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A Multidimensional System for Phosphopeptide Analysis Using TiO_2 Enrichment and Ion-exchange Chromatography with Mass Spectrometry

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Although offline enrichment of phosphorylated peptides is widely used, enrichment for phosphopeptides using TiO_2 is often performed manually, which is labor-intensive and can lead to irreproducible results. To address the problems associated with offline enrichment and to improve the effectiveness of phosphopeptide detection, we developed an automated online enrichment system for phosphopeptide analysis. A standard protein mixture comprising BSA, fetuin, crystalline, α-casein and β-casein, and ovalbumin was assessed using our new system. Our multidimensional system has four main parts: a sample pump, a 20-mm TiO_2-based column, a weak anion-exchange, and a strong cation-exchange (2:1 WAX:SCX) separation column with LC/MS. Phosphorylated peptides were successfully detected using the TiO_2-based online system with little interference from nonphosphorylated peptides. Our results confirmed that our online enrichment system is a simple and efficient method for detecting phosphorylated peptides.

Key Words : Phosphorylated peptides, TiO_2, Online enrichment, Strong cation-exchange, Weak anion-exchange

Introduction

Posttranslational modification (PTM) of proteins entails a change in chemical composition by proteolytic cleavage or addition of functional groups at certain amino acid residues. A change in protein composition or structure often leads to an alteration in function of the protein.[1] One of the most important PTMs in biological systems is phosphorylation, in which a phosphate group is attached to proteins or other molecules. Phosphorylation is often a switch for protein activities or cellular pathways.[2-4] In mammalian cells, more than 30% of proteins at any given time point are thought to be phosphorylated. A change in protein composition or structure often leads to an alteration in protein function. Abnormally phosphorylated proteins have been associated with the pathology of a variety of disease states including cancer, diabetes, and Alzheimer's disease.[5-8] Although numerous studies have identified the involvement of phosphorylation in biological pathways or processes,[9,10] the analysis of phosphorylation as a PTM is still a formidable biological and technical challenge for several reasons, including structural homogeneity and low relative abundance.[9-11]

The low abundance of phosphorylated proteins is particularly troublesome for proteomics. To overcome the low abundance of phosphorylated proteins, several enrichment strategies have been developed, including immobilized metal affinity chromatography,[11,12] chemical derivatization of phosphorylated residues,[13-18] metal oxide surfaces,[19-21] and strong cation-exchange chromatography.[22,23] Enriched phosphorylated peptides are generally identified using conventional mass spectrometry (MS)-based analysis methods such as surface-assisted laser desorption/ionization mass spectrometry (SALDI-MS),[24] MALDI-MS,[25-29] ESI-MS,[30] and ECD-MS/MS.[31,32]

Among these phosphorylated peptide enrichment strategies, several studies have found that titanium dioxide TiO_2-based enrichment has the highest efficiency.[33,34] However, a TiO_2-based offline enrichment strategy has several drawbacks. An offline method involves several fractionation steps of the eluted sample, each of which is followed by a tryptic digest. Therefore, it is hard to avoid the loss of peptides, and the procedure is very time-consuming. In other words, this lengthy procedure has low efficiency and requires well-trained personnel. In this study, we developed and optimized a method for TiO_2 online enrichment for the detection of phosphorylated peptides. To develop the system, we systematically combined a TiO_2 enrichment column, a strong cation-exchange (SCX) column, and a weak anion-exchange (WAX) column. For effective characterization of low abundant phosphorylated peptide mixtures, mass spectrometry was performed using nanoLC/LTQ-FTMS.

Materials and Methods

Materials. α-Casein, β-casein, bovine serum albumin (BSA), crystalline, fetuin, ovalbumin, ammonium acetate, and formic acid were obtained from Sigma (Seoul, Korea). TiO_2 was provided by GL Sciences (Tokyo, Japan). SCX and WAX were obtained from PolyLC Incorporated (Columbia, MD, USA). For enzymatic digestions, modified

porcine trypsin from Promega (Madison, WI, USA) was used. Water was purified with a Milli-Q system (Millipore, Bedford, MA, USA).

Preparation of TiO_2 and SCX/WAX Packed Capillary Columns. Fused-silica capillary TiO_2 columns (250-μm i.d.) and biphasic trapping columns (250-μm i.d., SCX/WAX, 1:1, 1:2, 2:1 ratios) were prepared by slurry packing using an in-house pressure vessel driven by He gas. To optimize enrichment conditions, TiO_2 packed columns of three different lengths (10, 20, and 30 mm) were prepared. The biphasic columns contained 2.0 cm of a mixture of 5-μm Partisphere SCX and PolyWAX LP (PolyLC Inc., Columbia, MD). A modified Kasil frit preparation method was used to prepare a fritted filter at the end of the TiO_2 and biphasic capillary columns to retain resins in the capillary. The on-line liquid chromatography setup consisted of six main parts: a sample pump, auto sampler, TiO_2 column, six-port switching valve, separation column, and LC pump (Surveyor, Thermo Electron, Bremen, Germany).

In-Solution Digestion of Protein Standards. Standard peptides were prepared by digestion of a protein standard mix that comprised 100 μg each of α-casein, β-casein, crystalline, fetuin, ovalbumin, and BSA. Proteins were reduced in NH_4HCO_3 (50 mM) containing dithiothreitol (10 mM) at 55°C for 30 min and carboxymethylated with 40 mM iodoacetamide at room temperature for 25 min in the dark. Tryptic digestion was performed by addition of 10 μg/mL trypsin in NH_4HCO_3 (50 mM) containing $CaCl_2$ (5 mM) at 37 °C for 12-16 hours, followed by lyophilization.

Mass Spectrometry. Lyophilized peptide mixtures were solubilized in 0.1% formic acid and loaded onto a micro-capillary column packed with capillary TiO_2 columns (250-μm i.d. 2 cm in length) and biphasic trapping columns (250-μm i.d., SCX/WAX, 1:1, 1:2, 2:1 ratios, 2 cm in length). The column was equilibrated with 5% buffer B for 15 min before the next run, when 20 μL of washing solution (50% acetonitrile containing 2% formic acid) was injected, followed by elution of phosphorylated peptides by injection of 20 μL of 25% ammonia solution (pH 11). Peptide elution was performed by gradient elution with buffers A (0.1% formic acid) and B (80% acetonitrile containing 0.1% formic acid). Peptides were eluted with 5% buffer B for 25 min, 20% for 5 min, 60% for 50 min, and 100% for 5 min at a flow rate of 300 nL/min. In the next 2-7 steps, 10 μL of salt (25, 50, 125, 250, and 500 mM ammonium acetate, respectively) was injected onto an autosampler by a gradient method as described for step 1. For identification, eluted peptides were subjected to MS analysis using a 7-Tesla Finnigan LTQ-FT MS spectrometer (Thermo Electron, Bremen, Germany) equipped with a nano-ESI source in positive ion mode at a spray voltage of 2.5 kV. Spectra were analyzed with Mascot Daemon version 2.0 (Matrix Science, London, UK) using the SwissProt database. Peptides were identified at a peptide tolerance of ± 50 ppm, fragment mass tolerance of ± 0.8 Da, two missed trypsin cleavages, oxidation of Met, and fixed modification of carbamidomethyl cysteine. The peptide score was $-10 \times \mathrm{Log}(P)$, where P signifies the probability that the observed match was a random event.

Results and Discussion

Our goal in this study was to determine the optimal online LC-MS conditions for our phosphorylated peptide detection system that comprised TiO_2 enrichment, WAX, and SCX columns. The experimental configuration of our system is described schematically in Figure 1. Phosphorylated peptides were obtained from tryptic digestion of a protein standard mix (α-casein, β-casein, crystalline, fetuin, ovalbumin, and BSA) containing a total of 60 pmol of protein (total 2.2 μg). BSA was used as a positive control because phosphorylated peptides are not generated from BSA, and because tryptically digested BSA has a general peptide sequence. Phosphorylated peptides were concentrated on a TiO_2 online enrichment column followed by enrichment on a WAX:SCX co-column. Phosphorylated peptides eluted through the WAX:SCX column were subjected to reverse-phase chromatography/MS analysis.

Optimization of the Length of the TiO_2 Column. We first optimized the length of the TiO_2 column, which has been shown to be the most effective parameter to adjust for enrichment of phosphorylated peptides. Phosphorylated peptides have to be enriched because of their low abundance in biological materials.[35] Three different TiO_2 column lengths, (10, 20, and 30 mm) were evaluated at an injection flow rate of 5 μL/min. As shown in Table 1, use of a 10 mm TiO_2 packed column resulted in the identification of eight unique phosphorylated peptides, while use of a 20 mm column resulted in the identification of nine unique phosphorylated peptides, and a 30 mm column seven.

Pressure increases with increasing TiO_2 enrichment column length. Increasing pressure causes abnormal effects such as excessive binding to the TiO_2 spheres and degradation. More phosphopeptides were detected after the washing step when we used the 20 mm or 30 mm columns than the 10 mm column. This suggests that phosphorylated peptides might not be sufficiently trapped or eluted by a 10 mm enrichment column. Based on the above considerations, we chose to use a 20 mm enrichment column.

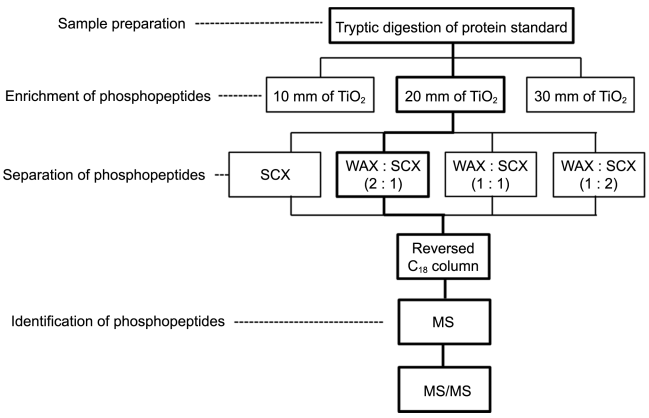

Figure 1. Experimental strategy for the detection of phosphorylated peptides.

Table 1. (a) Optimization of TiO_2 enrichment and (b) WAX/SCX separation for the identification of phosphorylated peptides

(a)

TiO_2 enrichment of phosphorylated peptides			
Length of TiO_2 enrichment column[a]	Average number of eluted phosphopeptides[b] (average ion score)[c]	Average number of eluted non-phosphopeptides	Ratio of phosphopeptides /non-phosphopeptides
10 mm	7 (64)	64	0.109
20 mm	8 (65)	58	0.138
30 mm	6 (61)	54	0.111

(b)

WAX/SCX separation of phosphorylated peptides			
Packing material	Average number of identified phosphopeptides[b] (average of ion score)[c]	Average number of eluted non-phosphopeptides	Ratio of phosphopeptides /non-phosphopeptides
SCX	5 (60)	49	0.102
WAX/SCX (2:1)	7 (59)	53	0.132
WAX/SCX (1:1)	6 (61)	53	0.113
WAX/SCX (1:2)	5 (62)	46	0.108

[a]Enrichment column was packed with TiO_2. [b]Elution solution was 25% ammonia solution (pH = 11). [c]Peptide ion score is $-10 \times \text{Log (P)}$, where P signifies the probability that the observed match is a random event. Phosphorylated peptides were generated from protein standard mix of α-casein, β-casein, and BSA. All protein standards were used at a final concentration of total 1.2 μg.

Optimization of the Ratio of WAX:SCX Packing Materials. We next optimized the ratio of WAX:SCX packing materials. In general, positively charged peptides bind well to SCX packing material under acidic conditions when the mobile phase contains an organic solvent.[36] However, for positively charged peptides that have poor retention on the SCX material,[37] complementary WAX material was used in series with SCX material (see Figure 1). To evaluate the WAX:SCX packing ratio, we compared the performance of 2:1, 1:1, and 1:2 WAX:SCX packing material ratios (Table 2). Although the differences among the three examined ratios were not significant, the 2:1 WAX:SCX packing material appeared to be most efficient for detection of phosphorylated peptides (eight phosphopeptides were identified). This result can be explained by the Donnan effect that is generated inside as column by a pair of opposite fixed charges. This is responsible for improved peptide recovery resulting from higher fluxes of salt cations and a lower population of salt anions proximate to the SCX resin. An improved WAX/SCX (2:1) packing ratio was achieved by combining increased retention of acidic peptides with moderately reduced retention of neutral-to-basic peptides by the added anion-exchange resin.[38] Subsequently, we used a 2:1 ratio of WAX:SCX for further optimization experiments.

Table 2. Phosphopeptide analysis using the TiO_2-WAX/SCX (2:1) system

Peptide no.	Sequence	No. of phosphorylation	Calculated mass, *m/z*	Observed mass, *m/z* (charge state)	Ion Score
1	NMAINPpSKENLCSTFCK (CAS. 40-56)	1	2092.8781	2092.8758 (1047.4452, +2)	73.96
2	YKVPQLEIVPNpSAEER (CAS. 119-134)	1	1950.9451	1950.9468 (976.4807, +2)	69.66
3	VPQLEIVPNpSAEER (CAS. 121-134)	1	1659.7869	1659.7894 (830.9020, +2)	56.05
4	KTVDMEpSTEVFTK (CAS. 152-164)	1	1593.6997	693.7011 (797.8578, +2)	75.60
5	TVDMEpSTEVFTK (CAS. 153-164)	1	1465.6047	1465.6076 (733.8111, +2)	67.39
6	TVDMEpSTEVFTKK (CAS. 153-165)	1	1593.6997	1593.7009 (797.8577, +2)	63.50
7	FQpSEEQQQTEDELQDK (CASB. 48-63)	1	2060.8212	2060.8175 (1031.4160, +2)	56.82
8	FDKLPGFGDpSIEAQCGTSVNVHSSLR (Ov. 60-85)	1	2900.3163	2900.3164 (967.7794, +3)	53.70
9	LPGFGDpSIEAQCGTSVNVHSSLR (Ov. 63-85)	1	2510.1260	2510.1289 (837.7169, +3)	70.25
10	EVVGpSAEAGVDAASVSEEFR (Ov. 341-360)	1	2087.9047	2087.9095 (1044.9620, +2)	94.58
11	RPFFPFHpSPpSR (CRY. 12-22)	2	1453.6656	1453.6659 (485.5626, +3)	48.88
12	LPpSNVDQSALSCSLSADGMLpTFSGPK (CRY. 120-145)	2	2761.2339	2761.2356 (921.4191, +3)	45.27
13	HTFpSGVASVEpSSSGEAFHVGK (FET. 313-333)	2	2198.9633	2198.9639 (733.9952, +3)	38.19

CAS, α-casein; CASB, β-casein; Ov, ovalbumin; CRY, crystalline; FET, fetuin. Phosphorylated peptides were generated from a protein standard mix of α-casein, β-casein, crystalline, fetuin, ovalbumin, and BSA. All protein standards were used at a final concentration of 60 pm (total 2.2 μg).

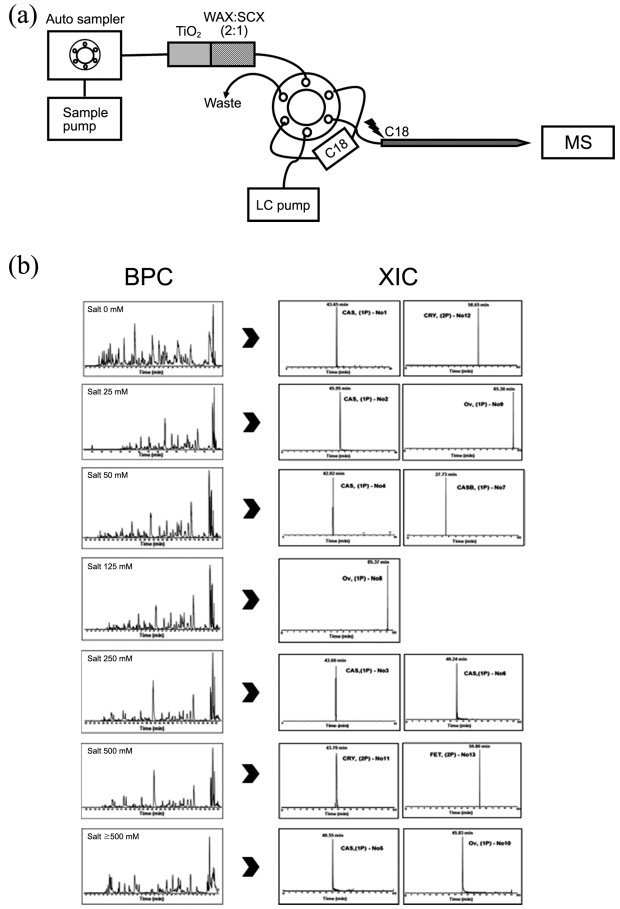

Figure 2. (a) Schematic diagram of the optimized online enrichment system for detection of phosphorylated peptides and (b) base peak chromatogram (BPC) and extracted ion chromatogram (XIC) of phosphopeptides obtained using the TiO_2-WAX/SCX (2:1) system. Designated numbers in chromatograms are the same as in Table 2. CAS, α-casein; CASB, β-casein; Ov, ovalbumin; CRY, crystalline; FET, fetuin.

Optimization of the Ion-Exchange Buffer Concentration for Separation of Phosphopeptides. Next, the concentration of the ion-exchange buffer was optimized using six different concentrations of ammonium acetate by eluting through the WAX:SCX (2:1) packed column. The number of phosphopeptides identified using different concentrations of ammonium acetate is shown in Figure 3. The number of detected phosphorylated peptides changed only slightly when the concentration of ammonium acetate was increased up to 500 mM. This clearly indicated that single-step washing was not sufficient and that a multistep washing strategy was needed for more efficient identification of phosphorylated peptides.

An online enriching column packed with TiO_2 was checked to confirm performance, as this is not a conventional method. Enriched phosphorylated peptides should not flow out of the column while they are being enriched by passing through TiO_2. Various concentrations of ammonium acetate were injected into the 20 mm TiO_2 column after a test sample was passed through. The injected salt volume was 20 μL. The test sample was a mixed solution of α-casein, β-casein, crystalline, fetuin, ovalbumin, and BSA. As can be seen in Supplementary Table 1, some phosphorylated peptides that eluted at 50 mM ammonium acetate were obtained without elution buffer (25% ammonia solution, pH 11). The identified phosphopeptides gradually decreased as 500 mM of salt was injected. However, after injection of TiO_2 elution buffer and several ammonium acetate salt steps, more than 10 kinds of phosphorylated peptides were identified. Therefore, we concluded that this was the most efficient combination in the elution system.

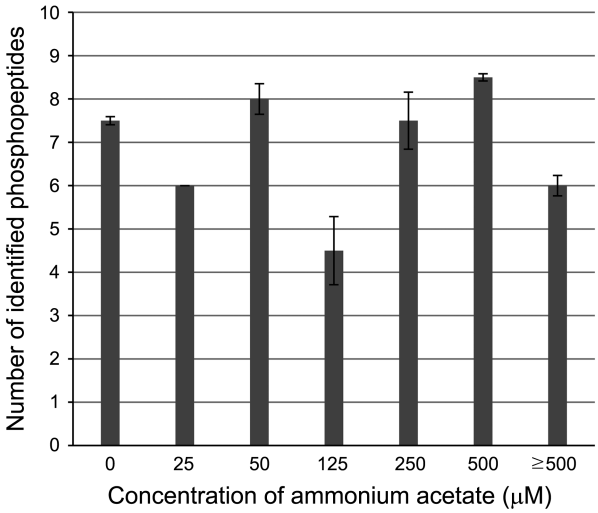

Figure 3. Number of phosphopeptides identified using WAX/SCX (2:1) at different concentrations of ammonium acetate.

Optimization of the Order of TiO_2 and the WAX:SCX Column. In this optimization study, we focused on optimizing several experimental parameters: the order of the TiO_2 and WAX:SCX columns, the combination ratio of WAX:SCX packing material, the ion-exchange buffer concentration, and the length the TiO_2 enrichment column. We examined the order of TiO_2 and WAX:SCX columns that was best for efficient identification of phosphorylated peptides. Two different setups were used: in the first, the order was TiO_2 (20 mm column) followed by WAX:SCX (2:1) columns, and in the second, the order was WAX:SCX (2:1) and TiO_2 (20 mm column). Table 2 shows the list of phosphorylated peptides identified with the optimized methods. Overall, using the TiO_2 column first followed by the WAX:SCX columns yielded a greater variety of phosphorylated peptides (Supplementary Table 2). Therefore, we used this order for all subsequent experiments (Fig. 2).

In conclusion, we developed a new analytical online titanium dioxide-based enrichment for the detection of phosphorylated peptides. In addition, we improved prefractionation and the specific enrichment of phosphorylated peptides to reduce sample complexity and enhance the accessible dynamic range.[39] Our system comprises a WAX:SCX (2:1) packed column and a TiO_2-packed column in an online configuration. This strategy is advantageous as it is easy to control and provides good efficiency in enriching phosphorylated peptides. This system has the potential to be

a powerful tool for phosphopeptide analysis of real biological samples.

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