Phosphopeptide Enrichment by Aliphatic Hydroxy Acid-modified Metal Oxide Chromatography for Nano-LC-MS/MS in Proteomics Applications*§

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We developed novel methods for phosphopeptide enrichment using aliphatic hydroxy acid-modified metal oxide chromatography (MOC). Titania and zirconia were successfully applied to enrich phosphopeptides with the aid of aliphatic hydroxy acids, such as lactic acid and β-hydroxypropanoic acid, to reduce the interaction between acidic non-phosphopeptides and the metal oxides. These methods removed the vast majority of non-phosphopeptides from phosphoprotein standard digests, and large numbers of phosphopeptides could be readily identified. The methods were coupled with nano-LC-MS/MS systems without difficulty. Recovery of phosphopeptides in MOC varied greatly from peptide to peptide, ranging from a few percent to 100%, and the average was almost 50%. Repeatability and linearity were satisfactory. In an examination of the cytoplasmic fraction of HeLa cells, more than 1000 phosphopeptides were identified using lactic acid-modified titania MOC and β-hydroxypropanoic acid-modified zirconia MOC, respectively. The overlap between phosphopeptides enriched by these two methods was 40%, and the combined results provided 1646 unique phosphopeptides. To our knowledge, this is the first successful application of a single MOC-based approach to phosphopeptide enrichment from complex biological samples such as cell lysates. Molecular & Cellular Proteomics 6:1103–1109, 2007.

Phosphorylation is a key event in cellular signaling networks (1), and an understanding of proteome-wide phosphorylation/dephosphorylation dynamics is important not only in relation to particular signal transduction pathways but also to obtain an overview of the whole network. MS is currently one of the most powerful techniques for proteome-wide experiments (2), but further improvements in protein identification efficiency are still needed (3). One of the key requirements for phosphoproteome analysis is to remove abundant non-phosphopeptides from complex mixtures, such as cell lysates, to detect low abundance phosphopeptides. Several approaches to enrich phosphopeptides prior to MS analysis, coupled with phosphate-specific MS acquisition techniques, have been reported. For phosphotyrosine-containing proteins, antibodies such as 4G10 and pY100 work adequately at both the protein and peptide levels (4, 5). In addition, precursor ion scanning specific to phosphotyrosine has been applied to filter out non-phosphopeptides (4). On the other hand, a similar approach has not been effective for phosphoserine- and phosphothreonine-containing proteins, although it has helped to identify new players in the signaling pathway in some cases (6). As more general approaches for phosphopeptide enrichment, IMAC (7–9), IMAC with methyl esterification (10, 11), strong cation exchange chromatography (12, 13), and metal oxide chromatography (MOC)† using titania (14–18), zirconia (19), and alumina (20) have been reported. In addition, precursor ion scanning in the negative mode (21), neutral loss-triggered MS3 (22, 23), pseudo-MS3 (24), and electron transfer dissociation (25) have been developed to detect phosphopeptides selectively. By combining these methods, it is currently possible to identify hundreds or thousands of phosphopeptides (8, 10, 12, 13, 22, 23, 26). Nevertheless current phosphopeptide enrichment methods are still not sufficiently specific (9), and samples generally contain large amounts of non-phosphopeptides even after enrichment. This causes ambiguity in peptide identification, although high accuracy measurement of precursor ions helps to reduce false positives (22). The situation at present is that improvement of phosphopeptide enrichment processes will lead directly to higher quality phosphoproteome analysis.

Titania was first applied as a chemoaffinity medium for organophosphates including phosphopeptides by Ikeguchi and Nakamura (14) and was subsequently made commercially.

† The abbreviations used are: MOC, metal oxide chromatography; HPA, β-hydroxypropanoic acid; DHB, 2,5-dihydroxybenzoic acid.

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available for proteomics applications (15–17). Zirconia also has affinity for phosphate (27) and has been used for phos-
phopeptide enrichment as well (19). Larsen et al. (18) showed that inclusion of o-hydroxybenzoic acid and its deriv-
atives, such as 2,5-dihydroxybenzoic acid (DHB), in the sample loading buffer was effective to remove acidic non-phosphope-
tides during phosphopeptide enrichment with titania for MALDI-MS analysis. However, this protocol is not directly applicable to LC-MS/MS analysis because residual DHB interferes with peptide detection, and the system becomes unstable because of precipitation of DHB around the orifice and in the LC system.

We developed new approaches for phosphopeptide enrichment using MOC modified with aliphatic hydroxy acids. The conditions were optimized for phosphopeptide enrichment from cell lysates followed by LC-MS/MS analysis to identify phosphopeptides.

**EXPERIMENTAL PROCEDURES**

**Materials**—Titania (titanium dioxide; particle size, 10 μm) was ob-
tained from GL Sciences (Tokyo, Japan). Zirconia (zirconium dioxide; particle size, 10 μm) was from ZirChrom Separations (Anoka, MN). Alumina for TLC (catalog no. 1.01702.0500) was purchased from Merck, Al(OH)₃·H₂O, bovine α-casein, bovine fetuin, chicken phos-
vitin, and DHB were from Sigma. β-Hydroxypropanoic acid (HPA) was obtained from Tokyo Kasei (Tokyo, Japan). C₈ and C₁₈ Empore disks were from 3M (St. Paul, MN). Modified trypsin was from Promega (Madison, WI). Water was obtained from a Millipore Milli-Q system (Bedford, MA). MS-grade Lys-C, boehmite, and all other chemicals

**Digestion of Standard Phosphoproteins**—α-Casein, fetuin, and phosvitin were individually reduced with DTT, alkylated with iodoac-
etamide, and digested with Lys-C followed by dilution and trypsin digestion as described previously (28). These digested samples were desalted using StageTips with C₁₈ Empore disk membranes (29). The eluates were mixed, and the peptide concentration was adjusted to 0.5 mg/ml with 0.1% TFA, 80% acetonitrile.

**Enrichment of Phosphopeptides from Standard Phosphoprotein Digests**—Custom-made MOC tips were prepared using C₈ StageTips and metal oxide bulk beads (3 mg of beads/200-μl pipette tip) as described for strong cation exchange (beads)-C₁₈ tips (30). Prior to loading samples, the MOC tips were equilibrated with 0.1% TFA, 80% acetonitrile with hydroxy acids as selectivity enhancers (solution A). As the enhancers, glycolic acid, lactic acid, malic acid, tartaric acid, and DHB were used at a concentration of 300 mg/ml, and HPA was used at 100 mg/ml. The digested standard phosphopeptide mixture (15 μl) was mixed with 100 μl of solution A and loaded on the MOC tip. After successive washing with solution A and solution B (0.1% TFA, 80% acetonitrile), 0.5% ammonium hydroxide was used for elution. The eluted fraction was acidified with TFA, desalted using C₁₈ StageTips as described above and concentrated in a vacuum evaporator followed by the addition of solution A for subsequent nano-
LC-MS/MS analysis.

**Analysis of HeLa Cell Cytoplasmic Fraction**—HeLa cells, cultured to 80% confluence in 15-cm diameter dishes, were homogenized with a Dounce homogenizer (10 strokes) after having been spiked with protein phosphatase inhibitor mixtures 1 and 2 (Sigma) and protease inhibitors (Sigma) in 100 mM Tris buffer (pH 8.0). The homogenate was centrifuged at 1500 × g for 10 min, and the supernatant was digested and desalted as shown above. Titania or zirconia MOC tips were preconditioned with 20 μl of solution A. The digested sample from a total of 200 μg of HeLa cytoplasmic proteins was diluted with 100 μl of solution A and loaded to the MOC tip. Washing was done with 20 μl each of solutions A and B, and then phosphopeptides were eluted with 20 μl of 0.5% ammonium hydroxide followed by acidification with 20 μl of 1% TFA. Subsequent procedures were the same as described for the standard phosphopeptides.

**Nanop-LC-MS System**—Nano-LC-MS/MS analyses were conducted by using a QSTAR system (QSTAR XL (AB/MDS-Sciex, Toronto, Canada), Agilent 1100 nanoflow pump (Waldbronn, Germany), and HTC-PAL autosampler (CTC Analytics, Zwingen, Switzerland) or an Orbitrap system (Finnigan LTQ-Orbitrap (Thermo Electron, Bremen, Germany), Dionex Ultimate3000 pump with FLM-3000 flow manager (Germering, Germany), and HTC-PAL autosampler). Repro-
Sil C₁₈ materials (3 μm, Dr. Maisch, Ammerbuch, Germany) were packed into a self-pulled needle (150-mm length × 100-μm inner diameter, 6-μm opening) with a nitrogen-pressurized column loader cell (Nikkyo Technos, Tokyo, Japan) to prepare an analytical column needle with “stone-arch” frit (31). A polytetrafluoroethylene-coated column holder (Nikkyo Technos) was mounted on an x-y-z nanospray interface (Nikkyo Technos), and a Valco metal connector with a magnet was used to hold the column needle and to set the appropriate spray position. The injection volume was 5 μl, and the flow rate was 500 nl/min. The mobile phases consisted of 0.5% acetic acid (A) and 0.5% acetic acid and 80% acetonitrile (B). A three-step linear gradient of 5−10% B in 5 min, 10−40% B in 60 min, 40−100% B in 5 min, and 100% B for 10 min was used throughout this study. A spray voltage of 2400 V was applied via the metal connector as described previ-
ously (31). The MS scan range was m/z 300−1500, and the top three (QSTAR) or top six (LTQ-Orbitrap) precursor ions were selected for subsequent MS/MS scans. A lock mass function was used for the LTQ-Orbitrap to obtain constant mass accuracy during gradient analysis (32).

**Database Searching**—Mass Navigator version 1.2 (Mitsui Knowledge Industry, Tokyo, Japan) was used to create peak lists on the basis of the recorded fragmentation spectra. All parameters used in this process are described in Supplemental Table S1. Peptides and proteins were identified by means of automated database searching using Mascot version 2.1 (Matrix Science, London, UK) against Uniprot/Swiss-Prot release 9.0 (October 31, 2006) with a precursor mass tolerance of 50 ppm (QSTAR) or 3 ppm (LTQ-Orbitrap), a fragment ion mass tolerance of 0.25 Da (QSTAR) or 0.8 Da (LTQ-Orbitrap), taxon-
omy of human, and strict trypsin specificity (33) allowing for up to two missed cleavages. Carbamidomethylation of cysteine was set as a fixed modification, and oxidation of methionines, and phosphorylation of serine, threonine, and tyrosine were allowed as variable modifica-
tions. Note that neutral loss products from both precursor and frag-
ment ions were considered for Mascot scoring in this phosphorylation modification setting, although no assignment was indicated for pre-
cursor-origin neutral loss peaks in the output results according to the supplier.

Peptides were considered identified if the Mascot score was over the 95% confidence limit based on the “identity” score of each peptide and at least three successive y- or b-ions with two and more y- or b-, and/or precursor-origin neutral loss ions were observed, based on an error-tolerant peptide sequence tag concept (34). A randomized decoy database created by a Mascot Perl program estimated a delta Mascot score of 5%. Peptides were considered identified if the Mascot score was over the 95% confidence limit based on the “identity” score of each peptide and at least three successive y- or b-ions with two and more y- or b-, and/or precursor-origin neutral loss ions were observed, based on an error-tolerant peptide sequence tag concept (34). A randomized decoy database created by a Mascot Perl program estimated a delta Mascot score of 5%. Peptides were considered identified if the Mascot score was over the 95% confidence limit based on the “identity” score of each peptide and at least three successive y- or b-ions with two and more y- or b-, and/or precursor-origin neutral loss ions were observed, based on an error-tolerant peptide sequence tag concept (34). A randomized decoy database created by a Mascot Perl program estimated a delta Mascot score of 5%.
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TABLE I

| MOC modifier | Identified peptides (p < 0.05) | No. of phosphopeptides | No. of non-phosphopeptides |
|--------------|--------------------------------|------------------------|---------------------------|
| Titania      |                                |                        |                           |
| None         | 8                              | 10                     |
| DHB          | 4                              | 3                      |
| Glycolic acid| 10                             | 11                     |
| Lactic acid  | 12                             | 0                      |
| Malic acid   | 6                              | 0                      |
| Tartaric acid| 8                              | 2                      |
| HPA          | 6                              | 0                      |
| Zirconia     |                                |                        |                           |
| None         | 9                              | 13                     |
| DHB          | 3                              | 3                      |
| Glycolic acid| 8                              | 4                      |
| Lactic acid  | 10                             | 1                      |
| Malic acid   | 6                              | 0                      |
| Tartaric acid| 5                              | 3                      |
| HPA          | 11                             | 0                      |
| Alumina      |                                |                        |                           |
| None         | 1                              | 17                     |
| DHB          | 0                              | 2                      |
| Glycolic acid| 0                              | 1                      |
| Lactic acid  | 0                              | 4                      |
| Malic acid   | 1                              | 4                      |
| HPA          | 1                              | 3                      |
| Al(OH)₃ₓH₂O |                                |                        |                           |
| None         | 8                              | 17                     |
| DHB          | 4                              | 8                      |
| Glycolic acid| 0                              | 3                      |
| Lactic acid  | 0                              | 4                      |
| Malic acid   | 0                              | 5                      |
| HPA          | 0                              | 7                      |
| Boehmite     |                                |                        |                           |
| None         | 0                              | 11                     |
| DHB          | 0                              | 6                      |
| Glycolic acid| 1                              | 8                      |
| Lactic acid  | 0                              | 7                      |
| Malic acid   | 0                              | 12                     |
| HPA          | 0                              | 10                     |

Identification of acidic peptides (8, 10, 18, 19). Without hydroxy acids, with 50–80% acetonitrile were effective to reduce the co-puri-

RESULTS AND DISCUSSION

Effect of Hydroxy Acids on Phosphopeptide Enrichment—A digested phosphoprotein standard mixture of α-casein, fetuin, and phosvitin was used to evaluate the effect of hydroxy acids in the loading buffer on selective enrichment of phosphopeptides with titania, zirconia, alumina, Al(OH)₃ₓH₂O, and boehmite MOC tips (Table I). All experiments were performed under acidic conditions in the presence of 0.1% trifluoroacetic acid and 80% acetonitrile because it has been reported that acidic conditions with 50–80% acetonitrile are effective to reduce the co-purifi-
cation of acidic peptides (8, 10, 18, 19). Without hydroxy acids, eight or nine phosphopeptides were identified with titania, zir-

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Recovery of phosphopeptides after enrichment with Titania MOC Tip—Recovery of phosphopeptides after enrichment with lactic acid-modified titania MOC tips was examined using the peak areas in extracted ion current chromatograms of phosphopeptide-enriched and non-enriched samples (Table II). Recovery of individual phosphopeptides ranged from a few...
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Tryptic digests of α-casein, fetuin, and phosvitin (2.5 μg/tip) were purified using lactic acid-modified titania MOC tips. Recovery was calculated based on peak areas in the extracted ion chromatograms of the phosphopeptide-enriched sample and sample without enrichment. Peak intensities were from samples without enrichment. Bold and underlined letters represent identified and ambiguous phosphorylated sites, respectively.

| Protein sequence | No. of phosphorylated sites | Charge | Recovery ± S.D. (n = 3) | Peak intensity |
|------------------|----------------------------|--------|-------------------------|---------------|
| α-Casein S1      |                            |        |                         |               |
| VNELSIK          | 1                          | 2      | 5.8 ± 1.5               | 2340          |
| VQPLEIVPNSAEER   | 1                          | 2      | 98.7 ± 15.1             | 6406          |
| YKVQPLEIVPNSAEER | 1                          | 3      | 12.4 ± 3.9              | 5982          |
| DIGSSESTDQAMEDIK  | 2                          | 2      | 147.5 ± 119.5           | 795           |
| α-Casein S2      |                            |        |                         |               |
| EQLSTSEENSK      | 1                          | 2      | 6.7 ± 0.8               | 1250          |
| EQLSTSEENKK      | 2                          | 2      | 2.2 ± 0.5               | 610           |
| NMAINPSK         | 1                          | 2      | 148.7 ± 13.5            | 12303         |
| TVDMESTEVFTK     | 1                          | 2      | 94.8 ± 11.1             | 3571          |
| Fetuin           |                            |        |                         |               |
| CDSSPSDAEDVRK    | 1                          | 3      | 30.3 ± 9.6              | 182           |
| HTFSGVASVESSGEAFHVKG | 2             | 3      | 41.1 ± 6.3              | 1493          |
| Phosvitin        |                            |        |                         |               |
| TTSFPHASAEGER    | 1                          | 3      | 78.3 ± 12.7             | 6371          |
| TTSFPHASAEGER    | 2                          | 2      | 41.2 ± 11.2             | 965           |
| TTSFPHASAEGERSVHEQK | 3                     | 3      | 20.5 ± 4.0              | 3669          |

The values of relative standard deviation of triplicate analysis for 13 peptides (Table II) ranged from 9.1 to 81% (average, 24%). Linearity was also investigated in the range from 0.125 to 25 μg of total loaded amount of digested peptides per titania MOC tip, with six data points, as shown in Supplementary Fig. S1. Five phosphopeptides with the smallest peak areas in Table II were selected to avoid saturation of the MS detector. The correlation coefficients of these five phosphopeptides ranged from 0.927 to 0.999, indicating that the method is satisfactory for quantitation.

Enrichment of Phosphopeptides in HeLa Cytoplasmic Extracts—There have been only a few reports of the application of phosphopeptide enrichment methods to real complex samples such as crude cell extracts including very recent large scale studies (40, 41). In those studies, ~10 mg of starting materials were used for strong cation exchange chromatography prior to phosphopeptide enrichment. To our knowledge there has been no report describing the successful use of a single MOC-based method for phosphopeptide analysis of cell lysates using less than 1 mg as the starting material. It is an extremely challenging task to enrich phosphopeptides from real complex mixtures with a high dynamic range. Therefore we examined the applicability of aliphatic hydroxy acid-modified MOC tips for phosphopeptide enrichment from the cytoplasmic fraction of HeLa cells with less than a 1-mg amount. Fig. 2 shows the number of identified phosphopeptides and non-phosphopeptides following enrichment with three titania MOC tips, i.e. a lactic acid-modified tip, a DHB-modified tip, and a tip without modifier. The ratio of the
Identification of phosphoproteins, phosphopeptides, and their phosphorylated sites in the cytoplasmic fraction of HeLa cells

Nano-LC-MS/MS was performed using the LTQ-Orbitrap. These data were obtained from four replicated experimental results with four nano-LC-MS/MS runs. The number of phosphorylated peptides was based on unique sequences containing missed cleavage products, oxidation of methionine, and phosphorylation of different sites. The number of phosphorylated sites was calculated from phosphopeptides with modification sites determined unambiguously. Note that 66 peptides of a total 1645 phosphopeptides were ambiguous for either phosphorylation or sulfation.

|                     | Titania/lactic acid | Zirconia/HPA | Total  |
|---------------------|---------------------|--------------|--------|
| No. of phosphorylated proteins | 626                 | 664          | 834    |
| No. of phosphorylated peptides      | 1100                | 1181         | 1645   |
| No. of phosphorylated sites         | 983                 | 1057         | 1467   |

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The on-line version of this article (available at http://www.mcponline.org) contains supplemental material.

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