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PhosphoShield: Improving Trypsin Digestion of Phosphoproteins by Shielding the Negatively Charged Phosphate Moiety

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Abstract

Protein phosphorylation is a post-translational modification that is essential to cellular signaling, cellular function, and associated disease progression. Bottom-up proteomics based on enzymatic digestion is the most widely used approach for identifying and quantifying phosphoproteins in complex biological samples. Researchers have largely optimized the experimental conditions for trypsin digestion and it is now routine. However, trypsin digestion is impaired by the presence of phosphorylated residues in the protein sequence. This impairment arises from the fact that there are commonly salt bridges between a negatively charged phosphate group and the side chain of protonated arginine or lysine. On average, 55% of all phosphopeptides have their phosphosites located less than three amino acid residues from a cleavage site. Salt bridges reduce the cleavage accessibility for trypsin by masking the basic site chain groups of arginine and lysine. Thus, there are frequent missed cleavage sites in the vicinity of phosphorylation sites, thereby lessening both the depth of proteome coverage and the quantification accuracy of phosphoproteomics. In this work, we propose a method termed PhosphoShield, to mitigate salt bridge formation by adding a digallium complex that exhibits high binding affinity to the phosphate group. We tested our method using quantitative mass spectrometry analyses of the phosphoproteome of human liver cancer cells (HepG2). PhosphoShield significantly enhances the cleavage frequency of at least 17% of the tryptic phosphopeptides that have cleavage sites close to the phosphate group.
Introduction

Phosphorylation is one of the most frequent posttranslational modifications (PTMs). [1, 2] It affects enzyme activities and plays an essential role in cell signaling by conveying external stimuli to guide internal cellular regulations. [3–5] as well as enzymes’ subcellular localization and interactions. [6] The biological importance of this PTM has motivated numerous efforts to develop robust and efficient mass spectrometry (MS) approaches for PTM characterization, including sequencing and identification of phosphorylated proteins.

Trypsin is the most commonly used proteolytic enzyme in MS-based proteomics due to its high cleavage specificity: C-terminally to arginine and lysine amino acid residues. [7] The digestion mechanism involves targeting these basic amino acids in a binding pocket and subsequently cleaving the C-terminal amide bond through a chemical reaction, which involves a serine residue on the protease. Trypsin’s substrate binding pocket is deep and narrow and features a negatively charged aspartate. This catalytic site determines trypsin’s high specificity to either arginine or lysine via ionic interactions with the aspartate. [8–10] However, the rate of tryptic digestion is sensitive to the type of amino acid residues and residue modifications in the vicinity of the cleavage site. Some sequence motifs can lower trypsin cleavage propensity by forming structures that are unfavorable for substrate recognition. Researchers have established this effect for KP, RP, and to lesser extent for KR, RK, RR, and KK motifs. [11] The same was observed for acidic residues, such as aspartic or glutamic acids, as well as neighboring arginine or lysine amino acid residues. [11]

Phosphate groups are negatively charged at physiological pH and found prolific in forming hydrogen bond networks and salt bridges with basic amino acid residues (arginine or lysine). [4, 6] These salt bridges in phosphorylated proteins obscure trypsin substrate recognition, which leads to missed cleavage. As a result, the frequency of miss-cleaved peptides (MCPs) is significantly larger from phosphorylated proteins compared with proteins that are not phosphorylated. [12–16]

From a proteomics viewpoint the presence of MCPs in the analyzed proteolytic mixtures reduces the (1) efficiency of protein identification and (2) quantification accuracy. Although longer peptides are in general more specific for protein identification, expanding the search space by including MCPs into the database results in a higher probability of false positive matches. [17] Thus, a researcher faces having higher rates of false positive or false negative identifications when choosing the number of missed cleavages for the database searches.

The larger presence of MCPs hampers quantification of phosphopeptides, since MCPs introduce a bias between tryptic peptides representing modified proteins and their non-modified counterparts. Indeed, protein quantification in bottom-up proteomics relies heavily on the efficiency and reproducibility of enzymatic digestion. As an example of this bias, MCPs among the non-specific cleavages are one of the major sources of unpredicted variations in absolute and relative protein quantification. [18] Moreover, the
presence of internal arginine and lysine in the sequences of MCPs renders fragmentation with vibrational excitation [e.g., higher-energy collisional dissociation (HCD) and collision-activated dissociation] less efficient due to stronger retention of the mobile proton at these basic residues.[19–22] This results in low-quality MS/MS spectra, which complicates reliable determination of the phosphorylation site.

To address these challenges, numerous efforts have focused on optimizing sample preparation protocols[16] and experimental workflow[13], as well as developing methods of phosphopeptide enrichment.[14] In this work, we addressed the problem of missed cleavage peptides induced by trypsin digestion of phosphoproteins. Our approach utilizes binding of a specifically designed digallium complex to the phosphate ester motif, thus effectively competing with the formation of salt bridges between the arginine/lysine side chains and the phosphate group of phosphorylated residues. Synthesis and characterization of the digallium complex have recently been reported.[23–25] Here, we investigated the MCP distributions and abundances for the treated samples compared with the untreated control in a phosphoproteome analysis of the human liver cell line HepG2.

**Experimental**

**Study design**

Figure 1 shows the study design. Five sample replicates of human liver cancer (HepG2) lysates were treated with the digallium complex and five sample replicates were left untreated. All of the samples were digested with trypsin, and then the samples were labeled with TMT10-plex (Thermo Fisher Scientific, San Jose, CA, USA). Sample replicates were pooled, and the phosphorylated peptides were enriched using immobilized metal affinity chromatography (IMAC). Enrichment was followed by liquid chromatography (LC)–MS/MS analysis. Both enriched and background samples were analyzed.

High-performance liquid chromatography–MS/MS datasets obtained in this study are available at ProteomeXchange[26] (http://www.proteomexchange.org/), identifier PXD018723.
Figure 1. Design of the experiment.

**Chemicals**

The digallium complex \([\text{Ga}_2(\text{bpbp})(\text{OH})_2(\text{H}_2\text{O})_2](\text{ClO}_4)_3\), where bpbp is 2,6-bis((N,N'-bis(2-picolyl)amino)methyl)-4-methylphenolate, was synthesized as described previously.[25]

**Sample preparation**

HepG2 cells (1 mg) were washed with 100 mM ammonium acetate buffer (pH 7), and resuspended in 200 µL of lysis buffer containing 100 mM Tris (pH 8.5), 6 M guanidinium hydrochloride (GdmCl), 5 mM tris(2-carboxyethyl)-phosphine (TCEP), 10 mM chloroacetamide, PhosSTOP (Roche), and cOmplete protease inhibitor cocktail (Roche). Samples were boiled at 95°C for 10 min. The mixture was then subjected to tip sonication on ice using a Bandelin Sonopuls HD2070 (Bandelin Electronic, Berlin, Germany) ultrasonic homogenizer (1 s on, 1 sec off for 2 min with an amplitude of 40%). Proteins were separated by ethanol/acetone precipitation. The protein pellet was resuspended in 50 mM triethylammonium bicarbonate (TEAB) buffer and the protein concentration was measured by Qubit fluorometric quantification (Thermo Fisher Scientific). Protein samples were split into 10 parts (100 µg of protein in each) and transferred to a 10.000 MWCO spin filter (Millipore, USA). Five samples of HepG2 proteins were incubated overnight with the digallium complex (1.67 mM) in a sample volume of 150 µL. Thereafter, excess digallium complex was discarded by washing the filter 3× with 350 µL of 50 mM TEAB buffer. All of the control samples were treated the same way in parallel, except for the addition of the digallium complex.

For enzymatic digestion, purified methylated trypsin prepared in-house[27] was added in a 1:100 w/w ratio and incubated for 2 h at 37°C. Then trypsin was added one more time in a ratio of 1:100 w/w and the solution was incubated overnight. After trypsin digestion the filters were spun down and washed with 350 µL of 50 mM TEAB buffer. The samples were incubated overnight in 10 mM Na$_4$P$_2$O$_7$ to remove the digallium complex.
Prior to labeling, all of the samples were desalted using Oasis cartridges for solid-phase extraction (Oasis HLB, 1 cm$^3$, 10 mg, 30-µm particle size, Waters) and dried. Samples were resuspended in 100 mM HEPES (pH 8.5), then labeled with the TMT10-plex kit (Thermo Fisher Scientific, San Jose, CA, USA) in accordance with the manufacturer’s instructions. The HepG2 samples treated with the digallium complex were labeled with TMT-126, TMT-127N, TMT-127C, TMT-128N, and TMT-128C tags; and control samples containing TMT-129N, TMT-129C, TMT-130N, TMT-130C, and TMT-131C tags. Small quantities of all labeled samples were pooled, desalted, and analyzed by LC–MS to check the labeling efficiency. The quantities of peptides in each labeling channel were adjusted to the same average concentration before mixing.

Each pooled sample was enriched using Fe$^{3+}$ IMAC. An UltiMate 3000 LC system (Thermo Scientific, Germering, Germany) was used, equipped with a freshly recharged IMAC column (ProPac IMAC-10, 2 × 50 mm, Thermo Scientific). The following solvents were used for chromatographic separation: (A) 0.1 v/v% trifluoroacetic acid (TFA); (B) 10 mM NH$_4$OH, pH 10 (adjusted using TFA); (C) 99.9 v/v% acetonitrile (ACN), 0.1 v/v% TFA. Supplemental Figure S1 shows the employed gradient. The HepG2 sample was diluted to 750 µL using a loading solvent (50 v/v% ACN, 0.1 v/v% TFA). The non-bound fraction was collected from 0.5–7 min and the enriched fraction (phosphorylated peptides) was collected from 12.15–16.5 min. Both fractions were dried in a SpeedVac and stored at −20°C until LC–MS/MS analysis.

**LC–MS/MS**

LC–MS/MS analysis was performed with an Orbitrap Q-Exactive HF mass spectrometer (Thermo Fisher Scientific, San Jose, CA, USA) coupled with a Dionex UltiMate 3000 nanoflow LC system (Thermo Fisher Scientific, Germering, Germany). The following solvents were used for chromatographic separation: (A) 0.1 v/v% formic acid (FA) in water; (B) 95 v/v% ACN, 0.1 v/v% FA in water. A trap column µ-Precolumn C18 PepMap100 (5 µm, 300-µm i.d., 5 mm, 100 Å; Thermo Fisher Scientific) and a homemade analytical column (75-µm i.d., 25 cm), filled with Inertsil ODS-3 2-µm sorbent (GL Sciences) packed by the FlashPack method[28], were employed for separations. Peptides were eluted at a 300 nL·min$^{-1}$ flow rate using a linear gradient from 5 v/v% to 30 v/v% B over the course of 76 min, then to 45 v/v% B over the course of an additional 13 min.

MS measurements were performed using data-dependent acquisition mode (Top 15). The MS spectrum was acquired at the resolution of 120,000 at m/z 200 in the mass range of m/z 300–1400. MS/MS data were acquired at a resolution of 60,000 using HCD at normalized collision energy of 33. The maximum injection time during MS/MS was 110 ms with an automated gain control value of 1 x 10$^5$. An isolation window of m/z 1.4 with m/z 0.2 offset and a dynamic exclusion period of 30 s were used for analyses.
**Data analysis**

Raw files were analyzed with Proteome Discoverer (version 2.3, Thermo Fisher Scientific) against the human SwissProt database (20,415 entries, accessed May 2019) coupled with a common contaminant database using SequestHT.[29, 30] The following search parameters were employed: trypsin cleavage rule with a maximum of three MCPs; precursor and fragment mass errors were set at 10 ppm and 0.02 Da, respectively; TMT (+229.16293 Da) was selected as a fixed modification for the peptide N-terminus and lysine; cysteine carbamidomethylation was selected as a fixed modification, and phosphorylation of serine, threonine, and tyrosine as well as oxidation of methionine were selected as variable modifications. Percolator was employed for post-searching validation. Peptide spectrum matches (PSMs) were filtered to a 1% false discovery rate (FDR).

Phosphorylation sites were localized using the IMP–ptmRS node.[31] PSMs with a localization probability greater than 0.75 were considered to be valid. Only PSMs with a co-isolation interference less than 50%, and the sum of the reporter ions’ signal-to-noise values greater than 100, were used for quantification. Only unique peptides were considered for the subsequent statistical analysis.

Principal component analysis (PCA) of quantified PSMs was performed to validate the quality and variance of the data (Supplemental Figure S2). Statistical analysis of the quantified and localized peptides was performed using PolySTest, [32] An unpaired LIMMA moderated t-test,[33, 34] which includes linear model estimation and empirical Bayes moderation of standard errors, was employed to assign altered peptide abundances. Calculated p-values were corrected for multiple testing in accordance with Storey FDR estimations.[35] The peptides satisfying FDR_{Storey} < 0.05 were determined as a signature of a differential change in the MCP rate.

**Public data**

Three publicly available datasets containing both phosphoproteomes and non-modified proteomes were selected from the PRIDE repository:[26] (1) mouse (*Mus musculus*) retina (PXD009981 and PXD009909);[36] (2) monkey (*Cercopithecus aethiops*) kidney COS-7 cells (PXD010006);[37] and (3) HeLa cells (PXD000612).[38] SwissProt mouse (17 025 entries, accessed November, 2019), Uniprot TrEMBL Chlorocebusaffectusabaeus canonical (19,490 entries, accessed April 2017, taken from the original study), and SwissProt human (20,415 entries, accessed May 2019) databases were used, respectively. TMT modification was switched off for the mouse and HeLa dataset. All of the other parameters were the same as in the present work.
Results and Discussion

Efficiency of trypsin digestion

Figure 2 shows the normalized distribution of MCPs from three publicly available phosphoproteomics data sets (mouse retina, monkey kidney, and human HeLa cell line). To study the rate of MCPs as a function of the distance between the phosphorylation site and the tryptic cleavage site, we divided the population of phosphorylated peptides into two groups: (1) peptides with a phosphorylated residue located at three or fewer residues from the cleavage site (arginine/lysine) in the sequence (termed adjacent peptides); and (2) peptides with more than three residues between the phosphorylation site and arginine and/or lysine (termed distant peptides). Additionally, we determined the observed “background” MCP rate for non-phosphorylated proteins (background peptides) from each dataset. Background peptides allow capturing the effect of missed cleavages sources, independent of phosphorylation. The three data sets display very similar distributions among the MCP frequency. Specifically, the adjacent phosphopeptides reveal a clear shift toward a greater number of missed cleavages compared with their distant counterparts. Note that the overall digestion efficiencies were different for different data sets, which is evident by the differences in MCP among background peptides. Mouse and monkey kidney datasets (Figure 2A, B) show approx. 15% of MCPs among the background peptides, whereas it was 26% for the HeLa dataset (Figure 2C). Such deviation in protein digestion efficiency is not uncommon since the yield is governed by many factors, including protein/enzyme concentrations and experimental conditions. As expected, all three data sets reveal a significant increase in MCPs for the adjacent relative to the background groups of peptides by factors of 3.6, 1.86, and 1.77 for the mouse, monkey, and HeLa datasets, respectively. The distant phosphopeptide groups exhibit similar MCP distributions as the background groups, indicating that salt bridge formation is prevalent only within the short distances between residues in sequences of phosphoproteins. This suggests that methods that compete with the native salt bridge formation should have the greatest impact on phosphopeptides of the adjacent group. Of the two groups, the adjacent group is the most abundant in proteomes and constitute from 51% to 58% of all the phosphorylated peptides in these datasets.
Figure 2. MCPs normalized distribution in background, adjacent, and distant peptides for three different datasets. A – Mouse retina; B – Monkey kidney; C - HeLa. Numbers in parentheses correspond to the size of the groups.

In order to compete with the salt bridge formation between the phosphate group and the K/R amino acid residues, we propose shielding the negative charge of the phosphate group with a digallium metal complex \[ [LGa_2(OH)_2(H_2O)_2]^{3+} \] (\( L = 2,6\)-bis((\( N, N'\)-bis(2-picolyl)amino)methyl)-4-tertbutylphenolate), which was characterized by very high binding affinity and free energy changes (\( K_{assoc} = 3.08 \cdot 10^6 \text{ M}^{-1} \), \( \Delta G = -8.99 \text{ kcal-mol}^{-1} \)) to the phosphate ester.[23] This renders the digallium complex considerably more prone to phosphate binding compared with ammonium and guanidinium cations from lysine and arginine side chains, which have Gibbs free energy changes of approximately −2 kcal-mol\(^{-1}\) upon salt bridge formation.[39]

Scheme 1 shows our proposed action of the PhosphoShield approach, using arginine and phosphoserine residues as an example. If an arginine (or lysine) participates in a salt bridge, trypsin cannot recognize the binding site, thus resulting in a missed cleavage (Scheme 1, A). In contrast, if the negative charge of the phosphate ester forms strong ionic bonds to the high-affinity digallium cation, the likelihood of a salt bridge is greatly reduced and the cleavage site becomes accessible to trypsin (Scheme 1, B).
Scheme 1. Proposed mechanism of action of the PhosphoShield approach, illustrated here as an example with arginine as a cleavage site neighboring a phosphorylated serine: (A) – Trypsin has limited access the site of the cleavage, if it participates in a salt bridge. (B) – Salt bridge formation is prohibited, thus providing access to the cleavage site for trypsin. The trypsin structure was adopted from rcsb.org (structure 2BY5).[40]

**Application of the digallium complex to a phosphoproteomics experiment.**

In the PhosphoShield approach, trypsin digestion of the HepG2 proteome occurs in the presence of the digallium complex bound to protein phosphate moieties. A pilot study (Figure S3) revealed that the digallium complex at a certain concentration led to trypsin inhibition. Thus, we removed excess digallium complex from the solution prior to trypsin digestion by repeated filter washes. After trypsin digestion, addition of excess pyrophosphate, which has a seven-fold higher binding affinity to the digallium complex,[23] caused release of the phosphate-bound digallium complexes from the peptide sample. Finally, we purified the sample by reverse-phase chromatography.

PCA confirmed excellent reproducibility between the sample replicates, and significant differences between treated and control (untreated) peptide groups (Figure S2). Importantly, for the background peptides (non-phosphorylated peptides), there was no separation between treated and control groups in either principal component. This result reveals that addition of the digallium complex does not affect the digestion efficiency of non-phosphorylated proteins to any significant degree.
Using TMT-10 multiplexing (five digallium-treated and five controls) allowed us to assess the MCP distribution for the HepG2 proteome (Figure 3). Similarly to the previous analyses, we observed a bias toward a higher number of MCPs for the adjacent phosphorylation group of peptides compared with the background peptides. The extent of MCPs was 25% and 70% among the background peptides, and adjacent phosphopeptides, respectively. As expected, the phosphopeptides of the distant group showed a MCP distribution similar to the distribution of the background peptides. Indeed, the observed changes in MCP distributions for adjacent and distant groups of phosphopeptides confirmed a strong dependence of salt bridge formation on the distance between the residues involved in the interaction.

![Figure 3. Normalized distribution of MCP in background, adjacent, and distant phosphopeptides of the PhosphoShield dataset.](image)

Next, we compared the results obtained for treated and control samples. We analyzed the adjacent group, which contains 4,266 phosphopeptides. A Volcano plot (Figure 4) shows phosphopeptides that significantly changed their abundances in the PhosphoShield experiment as a result of disruption of native salt bridges (Figure 4A). The number of significantly altered phosphopeptides was approximately 17%, with more phosphopeptides increasing their abundances rather than decreasing.

To further explain the observations shown in Figure 4A, we separately analyzed the MCP distributions of adjacent phosphopeptides with increasing and decreasing abundances (Figure 4B). This analysis shows that the phosphopeptides with decreasing abundances exhibited more missed cleavages than the group of phosphopeptides that experienced gains in abundances. This observation underscores that the distribution of these phosphopeptides was approaching the distribution of the background peptides, proving that PhosphoShield works as expected. Note, however, that the proposed approach did not prevent missed cleavages completely. One can observe this from the MCP distribution of phosphopeptides gaining abundance that should have been closer to the distribution of the background peptides.
Figure 4. A – Volcano plot for the adjacent group of phosphopeptides in the PhosphoShield dataset. B – Normalized MCP for phosphopeptides of the adjacent group (Red – increased abundance; Blue – decreased abundance).

The MCP distribution confirms indirectly that the PhosphoShield approach noticeably reduced the formation of salt bridges between arginine or lysine residues and the phosphate groups. However, there is still room for improving the efficiency of the approach, as the MCP distributions are still significantly different compared with the background peptides (non-phosphorylated peptides). This may be addressed by selecting alternative phosphate-specific reagents or further optimizing our sample preparation protocol. In this regard, a likely reason for the presence of MCPs among phosphopeptides is the size of the digallium complex. When bound to a phosphorylated residue, the digallium complex may reduce trypsin access to the cleavage site due to its bulkiness. We attempted to circumvent the sterically issues associated with the digallium complex by substituting it with (much smaller) uranyl nitrate. The uranyl cation (UO$_2^{2+}$) has considerable phosphate binding affinity but experiments aimed at reducing the MCP frequency were less convincing, including unintentional inhibition of trypsin digestion (data not shown). Well-known guanidinium ions could serve as another possible reagent in the PhosphoShield approach. It has been reported to disrupt salt bridges formation by interacting with the carboxylic acid side chains.[41, 42]. However, the association constants of guanidinium ion are 1.4 and 2.6 M$^{-1}$ with dihydrogenphosphate and hydrogenphosphate anions, respectively,[43] which are much lower than that of the digallium complex employed in our study. Since the guanidinium leads to protein denaturation, its influence on the trypsin activity should be taken into consideration as well. As a result, the utility of guanidinium ion in PhosphoShield requires further investigation.
Conclusion

The literature commonly refers to overrepresentation of miss-cleaved tryptic phosphopeptides, and this overrepresentation is an analytical challenge in phosphoproteome analyses. We addressed this challenge by using a novel approach termed PhosphoShield, which shields the negative charge of the phosphate ester moiety by using a digallium complex, and thus hinders strong internal salt bridge formation in phosphoproteins. Our use of the digallium complex improves the efficiency of tryptic digestion of phosphoproteins. Approximately 17% of the phosphopeptides from the group of peptides termed adjacent (those with a phosphate group located in the sequence at less than three residues from the cleavage site) significantly changed their abundances toward a population resembling that of the non-phosphorylated peptides. Our approach has potential in future phosphoproteome analyses, as it improves the quality of the product ion spectra and the consistency of the quantitative results. The results clearly demonstrate the feasibility of PhosphoShield to lessen the adverse effect of the phosphate group on trypsin digestion, and we expect that further investigations into our methodology could lead to additional improvements.

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Supplementary materials for

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IMAC enrichment gradient.

![Gradient for IMAC enrichment](image)

Figure S1. Gradient for IMAC enrichment.

PCA results

![PCA results](image)

Figure S2. PCA of A – background (non-phosphorylated) peptides, and B – phosphorylated peptides. Red color denotes treated samples, blue – non-treated.

Trypsin inhibition by digallium complex.

To investigate the influence of the digallium complex on the tryptic digestion an enzymatic assay of trypsin inhibitor (Sigma-Aldrich) was used (Figure S3). The procedure was performed according to manufacturer’s
instructions. The relative activity of trypsin was tested using BAEE (Nα-Benzoyl-L-arginine ethyl ester) as a substrate.

Figure S3. Time dependence of absorbance for different digallium complex concentrations.