DIONEX

Application Note 531

Phosphopeptide Enrichment Using a TiO_2 Nano Precolumn

INTRODUCTION

Phosphorylation of proteins, which occurs at serine, threonine, and tyrosine residues, is the most studied and best characterized of all post-translational modifications (PTMs). This PTM plays an important role in signal transduction, metabolic control, and gene regulation. The abundance of these phosphorylated peptides in samples is usually low, making detection difficult.

Metal affinity trapping and enrichment using either Ga(III) or Fe(III) as a ligand in a column such as the ProPac® IMAC-10 has been developed into an efficient technique for phosphoprotein analysis.[1] Another way of capturing phosphopeptides from a peptide mixture is by titanium dioxide (TiO_2). This technique was proposed and developed by Pinkse and co-workers[2] and further optimized by Larsen and co-workers.[3] In this study the performance of titanium dioxide columns, packed with spherical 5 µm particles, is investigated. The phosphopeptides are captured on the TiO_2 precolumn, eluted using ammonium bicarbonate (NH_4HCO_3), concentrated using a reversed-phase (RP) nano precolumn, separated using a RP nanocolumn, and directed to the mass spectrometer for identification.

EXPERIMENTAL

The TiO_2 nano precolumn consists of spherical 5 µm TiO_2 particles packed to a depth of 1 cm in a 15 cm long fused silica capillary with a 200 µm i. d. (P/N 164215).

Test samples were two single synthetic phosphopeptides (sequence and mass in Table 1) and a BSA digest spiked with the same synthetic phosphopeptides.

All solvents and water were LC-MS grade.

Table 1. Phosphopeptide Sequence and Mass

Amino Acid Sequence	MW (Da)
SVENLPEAGIpTHEQR	1758.8
ENIMRpSENSESQLTSK	1931.8

CONDITIONS

LC Conditions

LC system:	UltiMate™ 3000 Intelligent LC system
Analytical Column:	Nanocolumn: 75 µm x 15 cm, Acclaim® PepMap™ C18 PM100, 3 µm, 100Å (P/N 160321)
Nano Precolumns:	TiO_2 nano precolumn: 200 µm i.d. x 1 cm, packed with titanium dioxide, 5 µm particles (P/N 164215)
	RP nano precolumn: 100 µm i.d. x 1 cm, packed with Acclaim PepMap C18 PM100, 5 µm, 100Å (P/N 164197)
Mobile phases:	A: 0.05% trifluoroacetic acid (TFA) in water
	B: acetonitrile/water (80:20 v/v), 0.04% TFA
Loading solvents:	1: 0.05% TFA in water
	2: 0.05% heptafluorobutyric acid (HFBA) in water
Wash solvents:	1: acetonitrile/water (80:20 v/v), 0.1% HFBA
	2: acetonitrile/water (80:20 v/v), 0.1% HFBA, 2 mg/mL dihydroxybenzoic acid (DHB)
TiO_2 trap eluent:	250 mM NH_4HCO_3 in water titrated to pH 9.0 with NH_4OH
Gradient:	3-40% acetonitrile in 30 min
Loading flow:	8 µL/min
Flow-rate:	300 nL/min
Samples:	Single phosphorylated synthetic peptides
	BSA digest spiked with synthetic phosphopeptides
UV detector:	214 nm, 3 nL flow cell

MS Conditions

Mass spectrometer:	HCTultra™ (Bruker Daltonics)
ESI-MS:	Positive ion mode, mass range 200-2000 *m/z*

RESULTS & DISCUSSION

For this application two switching valves are required. The Flow Manager (FLM-3100) of the UltiMate 3000 system is equipped with two valves as shown in Figure 1. The TiO_2 column for phosphopeptide capture is connected to the right valve. This is the same position used for an ion-exchange (IEX) column in a two-dimensional liquid chromatography (2-D LC) experiment. The RP nano precolumn and separation column are connected to the left valve. The procedure for separating phosphopeptides from a peptide mixture is similar to an IEX-RP peptide separation. A part of the sample (i.e., the phosphorylated components) is captured by the TiO_2 phase based on the affinity of titanium for phosphate groups. In the case of 2-D LC with TiO_2 columns, there are only two LC runs necessary for complete sample analysis: sample loading and elution of the bound fraction. Phosphopeptides are eluted from the TiO_2 column with a 20 µL plug of basic solution.

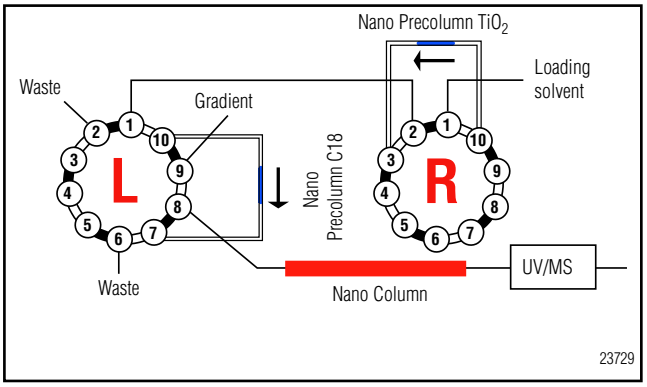

Figure 1. Experimental TiO_2 and RP preconcentration setup for phosphopeptide enrichment (FLM-3100).

Table 2 lists the valve switching program for Figure 1. In the first LC run the sample is injected onto the TiO_2 column and phosphopeptides are captured by the TiO_2 phase. The breakthrough consisting of non-phosphorylated peptides is trapped on the RP nano precolumn (100 µm x 1 cm) and eluted according to the gradient shown in the table. The second run can be an additional wash or direct elution of the phosphopeptides from the TiO_2 column by injecting a 20 µL plug of NH_4HCO_3, pH 9.0. After preconcentration, a gradient separation similar to the one applied to the non-phosphorylated peptides is performed. Both the TiO_2 column and the nano precolumn are loaded and eluted only in forward flush mode.

Table 2. Loading and Elution Conditions						
Time	%B[a]	Valve RP	Valve TiO_2	Flow Rate (µL/min)		Event
				Loading	Micro	
0.0	4	10_1	10_1	10	0.3	Elute phosphopeptides from TiO_2. Concentrate on C18 Precolumn.
6.0	4	10_1	1_2	10	0.3	Begin gradient separation.
12.0	13	1_2	1_2	10	0.3	
36.0	50	1_2	1_2	10	0.3	Complete gradient.
36.1	90	1_2	1_2	10	0.3	Clean columns and precolumns
42.0	90	1_2	1_2	10	0.3	
42.1	4	1_2	1_2	10	0.3	
57.0	4	10_1	10_1	10	0.3	Regenerate columns.
65.0	4	10_1	10_1	10	0.3	

[a]*Mobile phase B is acetonitrile/water 80:20 v/v. Thus 4% B is approximately 3% acetonitrile and 50% B is 40% acetonitrile.*

PHOSPHOPEPTIDE CAPTURE ON A TiO_2 NANO PRECOLUMN

A standard composed of 10 ng of a phosphopeptide with known sequence was used to determine the binding ability of the TiO_2 nano precolumn. The phosphopeptide was loaded onto the TiO_2 precolumn using 0.05% TFA, and the loading solution was analyzed. The chromatogram of the TFA (Figure 2A) showed no breakthrough, confirming the ability of the TiO_2 to trap this peptide. Figure 2B shows the successful separation and detection of the phosphorylated peptide after elution from the TiO_2 phase using a 20 µL plug of 250 mM NH_4HCO_3, pH 9.0.

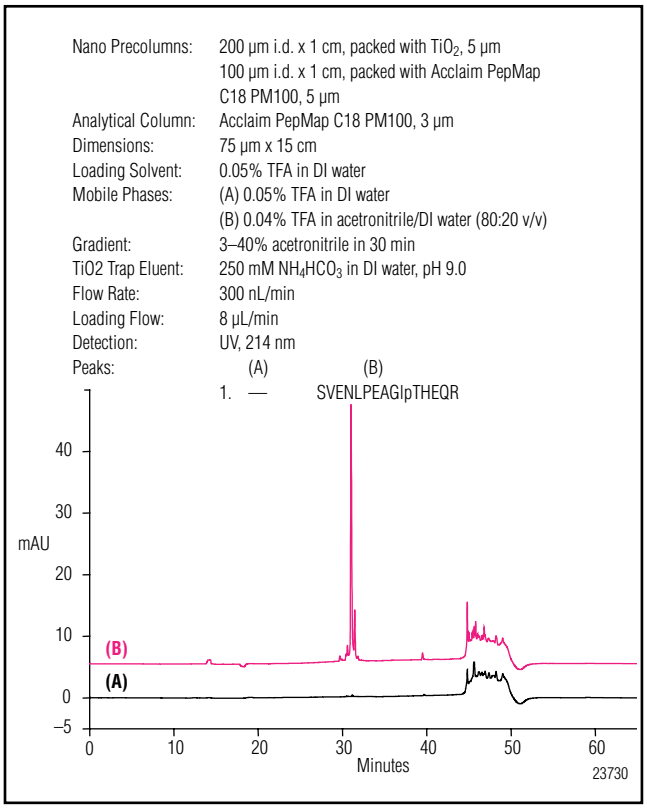

Figure 2. Capture of phosphopeptide SVENLPEAGlpTHEQR on a TiO_2 column. Gradient: 3–40% acetonitrile in 30 min. (A) Chromatogram of loading solution (B) Chromatogram of phosphopeptide eluted after NH_4HCO_3 plug.

ENRICHMENT OF PHOSPHOPEPTIDES FROM A COMPLEX DIGEST BY TiO_2 CAPTURE

To demonstrate phosphopeptide enrichment from a complex peptide mixture, a tryptic digest of the non-phosphorylated protein BSA, spiked with synthetic phosphopeptides, was loaded onto the TiO_2 precolumn. Each synthetic peptide contained one phosphate group. One of the peptides was phosphorylated at threonine, the other at serine (Table 1).

Previous studies have shown that the TiO_2 phase not only binds phosphorylated peptides, but also other acidic peptides. These elute from the TiO_2 at high pH together with the phosphopeptides, making spectral analysis more difficult and sometimes suppressing ionization of the phosphopeptide. Pinkse et al.[2] and Larsen et al.[3] demonstrated that the use of strong acids during loading of the TiO_2 column, followed by an acidic wash, is very effective in the removal of non-specific bound peptides. Acetic acid, trifluoroacetic acid (TFA), and dihydroxybenzoic acid (DHB) have been shown to assist with the removal of non-phosphorylated peptides. In this study, heptafluorobutyric acid (HFBA) was used for loading and washing the TiO_2 precolumn.

The breakthrough, composed of peptides without affinity for the TiO_2 phase, was eluted and separated on the analytical nanocolumn. The chromatogram of the breakthrough, Figure 3A, demonstrates the effectiveness

of HFBA in the removal of BSA peptides. A 20 µL plug of 250 mM NH_4HCO_3, pH 9.0 was then used to elute the bound fraction, which was separated using the same gradient (Figure 3B).

An additional wash with 20 mg/mL DHB, 80% acetonitrile, 20% DI water, and 0.1% HFBA, performed after loading, further aided the removal of acidic peptides from the TiO_2 precolumn (data not shown). This confirms the finding of Larsen et al.[3]

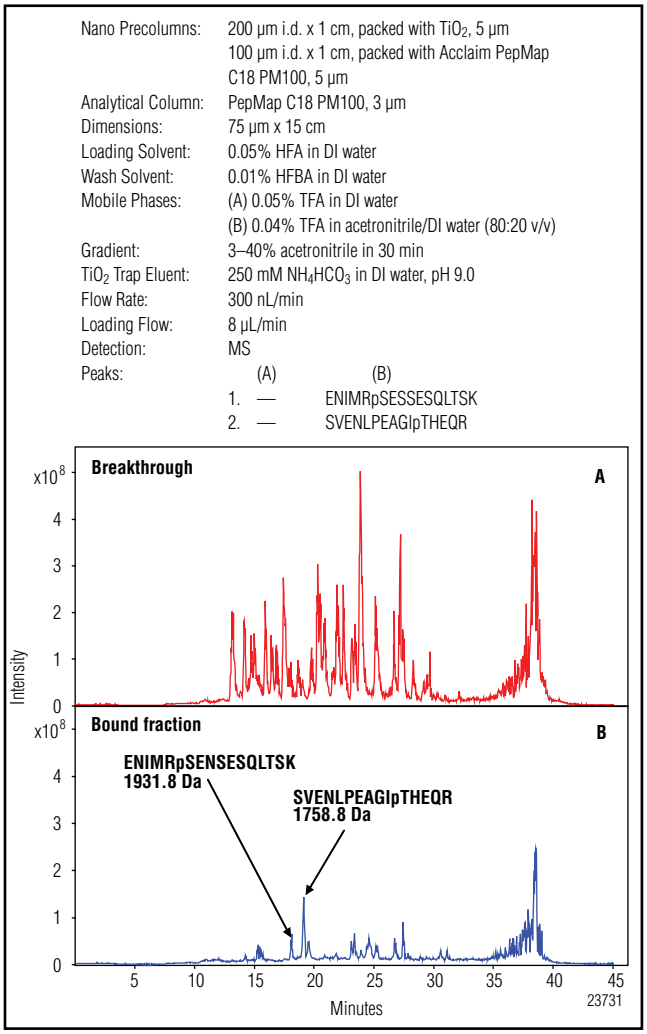

Figure 3. Isolation of two synthethic phosphopeptides (SVENLPEAGlpTHEQR and ENIMRpSENSESQLTSK) from a BSA tryptic digest, on a TiO_2 column. The loading solvent was 0.05% aqueous HFBA. Gradient: 3–40% acetonitrile in 30 min. Sample amount: 180 ng BSA digest containing 5 ng phosphopeptides. (A) Breakthrough of most of the BSA peptides after loading. (B) Release of the bound fraction containing the phosphorylated peptides and several acidic peptides from BSA, upon application of 20 µL NH_4HCO_3, pH 9.0.

Peptides resulting from both separations were analyzed on-line with a Bruker HCT/Ultra ion-trap mass spectrometer. ESI-MS/MS and MS(3) spectra were recorded. As generally occurs with labile post-translational modifications, the MS/MS fragmentation of phosphorylated peptides tends to first remove the phosphate group, leaving the amino acid backbone nearly intact. This process takes place because the energy needed to break the phosphate bond is much lower than that needed for an amide bond. Therefore, if peptide identification is desired, a higher order of fragmentation of the peptide is necessary. MS(3) spectra of phosphorylated peptides were selectively recorded based on the neutral loss of phosphoric acid (98, 49 and 32.6) for singly, doubly, and triply charged ions, respectively, which is characteristic for phosphopeptides.

In the example below the Fragmentation Only option of the Auto MS(3) algorithm was employed (Esquire control software, Bruker Daltonics, version 6.1). This option allows the acquisition of MS(3) spectra without isolation of the ions showing neutral loss of phosphoric acid. The resulting MS(3) fragments are added to the existing MS(2) fragments, increasing sensitivity and offering a more complete fragmentation spectrum of the peptide.

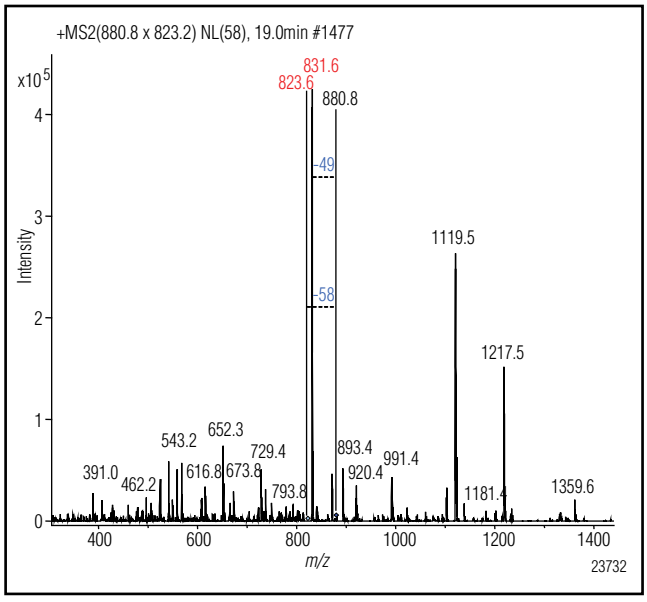

Figure 4. Fragmentation Only [MS(3) without isolation] of 5 ng of phosphopeptides SVENLPEAGlpTHEQR with mass 1758.7938 Da. The mass/charge ratio of the parent ion being fragmented is 880.8^{2+}. The detected neutral loss of 49 (phosphate) and 58 (phosphate and water) is indicated.

CONCLUSIONS

1. The newly designed TiO_2 nano precolumns successfully capture phosphorylated peptides.
2. In addition to phosphorylated peptides, TiO_2 also binds other acidic peptides, which are difficult to wash off the TiO_2 column. HFBA added to the loading solvent helps in removal of non-phosphorylated, acidic peptides.
3. Phosphopeptide enrichment on the TiO_2 column is easily performed in a 2-D LC-like set-up, with very low dead volume.

REFERENCES

1. Dionex Corporation. Automated Enrichment and Determination of Phosphopeptides Using Immobilized Metal Affinity and Reversed-Phase Chromatography with Column Switching. Technical Note 705; LPN 1763. Sunnyvale, CA, 2006.
2. Pinkse, M. W. H.; Uitto, P. M.; Hilhorst, M. J.; Ooms, B.; Heck, A. J. R. Selective Isolation at the Femto-mole Level of Phosphopeptides from Proteolytic Digests Using 2D-NanoLC-ESI-MS/MS and Titanium Oxide Precolumns. *Anal. Chem.* **2006**, 76, 3935-3943.
3. Larsen, M. R.; Thingholm, T.E.; Jensen, O.N.; Roepstorff, P.; Jorgensen, T. J. D. Highly Selective Enrichment of Phosphorylated Peptides from Peptide Mixtures Using Titanium Dioxide Microcolumns. *Mol. Cell. Proteomics* **2005**, 4, 873-886.

Passion. Power. Productivity.

DIONEX

Dionex Corporation
1228 Titan Way
P.O. Box 3603
Sunnyvale, CA
94088-3603
(408) 737-0700

North America
U.S. (847) 295-7500
Canada (905) 844-9650

South America
Brazil (55) 11 3731 5140

Europe
Austria (43) 1 616 51 25 Benelux (31) 20 683 9768 (32) 3 353 4294
Denmark (45) 36 36 90 90 France (33) 1 39 30 01 10 Germany (49) 6126 991 0
Ireland (353) 1 644 0064 Italy (39) 02 51 62 1267 Switzerland (41) 62 205 9966
United Kingdom (44) 1276 691722

Asia Pacific
Australia (61) 2 9420 5233 China (852) 2428 3282 India (91) 22 28475235
Japan (81) 6 6885 1213 Korea (82) 2 2653 2580 Singapore (65) 6289 1190

www.dionex.com

ISO 9001 REGISTERED

LPN 1898 PDF 04/07