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Evaluation of quantitative performance of sequential immobilized metal affinity chromatographic enrichment for phosphopeptides

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ABSTRACT

We evaluated a sequential elution protocol from immobilized metal affinity chromatography (SIMAC) employing gallium-based immobilized metal affinity chromatography (IMAC) in conjunction with titanium dioxide-based metal oxide affinity chromatography (MOAC). The quantitative performance of this SIMAC enrichment approach, assessed in terms of repeatability, dynamic range, and linearity, was evaluated using a mixture composed of tryptic peptides from caseins, bovine serum albumin, and phosphopeptide standards. Although our data demonstrate the overall consistent performance of the SIMAC approach under various loading conditions, the results also revealed that the method had limited repeatability and linearity for most phosphopeptides tested, and different phosphopeptides were found to have different linear ranges. These data suggest that, unless additional strategies are used, SIMAC should be regarded as a semiquantitative method when used in large-scale phosphoproteomics studies in complex backgrounds.

Reversible phosphorylation of serine, threonine, and tyrosine residues is critical for the regulation of many biological processes and is a highly dynamic aspect of the proteome. During recent years, mass spectrometry (MS)[1]-based phosphoproteomics has emerged as a useful tool to survey the phosphorylation state of a complex protein mixture in a large-scale and high-throughput fashion. However, given the fact that most phosphoproteins are in low abundance with phosphorylation in low stoichiometry, the use of an enrichment technique before MS analysis has become necessary to analyze phosphopeptides from a complex background such as a total cell lysate. Immobilized metal affinity chromatography (IMAC) based on ferric ions has long been used to capture phosphopeptides nonspecifically [1]. Over the years, new IMAC chemistries based on various multivalent metal cations, including gallium [2], zirconium [3], and titanium [4], have been introduced with varying selectivity and efficiency. Much attention has also been drawn to the use of metal oxide affinity chromatography (MOAC) for phosphopeptide enrichment due to its reported higher recovery rate and selectivity compared with IMAC [5–14]. Numerous MOAC protocols based on different multivalent metal oxides such as titanium dioxide (TiO_2) [15], zirconium dioxide (ZrO_2) [16], and aluminum oxide (Al_2O_3) [17] have been widely adopted. Interestingly, it was reported recently that IMAC is less efficient for enrichment of monophosphorylated peptides than for multiply phosphorylated species [18–20]. In contrast, MOAC was shown to be more efficient for capturing monophosphorylated peptides [21]. This is probably due to the fact that monophosphorylated peptides have poor retention on IMAC material, whereas MOAC provides interactions that are strong enough to capture monophosphorylation but make it difficult to elute multiply phosphorylated peptides. Recognizing this phenomenon, Thingholm and coworkers introduced a novel sequential elution protocol from IMAC, SIMAC (sequential immobilized metal affinity chromatography), using MOAC as the secondary enrichment step to capture monophosphorylated peptides that were not retained by IMAC enrichment [21,22]. The application of this SIMAC protocol on whole cell lysate from human mesenchymal stem cells provided more phosphopeptide identifications than using MOAC or IMAC alone [21]. Since then, this sequential combination of IMAC and MOAC enrichment has gained popularity in various large-scale phosphoproteomics studies [23–25].

Despite the fact that IMAC- or MOAC-based protocols have been used in large-scale phosphoproteomics studies during recent years, questions about the reliability of these methods remain. In

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[1] *Abbreviations used:* MS, mass spectrometry; IMAC, immobilized metal affinity chromatography; MOAC, metal oxide affinity chromatography; TiO_2, titanium dioxide; SIMAC, sequential immobilized metal affinity chromatography; DHB, 2,5-dihydroxybenzoic acid; LC, liquid chromatography; HPLC, high-performance liquid chromatography; ACN, acetonitrile; DTT, dithiothreitol; IAA, iodoacetamide; SDS, sodium dodecyl sulfate; BSA, bovine serum albumin; ABC, ammonium bicarbonate; FA, formic acid; TFA, trifluoroacetic acid; SPE, solid-phase extraction; ESI–Q-TOF, electrospray ionization–quadrupole time-of-flight; MS/MS, tandem MS; CV, coefficient of variation; ANOVA, analysis of variance; SILAC, stable isotope labeling by amino acids in cell culture.

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particular, there is very limited knowledge on whether metal-based affinity enrichment techniques can be used in quantitative phosphoproteomics scenarios. Attention has usually been given to testing the selectivity and sensitivity of the enrichment methods but not to the assessment of quantitative measures such as the repeatability, dynamic range, and linearity. In a typical large-scale phosphoproteomics study, a liquid chromatographic separation step (e.g., strong cation exchange [SCX], hydrophilic interaction liquid chromatography [HILIC], or electrostatic repulsion–hydrophilic interaction chromatography [ERLIC]) is performed as a peptide fractionation procedure to reduce the sample complexity prior to the isolation of phosphopeptides from each fraction using metal-based affinity chromatography. However, each fraction usually contains peptide subsets with different total peptide amounts and complexity. Such dynamic sample characteristics have made the estimation of the quantitative performance of metal-based affinity chromatography even more difficult in a real large-scale phosphoproteomics application.

In this study, we evaluated the repeatability, dynamic range, and linearity of metal-based affinity chromatography for quantitative phosphoproteomics applications. The testing protocol was modified from the SIMAC procedure in which IMAC and MOAC were performed sequentially as described by Thingholm and coworkers [21]. In the first step, a gallium-based IMAC method was selected because gallium has been shown to have higher selectivity and sensitivity than other metal-based IMAC methods [26]. The subsequent MOAC procedure was based on the most widely used TiO_2–MOAC protocol as described by Jensen and Larsen [18]. Glycolic acid was used to prevent nonspecific binding of non-phosphorylated peptides with acidic amino acid residues. Jensen and Larsen showed glycolic acid to be an effective alternative to 2,5-dihydroxybenzoic acid (DHB) as an acidic quenching agent [18]. Two experiments were performed. First, to test the enrichment repeatability of SIMAC from varying backgrounds, we constructed a series of peptide mixtures with a variety of loading amounts and complexity to mimic sample characteristics of peptide mixture as the result of liquid chromatography (LC) prefractionation. In the second experiment, a complex background was spiked with a series of phosphopeptide standard mixtures at different concentrations to estimate the linearity and dynamic range of the SIMAC method.

Materials and methods

Materials

High-performance liquid chromatography (HPLC)-grade acetonitrile (ACN), water, and acetic acid were obtained from Thermo Fisher (Waltham, MA, USA). Urea, dithiothreitol (DTT), iodoacetamide (IAA), sodium dodecyl sulfate (SDS), and 2DE Ready Prep cleanup kits were purchased from Bio-Rad (Hercules, CA, USA). Bovine serum albumin (BSA), α-casein and β-casein, ammonium bicarbonate (ABC), formic acid (FA), trifluoroacetic acid (TFA), glycolic acid, and ammonium hydroxide were purchased from Sigma (St. Louis, MO, USA). Sep-Pak solid-phase extraction (SPE) columns with a 200 mg of C18 resin were obtained from Waters (Milford, MA, USA). Spin columns with filters (cat. No. M105010S) were purchased from Boca Scientific (Boca Raton, FL, USA). Ga(II)–IMAC Nutip (part No. TT2GAA) from Glygen (Columbia, MD, USA) and Titansphere TiO_2 beads from GL Science (Tokyo, Japan) were used as enrichment media in all SIMAC experiments. Trypsin was purchased from Promega (Fitchburg, WI, USA). One phosphopeptide standard mixture (P33357) was purchased from Invitrogen (Carlsbad, CA, USA), and another phosphopeptide standard set (PHOSPHOSTD01) was obtained from Glygen. Table 1 provides a detailed description of these peptide standards.

Protein preparation and digestion

The protein mixtures in each experiment were dissolved in 25 mM ABC with 0.1% SDS and then directly reduced by 10 mM DTT for 1 h and alkylated by 40 mM IAA for 30 min in the dark. Alkylation was quenched by adding DTT to the final concentration of 20 mM. Protein was then precipitated using 2DE Ready Prep cleanup kits according to the manufacturer's protocol. The resulting protein pellet was reconstituted in 25 mM ABC and digested with trypsin at a 30:1 protein/protease ratio. Digestion was carried out at 37 °C for 5 h and stopped by acidification using TFA. Tryptic peptides were purified using a 200-mg C18 Sep-Pak SPE column and dried with a SpeedVac (Thermo Electron).

Experiment 1

The goal of this experiment was to estimate the repeatability of the SIMAC procedure with different loading backgrounds. BSA was chosen to create a non-phosphopeptide background because it is rich in acidic amino acids such as Asp and Glu that compete with phosphopeptides during SIMAC enrichment. For practical purposes, three different loading amounts (100, 200, and 500 μg) were tested. These values were selected because most large-scale phosphoproteomics studies start with 1–5 mg of total lysate and most LC prefractionation procedures generate 10–20 fractions. In each loading test, two different levels of sample complexity were created by mixing tryptic peptides from a caseins/BSA ratio of 1:49 or 1:99 (w/w), corresponding to the low stoichiometry of phosphoproteins typically seen in real samples such as cell lysate. In all

Table 1
List of phosphopeptide standards used in this study.

Name	Sequence	Number of phosphorylations	$(M+H)^{1+}$	$(M+2H)^{2+}$	$(M+3H)^{3+}$
P33357 mixture standard (Invitrogen)					
NP1	DRVYIHPF	0	1046.54	523.77	349.52
NP2	DRVYIHPFHL	0	1296.69	648.85	432.90
NP3	GKGRGLSLSRFSWGA	0	1578.85	789.93	526.95
P1	DHTGFL[pT]E[pY]VATR	2	1669.67	**835.34**	**557.23**
P2	TRDI[pY]ETDYYRK	1	1702.75	851.87	**568.25**
P3	VPIPGRFDRRV[pT]VE	1	1720.89	860.95	**574.29**
P4	DLDVPIPGRFDRRV[pS]VAAE	1	2192.09	1096.53	**731.37**
PHOSPHOSTD01 standard (Glygen)					
P5	WWGSGPSGSGG[pS]GGGK	1	1500.60	**750.80**	500.87
P6	WWGSGPSG[pS]GG[pS]GGGK	2	1580.58	**790.78**	527.53
P7	WWGSGP[pS]G[pS]GG[pS]GGGK	3	1660.53	830.77	**554.18**
P8	WWG[pS]GP[pS]G[pS]GG[pS]GGGK	4	1740.45	870.75	**580.82**

Note: The actual *m*/*z* species used in LC–MS label-free quantitation are shown in bold.