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Optimizing TiO_2-Based Phosphopeptide Enrichment for Automated Multidimensional Liquid Chromatography Coupled to Tandem Mass Spectrometry

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An automated online multidimensional liquid chromatography system coupled to ESI-based tandem mass spectrometry was used to assess the effectiveness of TiO_2 in the enrichment of phosphopeptides from tryptic digests of protein mixtures. By monitoring the enrichment of phosphopeptides, an optimized set of loading, wash, and elution conditions were realized for TiO_2. A comparison of TiO_2 with other resins used for phosphopeptide enrichment, Fe(III)-IMAC and ZrO_2, was also carried out using tryptic digests of both simple and moderately complex protein mixtures; where TiO_2 was shown to be superior in performance.

Protein phosphorylation is a widely used means of signal transduction in many different species, where many of these phosphorylation events have been shown to regulate a vast array of cellular and molecular processes.[1] Due to its fundamental role in biology, the identification of phosphorylation events in different biological contexts is a growing area of research. Shotgun-based liquid chromatography coupled to tandem mass spectrometry (LC−MS/MS) has become the predominant means of identifying phosphorylation sites in simple or complex protein mixtures, enabling phosphoproteomic studies to be carried out in many different areas of biological research.[2−6]

Shotgun-based protein identification generally involves the digestion of a protein mixture, followed by LC−MS/MS analysis resulting in the identification of peptides derived from the original protein mixture.[7] Identification of post-translational modifications (PTMs) by shotgun-based LC−MS/MS, in general, can be quite difficult depending on the sample being analyzed. Major factors that contribute to the difficulties in the analysis of PTMs in a sample include the following: (1) the complexity of the protein mixture being analyzed, (2) the dynamic range in abundances of all proteins in the sample, and (3) substoichiometric modification(s) of proteins. For simple protein mixtures (e.g., an extensively purified protein or protein complex), LC−MS methods alone are often sufficient to identify PTMs on the protein(s) of interest.[8,9] However, as a protein sample becomes more complex, the ability to identify PTMs decreases due to limitations in chromatographic resolution and in the sampling rate and sensitivity of the mass spectrometer. One solution to this problem is the use of PTM-specific enrichment strategies upstream of LC−MS analysis.[10−12]

One of the most common PTMs studied by mass spectrometry is phosphorylation. A number of techniques have been developed for the specific enrichment of phosphopeptides,[6] with immobilized metal affinity chromatography (IMAC)[13] being the most commonly used procedure. More recently, TiO_2 has been used as an alternative to IMAC,[14−18] and in one study, it was reported to be more effective than IMAC in the specific enrichment of phosphopeptides.[16] Unfortunately, there has been limited information published regarding the optimal buffer conditions to use for TiO_2-based enrichment of phosphopeptides,[16,18] relative to the extensive literature for IMAC-based enrichment procedures.[19] Due to the

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lack of these types of studies, it is difficult to conclusively compare the enrichment efficiencies of TiO_2 and IMAC. In this study, we optimize the buffer/solvent conditions for TiO_2-based enrichment of phosphopeptides using an online multidimensional LC−MS system, and we compare the effectiveness of TiO_2 relative to Fe-(III)-IMAC and ZrO_2 using samples of different complexity. In the end, an automated multidimensional phosphopeptide enrichment method is developed that has the capability for high-throughput sample analysis by mass spectrometry.

EXPERIMENTAL SECTION

α-Casein and β-Casein Digestion. α-Casein and β-casein (Sigma Aldrich), were originally made up into stock solutions of 2 and 1 mg/mL using H_2O. One hundred micrograms of each was digested using trypsin (Promega, sequencing grade) at an enzyme to substrate ratio of 1:10. Digestion was carried out separately for each protein in 2 M urea, 100 mM Tris pH 8.5 in a total volume of 200 μL for 6 h at 37 °C; the digests were then stored at −80 °C without further treatment. Aliquots of the digests were subsequently brought up in different concentrations of acetonitrile and formic acid for injection on the multidimensional liquid chromatography system (MDLC).

Recombinant Protein Purification and in Vitro Kinase Assay. pMBP-Dig1-FLAG was expressed in *Escherichia coli* strain BL21, which was cultured at room temperature in 1-L of 2× YT supplemented with 100 μg/mL carbenicillin and 1 mM $MgSO_4$. Upon reaching an OD_{600} of 0.6, expression was induced with 0.1 mM IPTG for 4 h. Cells were collected by centrifugation, resuspended in 15 mL of buffer C (20 mM Tris-HCl (pH 7.4), 200 mM NaCl) containing lysozyme, and frozen at −20 °C. Samples were then thawed in 15 mL of buffer A (20 mM Tris-HCl (pH 7.4), 200 mM NaCl, 10 mM EDTA, 10 mM EGTA, 10 mM BME, 1 mM AEBSF, 5 μg/mL leupeptin) and sonicated. Cell debris was removed by centrifugation for 30 min at 16 000 rpm at 4 °C. Four milliliters of amylose resin (New England Biolabs) was washed twice with buffer B (20 mM Tris-HCl (pH 7.4), 200 mM NaCl, 10 mM EDTA, 10 mM BME, 0.1 mM AEBSF) and added to the clarified lysate. The mixture was rocked for 2 h at 4 °C. Beads were washed four times in buffer B and then eluted with 12 mL of buffer B supplemented with 20 mM maltose for 30 min at 4 °C. DTT and glycerol were added to the eluate to a concentration of 1 mM and 20%, respectively.

Yeast strain YM1972 (*MAT***a** *bar1*Δ) was transformed with a plasmid encoding a protein A-tagged version of *FUS3* driven by the *GAL1* promoter[20] and grown overnight in YPAD media containing 0.2% glucose. The culture was then diluted to an OD_{600} of 0.6 with YPAD media containing 2% galactose to a final volume of 300 mL and cultured for an additional 6 h. The culture was then treated with 100 nM α-factor for 15 min. Cells were collected by centrifugation and snap-frozen. Pellets were resuspended in 600 μL of TENNI buffer (50 mM Tris-HCl (pH 7.4), 250 mM NaCl, 50 mM NaF, 5 mM EDTA, 0.1% Igepal, 15 mM 4-nitrophenyl phosphate, 30 mM $Na_2S_2O_5$, 0.1 mM Na_3VO_4, 80 mM β-glycerophospate, 1:100 Sigma phosphatase inhibitor cocktails 1 and 2, and 1:260 Sigma protease inhibitor cocktail), and zirconia/silica beads were added. Cells were disrupted in a Mini-Beadbeater 8 (Biospec products) by four cycles of disruption at full power for 1 min, cooling on ice for 2 min between each cycle. Extract was removed by pipetting, the beads were washed in 600 μL of TENNI buffer, and this material was added to the extract. Cell debris was removed by centrifugation at 14 000 rpm for 10 min.

IgG Sepharose (Pharmacia) was washed three times in TENNI buffer. A 30-μL sample was added to the clarified extract, and the mixture was rocked at 4 °C for 2 h. The beads were washed 4 times with 500 μL of TENNI buffer and twice with kinase buffer (50 mM Tris-HCl (pH 7.4), 10 mM $MgCl_2$, 1 mM DTT, 15 mM 4-nitrophenyl phosphate, 30 mM $Na_2S_2O_5$, 0.1 mM Na_3VO_4, 80 mM β-glycerophospate, 1:100 Sigma phosphatase inhibitor cocktails 1 and 2, and 1:260 Sigma protease inhibitor cocktail). A slurry of beads in kinase buffer was combined with 18 μg of purified Dig1, and the kinase reaction was initiated by adding ATP to a final concentration of 1 mM in a total reaction volume of 450 μL. Following incubation at 30 °C for 1 h, the beads were separated by centrifugation, and the supernatant was concentrated by precipitation with trichloroacetic acid (TCA). Based on western blot analysis of previous in vitro kinase assays, only about one-third of the added Dig1 remains soluble and is present in the supernatant after the in vitro kinase assay. Thus, only 6 μg of purified Dig1 is expected in the supernatant of the reaction analyzed.

The TCA precipitate was then solubilized in 50 μL of 8 M urea, 100 mM Tris pH 8.5. The mixture was brought to 5 mM TCEP and incubated at room temperature for 30 min. Iodoacetamide was then added to 10 mM and the resultant mixture incubated at room temperature for 30 min in the dark. The sample was then diluted to 4 M urea with 100 mM Tris pH 8.5. $CaCl_2$ was added to 2 mM and trypsin at 1:25 enzyme to substrate ratio. Digestion was carried out at 37 °C overnight. The digest was then split into nine equal aliquots. Each aliquot was subsequently brought up to 20% acetonitrile, 2% formic acid in a total of 100 μL, where 40 μL (∼270 ng, ∼3 pmol, of expected amount of substrate) was used per injection on the MDLC when comparing different chromatography resins.

Multidimensional Chromatography Coupled Tandem Mass Spectrometry (LC/LC−MS/MS). Tryptic digests were analyzed using the Ettan MDLC (GE Healthcare), which is essentially a combination of an autosampler, HPLC, and four-valve plumbing system in one instrument allowing for automated online LC/LC−MS/MS. The plumbing setup on the MDLC was in the "online salt step" configuration, (see Ettan MDLC manual or Supporting Information S-Figure 1) except that the analytical columns were removed from the "column switching valve" and were replaced with a single C18-packed pulled tip (see below) positioned at the interface with the ion trap mass spectrometer. Briefly, the sample flow path leads from the autosampler to the first dimension column, which consists of 5 cm of resin (e.g., TiO_2, see below for all resins used) packed in 250-μm fused silica. During loading, the flow-through is collected on one of two trap columns (C18, Gemini, 5 μm, 110 Å, Phenomenex) of 5 cm packed in 250-μm-i.d. fused silica. Ultimately, elution buffer is delivered from the autosampler over the first dimension column, and eluted peptides are collected on the second trap column, while buffer goes to waste. Peptides bound to each trap column can then be eluted

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using a reversed-phase gradient over the trap column and analytical column placed at the end of the plumbing line in a 75-μm-i.d. fused-silica pulled-tip containing 15 cm of C18 resin, 3 μm (Aqua, 125 Å, Phenomenex). However, in this study, the flow-through from loading on the first dimension column is typically not analyzed by LC−MS/MS. A micro-tee union (Upchurch) was used to connect the end of the plumbing line of the MDLC to the analytical column and to allow for the application of high voltage for electrospray as previously described.[21]

Resins used in the first dimension column position of the MDLC included the following: (1) TiO_2 (5 μm, Titansphere, GL Sciences), (2) TiO_2 (5 μm, 300 Å, Sachtopore-NP (labeled TiO_2-Sac in text), ZirChrom Separations), (3) ZrO_2 (5 μm, 300 Å, ZirChrom-PHASE, ZirChrom Separations), and (4) metal chelate resin (Poros 20 MC, 20 μm, Applied Biosystems).

IMAC was carried out using a packed Poros 20 MC column (as described above) placed in the first dimension column position of the MDLC. Fe(III) was then loaded on the resin via the MDLC essentially as described previously.[22] Briefly, the Poros 20 MC column was flushed with 100 μL of 50 mM EDTA pH 8, then flushed with 100 μL of H_2O, then loaded with 100 μL of 100 mM $FeCl_3$, and equilibrated with 0.1% formic acid. After sample loading and elution, the above procedure was repeated prior to subsequent sample loading.

MDLC buffers used for HPLC included buffer A (0.1% formic acid) and buffer B (84% acetonitrile, 0.1% formic acid). The MDLC method used for all multidimensional chromatography consisted of five steps. The first step loads a digested sample (10 μL for α- and β-casein digest or 40 μL for Fus3-Dig1 digest) onto the first dimension column via the autosampler with the flow-through going to trap column 1 (see S-Figure 1 for MDLC diagram). The second step injects 20 μL of wash buffer over the first dimension column via the autosampler and onto trap column 1. The third step injects 100 μL of elution buffer over the first dimension column and onto trap column 2 via the autosampler. The fourth step develops a reversed-phase gradient over trap column 2 and the analytical column. An optional step can follow that develops a reversed-phase gradient over trap column 1 and the analytical column in order to examine what did not bind to the first dimension column, though this step was not utilized in this study. The last step includes injecting 40 μL of buffer B, via the autosampler, over the first dimension column and trap column 1 to waste. A 2- and 3-h reversed-phase gradient was used for analyzing the α- and β-casein digest and the kinase assay digest, respectively. For "reversed phase only runs", the first dimension column was left out of the MDLC setup, and a digest was loaded directly onto trap column 2 followed by a reversed-phase gradient as described above. Flow rates used in the MDLC separations were as follows: 10 μL/min for loading of sample, wash buffer, and elution buffers onto the first dimension column, 15 μL/min for desalting (using buffer A) of trap columns after previously mentioned steps, and 100 μL/min for the reversed-phase gradient (due to the split flow within the MDLC and length of column, flow rate out of the analytical column tip was 200 nL/min). The MDLC was interfaced with an LCQ Deca ion trap mass spectrometer (Thermo Electron) as described above. Data-dependent MS/MS (MS2) acquisition was coupled with the above MDLC analyses as described previously.[23]

MS Data Processing. MS2 spectra were extracted from Xcalibur RAW files using the program Raw Extract (written by John Venable, T.S.R.I.) and poor quality spectra were removed using a previously described program.[24]

For the α- and β-casein samples, MS2 spectra were searched against a 10-protein database using SEQUEST.[25] The database contained α-casein, β-casein, albumin, and actin from bovine; lysozyme and ovalbumin from chicken, cytochrome *c* and apomyoglobin from horse; phosphorylase A from rabbit; and lectin from lentil. Serine and threonine were differentially searched for phosphorylation (+80) considering up to four modifications per peptide; no enzyme specificity was used, and a mass tolerance of ±3 amu for the precursor was used in the search. The SEQUEST results were then filtered using the program DTASelect[26] requiring XCorr cutoffs of 1.5, 2.5, and 3.5 for +1, +2, and +3 charged peptides, respectively, a DeltaCN cutoff of 0.08, and fully tryptic status of identified peptides. Peak areas of select phosphopeptides were calculated using the program CenSus (written by S.K.P., T.S.R.I., and will be published elsewhere).

For the kinase assay sample, MS2 spectra were searched against a database consisting of the *Saccharomyces cerevisiae* proteome (downloaded from SGD 12/16/05), *E. coli* K12 proteome (downloaded 7/20/06 from Integr8, EMBL-EBI), common protein contaminants, and the reversed sequence of these three databases. The SEQUEST search parameters were as above except tyrosine was also considered for phosphorylation, up to only two modifications per peptide were considered, cysteine was considered to have a static modification of +57 due to alkylation by iodoacetamide treatment, and only tryptic peptides (with a maximum of two missed internal cleavage sites allowed) were considered in the search. SEQUEST results were filtered based on determining false positive rates in a manner similar to that previously described.[27] Identifications were filtered based on the combination of XCorr and DeltaCN scores, separating unmodified and modified identifications each into additional categories of +1, +2, and +3 charged peptides, and separately calculating false positive rates. Unmodified and modified peptide identifications were set at a false positive cut off of 2 and 5%, respectively, using the latest version of DTASelect, DTA2.0 (written by Dave Tabb and Daniel Cociorva).

RESULTS AND DISCUSSION

An automated multidimensional liquid chromatography system (MDLC) coupled to ESI-based tandem mass spectrometry was used to optimize a set of buffer conditions for the enrichment of phosphopeptides using TiO_2. In all buffer optimization experi-

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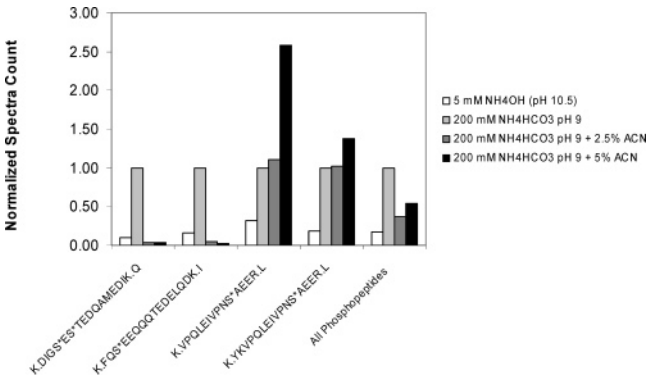

Figure 1. Comparison of elution buffers for phosphopeptide analysis using TiO_2. One picomole of a tryptic digest of α- and β-casein was loaded onto TiO_2 using an MDLC. Bound peptides were washed, eluted onto a C18 trap column, and subjected to online LC−MS/MS analysis; peptide−spectral matches were made using the SEQUEST algorithm. Four different elution buffers were tested. Spectra count results of the four most abundant phosphopeptides and the sum of all spectra counts from all identified phosphopeptides are presented. Spectra count results are normalized to those observed for the 200 mM NH_4HCO_3 pH 9 elution buffer. Non-normalized data of all identified phosphopeptides are included in Supporting Information (S-Table 7). The loading and wash buffers contained 10% acetonitrile and 1% formic acid.

ments, a tryptic digest of 1 pmol of both α- and β-casein was used, and duplicate runs were carried out. In short, the digest, in defined buffer, was loaded via the autosampler onto a TiO_2 column while the flow-through went to waste. The TiO_2 column was then washed by an injection of defined buffer using the autosampler with the flow-through going to waste. TiO_2-bound peptides were then eluted onto a trap column, again by autosampler injection, using a defined buffer. The eluted peptides were separated using a reversed-phase gradient over the trap column and analytical column that was coupled to ESI-based data-dependent MS/MS analysis (see Experimental Section and Supporting Information S-Figure 1 for further details).

Initially, four elution buffers were tested for the ability to elute α- and β-casein-derived phosphopeptides off of TiO_2 resin; two of which, NH_4HCO_3 pH 9 and NH_4OH pH 10.5, have been used in previous publications.[16,17] In this study, the concentration of NH_4HCO_3 used (200 mM) was reduced from that used by Pinkse et al.[17] (250 mM) only to reduce the high back pressure caused by this buffer. Using the MDLC, the α- and β-casein digest was loaded onto TiO_2 in 10% acetonitrile and 1% formic acid (as arbitrary loading buffer), and the column was washed with loading buffer. Bound peptides were then eluted with one of four buffers and analyzed by LC−MS/MS as described above. Figure 1 shows the results for each elution buffer used. In the analysis, overall, eight unique phosphopeptides were identified that derive from all three proteins in the mixture, α-S1, α-S2, and β-casein (S-Table 7). Spectra count information for the four most prominent and reproducibly identified phosphopeptides from this digested mixture are displayed in Figure 1; these include K.FQS*EEQQQTEDELQDK.I from β-casein and K.YKVPQLEIVPNS*AEER.L, K.VPQLEIVPNS*AEER.L, and K.DIGS*ES*TEDQAMEDIK.Q from α-S1 casein. The sum of spectra count numbers for all identified phosphopeptides (not just the four most prominent) are also reported for each elution condition in Figure 1. Previous work has shown that spectra count can be used as a means of quantification.[28,29] Thus, we use spectra count as a means of quantifying phosphopeptide abundance in this comparison of elution buffers and in subsequent experiments as well. As an additional means of determining phosphopeptide abundance, peak areas for the two most intense phosphopeptides, K.YKVPQLEIVPNS*AEER.L and K.FQS*EEQQQTEDELQDK.I., were determined from the resulting chromatograms of the experiment and are presented in Supporting Information (S-Table 1).

Due to large differences in spectra count numbers of the four most prominent phosphopeptides and for the sum of all phosphopetides identified, presented in Figure 1, it is clear that the use of 200 mM NH_4HCO_3 pH 9 is significantly better than 5 mM NH_4OH pH ∼10.5 as an elution buffer for TiO_2 using this automated multidimensional LC setup. Additionally, peak area data for the two most prominent phosphopeptides for this experiment (S-Table 1) correlate well with the spectra count data for the corresponding individual phosphopeptides. In contrast to these findings, it was recently reported that NH_4OH pH 10.5 is more of an effective phosphopeptide elution buffer than NH_4HCO_3 pH 9 for TiO_2.[16] One obvious reason for the discrepancy is the different means of chromatography used; where in the report from Larsen et al., the TiO_2 separation step was done offline using microcolumns packed in pipet tips.[16] Due to the limited buffering capacity of NH_4OH, the effective pH of a 5 mM solution may be significantly reduced once it reaches the TiO_2 column in our MDLC setup due to the primary running buffer (0.1% formic acid) in the autosampler and HPLC lines. If true, this could then explain the reduced elution efficiency of NH_4OH compared to NH_4HCO_3 and the difference in observed results between the two studies. Also, additions of 2.5 and 5% acetonitrile to 200 mM NH_4HCO_3 pH 9 were tested as elution buffers (Figure 1), since small concentrations are often added to elution buffers in order to enhance elutions by reducing hydrophobic interactions of peptides with the base material of resins. It is apparent that the addition of just a small concentration of acetonitrile (2.5%) significantly reduces the spectra count numbers for two of the four most prominent phosphopeptides. Again, peak area data (S-Table 1) for the two most prominent phosphopeptides correlate well with the corresponding spectra count data. The most likely explanation of these results is that the addition of acetonitrile to the elution buffer reduces the ability of the affected phosphopeptides to bind to C18 after being eluted. Correspondingly, the reduced capacity of some phosphopeptides to bind to C18 has been observed before and is often attributed to their general hydrophilic nature. Interestingly, the detection of K.VPQLEIVPNS*AEER.L was actually enhanced by the addition of 5% acetonitrile. This is in contrast to the aforementioned results of two other phosphopeptides and may be due to differences in overall hydrophobicity of the phosphopeptides. Altogether, we decided not to include acetonitrile in our elution buffer of 200 mM NH_4HCO_3 pH 9 used in further optimization experiments.

We next attempted to optimize the concentration of acetonitrile used in our loading buffer for TiO_2-based enrichment of phosphopeptides. Using the same digest as above, we tested a range

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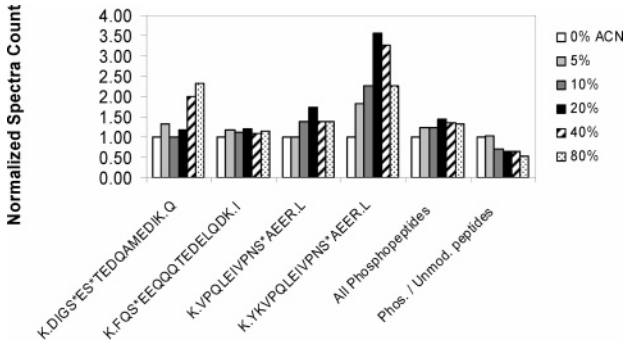

Figure 2. Affect of acetonitrile in TiO_2 loading buffer. One picomole of a tryptic digest of α- and β-casein was loaded onto TiO_2 using the MDLC similarly as in Figure 1, except that different concentrations of acetonitrile (ACN) were used in the loading buffer and wash buffer, and peptides were eluted from TiO_2 using 200 mM NH_4HCO_3 pH 9. A comparison of 0−80% ACN in both loading and wash buffers (all in the presence of 0.1% formic acid) is presented. Spectra count results of the four most prominent phosphopeptides, the summary data of all identified phosphopeptides, and the ratio of phosphopeptides to unmodified peptides are presented for each concentration of ACN used. Spectra count results are normalized to those observed for the 0% ACN buffer. Non-normalized data of all identified phosphopeptides are included in Supporting Information (S-Table 8).

Table 1. Results of the Use of Different Concentrations of ACN in Loading and Wash Buffers with 0.1% FA in a TiO_2-Based Enrichment of Phosphopeptides from a Tryptic Digest of α- and β-Casein

	peak area	
% ACN	K.YKVPQLEIVPN-S*AEER.L	K.FQS*EEQQQTE-DELQDK.I
0	1.7×10^9	7.5×10^9
5	2.8×10^9	1.3×10^{10}
10	5.6×10^9	1.8×10^{10}
20	1.1×10^{10}	1.9×10^{10}
40	1.0×10^{10}	1.8×10^{10}
80	4.4×10^9	2.1×10^{10}

of 0−80% acetonitrile with 0.1% formic acid in the loading buffer and wash buffers. For a particular sample, after loading, the TiO_2 column was washed with the same buffer used for loading. Subsequently, peptides were eluted from TiO_2 and analyzed as described above. As previously done, we report on the spectra count numbers for the four most prominent phosphopeptides and the sum of all phosophopeptides identified in Figure 2. Additionally, the unmodified spectra count was documented, and the ratio of phosphopeptide to unmodified spectra count is reported in Figure 2. This ratio allows one to easily see the effect of different buffers on the amount or numbers of unmodified peptides that bind and are subsequently eluted from the TiO_2 column, relative to phosphopeptides. Increasing the concentration of acetonitrile in incremental steps from 0 to 20% in the loading buffer generally results in a correlative increase in spectra count numbers for the four most prominent phosphopeptides as well as all phosphopeptides combined (Figure 2). Similar, but more dramatic, effects are seen in the peak area data for the two most prominent phosphopeptides (Table 1). Above this concentration, 40 and 80% acetonitrile, a pleateau and slight decrease in some of these numbers are seen (Figure 2 and Table 1). As for the ratio of phosphopeptide to unmodified peptide spectra count, a slight decrease was

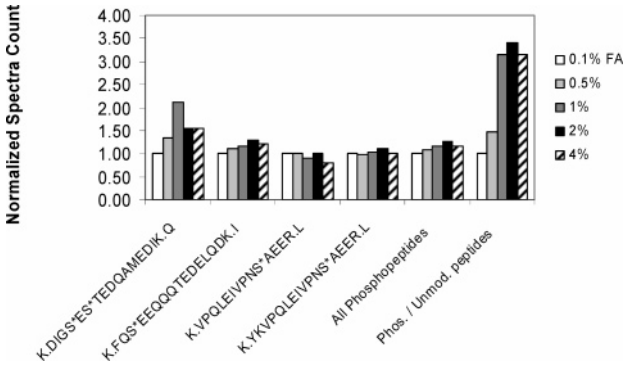

Figure 3. Affect of formic acid in TiO_2 loading buffer. One picomole of a tryptic digest of α- and β-casein was loaded onto TiO_2 using the MDLC and analyzed as in Figure 1, except that each mixture was loaded in 20% acetonitrile and varying concentrations of formic acid (FA). Bound peptides were washed with loading buffer and eluted with 200 mM NH_4HCO_3 pH 9. A comparison of 0.1−4% FA used in both loading and wash buffers is presented. Spectra counts are normalized to the results observed for 0.1% FA. Non-normalized data of all identified phosphopeptides are included in Supporting Information (S-Table 9).

observed correlating with increasing concentration of acetonitrile (Figure 2). This suggests that binding of unmodified peptides, as well as phosphopeptides, to TiO_2 is enhanced by acetonitrile. From these results, 20% acetonitrile was chosen as the concentration to use in the loading buffer for future experiments. This is viewed as a compromise between largely increasing the efficiency of phosphopeptide binding while only marginally increasing the binding of unmodified peptides. The retention of unmodified peptides on TiO_2 during this optimization process is of obvious concern because this activity will ultimately affect the number of phosphopeptides identified in a sample by LC−MS/MS through various means (e.g., suppression of ionization), and this will be especially important when dealing with more complex samples (see below).

We next set out to optimize the concentration of formic acid used in our loading and wash buffers. A range of 0.1−4% formic acid containing 20% acetonitrile was tested as a loading buffer and wash buffer. As in the previous optimization experiment, the same loading and wash buffer was used for each digested sample of α- and β-casein and duplicate runs were carried out, the results of which are displayed in Figure 3. A dramatic decrease in the number of unmodified peptides identified in the TiO_2 eluates due to the use of increasing amounts of formic acid in the load and wash buffers was observed. This is shown in Figure 3, where one can see a large correlative increase in the ratio of phosphopeptide to unmodified spectra with increasing concentrations of formic acid. There was a significant increase in spectra count for only one of the phosphopeptides in response to increasing formic acid, which may be due to the effect of reducing the number of unmodified peptides in the final eluate. Peak area data for the two most prominent phosphopeptides (S-Table 2), again, reflect the spectra count data. Based on all of the data, we chose 2% formic acid as the optimal concentration in our loading buffer containing 20% acetonitrile. Additionally, we note that these findings are generally in agreement with those observed by Larsen et al. and Schlosser et al. for TiO_2-based phosphopeptide enrichment.[16,18]

Table 2. Limit of Detection Experiment for Individual Phosphopeptides in a Tryptic Digest of α- and β-Casein[a]

	amount loaded				
phosphopeptides identified	1 fmol	10 fmol	100 fmol	1 pmol	10 pmol
K.FQS*EEQQQTEDELQDK.I	1	3	8	61.5	91.5
K.YKVPQLEIVPNS*AEER.L	1	1.5	4.5	30.5	52
K.VPQLEIVPNS*AEER.L	0	1.5	1	5	19.5
K.DIGS*ES*TEDQAMEDIK.Q	0	0	1	5.5	21
K.IEKFQS*EEQQQTEDELQDK.I	0	0	1	1	4
K.DIGS*ESTEDQAMEDIK.Q	0	0	0	1	0.5
K.VNELS*KDIGS*ESTEDQAMEDIK.Q	0	0	0	0.5	0.5
K.NMAINPS*KENLCSTFCK.E	0	0	0	0	1
K.TVDMES*TEVFTK.K	0	0	0	0.5	0

[a] Values presented are the average spectra count per peptide from two runs for 1 fmol up to 10 pmol. One femtomole was the lowest amount where at least one phosphopeptide was identified in both replicate MDLC runs.

We next tested the binding efficiency of TiO_2 in the context of our optimized loading buffer. This was done by carrying out an offline experiment where we loaded 1 pmol of digested α- and β-casein in a buffer containing 20% acetonitrile and 2% formic acid onto a TiO_2 column (of the same dimensions and using similar flow rates as in previous experiments). The flow-through was collected, diluted to a final concentration of 2% acetonitrile, and analyzed by reversed-phase LC−MS/MS, where no phosphopeptides were observed (data not shown). Thus, it appears that the majority of phosphopeptides from 1 pmol of digested α- and β-casein bind TiO_2 within our optimized loading buffer of 20% acetonitrile and 2% formic acid.

Last, we decided to test the effect of increasing the concentration of acetonitrile used in the wash buffer. To test this, the same digest was loaded in 20% acetonitrile and 2% formic acid onto TiO_2 via the MDLC, then washed with a range of 20−80% acetonitrile in 2% formic acid, and subsequently eluted and analyzed as above. The results (S-Figure 3) show a slight increase in phosphopetide spectra counts and also in the ratio of phosphopeptide to unmodified peptide spectra counts in correlation with the use of increasing concentrations of acetonitrile in the wash buffer. From these results, we chose to use 80% acetonitrile, along with 2% formic acid, in our wash buffer. Altogether, we determined the best concentrations of acetonitrile and formic acid to use for the enrichment of phosphopeptides using TiO_2 in our automated LC setup using a digest of a simple phosphoprotein mixture. Thus, our final conditions include the following: a loading buffer containing 20% acetonitrile and 2% formic acid, a wash buffer of 80% acetonitrile and 2% formic acid, and an elution buffer of 200 mM NH_4HCO_3 pH 9.

As mentioned above, there have been some reports on the analysis of different buffer conditions used for phosphopeptide enrichment using such resins as IMAC and TiO_2. The most thoroughly studied condition in these reports has been the acid content of the loading buffer. For TiO_2-based enrichment, it has been shown that increases in the concentration of a particular acid or replacing an acid with one of a lower pH in the loading buffer reduces nonspecific binding of unphosphorylated peptides, while enhancing the detection efficiency of phospopeptides.[16,18] The same trend has been reported with the use of IMAC and ZrO_2 as well.[15,19] Additionally, Kokubu et al. showed that increasing the concentration of acetonitrile in a loading buffer enhances the enrichment of phosphopeptides using IMAC.[19] However, this result is slightly confounded by the fact that the flow-through and IMAC-eluted peptides were not separated due to the presence of C18 resin adjacent to the IMAC resin in the columns used in the study.[19] In contrast to the report by Kokubu et al., Haydon et al. observed that there was no effect on nonspecific binding of unphosphorylated peptides to IMAC by increasing the concentration of acetonitrile in the loading buffer.[30] However, in the data presented above, we observed an increase in phosphopeptide binding while observing a smaller increase in unmodified peptide retention, due to an increase in acetonitrile concentration in the loading buffer (Figure 2).

We next wanted to determine the limits of detecting phosphopeptides in the α- and β-casein digest using TiO_2. We tested amounts from 1 amol up to 10 pmol of the digest, in 10-fold increments, on the MDLC using the aforementioned optimized buffer conditions, and each load amount was run in duplicate. Table 2 shows the results of the nine phosphopeptides identified in this experiment, for 1 fmol up to 10 pmol; 1 fmol is chosen as the lowest amount to display because this is the lowest amount loaded that resulted in at least one phosphopeptide being identified in both of the duplicate runs.

After optimized conditions were reached using our LC system, we attempted to compare TiO_2 with other resins used for phosphopeptide enrichment. Altogether, two different sources of TiO_2, ZrO_2, and Fe(III)-IMAC were compared using the MDLC. The same optimized loading, wash, and elution buffers for TiO_2 were used for the other phosphopeptide enrichment resins in the comparative analysis. Fe(III) loading, stripping, and regeneration of the IMAC column with the MDLC was carried out as described in the Experimetal Section. Three amounts (100 fmol, 1 pmol, 10 pmol) of the α- and β-casein digest were run in duplicate for each resin using the MDLC, as was a reversed-phase analysis alone (i.e., no enrichment). Figure 4 shows spectra count results for the four most prominent phosphopeptides, total phosphopeptides, and ratio of phosphopeptides to unmodified peptides from the analysis of 1 pmol of the digest (average of duplicate runs), though the 100-fmol and 10-pmol results showed similar trends (S-Table 11). From these results, shown in Figure 4, it is apparent that each resin shows similar differences for each of the four listed phosphopeptides and for the combined phosphopeptide category as well. Overall, TiO_2 (GL Siences) demonstrated the highest

(30) Haydon, C. E.; Eyers, P. A.; Aveline-Wolf, L. D.; Resing, K. A.; Maller, J. L.; Ahn, N. G. *Mol. Cell. Proteomics* **2003**, *2*, 1055−1067.

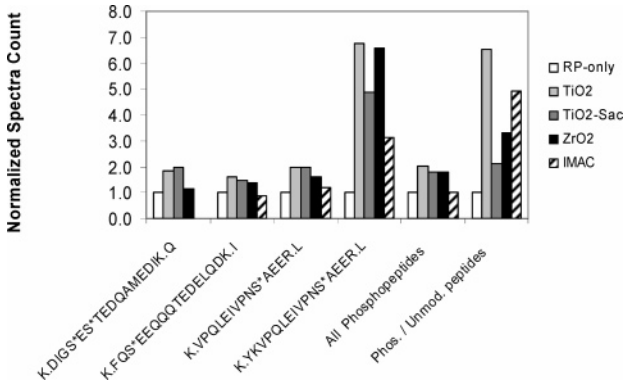

Figure 4. Comparison of different resins in the ability to enrich phosphopeptides from a tryptic digest of α- and β-casein. Results shown are the average of duplicate runs, loading 1 pmol (of each digested protein). The optimized buffer system using online two-dimensional liquid chromatography, as described in the text, was used for all chromatography runs except for the RP-only run (reversed phase only, single dimension, where no enrichment step is performed). All reported values represent average spectra counts or ratios of average spectra counts from duplicate runs and are normalized to the RP-only run, additional data presented in S-Table 11.

phosphopeptide spectra counts and the highest ratio of phosphopeptide to unmodified peptide spectra counts for all the resins. The other source of TiO_2 (TiO_2-Sachtopore, ZirChrom Separations) and ZrO_2 resins had only slightly less phosphopeptide spectra counts, overall, but had significantly lower spectra count ratios of phosphopeptides to unmodified peptides compared to TiO_2 from GL Sciences. The phosphopeptide spectra counts for the IMAC runs were significantly lower than the other resins and similar to the reversed-phase analysis alone, indicating that there is a decrease in phosphopeptide retention on the IMAC column relative to the other phosphopeptide enrichment resins. However, it was observed that the ratio of phosphopeptide to unmodified peptide spectra count for IMAC was relatively higher compared to that seen with the use of TiO_2-Sachtopore and ZrO_2. As carried out in the previous optimization experiments, peak areas of the two major phosphopeptides from the digest were also measured in this experiment (S-Figure 4); where these peak area intensities generally reflect the phosphopeptide spectra count data presented in Figure 4.

Last, we wanted to compare the same phosphopeptide enrichment resins using a more complex sample. The protein mixture chosen was an in vitro kinase assay using the kinase Fus3, expressed and purified from yeast, and its substrate Dig1, expressed and purified from *E. coli*. A silver-stained gel of the in vitro kinase assay and a western blot analysis of Dig1 before and after the kinase reaction (which shows a shift in molecular weight upon phosphorylation) are presented in Supporting Information (S-Figure 5). This protein mixture was digested with trypsin and used to compare the different resins using the optimized buffer conditions via the MDLC as above. Approximately 270 ng (~3 pmol), based on amount of Dig1, was used in each LC analysis and run in duplicate. The mixture was of moderate complexity; where, by reversed-phase analysis alone, 49 proteins were identified with at least two unique peptides; and in a less stringent filtering, 100 proteins were identified with one or more unique peptide. Within this list of proteins, Dig1 was the third most abundant protein based on spectra count, with a sequence coverage of 59% (S-Table 4, where the 14 most abundant proteins, based on spectra count, are listed). From the comparison of resins using this more complex sample (shown in Table 3), it was observed that TiO_2 (GL Sciences) retained the highest number of unique Dig1 phosphopeptides (15), whereas IMAC identified even fewer (1) than the reversed-phase analysis alone (3). This suggests, again, that Fe(III)-IMAC has less retention capabilities than TiO_2 using this online LC system. Both TiO_2-Sachtopore and ZrO_2 had slightly fewer Dig1 phosphopeptides identified (9 and 11, respectively) and a much lower phosphopeptide to unmodified spectra count ratio compared to that observed for TiO_2 (GL Sciences) (Table 3). Additional data from this experiment, including a list of all phoshopeptides identified, with corresponding spectra counts and SEQUEST XCorr scores, are presented in Supporting Information (S-Table 12). Altogether, the data suggest that TiO_2 (GL Sciences) outperforms ZrO_2, IMAC, and TiO_2-Sachtopore as a phosphopeptide enrichment resin for analyzing moderately complex protein digests. At this point, it is unclear why one source of TiO_2 worked better than another for enrichment of phosphopeptides, but may be due to undocumented modifications of the resins made by the respective manufacturers.

Similar results have been seen in the limited number of reports comparing phosphopeptide enrichment resins. Larsen et al. also found TiO_2 to be superior to Fe(III)-IMAC in the enrichment of phosphopeptides.[16] Interestingly, Larsen et al. observed a large difference in efficacy of phosphopeptide enrichment between the two resins when comparing a simple mixture (a digest of 3 phosphoproteins and 3 unphosphorylated proteins mixed 1:1) to that of a digest from a 1:50 mixture (phosphoproteins.unphosphorylated proteins) of the same proteins.[16] This is similar to what we observed between the analyses of the simple α- and β-casein sample (Figure 4) and the moderately complex in vitro kinase sample (Table 3). Additionally, though we found TiO_2 to be more effective than ZrO_2 in the enrichment of phosphopeptides, a previous study found less of a difference between the two resins;[15] however, the source of the resins, as mentioned before, may be of significant influence in the overall performances observed and may explain the difference in results between the two studies.

One final point of concern is whether the optimized buffer conditions determined for the digest of the simple mixture of α- and β-casein would also be optimal for a more complex mixture. Due to limited amounts of the above Fus3−Dig1 in vitro kinase sample, we decided to only test the buffer component that we felt would most likely affect a moderately complex sample differently from a simple mixture and that was the amount of acetonitrile used in the loading buffer. Thus, we tested increasing amounts of acetonitrile in the loading buffer for the TiO_2-based enrichment of phosphopeptides from a digest of the Fus3−Dig1 sample, as similarly done with the α- and β-casein (Figure 2), in the presence of 2% formic acid. Results from these experiments show that the addition of at least 5% acetonitrile dramatically enhances the detection of phosphopeptides from the substrate Dig1 (S-Table 5), where the use of 5−20% acetonitrile resulted in the same number of Dig1-derived phosphopeptides being identified (S-Table 6). However, further increases in the concentration of acetonitrile (40 and 80%) used in the loading buffer reduced the number of phosphopeptides identified and dramatically

Table 3. Comparison of Different Resins in the Ability To Enrich Phosphopeptides from a Tryptic Digest of a Moderately Complex Protein Mixture, an in Vitro Kinase Assay Containing the Purified Kinase Fus3 and Its Protein Substrate Dig1

Dig1 phosphopeptides identified	RP-only	TiO_2	TiO_2 Sac	ZrO_2	IMAC
K.DGNLAS*SNSAHFPPVANQNVK.S		+		+	
K.RVNDSYDS*PLSGTASTGK.T		+	+	+	
R.LRT*TAEDTSIAK.S		+		+	
R.NSSVGS*SANAGPTQQR.A		+	+		
R.NSSVGSS*ANAGPTQQR.A	+	+	+	+	
R.RSEEGSRNS*SVGSSANAGPTQQR.A		+		+	
R.SEEGSRNS*SVGSSANAGPTQQR.A		+			
R.SSWHEAEPNKDEEEGTDLAIEDGAVPT*PTFTTFQR.T	+	+	+	+	+
R.SSWHEAEPNKDEEEGTDLAIEDGAVPTPT*FTTFQR.T		+			
R.TS*QPQQQSPSLLQGEIR.L		+	+	+	
R.TSQPQQQS*PSLLQGEIR.L		+	+	+	
R.TTAEDTSIAKST*QDPIGDTEISVANAK.G		+	+	+	
R.VNDSYDS*PLS*GTASTGK.T		+			
R.VNDSYDS*PLSGTASTGK.T	+	+	+	+	
R.VNDSYDSPLS*GTASTGK.T		+	+	+	
Dig1 phosphopeptides	3	15	9	11	1
total phosphopeptides	4	29	13	17	3
total unmodified peptides	493	297	457	401	84
ratio phos/unmod peptides (norm)	1	12.1	3.5	5.2	4.4

increased the number of unmodified peptides that bound to TiO_2 and were subsequently eluted and identified (S-Table 5). Thus, the large excess of unmodified peptides in the eluate appears to decrease the number of phosphopeptides that can be identified. This is most likely due to the suppression of ionization of low-abundant phosphopeptides by more abundant, coeluting unmodified peptides and is due to the complexity of the eluate being analyzed by LC−MS/MS combined with the limited data-dependent sampling capabilities of the mass spectrometer. Altogether, the use of 5−20% of acetonitrile with 2% formic acid can be recommended as a loading buffer for samples of this complexity.

CONCLUSION

Using an automated online multidimensional LC system, we have been able to assess the effectiveness of using TiO_2 for the enrichment of phosphopeptides from protein digests. We demonstrated that the concentration of both acetonitrile and formic acid in the loading and wash buffers can be optimized to significantly enhance the performance of TiO_2 in the enrichment of phosphopeptides. Additionally, we demonstrated that TiO_2 outperforms Fe(III)-IMAC and ZrO_2 in phosphopeptide enrichment, using tryptic digests of both simple and moderate complexity. Together, we have optimized a system that will allow us to carry out higher-throughput analyses assessing phosphorylation states of multiple protein samples in a more efficient time scale.

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SUPPORTING INFORMATION AVAILABLE

Supplementary figures and tables provided as separate files. This material is available free of charge via the Internet at http://pubs.acs.org.

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