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Enrichment and analysis of phosphopeptides under different experimental conditions using titanium dioxide affinity chromatography and mass spectrometry

Uma K. Aryal[1]* and Andrew R.S. Ross [1‡]

National Research Council, Plant Biotechnology Institute, 110 Gymnasium Place, Saskatoon, Saskatchewan, Canada S7N 0W9.

* Corresponding author: E-mail: Uma.Aryal@nrc-cnrc.gc.ca
Fax: (306) 975-4839

‡Present address: Fisheries and Oceans Canada, Institute of Ocean Sciences, P.O. Box 6000, 9860 West Saanich Road, Sidney, British Columbia, Canada V8L 4B2.

Running title: Evaluation of titanium dioxide for phosphopeptide enrichment

Abstract

Titanium dioxide metal oxide affinity chromatography (TiO_2-MOAC) is generally acknowledged to be more selective than immobilized metal ion affinity chromatography (IMAC) for phosphopeptide enrichment; however, the widespread application of TiO_2-MOAC for biological samples is hampered by conflicting reports of the optimal experimental conditions. The performance of TiO_2-MOAC was evaluated under various loading and elution conditions. Loading and stringent washing of peptides under strongly acidic conditions ensured highly selective enrichment for phosphopeptides, with minimal carryover of non-phosphorylated peptides. Contrary to previous reports, the addition of glycolic acid to the loading solution was found to reduce specificity towards phosphopeptides. Base elution in ammonium hydroxide or ammonium phosphate provided optimal specificity and recovery of phosphorylated peptides. In contrast, elution with phosphoric acid resulted in an incomplete recovery of phosphopeptides, while inclusion of 2,5-dihydroxybenzoic acid in the eluant introduced a bias against the recovery of multiply phosphorylated peptides. We also found that phosphopeptides were highly stable at high pH. TiO_2-MOAC was found to be intolerant of many reagents commonly used as phosphatase inhibitors during protein purification. In a comparison, TiO_2-MOAC showed higher specificity than either immobilized gallium (Ga^{3+}) or iron (Fe^{3+}) affinity chromatography (IMAC) or zirconium dioxide (ZrO_2) affinity chromatography (MOAC) for phosphopeptide enrichment. Matrix-assisted laser desorption/ionization mass spectrometry (MALDI-MS) was most effective in

detecting larger, multiply phosphorylated peptides while liquid chromatography-electrospray ionization tandem mass spectrometry (LC/ESI-MS/MS) was most effective for smaller, singly phosphorylated peptides.

Keywords: Phosphorylation, Metal Oxide Affinity Chromatography, MALDI-MS, LC/ESI-MS/MS, 2,5-Dihydroxybenzoic acid, Glycolic acid

Reversible phosphorylation is one of the most common mechanisms for covalent modification of proteins and is found in as many as one third of eukaryotic gene products.[1,2] Although the number of cellular phosphoproteins is relatively high, the phosphorylated residues themselves are generally of low abundance due to the sub-stoichiometric nature of this modification.[3] The detection and sequencing of tryptic phosphopeptides derived from such proteins is an important aspect of many biological and biomedical research programs. However, the prevalence of non-phosphorylated peptides in protein digests has made it necessary to develop efficient separation and enrichment methods for phosphopeptide analysis.

Immobilized metal ion affinity chromatography (IMAC) has been widely used for the selective enrichment of phosphopeptides;[4,5] however, this method is prone to low recoveries and/or non-specific binding of non-phosphorylated peptides.[6] Metal oxide affinity chromatography (MOAC) using titanium dioxide (TiO_2) has been proposed recently as an alternative to IMAC.[7,8] This technique is based on the selective interaction of phosphopeptides with porous titanium dioxide microspheres (titanospheres) via bidentate binding at the TiO_2 surface.[9,10] Such interactions arise from the affinity of the negatively charged phosphate groups for positively charged metal ions in the MOAC resin.[3] According to established protocols, peptide mixtures are loaded onto the column under acidic conditions and the bound phosphopeptides eluted in basic solution.[7,8,11,12] The selectivity of different IMAC and MOAC methods for phosphopeptides has been studied extensively; however, many of the results presented in the literature are either contradictory or of limited practical use. In

the case of TiO_2-MOAC, peptide loading in the presence of 2,5-dihydroxybenzoic acid (DHB) or phthalic acid has been shown to increase selectivity for phosphopepetides,[8,13] but neither DHB nor phthalic acid are considered compatible with phosphopeptide analysis using liquid chromatography/electrospray ionization-tandem mass spectrometry (LC/ESI-MS/MS) as these compounds are assumed to cause the contamination of the LC system and the inlet of the mass spectrometer .[12,14,15] A desire to avoid potential losses during further purification of TiO_2-enriched phosphopeptides prior to MS analysis has led to the search for alternative 'non-phosphopetide excluders' that are compatible with both matrix-assisted laser desorption/ionization (MALDI) and ESI. Jensen and Larsen[14] recently proposed glycolic acid as an alternative to DHB or phthalic acid; however, this observation was contradicted by another recent observation by Sugiyama and co-workers[12] who reported increased non-specificity of TiO_2-MOAC in the presence of this reagent. Obviously, compatibility of enrichment method with mass spectrometric analysis without the requirement for further downstream purification of the enriched phosphopeptides is desired.[16] Compared with the extensive literature on IMAC-based methods, there is relatively little information available regarding optimal conditions for TiO_2-MOAC.[17] This makes it difficult to predict which method would be better suited to particular applications, or more compatible with the reagents used in cell biology or biochemical studies. For example, TiO_2-MOAC is known to be more tolerant than IMAC to certain reagents and buffers used in biological experiments[14] but its compatibility with protease and phosphatase inhibitors used in proteomic sample

preparation is unknown. This information is needed to develop faster and more efficient enrichment protocols that can be applied to a wide range of biological samples.

To address these uncertainties, the performance of TiO_2-MOAC has been evaluated under different loading and elution conditions, using a single make and model of commercially available TiO_2 column (NuTip^{TM} NT1TIO, Glygen Corp., USA) to minimize experimental variability. The ultimate goal of this study was to develop a procedure for phosphopeptide enrichment that would be sufficiently robust for generalized use. As well, the selectivity of TiO_2-MOAC was compared to zirconium dioxide (ZrO_2) affinity chromatography (ZrO_2-MOAC), and immobilized Ga^{3+} and Fe^{3+} affinity chromatography (Ga-IMAC, Fe-IMAC). The relative performances of MALDI-MS and LC/ESI-MS/MS for the detection of phosphopeptides isolated by those column affinity techniques were evaluated.

EXPERIMENTAL

Materials

Acetonitrile (ACN) and HPLC-grade water were obtained from Merck (Darmstadt, Germany). Trifloroacetic (TFA), formic (FA), phosphoric (PA) and acetic (AA) acids, ammonium bicarbonate (NH_4HCO_3), ammonium hydroxide (NH_4OH), bovine α- and β-caseins, mass calibrants (Angiotensin 1, ACTH clip 1-17 and 18-35), PhosphoProfile™ gallium silica spin columns (product no. P2873) and PHOS-select™ iron affinity gels (product no. P9740) were purchased from Sigma (St. Louis, MO). Modified porcine trypsin (sequencing grade) was obtained from Promega (Madison, WI) and 2, 5-dihydroxybenzoic acid (DHB) from Waters (Milford, MA). Porous titanium dioxide NuTips™ (product no NT1TIO), zirconium dioxide NuTips™ (NT1ZRO) and empty TopTips™ (TT2EMT) were purchased from Glygen Corp (Columbia, MD). PepClean™ C_{18} spin columns (product no. 89870) were purchased from Pierce (Rockford, IL), and C_{18} 3M high performance extraction disks from Empore (Empore Center, St. Paul, MA).

Preparation of tryptic digests

One hundred picomoles each of the model phosphoproteins, α-casein and β-casein, were dissolved separately in 100 μL of 0.1M NH_4HCO_3 (pH 8.5) containing 10 mM dithiothreitol (DTT) and incubated at 56°C for 30 min. Each protein was alkylated using 40 mM iodoacetamide for 1 h at room temperature in the dark. The reaction was quenched by addition of DTT to a final concentration

of 5 mM, and the protein digested with trypsin at 37°C overnight using an enzyme:substrate ratio of 1:50 (w/w). Each tryptic digest was dried in a vacuum centrifuge (model DNA 120; Thermo Savant, Colin Drive, NY), reconstituted in 100 μL of 0.1% aqueous TFA, and desalted using PepClean^{TM} C_{18} spin columns (Pierce) according to manufacturer's protocol. Briefly, each column was first conditioned by loading with 200 μL of 50% methanol, followed by centrifugation, and this was repeated once. The column was then equilibrated by loading with 200 μL of 0.5% TFA in 5% ACN, centrifuged and repeated once. One hundred μL of tryptic digest in 0.1% TFA was then loaded, centrifuged, and the collected solution reloaded and centrifuged again. After washing twice with 200 μL of 0.5% TFA in 5% ACN, the spin column was eluted twice with 40 μL of 70% ACN. The combined eluate (80 μL) was dried and reconstituted in 100 μL of 0.1% TFA, and stored at -20°C until further use.

For each experiment, 1 μL of the desalted α-casein digest was combined with an equal volume of β-casein digest and 98 μL of the appropriate loading solution (see below) to produce a 10 femtomoles/μL combined digest solution. This was divided into five 20 μL aliquots, each containing 200 femtomoles of digest, from which phosphopeptides were subsequently purified by MOAC or IMAC. Unless otherwise noted, 200 femtomoles of the combined α- and β-casein digest were used in all experiments.

Phosphopeptide purification by TiO_2 metal oxide affinity chromatography (TiO_2-MOAC)

Prior to sample loading, the disposable TiO_2 NuTips™ (Glygen) were conditioned/equilibrated with 20 μL of 1% TFA in 30% ACN using 10 aspirate/expel (A/E) cycles. To evaluate the effect of different organic acids on phosphopeptide binding, 1 μL volumes of both desalted casein digests were combined with 98 μL of 0.1, 1 or 5% concentrations of AA, FA or TFA and 30% ACN, and 20 μL (200 femtomoles) of this combined digest was loaded onto a TiO_2 tip using 50 A/E cycles. The effect of DHB on phosphopeptide binding was investigated by loading peptides in 20 μL of 1% TFA in 50% ACN, with or without DHB (130 mM).

After each loading, the tips were washed once with 20 μL of 1% TFA in 30% ACN, once with 20 μL of 1% TFA in 50% ACN, twice with 20 μL of 1% TFA in 75% ACN, and finally, twice with 20 μL of HPLC grade water, using 10 A/E cycles for each wash. Bound phosphopeptides were eluted with 20 μL of 0.4 M NH_4OH in 30% ACN, using 20 A/E cycles. The eluate was acidified immediately by adding 5 μL of aqueous 1% TFA and used for MS analysis (see below).

To investigate the efficiency of different eluants, TiO_2 tips were loaded using 1% TFA in 30% ACN, washed sequentially with various solutions as described above, and eluted with either 20 μL of 0.25 M NH_4HCO_3 (pH 9) in 30% ACN, 0.4 M NH_4OH (pH ~11) in 30% ACN, 100 mM $NH_4H_2PO_4$ (pH 10) in 30% ACN, or 1% PA (without or with 130 mM DHB) in 50% ACN.

The compatibility of TiO_2 with various reagents commonly used during phosphoproteomic sample preparation also was investigated. These reagents included 2 mM okadaic acid, 20 mM sodium fluoride, 2 mM sodium molybdate, 2 mM sodium orthovanadate, 2 mM sodium β-glycerophosphate, 100 mM imidazole, 100 nM calyculin A, 20 mM phenylmethylsulphonyl fluoride (PMSF), 20 mM Sigma protease inhibitor cocktail (product # P9599), and 30 mM polyethylene glycol which is used in two-phase partitioning procedures.

To compare performance using the different MOAC media, TiO_2 and ZrO_2 NuTips™ were equilibrated/conditioned with 20 μL of 1% TFA in 30% ACN and loaded with 20 μL (200 femtomoles) of the combined digest in 1% TFA in 30% ACN, using 50 A/E cycles. After washing, as previously described, bound phosphopeptides were eluted with 20 μL of 0.4 M NH_4OH in 30% ACN, using 20 A/E cycles. Each experiment was repeated at least 3 times under each experimental condition to check the reproducibility of the results.

Phosphopeptide purification by immobilized Ga^{3+} and Fe^{3+} metal affinity chromatography (Ga-IMAC, Fe-IMAC)

For Ga-IMAC, pre-packed PhosphoProfile™ gallium silica spin columns (Sigma) were first washed/equilibrated with 50 μL of 1% TFA in 30% ACN and then loaded with 20 μL (200 femtomoles) of the combined casein digest mixture in 1% TFA with 30% ACN, using a pipette to apply gentle pressure and ensure proper loading of the sample. After loading, columns were incubated at room temperature for 15 min before spinning at 1000 rpm for 1 min. The sample flow-

through was then re-loaded, and the incubation and centrifugation repeated. The columns were washed with 50 μL volumes of the same washing solutions used for TiO_2-MOAC, with centrifugation at 1000 rpm for 2 min during each washing step. Bound phosphopeptides were eluted by loading the column with 20 μL of 0.4M NH_4OH in 30% ACN, incubating at room temperature for 5 min, then spinning at 1000 rpm for 1 min. The eluate was immediately acidified by adding 5 μL of 1% aqueous TFA and used for MS analysis.

For Fe-IMAC, PHOS-Select™ iron affinity gel beads were carefully stirred until completely and uniformly suspended in the stabilizing buffer supplied. Five μL of the resulting slurry was then mixed with 5 μL of 1% TFA in 30% ACN and loaded into empty TopTips™ (Glygen), using a regular pipette tip with about 1 mm of the end cut off to allow unrestricted flow and uniform distribution of the suspended beads. The beads were washed/equilibrated by adding 50 μL of 1% TFA in 30% ACN and spun in a microcentrifuge for 1 min at 1000 rpm. This operation was repeated two more times to ensure complete removal of the stabilizing buffer (which contains glycerol). The flow-through was discarded and the Fe-IMAC tips were loaded with 20 μL (200 femtomoles) of the combined casein digest in 1% TFA with 30% ACN. To ensure proper loading, the tip was again fitted to a pipette and gentle pressure applied to push the sample into the gel beads. The tips were incubated at room temperature for 15 min before spinning at 1000 rpm for 1 min. The sample flow-through was reloaded and the incubation and centrifugation process repeated. Using the same procedure as for

Ga-IMAC, the Fe-IMAC columns were washed, eluted, and the eluate acidified and used for MS analysis (see below).

MALDI Mass Spectrometry

The acidified peptide eluates were vacuum-dried, reconstituted in 10 μL of 10% aqueous TFA and desalted with STAGE (STop And Go Extraction) tips prepared using Empore^{TM} C18 (octadecyl) extraction disks, according to established procedures.[18] The bound peptides were eluted using 1 μL of matrix solution containing 130 mM DHB in 10 mM $(NH_4)_2HPO_4$ and 75% ACN and deposited directly onto the MALDI target plate. MALDI-MS analysis was performed using a Voyager-DE STR instrument (Applied Biosystems, Framingham, MA) operating in the positive ion and reflectron modes with delayed ion extraction. Close external mass calibration was performed using Angiotensin 1, ACTH clip 1-17 and 18-35. Spectra were acquired over the *m/z* range 700-4000 and the results of 200 laser shots combined to produce a single averaged spectrum for each sample. The efficiency and selectivity of phosphopeptide binding under different loading and elution conditions were evaluated by comparing the number and type (i.e. single, multiple, or non-phosphorylated) and relative abundance of peptide ions detected by MALDI-MS.

Liquid Chromatography/ESI-Tandem Mass Spectrometry (LC/ESI-MS/MS)

For LC/ESI-MS/MS analysis, the eluates were dried, then reconstituted in 10 μL of 1% TFA in water and 6 μL of it was used for chromatographic separation.

LC/ESI-MS/MS analysis was performed using the nanoACQUITY UPLC system (Waters, Milford, MA, USA) interfaced to a quadrupole-time of flight (Q-TOF) Ultima Global hybrid tandem mass spectrometer fitted with a Z-spray nanoelectrospray ion source (Waters, Mississauga, ON, Canada). Chromatographic separation of peptides was accomplished by using Waters BEH130 C_{18} nanoAQUITY UPLC analytical column (75 μm × 100 mm, 1.75 mm) at a flow rate of 400 nL/min. Mobile phase A was 0.2% FA in water, and B was 0.2% FA in 100% ACN. The peptides were separated for 55 min by increasing the organic content of the mobile phase linearly from 0% to 45% over 45 min and then to 80% at 46 min, holding this concentration until 52 min and then reducing the ACN to 1% at 53 min. A 5 min seal wash with 10% ACN was also used after completion of each run. Mass calibration of the Q-TOF was performed using a product ion spectrum of Glu-fibrinopeptide B acquired over the *m/z* range 50-1900. The instrument was run in positive ion mode with source temperature of 80°C. Analysis was carried out using data dependent acquisition, during which peptide precursor ions were detected by scanning from *m/z* 400-1900 in TOF-MS mode. Multiply charged (2^+, 3^+, or 4^+) ions rising above pre-determined threshold intensity were automatically selected for TOF-MS/MS analysis, and product ion spectra were acquired over the *m/z* range 50-1900. The absence of singly-charged peptides was subsequently verified by means of extracted ion chromatograms generated for *m/z* values corresponding to every theoretical, singly-charged α- and β-casein peptide ion.

The MS/MS data were converted to a peak list (pkl) file format using MassLynx v2.15 software (Micromass) and searched against National Center for Biotechnology Information non-redundant (NCBInr) databases using an in-house Mascot server (v. 2.2). Phosphorylation of peptides identified as phosphopeptides via database searching was confirmed by manual inspection of the corresponding MS/MS spectra. Each validated phosphopeptide MS/MS spectrum was then reduced to single-charged, monoisotopic, centroided peaks using MaxEnt 3 (MassLynx v.4.1, Micromass), and *in silico* spectral fragmentation of the corresponding, database-matched phosphopeptide sequence obtained by using BioLynx software. The calculated fragment ion masses were then compared with the experimental values to confirm the position(s) of the phosphorylated residue(s).

RESULTS AND DISCUSSION

Effects of various organic acids on phosphopeptide binding

During affinity purification of phosphopeptides, acidic loading/washing solutions generally are effective in inhibiting non-specific binding of acidic and/or hydrophobic peptides, whereas high pH solutions are more efficient in recovering bound phosphopeptides.[11] There have been several studies to determine optimal sample loading conditions for TiO_2-MOAC;[3,7,8,12-14,19,20] however, the information presented in the literature still remains contradictory. For example, peptide loading in strong acids, such as TFA, was shown to reduce non-specific binding of peptides to TiO_2,[8,14] but this has been called into question by the same group in a recent report.[13] In view of these uncertainties, we have systematically re-investigated the effects of different organic acids and modifiers on TiO_2-MOAC selectivity towards phosphopeptides.

Direct analysis of a non-enriched peptide sample by MALDI-MS yielded only three phosphorylated peptides (labeled 8, 12 and 13; Fig. 1(a)), and these were at relatively low intensities. Many additional phosphopeptides were visible following TiO_2-MOAC enrichment (Fig. 1, b-d). Phosphorylated peptides detected after enrichment are indicated by numbers (1-25) while non-phosphorylated peptides are indicated by letters (A-G) in the spectra (Fig. 1). The majority of the multiply phosphorylated peptides appear in a cluster at the higher *m/z* region of the spectra (right side).

To evaluate the binding selectivity of the TiO_2 columns for phosphorylated peptides under different loading conditions, casein digests were loaded onto TiO_2 tips with either 0.1, 1 or 5% concentrations of AA, FA or TFA in 30% ACN and the bound peptides were eluted using 0.4 M NH_4OH in 30% ACN (Table 1). When samples were loaded in 0.1% concentrations of these acids, phosphorylated peptides along with several acidic non-phosphorylated peptides were detected in the eluants (Table 1). The number of non-phosphorylated peptides in the eluted sample decreased as the concentration of acid in the loading solution was increased (Table 1). As well, the number and relative intensities of non-phosphorylated peptides were much lower with TFA than for either AA or FA. The results obtained from repeated experiments indicate that the efficacy of these organic acids in inhibiting non-specific binding of peptides to TiO_2 is in the order of TFA > FA > AA.

TFA concentration effects on intensities of two non-phosphorylated peptide peaks A (*m/z* 1266.8) and D (*m/z* 1759.1) and relative intensities of phosphorylated peptide peaks were monitored (Fig. 1, b-d). In all cases, the relative intensities of peaks A and D were reduced when using 1% rather than 0.1% TFA in the loading buffer. At the same time, the relative abundances of multiply phosphorylated peptide peaks 16-20, 23, and 24/24" were greater for 1% than for either 0.1% or 5% TFA. The higher concentrations of TFA, i.e. 5% TFA and higher, appeared to reduce the specificity towards phosphopeptides; this was possibly because the relatively high concentrations of dissociated acid molecules competed with peptide phosphate groups for the available binding

sites. Similarly, increasing the proportion of ACN from 30% to 50% or 75% did not improve TiO_2 selectivity for phosphopeptides (data not shown). To summarize, peptides loaded with 1% TFA in 30% ACN (Fig. 1(c)), followed by stringent washing of the loaded peptides with 1% TFA in increasing concentrations of ACN (see Experimental section), provided the best result and this loading solution was used for all subsequent analyses.

Effect of different eluants on phosphopeptide recovery

Conventional protocols often use basic solutions to recover phosphopeptides from affinity columns.[16] However, acidic solutions containing phosphoric acid (PA) or a mixture of PA and DHB have been shown to act as efficient eluants for Fe-IMAC.[16,21-23] but their efficacy with TiO_2-MOAC has been unclear. Similarly, although NH_4OH is known to be an efficient eluant for both IMAC and MOAC [8,21,24] the suitability of other bases (e.g. NH_4HCO_3, $NH_4H_2PO_4$) for TiO_2-MOAC has yet to be explored. The efficiencies of these eluants with TiO_2-MOAC was investigated, using 1% TFA in 30% ACN as the loading solution, and elution with 0.4 M NH_4OH in 30% ACN (Fig. 2(a)) as the control comparison.

A number of singly phosphorylated and multiply phosphorylated peptides (peaks 11, 18, 20 and 24/24") were detected following elution with PA in 50% ACN (Fig. 2(b)); however, this was not as effective as the standard elution buffer (Fig. 2(a), 0.4 M NH_4OH in 30% ACN) and inclusion of DHB significantly reduced the number of multiply phosphorylated peptides detected, as exemplified by the low signal intensity for peak 24 (Fig. 2(c)). Repeated experiments clearly showed

that DHB in the eluant inhibited the recovery of multiply phosphorylated peptides from TiO_2 columns (Fig. 2(c)). Therefore, PA or PA with DHB were not efficient eluants for the recovery of phosphopeptides from TiO_2-MOAC.

With regard to basic eluants, 0.4 M NH_4OH in 30% ACN (Fig. 2(a)) and 100 mM $NH_4H_2PO_4$ in 30% ACN (Fig. 2(e)) gave better recovery of multiply phosphorylated peptides than 250 mM NH_4HCO_3 in 30% ACN (Fig. 2(d)); this is illustrated by the relative abundance of peak 24 in each spectrum and the fact that multiply phosphorylated peptide peaks 18, 19, 23 and 25 were detected after elution with NH_4OH and $NH_4H_2PO_4$, but not in NH_4HCO_3. The best results were obtained by eluting bound phosphopeptides with either 0.4 M NH_4OH in 30% ACN (pH 11) or 100 mM $NH_4H_2PO_4$ in 30% ACN (pH 10) (Fig. 1(b), (f)). Therefore, 1% TFA in 30% ACN was optimal as loading solution and 0.4 M NH_4OH in 30% ACN was optimal as an eluant, and was used for all subsequent analyses as control.

In order to have practical utility, TiO_2-MOAC must be sensitive enough to detect phosphopeptides at concentrations typically found in digests of 2-D gel spots, or in complex mixtures derived from whole cell lysates or sub-cellular fractions. Sensitivity was therefore evaluated by loading 100, 50 and 25 femtomoles of the combined casein digests onto the TiO_2 tips using 1% TFA in 30% ACN, eluting with 0.4 M NH_4OH in 30% ACN and analyzing the peptides by MALDI-MS (Supplementary Fig. S1). The majority of casein phosphopeptides were detected in all cases, showing that this procedure should be sufficiently

sensitive for phosphoproteomic studies involving biologically relevant quantities of samples.

The sensitivity and specificity of TiO_2-MOAC enrichment for phosphopeptides also was examined using *Arabidopsis thaliana* leaf proteins subjected to two-dimensional gel electrophoresis (2-DE). Two isoforms of CA1 (carbonic anhydrase 1), previously found to be phosphorylated (Aryal *et al.* unpublished; Supplementary Table S1), were identified and pooled from the gel (Fig. 3(a); white circles). This map represents a cropped 2-D gel image generated using a 17-cm 3-10 NL immobilized pH gradient (IPG) strip with 200 μg of leaf extract in 8 M urea, 2 M thiorea, 2% CHAPS, 0.05% Pharmalyte buffer. The CA1 spot towards the more acidic side had 4 phosphorylation sites (Thr-69, Ser-98, Ser-200, and Ser-211), whereas the other spot had only one phosphorylation site (Ser-200) (Supplementary Table S1); the displacement towards more acidic region on the gel of the multiply phosphorylated isoform of CA1 was predicted by experimental evidence that each phosphorylation event reduces the *pI* of the protein and therefore causes a shift of the protein spot towards the acidic side along the isoelectric focusing (IEF) dimension in 2-D gel. To evaluate the sensitivity and specificity of the TiO_2-MOAC protocol, tryptic digests were prepared from each protein spot and then combined. Four μL of sample was run on the Q-TOF using LC/ESI-MS/MS without prior TiO_2-MOAC enrichment, while the remainder of the digest was vacuum-dried, re-constituted in 15 μL of 1% TFA in 30% ACN, and then purified by TiO_2-MOAC. Without prior enrichment 11 non-phosphorylated peptides and 4 singly phosphorylated

peptides were identified (Fig. 3(b)). When purified by TiO_2-MOAC, the eluate contained those same 4 singly phosphorylated peptides, but only one non-phosphorylated peptide (YMVFACSDSR), while the flow-through fraction contained all 12 of the non-phosphorylated peptides (and no phosphopepetides) (Fig. 3(b), Supplementary Table S1). In addition, the ion scores of identified phosphopeptides improved in the enriched sample than in the non-column purified fraction (see Supplementary Table S1). The TiO_2-MOAC method is clearly sensitive and specific enough to enrich for phosphopeptides from biologically appropriate quantities of peptide samples.

Stability of phosphopeptides at high pH

Phosphopeptides are expected to undergo alkaline hydrolysis at high pH,[27] and it has become common practice to acidify basic eluates with TFA following IMAC or MOAC. Matsumoto and co-workers[27] have shown that phosphosyntide-2, a phosphorylation product of calcium/calmodulin-dependent protein kinase type II (CaMK II), is stable at pH 11.5. The long-term stability of phosphopeptides in basic eluents has not been thoroughly investigated, and although hydrolysis may be negligible during IMAC or MOAC enrichment, it could become significant during storage or protracted analytical procedures involving multi-dimensional fractionation and/or separation of phosphopeptides prior to MS.[28,29]

To verify the long-term stability of phosphopeptides at high pH, TiO_2 columns loaded with 200 femtomoles of the combined casein digests in 20 μL of

1% TFA in 30% ACN were eluted using 0.4 M NH_4OH (pH ~11) in 30% ACN, and the basic eluate incubated at room temperature (~24°C) without added TFA. MALDI mass spectra of the eluate at 0 h (acidified immediately after elution), and at 1, 2 or 3 days of incubation under basic conditions are shown in Fig. 4; a-d. No visible peaks corresponding to the de-phosphorylated forms of any casein phosphopeptides were observed in any of the incubated alkaline samples (Fig. 4 (b-d)), showing these to be stable under these conditions.

This was further verified using tryptic peptides of the proteins DREPP (Plasma membrane polypeptide family protein), PSAD-1 (photosystem I subunit D-1), PSBP-1 (oxygen evolving enhancer protein 2) and ATPase (ATP synthase delta chain) that had been extracted from *Arabidopsis* thaliana leaves and separated by 2-DE as mentioned earlier. All four of these proteins had been confirmed previously as phosphorylated proteins (Aryal *et al.* unpublished). Each protein digest was re-suspended in 15 μL of 1% TFA in water, and 4 μL was analyzed by LC/ESI-MS/MS without an alkaline treatment as the control (0 h sample). The remaining amounts of each peptide solution were vacuum-dried and reconstituted in 20 μL of 0.4 M NH_4OH in 30% ACN (pH ~11) and incubated for 16 h at room temperature (16 h alkaline treatment). After 16 h of incubation, the solution was acidified by adding 5 μL of 1% TFA in water, and vacuum-dried again. The digests were re-suspended in 10 μL of 1% TFA in water, and 6 μL used for LC/ESI-MS/MS analysis. The identified phosphopeptides from each sample prior to, or after the 16 h incubation with NH_4OH are shown in Fig. 4(e). Interestingly, all the phosphopeptides, except KQLEDIASQLELGEIQLA$_p$T, that

were detected before alkaline treatment were also detected after the treatment, confirming our previous observation that phosphopeptides are stable at alkaline pH. Nevertheless, we took the precaution of acidifying all basic MOAC and IMAC eluants immediately after elution with TFA in this study.

Use of non-phosphopeptide excluders in loading solutions to improve selectivity

Loading TiO_2 columns with 1% TFA in 30% ACN provides high selectivity towards phosphopeptides for relatively simple samples; however, Jensen and Larsen[14] have suggested that a 'non-phosphopeptide excluder' should be used to optimize selectivity for the enrichment of phosphopeptides from complex samples such as cell lysates. DHB and phthalic acid have been used successfully for this purpose,[8,13] but tend to inhibit analysis of the recovered phosphopeptides using LC/ESI-MS/MS due to contamination of LC system and the inlet of the mass spectrometer.[12,14,15] Jensen and Larsen[14] have recently shown that 1M glycolic (hydroxyacetic) acid is equally effective as a non-phosphopeptide excluder, and is compatible with both MALDI-MS and LC/ESI-MS/MS analysis. However, Sugiyama and co-workers found that using 300 mg/mL (~ 4M) glycolic acid in the loading solvent resulted in non-specific binding of peptides.[12] In view of this uncertainty we decided to re-investigate the use of DHB and glycolic acid as non-phosphopeptide excluders for MOAC.

Based on previous investigations involving α- and β-casein,[23] DHB was added to the MOAC loading solution at a concentration of 130 mM, whereas

glycolic acid was included at 1, 0.5 or 0.25 M to investigate whether reducing (rather than increasing)[12] the concentration of this reagent might enhance specificity. MALDI mass spectra of casein digests enriched by TiO_2-MOAC with DHB or glycolic acid in the loading solution are shown in Fig. 5. The specificity obtained using 1% TFA with 30% ACN alone as the loading solution is sufficient to eliminate non-phosphorylated peptides from the spectrum (Fig. 5(a)). However, addition of DHB appeared to enhance overall recovery and detection of phosphopeptides, particularly those carrying multiple phosphorylations, as illustrated by the increase in relative intensity of peak 24 in the presence of DHB (Fig. 5(b)) .

When 1 M glycolic acid was used in the loading solution several non-phosphorylated peptides (labeled A, B, D, E, and F/F') were detected at significant levels (Fig. 5(c)). The relative abundances of these peptides decreased as the concentration of glycolic acid decreased from 1 to 0.5 or 0.25 M glycolic acid (Figs. 5 (c-e)), suggesting that the addition of glycolic acid to the loading solution actually increased non-specific binding of peptides to TiO_2 columns. No enhancement in the relative abundance of multiply phosphorylated peptides was observed using glycolic acid.

We also evaluated the effects of DHB and glycolic acid as non-phosphopeptide excluders for ZrO_2-MOAC. The addition of DHB to the loading solution reduced the binding of non-phosphorylated peptides to ZrO_2 columns while again enhancing the relative abundance of multiply phosphorylated peptides (data not shown). As well, the use of glycolic acid appeared to increase

non-specific binding of peptides to ZrO_2 columns, and gave no enhancement in the relative abundance of multiply phosphorylated peptides (data not shown). These observations are in direct contrast with those of Jensen and Larsen[14], who found 1M glycolic acid to be an efficient non-phosphopeptide excluder. Our results complement those obtained by Sugiyama and co-workers[12] who used a single, relatively high concentration (~4M) of glycolic acid.

Hydroxy acids bind to metal oxides by forming a cyclic chelate,[12, 30,31] whereas phosphate anions do so by bridging two metal ions.[9] Thus, hydroxy acids such as DHB should bind to metal oxides more weakly than a phosphate group but more strongly than the carboxylic groups of acidic non-phosphorylated peptides. It is unclear why glycolic acid (hydroxyacetic acid) should have been ineffective in displacing non-phosphorylated peptides from TiO_2 during our study. One possibility is that the structure and retention properties of titania, which (for saccharides at least) is strongly dependent upon the calcination temperature of the beads, [32,33] may differ from one study to another. In any event, it appears that loading samples with glycolic acid may actually increase non-specific binding of peptides during MOAC, depending on the materials and conditions used.

Effect of phosphatase inhibitors on phosphopeptide enrichment

During proteomic analysis a number of reagents may be used to solubilize hydrophobic proteins and to inhibit protease and phosphatase activities. TiO_2-MOAC is known to be compatible with most of the buffers and detergents used in biological experiments,[14] however, the tolerance of TiO_2-MOAC to other reagents,

including protease and phosphatase inhibitors, has not been widely explored. This information would be helpful in deciding what purification steps might be necessary prior to enrichment by TiO_2-MOAC and MS analysis, especially when using 'gel-free' proteomic approaches.

Sodium fluoride, sodium molybdate, sodium orthovanadate, sodium β-glycerophosphate, okadaic acid, imidazole and calyculin A are some of the more commonly used phosphatase inhibitors, and were therefore included in our study. These reagents were added to the 1% TFA in 30% ACN loading solution at concentrations normally used in biological experiments as summarized in Table 2. Table 2 also lists the number of phosphorylated and non-phosphorylated peptides detected following TiO_2-MOAC enrichment in the presence of these reagents. An example of the MALDI mass spectra from which these results were obtained is also shown in Fig. 6. By way of comparison, Fig. 6(a) shows the control spectrum obtained without including any of these reagents in the loading solution.

Several of the reagents tested were found to be incompatible with TiO_2 affinity chromatography. The most dramatic effect was observed with sodium molybdate and sodium β-glycerophosphate, which completely removed specificity towards phosphopeptides, as indicated by the detection of only 2 phosphorylated peptides (peaks 8 and 12) but several additional non-phosphorylated peptides in the eluant (Fig. 6 (d)(e)). Sodium orthovanadate also had a negative impact on specificity, as illustrated by the number of non-phosphorylated peptides co-purified with phosphopeptides (Fig. 6(f)). Although

TiO_2-MOAC appeared to be somewhat tolerant of sodium fluoride at first glance (Fig. 6(b)) its presence again reduced specificity and recovery of certain singly (peaks 5, 7, 8) and multiply phosphorylated peptides (peaks 24 & 25), as compared with the control (Fig. 6(a)). However, okadaic acid appeared to have negligible impact on the performance of TiO_2-MOAC, as shown by Fig. 6(c).

In addition to the above, the effects on phosphopeptide enrichment of other additions to the loading solutions were tested: imidazole, calyculin A, PMSF, polyethylene glycol (PEG), and a protease inhibitor cocktail from Sigma (product # P9599) containing 4-(2-aminoethyl)benzenesulfonyl fluoride (AEBSF), bestatin, pepstatinA, E-64, leupeptin and 1,10-phenanthroline, which are used routinely for plant protein extractions. The results of these experiments are again summarized in Table 2. We included PEG in our investigation because it has been found to improve detection of low-abundance proteins by selectively precipitating RuBisCo from plant protein extracts. [26,34-36] It is also used to purify plasma membrane proteins by aqueous two phase partitioning procedure. Our results show that TiO_2 affinity chromatography is somewhat tolerant of most these reagents in the loading solution, but that the presence of imidazole, PMSF, calyculin A or the Sigma protease inhibitor cocktail reduces the number and relative intensities of multiply phosphorylated peptide peaks. Therefore, purification of tryptic peptides to remove some additives prior to loading onto TiO_2-MOAC may be required for the highest performance of TiO_2 tips for phosphopeptide enrichment, especially for gel-free proteomic approaches.

Comparison of TiO_2 with other phosphopeptide enrichment methods

Both MALDI-MS and LC/ESI-MS data were obtained for the comparison of TiO_2-MOAC with ZrO_2-MOAC, Ga-IMAC and Fe-IMAC for efficient phosphopeptide enrichment. A list of the phosphorylated peptides with their molecular masses found in the combined α- and β-casein digests using various enrichment and analytical methods are given in Table 3. In all cases, samples were loaded in 1% TFA with 30% ACN and eluted with 0.4 M NH_4OH in 30% ACN. Both MS techniques detected more multiply phosphorylated peptides in the TiO_2 eluant than the ZrO_2 eluant, although the numbers of singly phosphorylated peptides were similar for each enrichment method (Table 3). These results are in keeping with previous studies that also found TiO_2-MOAC to be more efficient at recovering multiply phosphorylated peptides than ZrO_2-MOAC.[14,24] Unlike Kowen and Håkansson[24], however, we did not find ZrO_2 to be better than TiO_2 for recovery of singly phosphorylated peptides.

On the other hand, Fe-IMAC showed more bias towards the enrichment of multiply phosphorylated peptides whereas Ga-IMAC showed balanced recovery of both singly and multiply phosphorylated peptides than Fe-IMAC (Figs. 7(c) and 7(d)). However, considering the selectivity, both IMAC methods appear less selective for phosphopeptides than TiO_2-MOAC as non-phosphorylated peptides observed in MALDI-MS spectra from IMAC purified samples were not observed in TiO_2-MOAC enriched samples. But, signal intensities of multiply phosphorylated peptides were higher in IMAC than in TiO_2-MOAC as shown by the corresponding MALDI mass spectra (Figs. 7(a), 7(c) and 7(d)), supporting

previous claims that multiply phosphorylated peptides are purified with higher abundances using IMAC methods.[8] For example, peaks 20, 24 and 25 were generally of higher abundances in IMAC than in TiO_2 spectra, whereas peaks 15 and 22 were detected by MALDI-MS for both IMAC methods but not for TiO_2. Similarly, the tetra-phosphorylated peptide 21 was detected by LC/ESI-MS/MS in both Ga- and Fe-IMAC eluants but not for TiO_2 (Table 3). This might be possibly due to differences in strength of interaction between multiply phosphorylated peptides and the affinity media, not the higher specificity of IMAC. The interaction between TiO_2 and multiply phosphorylated peptides could be particularly strong, and may require a more stringent elution protocol than used in this comparative study. Furthermore, TiO_2-MOAC appeared to show even greater selectivity in this study than previously observed.[8] For example, LC/ESI-MS/MS detected just two non-phosphorylated peptides (peaks A and D) in TiO_2-MOAC eluates whereas four non-phosphoylated peptides were detected in IMAC-enriched sample (Supplementary Table S2). Non-selective binding of peptide D (*m/z* 1760) to TiO_2 and IMAC columns has been reported elsewhere.[6,8,11,14.] In conclusion, TiO_2-MOAC showed the highest specificity than either Ga- or Fe-IMAC or ZrO_2-MOAC for phosphopeptides enrichment.

Comparison of MALDI-MS and LC/ESI-MS/MS for analysis of affinity-purified phosphopeptides

LC/ESI-MS/MS analysis of the un-purified casein digest matched a total of 12 peptides (4 phosphorylated, 8 non-phosphorylated) to the first (αS1) sub-unit of

α-casein, 11 peptides (5 phosphorylated, 6 non-phosphorylated) to the second (αS2) sub-unit, and 5 peptides (2 phosphorylated, 3 non-phosphorylated) to β-casein (data not shown). When purified using the TiO_2-MOAC, 9 peptides (7 phosphorylated, 2 non-phosphorylated) were matched to αS1, 8 peptides (all phosphorylated) to αS2, and 3 phosphopeptides to β-casein (Table 3 and Supplementary Table 2). Hence, TiO_2 enrichment increased the number of matched phosphopeptides as well as their confidence levels (ion scores) when compared with the un-extracted samples. On the other hand, there were no non-phosphorylated peptides detected in TiO_2-MOAC enriched samples using MALDI-MS (Fig. 1(b)). This difference could be due to the inherent sensitivity of LC/ESI-MS/MS and of the Q-TOF instrument, which is greater than that of the Voyager-DE STR instrument used for MALDI-MS analysis. It is important to note that the two non-phosphorylated peptides (A and D) detected by LC/ESI-MS/MS in TiO_2-enriched samples were also the most abundant peptides detected by MALDI-MS in the un-purified digest (see Fig. 1(a)). Regardless of which MS technique was used, fewer non-phosphorylated peptides were detected in TiO_2-MOAC enriched samples than in any other extracts (Supplementary Table S2), further confirming TiO_2-MOAC as the most selective method for phosphopeptide enrichment.

With regard to phosphopeptide analysis, more singly and double phosphorylated peptides were detected using LC/ESI-MS/MS than MALDI-MS (Table 3). In fact, with the exception of peptide 9 all singly or doubly phosphorylated peptides observed using MALDI-MS were also detected by LC/ESI-MS/MS, while peptides

1-4 and 14 were only detected using LC/ESI-MS/MS. In contrast, more peptides carrying three or more phosphate groups were detected using MALDI-MS than with LC/ESI-MS/MS. For example, multiply phosphorylated peptides 15, 17-21, and 25 were only observed using MALDI-MS whereas the only multiply phosphorylated peptide detected by LC/ESI-MS/MS alone was peptide 21 (a missed-cleavage product incorporating peptide 19). Larsen *et al.*[8] and Gruhler *et al.*[37] also reported a bias against the detection of multiply phosphorylated peptides when using LC/ESI-MS/MS. Using our TiO_2-MOAC enrichment method, we were able to detect more phosphorylated peptides (including peptides 16', 22 and 24) than reported by others using LC/ESI-MS/MS,[8] and with less material (200 femtomoles of combined α- and β-casein digests), confirming the high efficiency of the TiO_2 tips. LC/ESI-MS/MS was more efficient in detecting singly phophorylated peptides than MALDI-MS across all chromatography methods, while the converse was true for multiply phosphorylated peptides where MALDI-MS more efficient (Table 3). Therefore, MALDI-MS was most effective in detecting larger, multiply phosphorylated peptides while LC/ESI-MS/MS was most effective for smaller, singly phosphorylated peptides.

CONCLUSIONS

The results presented here clearly show that TiO_2-MOAC is highly selective and sufficiently sensitive for the enrichment of phosphopeptides from biologically relevant quantity of tryptic digests. Optimal selectivity was achieved by loading peptides with 1% TFA in 30% ACN and washing sequentially with acidic

solutions (1% TFA) of increasing organic content (i.e. 30 to 75 % ACN). Glycolic acid was found to be ineffective as a non-phosphopeptide excluder in the loading solution. With the exception of okadaic acid additions, TiO_2-MOAC was found to be intolerant of many commonly used phosphatase inhibitors in the loading solution, and this has implications for phosphoproteomic studies that do not involve gel separation prior to MOAC. Base elution in 0.4 M NH_4OH or 100 mM $NH_4H_2PO_4$ gives efficient and balanced recovery of singly and multiply phosphorylated peptides and is compatible with MALDI-MS and LC/ESI-MS, both of which may be necessary to ensure detection of all phosphopeptides recovered using TiO_2-MOAC. In addition, using tryptic peptides from standard phosphoproteins as well as biological samples of plant origin, we have shown that phosphopeptides are stable in alkaline pH. Ga-IMAC and Fe-IMAC gave slightly better recovery of multiply phosphorylated peptides than MOAC but were less specific than TiO_2 for phosphopeptide enrichment.

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