Published in final edited form as:

Anal Biochem. 2011 February 1; 409(1): 81–88. doi:10.1016/j.ab.2010.10.003.

Discontinuous pH gradient-mediated separation of TiO₂-enriched phosphopeptides

Sung-Soo Park and Stuart Maudsley *

Receptor Pharmacology Unit, National Institute on Aging, National Institutes of Health, 251 Bayview Blvd., Biomedical Research Center, Baltimore, MD 21224, USA

Abstract

Global profiling of phosphoproteomes has proven a great challenge due to the relatively low stoichiometry of protein phosphorylation and poor ionization efficiency in mass spectrometers. Effective, physiologically-relevant, phosphoproteome research relies on the efficient phosphopeptide enrichment from complex samples. Immobilized metal affinity chromatography and titanium dioxide chromatography (TOC) can greatly assist selective phosphopeptide enrichment. However, the complexity of resultant enriched samples is often still high, suggesting that further separation of enriched phosphopeptides is required. We have developed a pH-gradient elution technique for enhanced phosphopeptide identification in conjunction with TOC. Using this process, we have demonstrated its superiority to the traditional ‘one-pot’ strategies for differential protein identification. Our technique generated a highly specific separation of phosphopeptides by an applied pH-gradient between 9.2 and 11.3. The most efficient elution range for high-resolution phosphopeptide separation was between pH 9.2 and 9.4. High-resolution separation of multiply-phosphorylated peptides was primarily achieved using elution ranges > pH 9.4. Investigation of phosphopeptide sequences identified in each pH fraction indicated that phosphopeptides with phosphorylated residues proximal to acidic residues, including glutamic acid, aspartic acid, and other phosphorylated residues, were preferentially eluted at higher pH values.

Keywords

titanium dioxide; phosphoproteomics; mass spectrometry; pH-dependent elution

Introduction

Protein phosphorylation is one of the most important and dynamic post-translational modifications required for the regulation of most intermediary metabolism signal transduction cascades [1,2]. An enormous range of protein kinases and phosphatases control the phosphorylation status of proteins that result in changes of protein-protein interactions, regulation of enzymatic activities and control of dynamic subcellular localization changes [2]. These multiple aspects of cellular protein biochemistry underpin almost all biological functions. Rapid and comprehensive identification of protein phosphorylation sites is essential for understanding cellular biology and signal transduction connectivity [3]. The broad and unbiased appreciation of complex protein phosphorylation networks has been

*Corresponding author: Dr. Stuart Maudsley, Phone: +1-410-558-8472, Fax: +1-410-558-8323, maudsleyst@mail.nih.gov.

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greatly enhanced through the employment of high throughput LC-MS/MS instruments and efficient phosphopeptide enrichment strategies [4,5]. Existing methodologies for phosphoproteome analysis include: fractionation of tryptically-digested complex peptide mixtures by strong cation exchange (SCX) chromatography; phosphopeptide enrichment by titanium dioxide (TiO₂) chromatography, immobilized metal ion affinity chromatography (IMAC), or chemistry-based modification of phosphate groups [4–7]. Indicative of the extent of interest in metal ion-mediated phosphoproteomic enrichment techniques many diverse strategies have recently been developed including the use of spectra cross-validation [8], pH modification [9], mesoporous TiO₂ [10] and even nanocrystalline TiO₂ films [11]. Employing these multiple experimental platforms, thousands of phosphopeptides and protein phosphorylation sites can be analyzed from a single experimental cell signaling study, however comprehensive and differential identification of distinct groups of phosphoproteins is still slightly beyond current technologies [12].

Phosphopeptide enrichment methods represent one of the most important breakthroughs for phosphoproteomics and cellular signaling analysis. The coherent combination of this technique with quantitative tools has facilitated the global understanding of phosphoproteomic signaling patterns [5–7]. For the chemistry-based methods, phosphate groups are modified via β -elimination and Michael addition or phosphoramidate chemistry and attached to solid support for isolation [13,14]. The basic chemical principle of phosphopeptide enrichment without chemical modification is that phosphate groups in phosphorylated peptides have very low pK_a values compared with functional moieties in other amino acids so that it can sustain its negative charge even in an acidic environment. To achieve efficient and selective capture of phosphorylated peptides, solid matrices with extant positive charges under acidic conditions were needed. A matrix that can achieve this involves immobilized metal ions like iron (Fe³⁺) or TiO₂ particles. However, the binding of non-phosphorylated peptides with multiple acidic residues, such as glutamic acid or aspartic acid, hindered the efficiency of the enrichment step and subsequently the number of phosphopeptides identified via MS/MS. TiO₂ has been demonstrated to possess a capacity to engender a highly specific enrichment of phosphopeptides [15]. Such TiO₂-based techniques have subsequently been further improved by the use of a so-called ‘non-phospho excluder’. Several additives, such as 2,5-dihydroxybenzoic acid, phthalic acid, and glycolic acid, have been tested in order to reduce non-specific binding and indeed have shown dramatic enrichment improvements although their exact physico-chemical mechanism is largely unknown [16].

Adsorption of phosphate ions onto TiO₂ is known to occur primarily via a very strong bidentate attachment and is highly sensitive to the environmental solution’s pH [17]. Hence minimal binding is seen at pH 11 while at lower pH values there is a continuous variation of binding strength [18]. The strong binding of phosphopeptides to TiO₂ particles is expected to efficiently occur in buffer conditions from acidic to slightly basic (pH 8.6) conditions. To rapidly elute phosphorylated peptides from a TiO₂ particle basic ammonia solutions are commonly employed. We have hypothesized that the bound phosphopeptides could be separated further with a high resolution through ascending pH gradients as the charge states of all the residues in the specific peptide and also the TiO₂ particle depend on the environmental solution’s pH. This was partially demonstrated with methyl-esterified CDK2 peptides, which showed the separation of monophosphorylated peptides from multiply phosphorylated peptides using two step pH elutions. Although the purified protein was used for this study, they showed that phosphorylated peptides could be further separated in TiO₂ chromatography. Phosphopeptide separation in TiO₂ chromatography with more complex sample has not been studied and would be more complicated without methyl esterification.

In our current study, we have undertaken a systematic study of the detailed behavior of pH-dependent phosphopeptide elution from TiO₂. We have demonstrated the differential and specific effect of pH step gradients on phosphopeptide elution from TiO₂ column using a complex, neural whole-cell lysate. Our purification and separation strategy effected an efficient separation of phosphopeptides after TiO₂ enrichment and also simultaneously indicated a biologically-relevant advantage of identifying idiosyncratic multiply-phosphorylated peptides.

Materials and methods

Cell culture and protein extraction

Human neuroblastoma SH-SY5Y cells were maintained in media which was a 1:1 mixture of Advanced MEM and F-12 + GlutaMAXTM-1 supplemented with 10% Fetal Bovine Serum and 1% Penicillin-Streptomycin (Gibco). Upon reaching 60–70% confluence, cells were removed from dishes and washed twice with ice-cold phosphate buffered saline (PBS). Cell pellets were suspended in freshly prepared 8 M urea (Invitrogen) with 50 mM NH₄HCO₃ (Fluka) and completely disrupted using a Sonic Dismembrator (Model 100; Thermo Fisher Scientific). Protein concentrations were determined by BCA protein Assay Kit (Thermo Fisher Scientific). Extracted proteins were immediately digested with trypsin as described in the following section.

Sample preparation

Disulfide bonds of proteins were reduced in 12.5 mM Tris(2-carboxyethyl)phosphine (Sigma) at room temperature (RT) for 1hr and alkylated with 25 mM iodoacetamide (Sigma) at RT for 30min in the dark. The reaction was quenched with an additional incubation in 15 mM dithiothreitol (Sigma) at RT for 15min. Protein extracts were diluted in 50 mM NH₄HCO₃ to a final concentration of 1 M urea and were digested at 37°C using sequencing grade trypsin (Promega) at 1:75 (trypsin/protein, w/w) ratio. After overnight digestion, formic acid (Fluka) was added to a final concentration of 0.5% (v/v) and the peptide samples were desalted using Sep-Pak Vac tC18 Cartridges 3cc/200mg (Waters). Cartridges were washed with 3 mL of acetonitrile (ACN; Thermo Fisher Scientific) and equilibrated with 3 mL of 0.1% formic acid. After loading peptides (1.5 mg per one cartridge), cartridges were washed with 6 mL of 0.1% formic acid. Peptides were then eluted with 1 mL of 80% ACN 0.1% formic acid, dried to below 50 µL but not completely in Savant SpeedVac (Thermo Fisher Scientific), and stored at –80°C. When needed, partially dried peptide solutions were compensated with water up to 50 µL and peptide concentrations were measured with a BCA assay. Subsequently, one volume (50 µL) of washing buffers in Table 1 was added, which results in the composition of loading buffer in Table 1.

Column preparation

All the columns used in this study were made using 360-µm o.d. (outer diameter) fused silica capillary (Polymicro Technologies). For TiO₂ column, 150-µm i.d. (inner diameter) capillary was cut by 20 cm and one end was placed in Kasil solution (Next Advance) to allow the solution to enter the capillary to a length of 3–4 cm by capillary action. Frit formation was then completed by heating overnight at 70°C. After cutting out excess frit with 1 cm remaining, the capillary was packed with TiO₂ particles (Titansphere TiO₂ 5µm; GL Sciences) which were suspended in 50% methanol (J.T.Baker). All the procedures with capillary columns, such as packing, washing, and sample loading and elution, were carried out manually using a Next Advance pressure-loader (Model PC 77) and the flow rate of each step was controlled by adjusting the applied pressure. The amount of packed particles was measured by weighing the column before and after packing using a microbalance (Sartorius Instruments) in dry condition and 6 cm packing was equal to 2 mg of particles.

The analytical C18 column with integrated electrospray emitter tip was prepared on a laser puller (P2000; Sutter Instrument) with 75- μ m i.d. capillary using a previously developed protocol [19]. C18 resins (Magic C18, 5 μ m, 300 Å; Michrom Bioresources) were packed to 8.5 cm of 10.5 cm fritless capillary column on pressure-loader and then the resulted C18 capillary column was used for analytical column and also an electrospray interface for LC-MS/MS [20].

Phosphopeptide enrichment

The TiO₂ capillary column was washed first with 1% ammonia (Fluka) and then with a specific washing buffer (WB; Table 1) for 10 min each at 1.5 μ L/min flow rate on pressure-loader. Peptide samples were mixed 1:1 with the specific WB and loaded to the column at 0.8 μ L/min. The loaded amount was 100 μ g for experiment of measuring loading capacity of TiO₂ particles and 150 μ g for other. The TiO₂ column was then washed with WB for 10 min at 1.5 μ L/min. The bound peptides were eluted with 1% ammonia (pH 11.3) for 5 min at 1.5 μ L/min and immediately acidified with 5 μ L of 2% trifluoroacetic acid (TFA; Fluka). For pH step gradient elution, elution buffers were freshly prepared with 100 mM NH₄HCO₃ and the pH of each respective elution buffer was adjusted accordingly with 20% ammonia. All the samples were prepared as three replicates from tryptic digestions and were stored at -80°C until needed.

Mass spectrometric analysis

LC-MS/MS experiments were performed using an LXQ linear ion trap mass spectrometer coupled with Surveyor LC system (Thermo Fisher Scientific). Peptide samples were loaded onto pre-equilibrated analytical columns on pressure-loader, in which the measured flow rates were 200–400 nL/min. The capillary-tip-columns were affixed to the ion-source in MS system and reversed-phase chromatographic separations were carried out using the following two gradient settings. For the 'one-pot' eluted samples, 100% A (0.1% formic acid in water) for 10 min; 0–50% B (0.1% formic acid in 100% ACN) for 100 min; 50–70% B for 10 min; 70% B for 5 min. For the samples eluted by pH step gradient, 100% A for 5 min; 0–50% B for 60 min; 50–70% B for 5 min; 70% B for 5 min. Pump flow rates were controlled to deliver 200 nL/min to the analytical column. This flow rate was consistently and reliably achieved by splitting pump flow at a 1:1000 ratio. LXQ settings were as follows: spray voltage, 1.6 kV; 1 microscan for MS scans at maximum inject time 10 ms with mass range 400–1400 m/z, 3 microscans for MS/MS at maximum inject time 100 ms with automatic mass range. The LXQ was operated in a data-dependent mode, that is, one MS scan for precursor ions followed by four data-dependent MS/MS scans for precursor ions above a threshold ion count of 500 with normalized collision energy value of 35%.

MS/MS data analysis

MS/MS data processing and database searches were performed on BioworksBrowser v3.3.1 SP1 (Thermo Fisher Scientific). DTA files were generated from LC-MS/MS raw files with the following options: precursor ion tolerance 1.5 amu, group scan 1, minimum group count 1, minimum ion count 20, and filtering through charge state analysis. The generated DTA files were searched against a composite target-decoy database [21] that was composed of human complete proteome (UniProt release 15.8 which has 59,155 protein entries) and its reversed-sequence version with the following criteria: enzyme, trypsin (KR/P); full enzymatic cleavage; missed cleavage sites, 3; peptide tolerance, 2.0 amu; fragment ions tolerance, 1.0 amu; variable modifications, carbamidomethylation (+57Da), methionine oxidation (+16Da), STY phosphorylation (+80Da); modifications per peptide, 4. The search result was filtered with Xcorr versus charge state (Xcorr of 1.5, 2.0, 2.5, and 3.0 for +1, +2, +3, >+3 charges, respectively) and delta CN 0.08. To ensure the false discovery rate (FDR) of peptide-spectral matches (PSM) remained below 0.5%, the search results were further

filtered with various Sf values and in this study a Sf value of 0.60 was used for FDR 0.49% for the overall results. Fragmentation spectra for peptides with only a single observation and for multiply phosphorylated peptides were verified through manual inspection according to the protocol used by reference [22].

Venn diagram, motif search, and sequence logo generation

Venn diagrams were generated using a web-based Venn diagram generator, *VENNY* [23]. Sequence motifs for phosphorylation sites were extracted using a web-based tool, *Motif-X*, with the following default setting: foreground format, MS/MS; width, 13; occurrences, 20; significance, 0.000001; background, IPI human proteome [24]. Sequence logos for doubly phosphorylated serine peptides were generated on WEB-based tool WebLogo v2.8.2 with default setting except small sample correction off [25].

Results and discussion

The processes we have employed for phosphopeptide enrichment are depicted in the schematic in Figure 1. TiO₂ particles were packed into capillary column and tryptic peptides were loaded and phosphopeptides were eluted with one-pot or pH-gradient format (details in Materials and method section). At first we measured the specific peptide-binding capacity of the particle in our microcolumn format with a conventional 'one-pot' elution to enhance our appreciation of the physico-chemical binding capacities of TiO₂ particle. Capillary columns of 150- μ m i.d. were packed with 6 cm of TiO₂ particles that effectively contained 2 mg of particles. These were then used for phosphopeptide enrichment from 100 μ g of peptide samples in various experimental conditions shown in Table 1. We employed this standardized 100 μ g of loaded peptide samples across multiple experimental conditions to specifically investigate differential elution techniques. It is possible that future modifications of the peptide loading volume may also be used to beneficially affect elution profiles. In our current study all the eluted samples were analyzed by LC-MS/MS and also their amounts were measured. Analysis of these revealed that the majority of identified peptides in Condition 1 (C1) and 2 (C2) (Table 1) were not phosphorylated (data not shown). Condition 3 (C3) resulted in about 2 μ g peptide recovery from the initial 100 μ g that were subsequently identified to be phosphorylated above 95% as shown in the following sections, while C1 showed 8 μ g peptide recovery. Therefore it appeared that the TiO₂ particles used in this study were able to adequately bind 4 μ g of sample per mg in acidic conditions. Additional organic solvents and glycolic acid were essential to minimize non-specific binding to the TiO₂ particle. Different bound peptide amounts between each solvent condition appeared to represent the non-specific binding washed out by the additional buffer components. In our enrichment strategy (C3) using 15% glycolic acid as a nonphosphopeptide excluder, approximately 2% of total tryptic peptides were recovered from the TiO₂ particle and most of them were identified to be phosphorylated.

LC-MS/MS analyses were performed with three independent C3 samples. A total of 328 distinct peptides were identified through these analyses. Only 12 of these identifications were non-phosphorylated, demonstrating a selectivity of 96.5% phosphopeptides. Across experimental triplicates we detected almost forty percent of the identical peptides in two or more of three replicates (Figure 2). We applied strict criteria for each of the identified peptides in the study, *i.e.* the FDR of these dataset was 0.5% with Sf 0.6 filtering and manual inspection of MS/MS spectra with a single observation or for multiply phosphorylated peptides was performed. The sample used to evaluate our protocols adequately represents the high complexity typically encountered from phosphorylation analysis of whole-cell protein mixtures. As the majority of MS processes identify peptides in a random manner the eventual functional correlation of identified peptides is of paramount importance. Using our experimental MS/MS conditions, the total matched spectral counts of

each analysis were in a relatively narrow range of 240 ± 29 . As spectral counting is a well-demonstrated non-isotopic quantitative tool in global proteome analysis [26,27], independently eluted peptide replicate samples contained approximately the same amount of phosphopeptides despite the large complexity of input sample.

To evaluate the effect of pH step gradient elution, we studied the phosphopeptide elution from the TiO_2 particles using different pH solutions. The identified peptides in each pH fraction (repeated in triplicate) were listed together for the comparison with each other fraction and also with 'one-pot' elution. Initially, elution buffers of pH 9.0, 10.0, and 11.3 were applied sequentially to TiO_2 column. At least 20 column volumes of buffer were applied between different sequential pH buffers to ensure the pH-specificity of peptide elution. Using these three elution buffers it was demonstrated that few peptides were eluted at pH 9.0 with almost all of these being non-phosphorylated peptides. With respect to the peptide amount based on spectral counting, the majority of peptides were recovered after pH 10.0 elution. A smaller number of peptides were then subsequently eluted at pH 11.3 (data not shown). To increase resolution, elution solutions buffered to intermediate pH environments between 9.0 and 10.0 were used, *i.e.* solutions buffered at 9.2, 9.3, 9.4, 9.5 and 9.7 (Figure 3). In Figure 3A, the relative amount of peptides in each pH gradient fraction and corresponding 'one-pot' elution were directly compared using spectral counts. Using an elution buffer of pH 9.1 no significant phosphopeptide elution was noted. Phosphopeptides were however efficiently eluted from pH 9.2 elution buffers onwards. A large majority of the eluted phosphopeptides were observed to be released from the column between pH 9.2-9.4. However, significant phosphopeptide elution was still observed above pH 9.5 (Figure 3A). This result suggested that the physico-chemical surface properties of the TiO_2 particles profoundly changed from pH 9.2 but still retained some positive charge even at these more basic conditions.

We were able to identify 15% more distinct phosphopeptides using our pH step gradient elution compared to the standard 'one-pot' elution strategy (Table 2). The reproducibility of peptide identification in both pH step gradient and 'one-pot' elution were very similar as an almost identical percentage of the eluted peptides were identified commonly in both methods using the complex SH-SY5Y whole-cell lysate. The distribution of phosphopeptides with unique peptide sequences including data-determined phosphorylation site assignments is represented in Figure 3B. The diagram represents the degree of high peptide resolution attainable with the step-gradient process. The Venn diagram distribution demonstrated both partial overlaps between multiple pH step gradient elution conditions as well as pH-unique peptide sets. Therefore, this strategy achieved reproducible selectivity of a distinct subset of peptides and our pH step gradient elution process represents a viable tool for fractionating or separating phosphopeptides from TiO_2 columns. Upon inspection of the pI distribution of the eluted peptides, there appeared to be no significant correlation based on the elution profile and the theoretical pI of the peptide.

Along with the rapid and efficient capacity to identify phosphopeptides, our pH gradient elution process also yielded significantly more multiply-phosphorylated peptide identifications compared to the standard 'one-pot' elution process. Specifically, using our pH step gradient process we identified 47 distinct multiply-phosphorylated peptides, compared to only 20 multiply-phosphorylated peptides discovered with the 'one-pot' elution approach. Of these 67 multiply-phosphorylated peptides, 14 were commonly detected between the two approaches (Table 2). Of these multiply-phosphorylated peptides 64 demonstrated 2 sites of phosphorylation, while three were triply phosphorylated. Inspection of the pH-dependent distribution of the spectral counts of doubly-phosphorylated phosphopeptides in each fraction revealed that the multiply-phosphorylated peptides were eluted in more basic conditions than mono-phosphorylated peptides, with most of them demonstrating elution in

the pH 9.7 fraction (Figure 4). Using the 'one-pot' elution process, only 3.6% of eluted peptides were doubly-phosphorylated peptides. Remarkably, 46.4% of recovered peptides in the specific pH 9.7 elution fraction were doubly-phosphorylated. The proportion of doubly-phosphorylated peptides was at least 12% in the fractions above pH 9.4. These observations suggested that charge states of TiO₂ particles begin to profoundly change from pH 9.2 but still retain an effective positive charge density so that more negatively charged peptides such as the multiply phosphorylated peptides are able to remain bound.

Upon further investigation of the nature of the doubly-phosphorylated peptides and their pH-dependent elution from TiO₂, we noted a strong relationship between elution and phosphoresidue sequence. We noted a strong relationship between the pH of elution buffers and the proximity of the two phosphorylated residues in the peptide sequence. As demonstrated in the lower panel of Figure 4, the distribution of phosphoresidue proximity in peptides of each fraction was different from that of 'one-pot' elution. The closer the relative positioning of the two phosphorylated residues in peptide sequence, the more basic an elution condition was required to elute these peptides. The distribution of phosphoresidue proximity in the pH 9.7 fraction was significantly biased to a 'zero' phosphoresidue separation, *i.e.* side-by-side phosphorylation sites in the eluted peptide. Peptides with a phosphoresidue separation of 'one' (phosphorylation sites separated by one non-phosphorylated residue) were eluted from pH 9.4 to pH 9.7. Conversely, peptides with a phosphoresidue separation of three or above were predominantly eluted below pH 9.4. Interestingly, this specific form of elution profile was similar to that for monophosphorylated peptides. Peptide binding to TiO₂ particles occurs primarily via the electrostatic association of negatively charged moieties in peptide and the positively charged TiO₂ surface, however this binding may be also strengthened locally by proximal acidic residues in the peptide and not as much by the total charge state of peptide (represented with peptide pI).

From our investigation, a sequence-dependent elution profile was observed for multiply phosphorylated peptides. We employed sequence motif analysis to elucidate the relationships between primary peptide sequence and pH-dependent elution profile. Using specifically serine-phosphorylated peptides, which represent the predominant phosphoproteins in most cells, we investigated any sequence commonalities amongst pH-dependent eluted peptides. The consistent presence of an XXsPXX motif (X = any residue) was observed with the criteria (probability) $p \leq 10^{-6}$ from peptides identified exclusively with a 'one-pot' elution, whereas a XXsDXX motif was additionally generated using exclusive peptides from the pH step gradient elution (Table 3). In addition to demonstrating improved detection of multiply phosphorylated peptides, this method also seemed to be ideal for purification of acidic site-specific phosphorylated peptides that bind strongly to TiO₂ particles. The sequence properties of doubly-phosphorylated peptides were also investigated using sequence logos instead of motif searches as their occurrence was relatively low compared to the monophosphorylated species. Serine-centered sequence logos were generated with 8 doubly phosphorylated peptides of 'zero' phosphoresidue separation (XXXssXXX, Figure 5A) and 17 doubly phosphorylated peptides with a phosphoresidue separation of 'one' (XXXsXsXXX, Figure 5B). The XXXsPXXX and XXXsDXXX motifs were reliably and consistently recognized and peptides with increased glutamate residues located closely to phosphoserine were dominant in both logos. An interesting relationship demonstrated in our analysis indicated that peptide sequences with a phosphoresidue separation of 'one' possessed more basic residues (lysine and arginine) in C-terminal region of the peptide compared to the 'zero' phosphoresidue separation sequences. This distinction therefore may result in a broader elution pattern of XXXsXsXXX peptides as lysine or arginine in the C-terminal region of phosphopeptides could reduce the strength of total negative charges. It seems especially for multiply-phosphorylated sequences that pH-gradient elution of phosphopeptides from TiO₂ column is capable of separating

phosphopeptides with distinct physico-chemical properties that were derived from adjacent acidic and basic residues. Using such information may facilitate the creation of purification processes that are specific for particular phosphorylation sites.

In this study, we investigated the ability of a pH-gradient elution strategy for bound phosphopeptides on TiO₂ for enhanced enrichment and separation compared to a conventional 'one-pot' elution technique. Phosphopeptide separation by pH-gradient elution enhances the qualitative identification of more phosphopeptides from a complex biologically-relevant mixture. In addition to this, a pH gradient-dependent elution strategy also generates a greater depth of information concerning the structure and sequence of multiply phosphorylated peptides. While our pH gradient-dependent elution process can assist the identification of specific diverse populations of phosphopeptides, it is also likely that additional complementary fractionations after TiO₂ loading, *e.g.* strong cation exchange, may also be useful for the functional separation of phosphopeptides. Such alternative techniques are also therefore likely to generate useful distinct elution profiles compared to our pH gradient process. The use therefore of multiple synergistic phosphopeptide elution techniques may eventually prove to be the norm for future analyses of complex phosphoproteomic mixtures.

The ability to analyze in-depth the phosphorylation-dependent signaling activity in neuronal tissue will greatly assist our appreciation of pathophysiological processes, drug-receptor functionality and also the nature of signaling within the cellular network structure itself [28–31]. Our methodology generated phosphopeptide elution profiles commensurate with other differential purification techniques that employed different primary matrices and loading parameters [9,32] suggesting that there may be recognizable relationships between small charged peptides and multiple high-charge density surfaces. A greater understanding of such physico/electro-chemical interactions may facilitate even more sophisticated and specific purification processes. This is especially important with respect to cell signaling activity as specific kinase substrates may be differentially purified and separated. For example, the 'one-pot' and the pH gradient processes may be used to differentially separate peptides phosphorylated by mitogen activated protein kinases such as extracellular signal-regulated kinase (ERK) [33] and acidic-targeted kinases, *e.g.* G protein coupled receptor kinase (GRK) [34]. The 'one-pot' format selectively enriched for proline-targeted peptides that are likely to be ERK substrates, while the more acidic peptides that are likely to be GRK substrates were preferentially enriched using the pH gradient process. In addition to this enhanced resolution for monophosphorylated peptides the pH gradient process was especially ideal for identification of multiply phosphorylated sites that may be linked to receptor signaling process involving, ERK, p38 or c-Jun N-terminal mitogen-activated kinase systems as these kinases often require multi-site phosphorylations to activate them [35,36].

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This work was carried out entirely with the support of the Intramural Research Program of the National Institutes of Health.

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Appendix A. Supplementary data

The amino acid composition of the identified peptides is shown in Supplementary Table 1 including detailed Sequest search results and spectral counts in each fraction.

Figure 1. Experimental workflow

TiO₂ columns were constructed from 360-µm o.d. 150-µm i.d. fused silica capillaries. A Kasil frit was formed in one end, and then 5 µm TiO₂ particles were packed in with a pressure-loader. TiO₂ chromatography was also performed with a pressure-loader. Peptide samples were loaded into equilibrated TiO₂ column and unbound ones were washed out. Peptides were eluted using one of two different mechanisms: a) by 1% ammonia at pH 11.3 and b) pH step gradient using 100 mM ammonium bicarbonate buffer of various pH values. Eluted peptides were analyzed via LC-MS/MS.

Figure 2. Venn diagram of reproducibility of one-pot elution

Whole-cell tryptic digest (150 µg) was loaded to TiO₂ column and peptides were recovered by experimental condition 3 (C3) described in Table 1, eluted with a pH 11.3 elution buffer of 1% ammonia and analyzed via LC-MS/MS. Three replicates (I, II, III) of analyses were compared to evaluate MS/MS-level reproducibility. Forty percent of the peptides were detected in two or more separate analyses. Each number represents distinct phosphorylated peptides and non-phosphorylated peptides are in parentheses.

A **B** **Figure 3. Distribution of identified peptides**

Whole-cell tryptic digest (150 µg) was loaded to TiO₂ column and peptides were eluted with 100 mM ammonium bicarbonate buffer with various pH values and analyzed via LC-MS/MS. Three replicates of analyses were summed to calculate total spectral counts. (A) Total spectral count of phosphopeptides and nonphosphopeptides identified in each elution. The majority of peptides were eluted below pH 9.3. (B) Venn diagram of number of distinct phosphopeptides eluted by each pH step. Number of multiply phosphorylated peptides are shown in parentheses

Figure 4. Spectral counts of multiply phosphorylated peptides in each elution

The relationship of multiply-phosphorylated peptide's primary sequence and pH-dependent elution was further analyzed. The upper panel indicates the total spectral counts of multiply-phosphorylated peptides and their percentages in each elution (in parentheses). The specific elution profile of doubly-phosphorylated peptides in the lower panel is shown by the distribution of spectral counts of each of the doubly serine-phosphorylated sequence patterns indicated in the left panel. The pH-dependent elution of these doubly-phosphorylated peptides demonstrates that the closer the relative positioning of the two phosphorylated residues in peptide sequence, the more basic an elution condition was required.

Figure 5. Sequence logos of doubly serine-phosphorylated peptide set

The identified phosphopeptides amino acid sequences of doubly serine-phosphorylated peptides with XXXssXXX and XXsXsXX patterns were specifically chosen and used to generate sequence logos with the WebLogo algorithm [19]. (A) Sequence logo generated with 8 nonredundant peptides with XXXssXXX patterns. (B) Sequence logo generated with 17 nonredundant peptides with XXsXsXX patterns.

Table 1Effect of buffer composition on peptide loading capacity of TiO₂ particles

	Loading buffer^a	Washing buffer	Total amount of eluted peptides (µg/mg particle)^b
condition 1	2% TFA	80% ACN, 2% TFA	4.0 ± 0.6
condition 2	40% ACN, 1% TFA	80% ACN, 2% TFA	1.9 ± 0.4
condition 3	40% ACN, 1% TFA 0.5M glycolic acid	80% ACN, 2% TFA 1M glycolic acid	0.9 ± 0.2

^aPeptide samples, which were eluted from desalting cartridge with 80% ACN 0.1% formic acid and dried partially in SpeedVac, were mixed with each washing buffer in 1:1 ratio except condition 1.

^bThe amount of particles 6 cm packed in 150 µm i.d. capillary was about 2 mg (dry-weight).

Table 2

Comparison of phosphopeptides and phosphorylation sites identified in two methodologies

	pH gradient	Common	One-pot elution	Total
Identified phosphopeptides ^a	149	200	106	455
Phosphorylation sites ^b	162	203	100	465
pS	146	186	88	420
pT	15	17	12	44
pY	1	0	0	1
Multiply phosphorylated peptides ^a	33	14	6	53

^a Number of distinct peptides which have redundant sequences but have unique modification such as oxidation and alkylation.^b Nonredundant phosphorylation sites

Table 3

S-centered motifs extracted data set.

Data set	Motif ^a	Score	Phospho data set (Matches/Size)	Background data set (Matches/Size)
One-pot	sP.....	16.00	79	215
				84,335
				1,094,911
	sP.....	16.00	90	306
				84,335
				1,094,911
pH gradient	sD.....	12.63	39	216
				46,119
				1,010,576

^aLowercase letters denote phosphorylated residues.