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Development of an off-line capillary column IMAC phosphopeptide enrichment method for label-free phosphorylation relative quantification

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ABSTRACT

Immobilized metal affinity chromatography (IMAC) and metal oxide type affinity chromatography (MOAC) techniques have been widely used for mass spectrometry-based phosphorylation analysis. Unlike MOAC techniques, IMAC requires rather complete removals of buffering reagents, salts and high concentrations of denaturant prior to sample loading in order for the successful enrichment of phosphopeptides. In this study, a simple off-line capillary column-based IMAC phosphopeptide enrichment method can shorten sample preparation time by eliminating the speed-vac step from the desalting process. Tryptic digest peptide samples containing 2 M urea can be directly processed and the entire IMAC procedure can be completed within 6 h. When tryptic digest peptide samples prepared from mouse whole brain tissues were analyzed using our method, an average of 249 phosphoproteins and 463 unique phosphopeptides were identified from single 2-h RPLC–MS/MS analysis (~88% specificity). An additional advantage of this method is the significantly improved reproducibility of the phosphopeptide enrichment results. When four independent phosphopeptide enrichment experiments were carried out, the peak areas of phosphopeptides identified among four enrichment experiments were relatively similar (less than 16.2% relative standard dev.). Because of this increased reproducibility, relative phosphorylation quantification analysis of major phosphoproteins appears to be feasible without the need for stable isotope labeling techniques.

1. Introduction

Immobilized metal affinity chromatography (IMAC) technique combined with mass spectrometric analysis has been widely used in protein phosphorylation analysis [1–8]. Both global and targeted phosphorylation analyses have often employed IMAC techniques to specifically enrich sub-stoichiometric phosphopeptides from tryptic digest peptide samples. Most early IMAC reports focused on the identification of novel phosphorylation sites, but phosphorylation analysis research using IMAC techniques has now been expanded to encompass the quantification of biologically important phosphoproteins [9–12].

As the application of IMAC techniques becomes routine, there have been many studies aimed at increasing both the phosphopeptide trapping efficiency and the analysis speed of IMAC. Methyl esterification of acidic amino acids increases the phosphopeptide enrichment efficiency of IMAC by eliminating non-specific binding [1,6,13]. IMAC phosphopeptide enrichment followed by enzymatic de-phosphorylation strategies has been developed to increase the coverage of phosphoproteins in actual protein samples [14,15]. Sequential elution strategies aimed to separate mono-phosphorylated peptides from multi-phosphorylated peptides by employing different acid and base elution techniques have been also developed, expanding the coverage of phosphopeptides and phosphoproteins [16]. Other studies of the effects of pH and various acids have been designed to reduce sample loss, and to increase the enrichment efficiency of IMAC [17,18]. Recently, biphasic micro-columns using IMAC type column material and C18 reversed phase column material have been shown to be very effective for the rapid enrichment of phosphopeptides from very complex biological samples [19,20]. Several studies focused on automated on-line IMAC phosphorylation analysis were also reported [21–23]. Phosphopeptide enrichment process by IMAC was optimized, and the IMAC process was successfully connected to RPLC analysis. All these studies show significantly improved sensitivity and throughput for IMAC based phosphopeptide analysis.

One of the main limitations of using IMAC in the protein phosphorylation analysis, however, is that it requires a rather long sample preparation procedure for tryptic digest samples containing high concentrations of denaturant or salts. The sample preparation procedure is mainly designed to remove chemical contaminants from the sample. In general, samples containing complex protein mixtures are enzymatically digested using high concentrations of denaturing reagents that interfere with the binding of

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phosphopeptides to the immobilized metal ions on the IMAC columns. Hence, high concentrations of urea, buffering reagents and salts need to be completely removed prior to the IMAC phosphopeptide enrichment procedure. A desalting step, combining solid phase extraction techniques with a speed-vac process, has almost become a prerequisite for IMAC sample preparation procedure. This step usually takes from several hours to nearly half of a day. Furthermore, long and laborious sample preparation procedures hamper the reproducibility of the phosphopeptide enrichment experiments. This is probably the reason why reproducible IMAC phosphopeptide enrichment experiments using urea-containing protein samples have not been frequently demonstrated before.

In this study, we demonstrate that an off-line capillary column-based IMAC method can significantly shorten IMAC sample preparation time by eliminating the speed-vac step from the desalting process. Two capillary columns, one for desalting and the other for IMAC phosphopeptide enrichment/trapping, along with sequential elution steps are employed to carry out the desalting and phosphopeptide enrichment processes. Notably, tryptic digest peptide samples prepared using 2 M urea can be directly processed. In order to test the phosphopeptide enrichment capability of the new method, samples of mouse whole brain tissue tryptic digests were analyzed. To demonstrate the improved reproducibility of our off-line capillary column-based IMAC method, a series of phosphopeptide enrichment experiments were conducted. The retention time and peak profiles of selected phosphopeptides in all four independent enrichment experiments were compared.

2. Experimental methods

2.1. Materials

Bovine α-casein, ammonium bicarbonate, calcium chloride, dithiothreitol (DTT), iodoacetamide (IAA), sodium chloride, sodium orthovanadate (Na_3VO_4), sodium fluoride (NaF), phenylmethylsulfonyl fluoride (PMSF), sodium β-glycerophosphate, urea and ammonium dihydrogen phosphate ($NH_4H_2PO_4$) were purchased from Sigma (St. Louis, MO). Formic acid (FA) and trifluoroacetic acid (TFA) were obtained from Merck (Darmstadt, Germany). HPLC grade acetonitrile (ACN), methanol and deionized water were all purchased from Fisher Science. Sequencing grade modified trypsin was obtained from Promega (Madison, WI). Aqua C18 (particle size 5 μm) reversed-phase column material was purchased from Phenomenx (Torrance, CA). The IMAC POROS 20 MC was purchased from Applied Biosystems (Forster city, CA). All synthetic phosphopeptides (HPNIT*QGADTHEYANSNLNR, HPNIT*QGADT*HEYANSNLNR and HAS*SLSGSIIR) were purchased from Anygen (Gwangju, Korea).

2.2. Sample preparation

Eight-week-old, ICR male mice were obtained from Samtako Bio, Korea, and sacrificed by cervical dislocation without anesthesia. Whole brains were dissected immediately, and washed with ice-cold 1× PBS. The samples were immediately frozen in liquid nitrogen and transferred to a −80 °C freezer. The tissue was ground using a mortar and pestle, and the powder was collected in eppendorf tubes that had been previously weighed. The final weight of the tissue was then calculated. To make the protein extracts, the powder was dissolved in an appropriate volume of extraction buffer containing 50 mM Tris–HCl pH 8.2, 8 M urea, 75 mM NaCl, 1 mM NaF, 1 mM β-glycerophosphate, 1 mM sodium orthovanadate, 1 mM sodium pyrophosphate, 1 mM PMSF and one tablet of protease inhibitor cocktail (complete mini, EDTA-free, Roche) per 10 ml of solution. The sample was incubated with rotation at 4 °C for 1 h and sonicated for 3 × 60 s at 4 °C with a 2-min rest between each pulse, using an output of 15%. In order to remove insoluble tissue debris from the suspensions, the samples were centrifuged at 100,000 × *g* for 1 h. The supernatant was transferred into separate tubes, and the protein concentration determined using a BCA Protein Assay Kit (Thermo Scientific, Cramlington, UK). The proteins (100 μg) were subjected to disulfide reduction using 5 mM DTT (56 °C, 25 min), and alkylation with 15 mM iodoacetamide (room temperature, 30 min in the dark). Excess iodoacetamide was captured with 5 mM DTT (room temperature, 15 min in the dark). After 4-fold dilution with 25 mM Tris–HCl (pH 8.2), The proteins were digested with sequencing grade trypsin at an enzyme:substrate ratio of 1:50 in the presence of calcium chloride (1 mM) at 37 °C overnight. Digestion was stopped by the addition of FA to 1%.

2.3. Off-line capillary column based IMAC phosphopeptide enrichment

Fig. 1 shows a schematic overview of our off-line IMAC phosphorylation analysis system. The phosphopeptide enrichment procedure consists of two steps, a desalting step and a phosphopeptide enrichment step. Capillary column packing, desalting and off-line IMAC enrichment experiments were carried out using a home-made high-pressure gas bomb. A pressure range from 400 to 600 psi was used to control different flow rates in the desalting and IMAC phosphopeptide enrichment processes. For the desalting step, a fused-silica capillary (250 μm i.d. × 360 μm o.d.; Polymicro Technologies, Phoenix, AZ, USA) was packed with 12 cm of AQUA C18. Inline MicroFilters (Upchurch Scientific, Oak Harbor, WA, USA) served as frits for both the desalting column and the phosphopeptide enrichment/trapping column (RP/IMAC). The packed desalting column was sequentially washed with 100 μl methanol, Solvent B (0.1% FA/80% ACN) and 1% FA aqueous solution. The peptide samples were loaded onto the column at a flow rate of 2 μl/min, and then the column was washed with more than five sample volumes of 1% FA at a constant pressure of 600 psi. For the phosphopeptide enrichment step, the biphasic phosphopeptide enrichment/trapping column comprised 3 cm of AQUA C18 and 10 cm of IMAC POROS 20 MC packed into a fused-silica capillary (same dimensions as above). The RP/IMAC column was washed once with 200 μl Solvent B and twice with 200 μl IMAC binding buffer (40% ACN/0.1% FA), before connection to the desalting column. The bound peptides were eluted from the desalting column to the RP/IMAC column with 100 μl IMAC binding buffer at a flow rate of 2 μl/min. The desalting column was then detached from the RP/IMAC column. The RP/IMAC column was washed three times with 200 μl IMAC binding buffer to wash out non-specifically bound peptides, and equilibrated with 60 μl 0.1% TFA at a flow rate of approximately 2 μl/min. The peptides bound to the IMAC resin were eluted to the 3 cm of AQUA C18 reversed phase with 100 μl IMAC elution buffer (200 mM $NH_4H_2PO_4$) at a flow rate of approximately 1 μl/min, and then the RP/IMAC column washed with 400 μl of 0.1% FA at a constant pressure of 700 psi. Lastly, a pre-conditioned analysis column (fused-silica capillary 100 μm i.d. × 360 μm o.d.; packed with 7 cm of AQUA C18) was connected with the RP/IMAC column for micro RPLC–MS/MS analysis.

2.4. Micro RPLC–MS/MS analysis

The analyses of the peptide samples were performed using an Agilent 1100 Series high-performance liquid chromatography (HPLC) pump (Agilent Technologies) coupled to a linear ion trap mass spectrometer (LTQ, Thermo Finnigan, San Jose, CA, USA) using an in-house-manufactured nano-ESI interface. The columns were

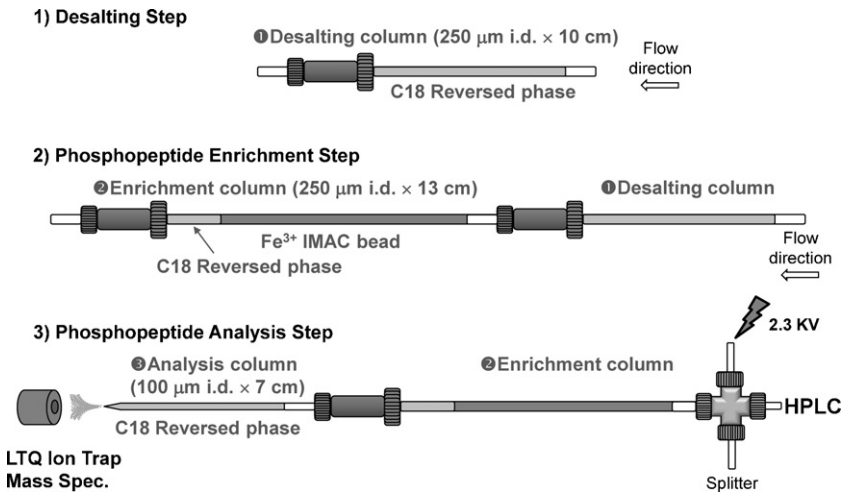

Fig. 1. An experimental scheme of capillary-column based rapid IMAC phosphorylation analysis method.

placed in line with an Agilent HP 1100 quaternary LC pump, and a splitter system was used to achieve a flow rate of 250 nl/min. Solvent A (0.1% FA) and Solvent B (0.1% FA/80% ACN) were used to make a 120-min gradient. The gradient profile started with a 5-min gradient from 0% to 5%, followed by a 67-min gradient from 5% to 30% buffer B followed by a 13-min gradient from 30% to 100% buffer B, and a 35-min gradient of 100% buffer B. The instrument was operated in data-dependent mode with an m/z range of 400–2000 and in the positive ion mode with a spray voltage of 2.5 kV. The 10 most abundant ions from each MS scan were selected for further MS/MS analysis by using normalized collision energy of 35%. A dynamic exclusion of 30 s was applied to avoid repeated analysis of the same abundant precursor ion.

2.5. Data analysis and validation

The MS/MS spectra obtained from the RPLC–MS/MS analysis were searched against a composite protein database containing the IPI mouse protein database (v.3.28), and its reversed complement and bovine alpha casein sequences, using Sorcerer-SEQUEST (V.3.4, Sage-N research, CA). The differential modification search option for phosphorylation modification (+80 on Ser, Thr, Tyr), and oxidation (+16 on Met), were considered in the search, and the maximum number of modifications allowed per peptide was seven (the maximum number of modification per type was five). Scaffold (version Scaffold-01_07_00, Proteome Software Inc., Portland, OR) was used to validate MS/MS based peptide and protein identifications. 'Peptide prophet' and 'Protein prophet' scores of 90% with a requirement of at least 1 identified peptide per protein were used to filter the search results. All filtered search results were then manually validated using the criteria mentioned previously [24]. Briefly, the presence of neutral loss of phosphoric acid was used first as a way of confirming Ser- and Thr-phosphorylated peptides, and major fragment ions (b and y series ions, N-Terminal side fragment ions of Pro-containing peptides, neutral loss ion of oxidized Met-containing peptides) of the phosphorylated peptide sequences were then manually assigned and checked for their intensities. The requirement of neutral loss of phosphoric acid was not applied to Tyr phosphorylated peptides. Manual validations of all MS2 spectra were used to confirm the search results because the number of MS2 spectra removed by manual validation appears to be higher than by estimation of the FDR. False discovery rates (FDR) were also calculated by comparing search results between forward and reversed protein databases and average FDR (reversed hits/forward hits) was 0.022.

3. Results

IMAC does not give good phosphopeptide enrichment results if samples contain high concentrations of denaturant, salts, or buffering reagents. IMAC phosphopeptide enrichment experiments for mouse whole brain–tissue tryptic digest peptide samples prepared using 2 M urea either with, or without, a desalting step were performed to test the effects of denaturant on the phosphopeptide enrichment results. The desalting step was carried out separately using solid phase extraction and speed-vac. LC–MS chromatograms in both experiments show clearly different peptide elution patterns (Supplementary Fig. S1). In the experiment without a desalting step, protein database search results using SEQUEST suggest a low level of phosphopeptide enrichment (~2.9% specificity) and significantly reduced phosphopeptide identification (~22 phosphopeptides) (Supplementary Table S1). Besides, most of the peptides identified contain relatively high number of acidic amino acid residues. When the tryptic digest peptide samples were desalted prior to IMAC, however, the phosphopeptide search results dramatically improved (~66% specificity and 498 phosphopeptides) (Supplementary Table S1).

In order to enrich the phosphopeptides from the tryptic digest peptides samples prepared using high concentrations of urea, we developed a two capillary column-phosphopeptide enrichment strategy; one capillary column, containing C18 reversed column material for desalting purposes (desalting column), and a second, containing both IMAC resin and C18 reversed phase column material for phosphopeptide enrichment and trapping purposes (phosphopeptide trapping column) (Fig. 1). Tryptic digest peptide samples containing 2 M urea were directly loaded onto the desalting column, which was subsequently washed to remove urea, salts and buffering reagents. After washing, the phosphopeptide trapping column was connected to the desalting column. Sample loading solution of IMAC was then used to elute the peptides from the desalting column, and to load them onto the phosphopeptide trapping column. Once the loading step of the phosphopeptide trapping column was finished, the desalting column was disconnected, and the phosphopeptide trapping column was used to carry out a typical IMAC phosphopeptide enrichment procedure. Details of entire phosphopeptide analysis procedure are described in the method section. The entire process, from sample loading to RPLC–MS/MS analysis, takes generally less than 8 h.

To test the phosphopeptide enrichment performance of our rapid phosphorylation analysis system, four sets of independent phosphopeptide enrichment experiments were carried out. Tryptic digest peptide samples were prepared from mouse whole

Table 1
Phosphopeptide and phosphoprotein identification results from four independent IMAC phosphopeptide enrichment experiments of mouse whole brain tissue lysates.

	Exp #1	Exp #2	Exp #3	Exp #4
Number of phosphoproteins identified ($p \geq 0.9$[a] and manual validation)	272	254	222	246
Number of unique phosphopeptides identified ($p \geq 0.9$[b] and manual validation)	501	457	406	488
Number of phosphorylation sites	549	478	426	523
Number of phosphopeptides identified/number of peptides identified	501/560	457/496	406/467	488/587

[a] Minimum protein probability threshold calculated by the protein prophet.
[b] Minimum peptide probability threshold calculated by the peptide prophet.

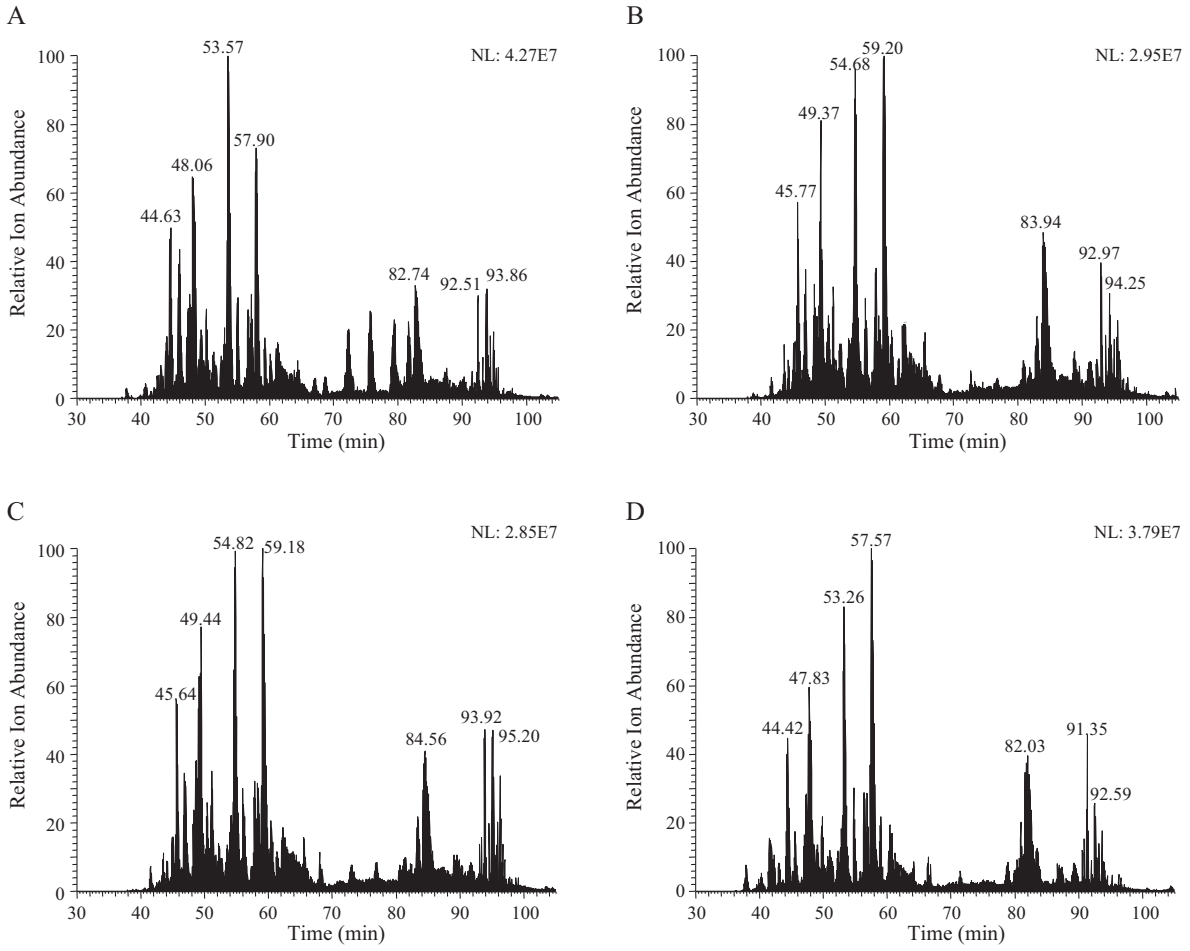

Fig. 2. LC–MS/MS chromatograms obtained from four independent off-line capillary column based IMAC phosphopeptide enrichment experiments. (A) First experiment, (B) second experiment, (C) third experiment, and (D) fourth experiment. Capillary columns and packing materials in each enrichment experiment were not re-used.

brain–tissue homogenates using 2 M urea. Four aliquots, corresponding to 400 μg of protein, were used and 0.5 μg of bovine alpha casein tryptic digest peptides was added to each aliquot as an internal standard. Fig. 2 shows the LC–MS chromatograms from four different IMAC phosphopeptide enrichment experiments. Although there were some minor differences in the ion intensities of the major peaks, the phosphopeptide elution profiles look similar. The phosphopeptide and phosphoprotein identification results from the four independent phosphopeptide enrichment experiments are summarized in Table 1. More than 1000 phosphopeptides were identified in each analysis. The average number of unique phosphopeptides and phosphoproteins identified was 463 and 249, respectively. A list of unique phosphopeptides identified from four independent enrichment experiments along with detailed information is provided in Supplementary Table S2. The phosphopeptide enrichment efficiency was estimated from the ratio of phosphopeptide to total peptide. This ratio ranged from 83.1% to 92.1%. The number of phosphoproteins redundantly identified in all four independent experiments was 107, and the ratio of redundantly identified phosphoproteins to total phosphoproteins identified in each experiment ranged from 39.3% to 48.1%. An average of 24.7% was observed for the multi-phosphorylated peptides (Supplementary Table 3).

The reproducibility of our rapid phosphorylation analysis method was examined by comparing peak areas of mouse brain phosphopeptides identified in four independent experiments. Tryptic digests of bovine alpha casein were co-injected with the mouse brain tissue phosphopeptide mixture samples as internal standards (10 pmol bovine alpha casein phosphopeptides vs. 100 μg of mouse brain protein tryptic digests). The peak areas of commonly identified phosphopeptides in all four independent experiments was calculated and ranked (Supplementary Table S4). More than two hundred phosphopeptides were listed and 90% of the phosphopeptides show relative standard deviations lower than 20%. An average relative standard deviations of all phosphopeptides quantified was 10.9%. In order to see any relationship between

Table 2
Integrated peak areas of three different intensity level phosphopeptides from four independent IMAC phosphopeptide enrichment experiments of mouse whole brain tissue lysates.

Phosphopeptide mass (charge state)	Exp #1		Exp #2		Exp #3		Exp #4		Average (std.)
	RT (min)	Peak area	RT (min)	Peak area	RT (min)	Peak area	RT (min)	Peak area	
10 largest peak area phosphopeptides									
SAKDS*DDEEEVVHVDR 637.70 (+3)	57.19	3.05 × E8	58.43	2.77 × E8	58.30	2.89 × E8	56.91	2.92 × E8	2.91 × E8 (0.11)
IEDVGS*DEEDDSGKDKK 649.79 (+3)	47.7	2.95 × E8	48.8	2.29 × E8	48.7	2.77 × E8	47.4	2.34 × E8	2.58 × E8 (0.32)
FGIHVYQFPECDS*DEDEDFKQQDR 1029.26 (+3)	87.41	1.79 × E8	88.86	1.81 × E8	89.56	1.85 × E8	87.00	1.34 × E8	1.70 × E8 (0.22)
DGS*PDAPAT*PEKEEVAFSEYK 1214.25 (+2)	79.9	1.38 × E8	80.9	1.38 × E8	81.4	1.46 × E8	78.0	1.27 × E8	1.37 × E8 (0.08)
S*PVEEVKPKPEAK 759.58 (+2)	48.11	1.32 × E8	49.26	1.31 × E8	49.18	0.98 × E8	47.89	0.98 × E8	1.14 × E8 (0.19)
IEDVGS*DEEDDSGKDK 909.58 (+2)	51.2	1.20 × E8	52.3	1.12 × E8	52.2	1.11 × E8	50.8	1.05 × E8	1.12 × E8 (0.06)
IEDVGS*DEEDDSGKDKK 974.65 (+2)	47.65	1.30 × E8	48.79	1.20 × E8	48.77	0.91 × E8	47.43	1.00 × E8	1.11 × E8 (0.18)
VARPQILEPRPQS*PDLCDDDVEFR 978.74 (+3)	59.33	0.93 × E8	60.43	1.03 × E8	60.51	0.94 × E8	59.01	1.02 × E8	0.98 × E8 (0.05)
LDPYDS*SEDDKEYVGFATLPNQVHR 993.06 (+2)	87.30	0.86 × E8	88.74	0.93 × E8	89.18	0.95 × E8	86.82	0.86 × E8	0.90 × E8 (0.05)
GTDDS*PKNSQEDLQDR 943.18 (+2)	50.66	0.99 × E8	51.77	0.80 × E8	51.77	0.89 × E8	50.27	0.73 × E8	0.85 × E8 (0.11)
10 smallest peak area phosphopeptides									
SPSEAKS*PAEAKS*PAEAK 973.10 (+2)	45.31	3.36 × E6	46.26	3.44 × E6	46.28	3.57 × E6	44.89	3.11 × E6	3.37 × E6 (0.19)
ETNVSKEDT*DQEEK 866.47 (+2)	41.63	3.52 × E6	42.41	3.20 × E6	42.41	3.44 × E6	41.44	2.61 × E6	3.20 × E6 (0.41)
RGS*ETmAGAAVK 637.67 (+2)	41.69	3.66 × E6	42.76	2.62 × E6	42.80	2.73 × E6	42.25	3.50 × E6	3.13 × E6 (0.53)
GSSDGRGS*DSESDLPHR 614.45 (+3)	48.94	3.23 × E6	50.11	2.38 × E6	50.13	2.73 × E6	48.49	3.72 × E6	3.02 × E6 (0.59)
VRPNSDLSNSTGQS*PHHK 681.20 (+3)	44.41	2.12 × E6	45.44	2.45 × E6	45.48	1.94 × E6	44.15	2.64 × E6	2.29 × E6 (0.31)
AEGAGTEEEGT*PK 678.58 (+2)	43.24	2.16 × E6	43.76	2.44 × E6	44.22	2.21 × E6	43.18	2.03 × E6	2.21 × E6 (0.17)
GHGHS*DEEDEEQPR 851.36 (+2)	40.46	1.99 × E6	41.48	2.17 × E6	41.74	1.82 × E6	40.33	1.95 × E6	1.98 × E6 (0.14)
TQSPHS*PKEESER 796.72 (+2)	40.76	1.98 × E6	40.82	1.81 × E6	41.69	2.00 × E6	39.53	1.74 × E6	1.88 × E6 (0.13)
RPmEEDGEEKS*PSK 572.57 (+3)	39.49	1.24 × E6	40.33	1.32 × E6	40.35	1.15 × E6	39.24	1.41 × E6	1.28 × E6 (0.11)
SPGSVSTHHSS*PR 472.95 (+3)	39.12	1.10 × E6	40.27	1.40 × E6	40.05	1.18 × E6	39.3	1.33 × E6	1.25 × E6 (0.13)

Extracted ion chromatograms corresponding to each phosphopeptide mass were constructed and integrated peak areas were calculated by built-in functions of Xcalibur software (ver. 2.0).
* denotes phosphorylation site and m denotes oxidized methionine.

Table 3
Integrated peak areas of co-injected alpha casein phosphopeptides from four independent IMAC phosphopeptide enrichment experiments of mouse whole brain tissue lysates.

Alpha casein phosphopeptides	Exp #1		Exp #2		Exp #3		Exp #4		Average (std.)
	RT (min)	Peak area	RT (min)	Peak area	RT (min)	Peak area	RT (min)	Peak area	
TVDMES*TEVFTK	75.71	3.00 × E7	76.6	2.46 × E7	77.04	2.55 × E7	75	3.04 × E7	2.76 × E7 (0.26)
EQLS*TS*EENSK	49	1.60 × E8	49.8	1.29 × E8	49.9	1.21 × E8	48.2	1.78 × E8	1.47 × E8 (0.23)
YKVPQLEIVPNS*AEER	82.9	6.80 × E8	84	7.36 × E8	84.6	6.51 × E8	82	7.41 × E8	7.02 × E8 (0.38)

* denotes phosphorylation site.

ion intensity and quantitation accuracy, two groups of phosphopeptides were selected based on their peak area. Top 10 phosphopeptides showing the largest peak areas were selected as one group and their peak areas were compared in four independent experiments. Bottom 10 phosphopeptides showing the smallest peak areas were also selected as another group and their peak areas were compared in the same way as the top 10 phosphopeptides (Table 2). The peak area difference between the largest and the smallest peak area phosphopeptides was more than two orders of magnitude. Peak area relative standard deviations calculated for the top 10 largest peak area phosphopeptides ranged from 4.0% to 16.9% with an average of 9.7%. Relative standard deviations calculated for the bottom 10 smallest peak area phosphopeptides ranged from 5.7% to 19.5% with an average of 11.0%. Lastly, the relative standard deviations calculated from three phosphopeptides of co-injected alpha casein were less than 15.7% (Table 3).

The recovery and specificity of our method was tested using several synthetic phosphopeptide standards spiked in the mouse brain tissue phosphopeptide samples (100 μg of mouse brain protein tryptic digests) in different concentrations from 1 pmol down to 1 fmol. These synthetic phosphopeptides were HPNIT*QGADTHEYANSNLNR, HPNIT*QGADT*HEYANSNLNR and HAS*SLSGSIIR. Tryptic digests of bovine alpha and beta caseins were also co-injected with these synthetic phosphopeptides to increase the number of phosphopeptides tested. The phosphopeptide enrichment experiment results are summarized in Supplementary Table S5. All three synthetic phosphopeptides were successfully enriched with the spike-in amount of 1 pmol and two synthetic phosphopeptides were identified at 100 fmol. Only one synthetic phosphopeptide was detected at 10 fmol. Singly phosphorylated peptide from bovine beta casein was detected as low as 10 fmol and two phosphopeptide from bovine alpha casein was identified as low as 100 fmol.

4. Discussion

Since tryptic digest peptide samples prepared under high concentrations (1–2 M urea or guanidine hydrochloride) of denaturants are not directly compatible with IMAC based phosphopeptide enrichment experiments, a desalting step involving a time-consuming speed-vac process is usually used to obtain good results. Previously reported biphasic column approaches combining IMAC type column materials and reversed phase column material have been demonstrated only with the desalted form of tryptic digest peptide samples [19,20]. Even metal oxide type phosphopeptide enrichment techniques such as TiO_2 appear not to tolerate high concentrations of salts or chemical denaturants [25]. This is the main reason why we put an additional C18 reversed phase column (desalting column) in front of our IMAC column for desalting purposes. One more thing that should be noted is that we use an off-line phosphopeptide enrichment approach instead of on-line approach. In our method, the desalting column is connected with the enrichment column after washing the desalting column because washing solutions containing urea affect the phosphopeptide enrichment

efficiency of the IMAC bead in the enrichment column (data not shown). Ficarro et al. reported a fully automated online IMAC/nano-LC/ESI-MS platform, of which column setups are similar to ours [21]. An additional reversed phase column was placed in front of the IMAC column to remove high concentrations of denaturant. Highly reproducible and sensitive phosphopeptide enrichment was demonstrated; however, complicated experimental setups involving 5 additional valve systems, multiple pumps and home-made control software were required to automate the whole sample preparation and IMAC procedure. Although our approach cannot be operated in a fully automated manner, rapid and reproducible phosphopeptide enrichment results can be easily obtained without the need for complicated experimental setups.

The average number of unique phosphopeptides and phosphoproteins identified from four independent phosphopeptide enrichment experiments was 463 and 249, respectively. These numbers may not be quite comparable with reports of other large scale phosphorylation analyses; however, they were obtained from single 120 min RPLC–MS/MS analysis using 100 μg of mouse–brain proteins [9,10,17]. Most reports of large scale phosphorylation analysis have used at least 200 μg of protein and employed sequential elutions from the IMAC column using two different elution solutions, or additional LC separation techniques such as strong cation exchange (SCX), to increase the number of phosphopeptides identified. We are currently working on combining a MudPIT system with our rapid phosphorylation analysis approach to expand the coverage of the phosphoproteome. The number of phosphopeptides versus total peptides identified can give an estimate of the phosphopeptide enrichment efficiency of our approach, since these percentiles are often interpreted as the phosphopeptide enrichment efficiency of the experiments [17]. The average enrichment efficiency was around 88%, which is relatively high considering the simplicity of the sample preparation/enrichment procedure. The number of proteins co-identified in all four experiments was around 45%, and these proteins turned out to have at least two matched phosphopeptides in their protein amino acid sequences. It can be speculated that most of the major phosphoproteins in mouse whole-brain tissue samples are redundantly identified, and our approach gives relatively reproducible phosphopeptide enrichment results.

Reproducibility is one of the key issues for analytical methods. Although off-line IMAC approach has been used in numerous cases, the reproducible isolation of phosphopeptides from repeated IMAC experiments has been demonstrated by only a few research groups [26]. Owing to laborious sample processing steps, and to the various enrichment conditions used for IMAC, many researchers have had difficulties in obtaining reproducible results. This is why metal oxide-type affinity purification techniques such as those using TiO_2 have often been suggested as an alternative [25,27–29]. Aebersold et al. tested the reproducibility, specificity, and comprehensiveness of the three most widely used phosphopeptide isolation techniques, and showed that IMAC can reproducibly and specifically isolate phosphopeptides [26]. In their report, 1.5 mg of previously desalted tryptic digest peptides was used in each enrichment experiment, and the IMAC resins were packed in micro spin column-type cartridges. The retention time and the intensity of overlapping phosphopeptides in two LC–MS analyses from three different IMAC enrichment experiments were compared to assess reproducibility. In our study, only 100 μg of tryptic digest peptides containing 2 M urea was used in each experiment and IMAC phosphopeptide enrichment was carried out using capillary columns. Instead of comparing all overlapping phosphopeptide mass spectral features (MS1) within any two LC–MS/MS analyses, we selected commonly identified phosphopeptides in four independent experiments including tryptic digest bovine alpha casein phosphopeptides and the peak areas of all phosphopeptides in four independent experiments were compared. Relatively low deviations in the phosphopeptide peak areas with an average of 10.9% were observed in four independent enrichment experiments, suggesting a high level of reproducibility. Notably, the level of peak area deviations for the low intensity phosphopeptides was not as high as expected. The presence of co-eluting phosphopeptides appears to affect the accuracy of the peak area measurement more than the amount of phosphopeptides. Hence, optimizing RPLC gradient conditions by focusing on the main region of phosphopeptide elution or employing longer separation conditions (−240 min separations), could improve the level of deviation in the peak area measurements. Recently, isotope labeling-free protein quantification results have been demonstrated using reproducible RPLC- or LC/LC–MS/MS analysis [30–33]. Relative protein quantification analyses of two, or more, different biological samples have been routinely carried out using spectrum counting, or peak area measurement, of the peptides [34]. Successful applications of isotope labeling-free protein quantification technique imply that reproducible enrichment of phosphopeptides combined with RPLC- or LC/LC–MS/MS analysis could be also used to compare the phosphorylation levels of specific proteins in two or more different samples [35]. And our results corroborate the potential use of our off-line capillary column IMAC method in the isotope-labeling-free phosphorylation quantification analysis. Unlike isotope-labeling technique based phosphorylation quantification analysis, isotope-labeling-free phosphorylation quantification is directly applicable to any types of biological sample, such as tissues or primary cell culture samples, without additional costs.

5. Conclusions

Our rapid and reproducible off-line IMAC phosphopeptide enrichment method is on a capillary-scale, requiring less than 100 μg of trypsin-digested proteins and is directly applicable to samples containing high concentrations of denaturants. Since both the sample preparation and IMAC process are carried out in capillary columns, the entire procedure is simplified and does not require complicated instrumental setups. Furthermore, improved reproducibility means that off-line IMAC enrichment experiments can be used to perform relative phosphorylation quantification analysis of major phosphoproteins without using expensive stable isotope-labeling techniques such as SILAC or SILAM. Lastly, rapid and reproducible phosphorylation analysis of tryptic digest samples containing urea using an off-line capillary column based IMAC phosphopeptide enrichment method has not been reported before as far as we are aware.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jchromb.2011.08.035.

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