Sequential enrichment from Metal Oxide Affinity Chromatography (SMOAC), a phosphoproteomics strategy for the separation of multiply phosphorylated from monophosphorylated peptides.

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ABSTRACT

Purpose: We explored sequential methods for complementary phosphopeptide enrichment to find an effective strategy for enrichment of multiply phosphorylated peptides. We also evaluated fractionation strategy with the phosphopeptide enrichment method to show the comprehensive phosphoproteomes.

Methods: We implemented a SMOAC strategy. One milligram of Nocodazole-arrested HeLa cell digest sample is our starting sample. We use a titanium dioxide (TiO_2) chromatography first for phosphopeptide enrichment. Flow-through and wash fraction s from TiO_2 were pooled and used for Fe-NTA chromatography for the second enrichment. We subsequently used high pH RP fractionation. We expanded our analytical depth by performing fractionation with pooled phosphopeptide eluates from both TiO_2 and Fe-NTA performed in series.

Results: The SMOAC strategy enabled effective enrichment of multiply phosphorylated peptides and emphasizes the utility of SMOAC with fractionation to elucidate the comprehensive phosphoproteome.

INTRODUCTION

The identification of multiply phosphorylated peptides in a complex biological sample has been a major challenge. Previously, a SIMAC (Sequential elution from IMAC) strategy was deployed for sequential separation of monophosphorylated peptides and multiply phosphorylated peptides from a complex sample with a significant increase in recovery of multiply phosphorylated peptide (1). We recently launched two phosphopeptide enrichment kits, Thermo Scientific™ Pierce™ HiSelect™ Fe-NTA and Thermo Scientific™ Pierce™ HiSelect™ TiO_2 phosphopeptide enrichment kits with newly optimized buffer conditions. Here we evaluated a SMOAC (Sequential enrichment of Metal Oxide Affinity Chromatography) method where phosphopeptides were enriched by TiO_2 first and the TiO_2 flow-through (FT) and wash fraction were pooled and subjected to Fe-NTA. In parallel we evaluated SIMAC (Sequential enrichment of Immobilized Metal Affinity Chromatography) where the order of phosphopeptides enrichment was reversed. We also performed deep phosphoproteome analysis by using high pH RP fractionation after the enrichment of phosphopeptides with SMOAC.

MATERIALS AND METHODS

Sample Preparation

HeLa S3 cells were cultured in S-MEM/glutamate/10% FBS media and treated with nocodazole (2.5 µl/mL) for 16 hours to achieve homogeneous mitotic arrest. Cells were harvested, lysed in 100mM TEAB/8M Urea containing Thermo Scientific™ Halt™ phosphatase inhibitor (PN#78427) followed by sonication, reduced, alkylated, digested with Thermo Scientific™ Pierce™ Trypsin/Lys-C (PN#90059; 90307) overnight, and desalted. For the SMOAC method, one milligram of Nocodazole-treated HeLa tryptic digest was subjected to Thermo Scientific™ Pierce™ HiSelect TiO_2 phosphopeptide enrichment kit (PN#A32993) and the TiO_2 eluent was saved for MS analysis. The TiO_2 flow-through (FT) and wash fractions were pooled, and the phosphopeptides were enriched by Thermo Scientific™ Pierce™ HiSelect Fe-NTA phosphopeptide enrichment kit (PN#32992). For the SIMAC method, another milligram (1 mg) of Nocodazole-treated HeLa tryptic digested peptides was subjected to HiSelect Fe-NTA phosphopeptide enrichment kit. The Fe-NTA FT and wash fractions were combined and applied to the HiSelect TiO_2 phosphopeptide enrichment kit. After SMOAC, phosphopeptides were fractionated with the Thermo Scientific™ Pierce™ high pH reversed-phase peptide fractionation kit (PN#84868). All eluents were quantitated with Thermo Scientific™ Pierce™ quantitative colorimetric peptide assay. The eluents were analyzed by the Thermo Scientific™ Orbitrap Fusion™ Tribrid™ instrument and the phosphorylation sites were localized.

For Thermo Scientific™ TMTzero™ (PN#90060) reagent labeling, 100 µg of desalted peptides were resuspended in 100 µl of 100 mM TEAB, pH8.5 (PN#90115). TMTzero reagent (0.8 mg) was dissolved in acetonitrile (41 µl). TMTzero labeling reaction was initiated by mixing the desalted peptides with TMTzero reagent (total 141 µl) and incubated for 1 hr. Multiple reactions were set up for scaling up. The reaction was quenched with hydroxylamine (PN#90115) to a final concentration of 0.27% v/v. The TMTzero reagent labeled samples were pooled and desalted.

LC-MS and Data Analysis

For the LC-MS analysis, 1~2 ug was injected onto a 50cm Thermo Scientific™ EASY-Spray™ C18 LC column (3 µm particle size) to separate peptides with a 5-25% acetonitrile gradient over 180 min at a flowrate of 300 nL/min. Spectra were acquired on an Thermo Scientific™ Orbitrap Fusion™ Tribrid™ mass spectrometer at top speed using the following parameters: FTMS full scan at 120,000, AGC 4e5, IT 50ms followed by IT MS2 scans at 1.6 isolation, HCD 30% collision energy, rapid, AGC 134, IT 50ms. For data analysis, Thermo Scientific™ Proteome Discoverer™ 1.4 software using the SEQUEST®HT search engine was used with a precursor mass tolerance of 10 ppm and fragment mass tolerance of 0.02 Da. Carbamidomethylation (+57.021 Da) for cysteine was used as a fixed modification with methionine oxidation (+15.996 Da) and phosphorylation (+79.966 Da, T, Y, S) as variable modifications with phosphoRS for site localization. Data was searched against a Swiss-Prot® human database with a 1% FDR criteria using Percolator.

RESULTS

Figure 1. SMOAC vs. SIMAC strategy. One milligram of Nocodazole-arrested HeLa cells protein digested peptide mixture was used as a starting sample. Details are given in the text.

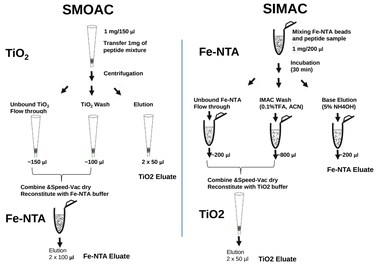


Figure 2. Analysis of peptides from Nocodazole-arrested proteins from HeLa using TiO_2 chromatography followed by Fe-NTA with flow-through/wash fraction from TiO_2 (SMOAC), and conversely Fe-NTA first followed by TiO_2 (SIMAC).

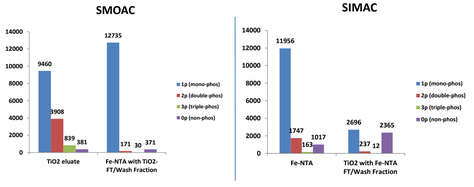
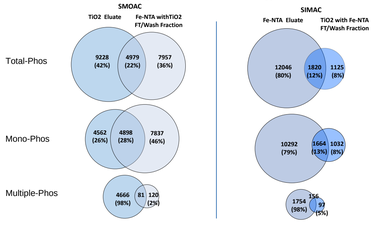


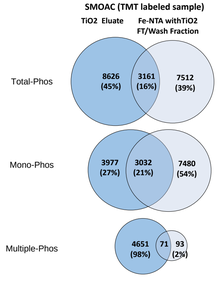
Figure 3. Venn diagram illustrating the overlap of phosphorylated peptides, mono-Phosphorylated peptides, or multiply phosphorylated peptides betweenTiO_2 and Fe-NTA in the SMOAC strategy or in the SIMAC strategy.



One milligram of Nocodazole-treated HeLa tryptic digest peptides were subjected to TiO_2 tip (Figure 1. SMOAC). The phosphopeptides eluted from the tip yielded a total of 9460 monophosphorylated peptides, 3908 double phosphorylated peptides, 839 triple phosphorylated peptide and 381 nonphosphorylated peptides (Figure 2 SMOAC). Thus 97% of the peptides identified were phosphorylated. Next the TiO_2 flow-through and wash fraction were pooled, dried, and the pooled samples were subjected to Fe-NTA (Figure 1. SMOAC). This serial Fe-NTA enrichment yielded 12735 monophosphorylated, 171 double phosphorylated, 30 triple phosphorylated, and 371 nonphosphorylated. (97% phosphopeptide selectivity). The serial Fe-NTA enrichment identified 36% additional unique peptides compared to TiO_2 alone. Interestingly, 98% of multiply phosphorylated peptides were identified from TiO_2. Overall, the results clearly indicate the benefit of the SMOAC strategy for the analysis of the FT and wash fraction of TiO_2 with Fe-NTA (Figure 3. SMOAC).

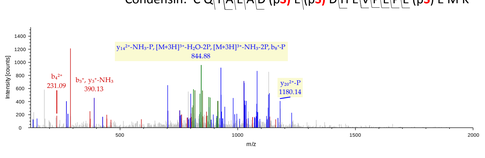
We also evaluated the SIMAC method (Figure 1. SIMAC). One milligram of Nocodazole-treated HeLa tryptic digest peptides were subjected to Fe-NTA column (Figure 1. SIMAC). We identified The 11956 monophosphorylated peptides, 1747 double, 163 triple phosphorylated peptides, and 1017 nonphosphorylated peptides were identified by the Fe-NTA (Figure 2. SIMAC). Thus 93% of the peptides identified were phosphorylated. Unlike the SMOAC method, the SIMAC method with these kit buffers and columns identified fewer multiply phosphorylated peptides and relatively few additional phosphopeptides. Only 8% additional unique peptides were identified, indicating a minimum benefit of the SIMAC strategy for the analysis of the Fe-NTA FT and wash fraction with TiO2 (Figure 3. SIMAC).

Figure 4. Venn diagram illustrating the overlap of TMT labeled phosphorylated peptides, mono-phosphorylated peptides, or multiply phosphorylated peptides betweenTiO_2 and Fe-NTA in the SMOAC strategy.



We also evaluated the SMOAC method with a TMT labeled sample. One milligram of Nocodazole-treated HeLa digest peptides was labeled with TMTzero reagent and subjected to the SMOAC strategy. We identified 11789 and 10673 phosphorylated peptides withTiO_2 and Fe-NTA in series, respectively, and 39% unique phosphorylated peptides were identified from Fe-NTA. Like the SMOAC data with native peptides, 98% of multiply phosphorylated peptides were identified TiO_2 (Figure 4), showing that majority of TMTzero reagent-labeled multiply phosphorylated peptides were enriched in TiO_2.

Figure 5. MS/MS peptide spectra of Condensin Ser-975, Ser-97, Ser-984 triple phosphopeptide CQTAEAD(pS)E(pS)DHEVPEPE(pS)EMK



One example of multiply phosphorylated protein identified by SMOAC, but not by SIMAC, was from the condensin protein. At the onset of prophase, most of condensin is associated with the chromosome arm and phosphorylated by Cdk1, leading to chromosome condensation as a preparatory step toward chromosome alignment at metaphase (2). The SMOAC identified Ser-973, Ser-975, and Ser-984 in one peptide (Figure 5). Previously published mitotic phosphorylation studies by mass spectrometry reported Ser-975 and Ser-977 (3,4), but not Ser-984. Nocodazole-arrested cells do enter prophase but cannot form metaphase because Nocodazole-arrested cells arrest at prometaphase. Thus it would be interesting to see whether newly identified Ser-984 has a specific function at prometaphase.

Figure 6. Fractionation with high pH reversed-phase chromatography of the combined phosphopeptide eluates from the SMOAC strategy.

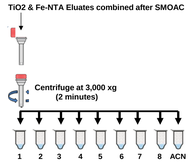


Figure 7. Analysis of peptides from fractionation from combined phosphopeptide sample after the SMOAC strategy.

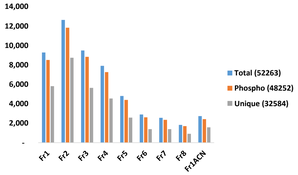


Table 1. Table showing number of phosphorylated and multiply phosphorylated peptides between SMOAC strategy and SMOAC followed by fractionation.

	Multiple-Phos	Mono-phos	Total Unique Phos
SMOAC	4041	18124	22165
SMOAC + Fractionation	5065	27520	32584

Next we fractionated phosphopeptides eluted from after SMOAC by using a high pH reversed-phase spin column (Figure 6). Fractionation reduced complexity of the phosphopeptide sample and increased peptide identification by LC/MS analysis. The two eluates from the SMOAC method were combined, dried, and reconstituted in 0.1% TFA. The combined eluates were subjected to fractionation, and 9 fractions were collected and analyzed in LC/MS (figure 7). Fractionation identified an additional ~10,000 phosphopeptides compared to the SMOAC method alone (Table 1).

Figure 7. CDC25C protein sequence and identification of multiple phosphorylation sites previously known colored in red and newly identified by this study colored in blue.

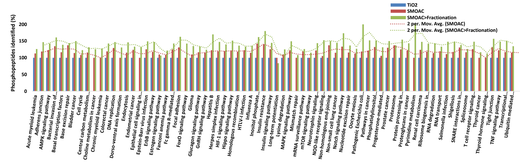
MSTELFSSTR EEGSSGSGSPS FRSNQRKMLN LLLERDTSFT VCPDVPRTPV
GKFLGDSANL SILSGGTPKR CLDLSNLSSG EITATQLTTS ADLDETGHLD
SSGLQEVHLA GMNHDQHLMK CSFAQLLCST PNGLDRGHRK RDAMCSSSAN
KENDNGNLVD SEMKVLGSPI TTVPKLDKNP NLGEDQAEEI SDELMEFSLK
DQEAKVSRSG LYRSPSMPEN LNRPRLKQVE KFKDNTIPDK VKKKYFSGQG
KLRKGLCLKK TVSLCDITIT QMLEEDSNQG HLIGDFSKVC ALPTVSGKHQ
DLKYVNPETV AALLSGKFQG LIEKFYVIDC RYPYEYLGGH IQGALNLYSQ
EELFNFFLKK PIVPLDTQKR IIIVFHCEFS SERGPRMCRC LREEDRSLNQ
YPALYYPELY ILKGGYRDFF PEYMELCEPQ SYCPMHHQDH KTELLRCRSQ
SKVQEGERQL REQIALLVKD MSP

Multiply phosphorylated CDC25 illustrates the effectiveness of SMOAC with fractionation to reduce complexity of the phosphopeptide sample. CDC25 is a serine/threonine and tyrosine dual-specificity phosphatase. CDC25 is a family of phosphatases such as CDC25A, CDC25B, and CDC25C. Phosphatase activity is required for transition from G2 to prophase, specifically for removal of inhibitory dual phosphorylation on CDK1. Multiple kinases phosphorylate CDC25 at Thr-48, Thr-67, Ser-122, Thr-130, Ser-168 and Ser-214 for its activation (Figure 8). TiO_2 enrichment identified a unique Ser-15 site, SMOAC identified Thr-48, Ser-168, and a Ser-263 site. Fractionation yielded phosphorylation sites identified by TiO_2 and SMOAC, plus two additional sites,Thr-67 and Ser-168 (Table 2). The Ser-15 site has never been reported before, whereas Ser-263 is a previously reported (4) phosphorylation site by checkpoint kinase (Chk1). We also analyzed other CDC25 paralogues, CDC25A and CDC25B. We observed similar trend in that SMOAC revealed additional unique phosphopeptides, and even more with fractionation (Table 2).

Table 2. List of phosphorylated peptides in CDC25C identified from TiO2 chromatography, SMOAC strategy, and SMOAC followed by fractionation.

	TiO2	SMOAC	SMOAC > Fractionation
CDC25A		LLFAcsPPPASQPVVK MGSSESTDSGFcLDsPGPLDSK LFDsPSLcSSSTR	MGSSESTDSGFcLDsPGPLDSK LLGcsPALK LFDsPSLcSSSTR AHETLHQSLSLAcsSPK SHSDsLDHDIFQLIDPDENK
CDC25B	sLcHDEIENLLDSDHR	sLcHDEIENLLDSDHR	sLcHDEIENLLDSDHR sVTPPEEQQEAEEPK MEVEELsPLALGR LLGHsPVLR
CDC25C	EEGSsGSGPSFR	DTSFTVcPDVPRtPVGK (Thr-48) YLGsPITTVPK (Ser-168) TVSLcDITITQMLEEDSNQGHLIGDFSK	EEGSsGSGPSFR DTSFtVcPDVPRtPVGK (Thr-48) FLGDSANLSILsGGTPK (Thr-67) YLGsPITTVPK (Ser-168) SGLYRsPSMPENLNRPR (Ser-214) TVSLcDITITQmLEEDSNQGHLIGDFSK

Figure 8. Unique phosphopeptides (%) identified by SMOAC strategy, SMOAC followed by fractionation after normalized by number of phosphopeptides identified by TiO_2



We also performed a cellular pathway analysis based on phosphoproteins identified. We used the Database for Annotation Visualization and Integration Discovery (DAVID) tool to visualize phosphoproteins identified by SMOAC on KEGG pathways. For example, TiO_2 identified 35 phosphorylated proteins in the AMPK signaling pathway. The SMOAC strategy identified 8 additional phosphorylated proteins, then fractionation added another 7 phosphorylated proteins in the pathway. Then we analyzed 73 additional signaling pathways to see how many additional proteins are identified. Overall, the SMOAC strategy identified ~25% more phosphoproteins than TiO_2 in the 74 cellular signaling pathways. Furthermore, fractionation added ~50% more phosphoproteins than TiO_2, demonstrating the benefit of SMOAC method followed by fractionation for deep phosphoproteome analysis (Figure 9).

CONCLUSIONS

- We find that the SMOAC strategy effectively enriched multiply phosphorylated peptides.
- The SMOAC strategy enabled enrichment of multiply phosphorylated TMT-labeled peptides.
- Using SMOAC followed by fractionation strategy enabled the comprehensive analysis of the proteome and phosphoproteome.

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TRADEMARKS/LICENSING