SHIMADZU

Application News

MALDI-TOF Mass Spectrometry No.B07

Phosphorylation Analysis by MALDI-TOF MS (3) Phosphopeptide Enrichment Technique Using TiO_2

Phosphorylation of proteins is one type of post-translational modification (PTM) which is important in the control of biological functions. Recently, mass spectrometry is being applied to the analysis of phosphorylation sites. However, due to the low ratio of phosphorylation as well as the marked decrease in ionization efficiency when phosphorylation is present, it is often difficult to conduct analysis of mixtures in their original state. Over the past several years, great achievements have been realized in phosphorylation research due to research into specific phosphopeptide enrichment techniques using IMAC (Immobilized Metal Affinity Chromatography) and titanium dioxide (TiO_2). Here we describe phosphorylation analysis using a combination of TiO_2-based phosphopeptide enrichment and MALDI-MS/MS (seamless PSD).

Fig. 1 shows the typical enrichment protocol using TiO_2. The principle underlying the affinity of TiO_2 to the phosphate group is illustrated in Fig. 2. The phosphopeptides can be enriched by washing them with an alkaline solvent after the phosphopeptides in the mixture are trapped with TiO_2. However, since TiO_2 also shows some affinity for acidic amino acids, a process is required that will exclude adsorption to non-phosphopeptides that contain acidic amino acids. For that, a high concentration acid and high concentration organic solvent (acetonitrile) are required.
Fig. 3 shows the effectiveness of TiO_2 enrichment. The phosphopeptides were enriched with specificity, and even the phosphopeptide (*m/z* 1660) that could not be detected using desalting alone was observed.

Step	Solution
Conditioning	80 % ACN, 2 - 2.5 % TFA
Sample preparation	80 % ACN, 2 - 2.5 % TFA
Adsorption	Above "Sample preparation solution"
Washing	80 % ACN, 2 - 2.5 % TFA
Elution	NH4OH (> pH 10), ACN
Desalting	Ordinary desalting by ZipTip (Millipore), etc.

Fig. 1 Enrichment Protocol Using TiO_2

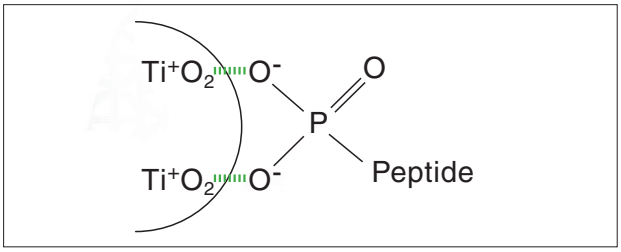

Fig. 2 TiO_2 Affinity for Phosphate Group

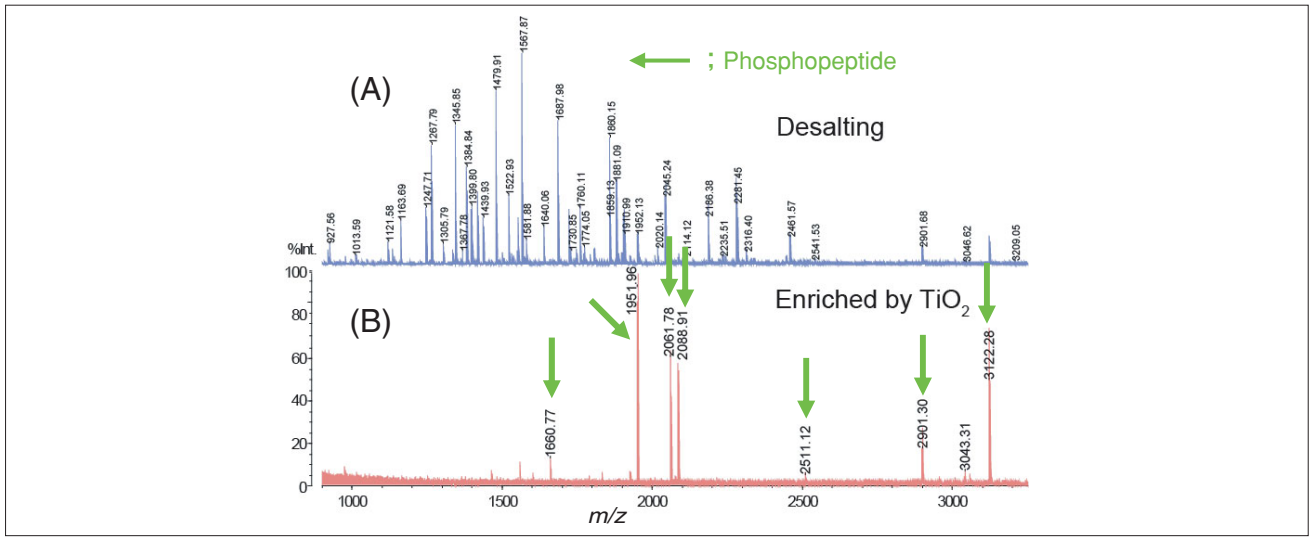

Fig. 3 Example of Phosphopeptide Enrichment Using TiO_2 (A) Desalted by C18 ZipTip (B) Enriched by TiO_2

＜Analytical Conditions＞
Instrument：AXIMA-Performance
Matrix ：2, 5-DHB (dihydroxybenzoic acid) 10 mg/mL (50 % acetonitrile, 0.1 % TFA)
Sample ：Tryptic digest mix (BSA, α-casein, β-casein, ovalbumin)

Fig. 4 shows the results of electrophoretic analysis of a whole cell lysate with abundant phosphoproteins. MS/MS (sPSD, or seamless Post Source Decay) measurement of 17 spots was conducted, and some of the results are shown in Fig. 5. The proteins of all the spots were identified, and the phosphorylation sites were specified.

Particularly noteworthy is that sequence analysis including phosphorylation sites was possible by sPSD, as shown in Spot 11.
Thus, enrichment using TiO_2 in combination with sPSD can be considered to be an effective method for conducting phosphorylation research.

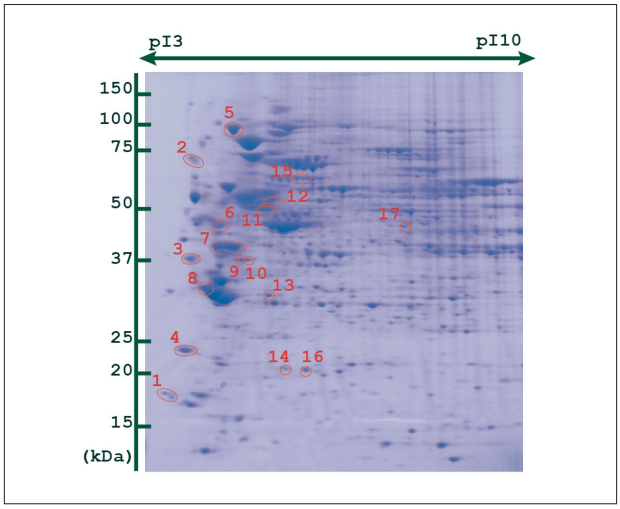

Fig. 4 Two-Dimensional Electrophoretic Profile of A431 Whole Cell Lysate (CBB-stained)

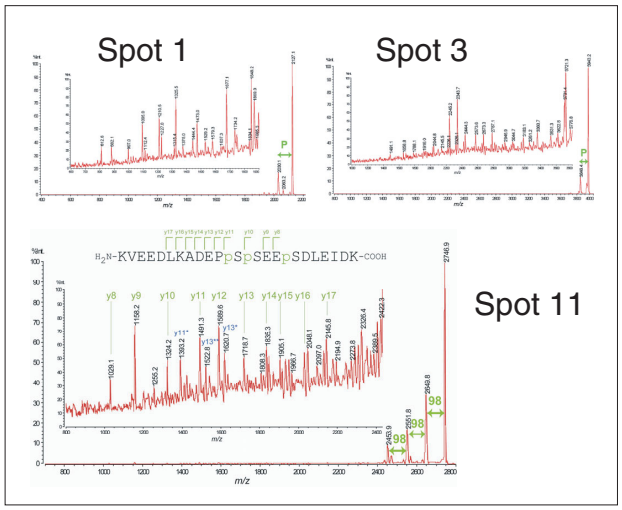

Fig. 5 sPSD Spectra of Phosphopeptides Extracted from Spots 1, 3 and 11

Table 1 Identified Phosphoproteins and Phosphorylation Sites

Spot No.	protein description	score	mass	sequence
1	60S acidic ribosomal protein P1	45	11665	K.KEEpSEEpADDDMGFGLFD.-
2	IQ domain-containing protein E	31	77696	R.VPpSPIAQApTGpSPVQEEAIVIIQpSALR.A
3	Nascent polypeptide-associated complex subunit	56	23384	K.VOGEAVSNIQENTQTPTVQEEpSEEEEVDETGVEVK.D
4	Prostaglandin E aynthase 3 - Homo sapiens	55	18982	K.DWEDDpSDEDMSNFDR.F
5	Endoplasmin precursor - Homo sapiens	17	92753	K.VEEEPEEEPEETAEDTTEDTEQDEDEEoxyMDVGpTDEEEETAK.E
6	CaM kinase-like vesicle-associated protein	26	54695	R.ATPATEESpTVPTTQSSAoxyMLATK.A
7	Hepatoma-derived growth factor	56	26902	K.GNAEGpSpSDEEGKLVIDEPAK.E
8	Elongation factor 1-beta	135	24935	K.DDDDIDLFGpSDDEEESEEAK.R K.DDDDIDLFGpSDDEEESEEAKR.L
9	Elongation factor 1-delta	44	31236	K.KPATPAEDDEDDDIDLFGpSDNEEEDKEAAQLR.E
10	Elongation factor 1-delta	73	31236	K.KPATPAEDDEDDDIDLFGpSDNEEEDKEAAQLR.E
11	Hsc70-interacting protein	47	41502	K.KVEEDLKADEPpSpSEEpSDLEIDK.E
12	Probable phospholipid-transporting ATPase IK	33	149626	R.STRAGPEPpSPAPPGPGDpTGDSDVTQEGSGPAGIRGGETVIR.A
13	Proteasome subunit alpha type-3	34	28661	K.ESLKEEDEpSDDDNoxyM.-
14	(Stathmin)			
15	Ras GTPase-activating protein-binding protein 1	108	52221	K.SpSSPAPADIAQTVQEDLR.T
16	Stathmin	73	17302	K.ESVPEFPLpSPPK.K R.ASGQAFELILpSPR.S
17	Septin-2	34	41715	K.IYHLPDAEpSDEDEDFKEQTR.L

[References]
1) Nature Methods., 4 (3), 231 (2007)

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