyl (C); Oxidation (M)
<input checked="" type="checkbox"/>	137	656.75	1311.48	1310.52	0.96	0	(24)	0.00086	1	K.GLFCMDCHQK.L + 2 Carbamidomethyl (C); Oxidation (M)
<input checked="" type="checkbox"/>	180	500.61	1498.82	1499.88	-1.06	0	(39)	2.4e-005	1	R.LIVNHLHLVNSLK.D + Deamidation (NQ)
<input checked="" type="checkbox"/>	181	750.43	1498.84	1498.90	-0.06	0	84	8.8e-010	1	R.LIVNHLHLVNSLK.D
<input checked="" type="checkbox"/>	182	500.94	1499.81	1499.88	-0.07	0	(41)	1.1e-005	1	R.LIVNHLHLVNSLK.D + Deamidation (NQ)
<input checked="" type="checkbox"/>	183	750.92	1499.83	1499.88	-0.05	0	(62)	9.4e-008	1	R.LIVNHLHLVNSLK.D + Deamidation (NQ)
<input checked="" type="checkbox"/>	184	750.96	1499.90	1499.88	0.02	0	(66)	3.6e-008	1	R.LIVNHLHLVNSLK.D + Deamidation (NQ)
<input checked="" type="checkbox"/>	188	510.24	1527.69	1527.75	-0.07	0	(63)	5.2e-008	1	R.NDTTELLLTESH.R.I
<input checked="" type="checkbox"/>	189	764.86	1527.71	1527.75	-0.05	0	(89)	1.1e-010	1	R.NDTTELLLTESH.R.I
<input checked="" type="checkbox"/>	190	510.56	1528.66	1528.74	-0.07	0	(54)	6e-007	1	R.NDTTELLLTESH.R.I + Deamidation (NQ)
<input checked="" type="checkbox"/>	191	765.36	1528.70	1528.74	-0.04	0	93	8.2e-011	1	R.NDTTELLLTESH.R.I + Deamidation (NQ)
<input checked="" type="checkbox"/>	192	765.38	1528.75	1527.75	1.00	0	(34)	5.6e-005	1	R.NDTTELLLTESH.R.I
<input checked="" type="checkbox"/>	204	786.40	1570.78	1571.82	-1.04	0	(63)	2.1e-007	1	R.VVGNESPAVTNLEPK.N + Deamidation (NQ)
<input checked="" type="checkbox"/>	205	524.60	1570.78	1570.84	-0.06	0	(55)	1.2e-006	1	R.VVGNESPAVTNLEPK.N
<input checked="" type="checkbox"/>	206	786.90	1571.78	1572.80	-1.02	1	40	4e-005	1	K.ITNENKDLIEQQK.N + Deamidation (NQ)
<input checked="" type="checkbox"/>	207	786.93	1571.84	1571.82	0.02	0	(58)	6.8e-007	1	R.VVGNESPAVTNLEPK.N + Deamidation (NQ)

<input checked="" type="checkbox"/>	19	517.78	1033.55	1032.50	1.05	0	(9)	0.024	1	K.VPLPSTPSR.G + Phospho (STY)
<input checked="" type="checkbox"/>	20	522.68	1043.34	1043.41	-0.07	0	34	4.2e-005	1	R.SRCPSPFRK.V + Carbamidomethyl (C); Phospho (STY)
<input checked="" type="checkbox"/>	21	522.68	1043.35	1043.41	-0.06	0	(27)	0.00019	1	R.SRCPSPFRK.V + Carbamidomethyl (C); Phospho (STY)
<input checked="" type="checkbox"/>	23	523.22	1044.42	1043.41	1.00	0	(7)	0.033	1	R.SRCPSPFRK.V + Carbamidomethyl (C); Phospho (STY)
<input checked="" type="checkbox"/>	26	536.78	1071.55	1071.62	-0.07	1	33	0.00016	1	K.VLLEEDVKK.L
<input checked="" type="checkbox"/>	28	562.66	1123.30	1123.38	-0.08	0	(19)	0.0018	1	R.SRCPSPFRK.V + Carbamidomethyl (C); 2 Phospho (STY)
<input checked="" type="checkbox"/>	33	574.25	1146.48	1146.55	-0.07	0	49	3.7e-006	1	K.NDIEQLNSK.V
<input checked="" type="checkbox"/>	34	581.74	1161.46	1161.55	-0.09	1	(29)	5.9e-005	1	K.KFSFVETPK.G + Phospho (STY)
<input checked="" type="checkbox"/>	35	581.74	1161.46	1161.55	-0.08	1	51	3.7e-007	1	K.KFSFVETPK.G + Phospho (STY)
<input checked="" type="checkbox"/>	38	597.72	1193.43	1192.43	1.00	0	(1)	0.039	1	K.VPLPSTPSR.G + 3 Phospho (STY)
<input checked="" type="checkbox"/>	41	613.24	1224.47	1224.55	-0.08	0	61	1.9e-007	1	K.SVNTHLSPYK.S + Phospho (STY)
<input checked="" type="checkbox"/>	42	616.23	1230.45	1230.52	-0.07	0	37	4.1e-005	1	K.TSPSSIMSTPK.R + Oxidation (M); Phospho (STY)
<input checked="" type="checkbox"/>	44	621.72	1241.42	1241.51	-0.09	1	(31)	4e-005	1	K.KFSFVETPK.G + 2 Phospho (STY)
<input checked="" type="checkbox"/>	47	630.32	1258.62	1258.73	-0.11	0	40	1.9e-005	1	K.NLSVVFAPTLAK.D
<input checked="" type="checkbox"/>	48	630.32	1258.63	1259.71	-1.08	0	(39)	2.8e-005	1	K.NLSVVFAPTLAK.D + Deamidation (NQ)
<input checked="" type="checkbox"/>	51	657.27	1312.52	1312.62	-0.10	0	54	6.5e-007	1	R.IPNSESTQALR.I + Phospho (STY)
<input checked="" type="checkbox"/>	58	714.80	1427.58	1427.65	-0.07	1	(4)	0.089	1	R.AGLKSVSPPPK.V + 2 Phospho (STY)
<input checked="" type="checkbox"/>	66	754.78	1507.54	1507.61	-0.07	1	29	6.8e-005	1	R.AGLKSVSPPPK.V + 3 Phospho (STY)
<input checked="" type="checkbox"/>	68	510.22	1527.62	1527.75	-0.13	0	(55)	3e-007	1	R.NDTTELLLTESH.R.I
<input checked="" type="checkbox"/>	69	764.84	1527.66	1527.75	-0.10	0	87	1.9e-010	1	R.NDTTELLLTESH.R.I
<input checked="" type="checkbox"/>	73	826.34	1650.66	1650.80	-0.15	0	(13)	0.014	1	R.VVGNESPAVTNLEPK.N + Phospho (STY)
<input checked="" type="checkbox"/>	74	826.35	1650.69	1650.80	-0.11	0	(74)	8.9e-009	1	R.VVGNESPAVTNLEPK.N + Phospho (STY)
<input checked="" type="checkbox"/>	75	826.85	1651.68	1651.79	-0.11	0	(55)	1e-006	1	R.VVGNESPAVTNLEPK.N + Deamidation (NQ); Phospho (STY)
<input checked="" type="checkbox"/>	76	826.88	1651.74	1651.79	-0.04	0	77	5.6e-009	1	R.VVGNESPAVTNLEPK.N + Deamidation (NQ); Phospho (STY)
<input checked="" type="checkbox"/>	77	554.90	1661.68	1661.80	-0.12	1	(41)	4.4e-006	1	K.VPLPSTPSR.G + Phospho (STY)
<input checked="" type="checkbox"/>	78	831.86	1661.71	1661.80	-0.09	1	65	1.5e-008	1	K.VPLPSTPSR.G + Phospho (STY)
<input checked="" type="checkbox"/>	79	832.40	1662.79	1661.80	0.99	1	(16)	0.0012	1	K.VPLPSTPSR.G + Phospho (STY)
<input checked="" type="checkbox"/>	81	563.51	1687.52	1687.65	-0.13	0	(23)	0.0016	1	R.ENEYSDHLSR.H + Phospho (STY)

Fig 1. Mascot mapping results. TiO₂ IMAC capture flow through (top panel) and 0.5% NH₃OH eluant (bottom panel).

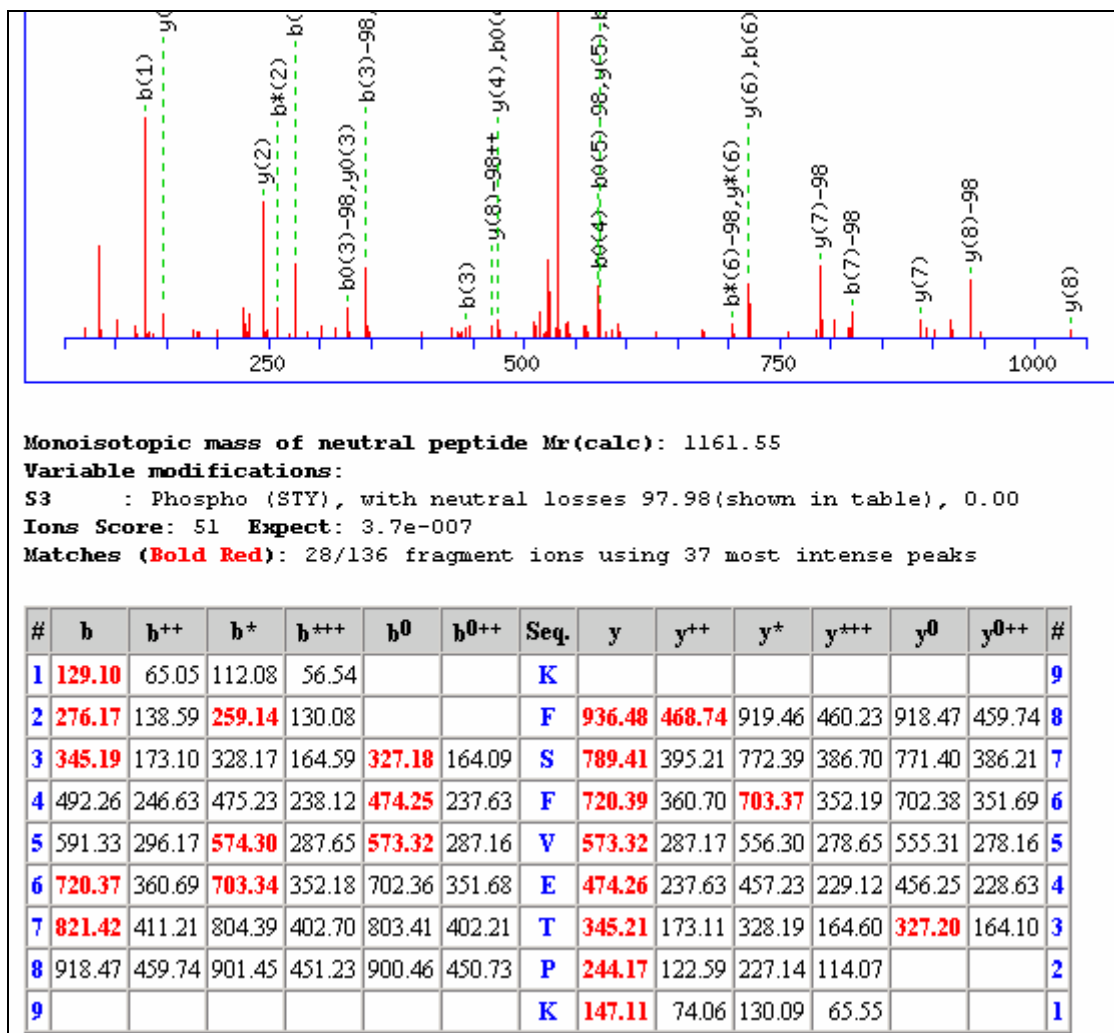


Fig 2. Peptide view for the CID fragmentation of **KFS_pFVETPK**. Beautiful spectrum; long run of y ion series; Loss of H₃PO₄ from y₃ fragment after β-elimination was evidently observed.

Conclusion

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

References

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